Identification of Juvenile Hormone 3 as the Only JH Homolog in All Developmental Stages of the Honey Bee

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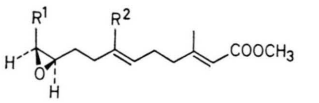
Total extracts from worker larvae, pupae, and adults of the honey bee were analyzed for their juvenile hormone contents. Using the 10-(heptafluorobutyrate)-11-methoxy derivative, the hormone was isolated in yields of 20–50% in nanogram quantities as calculated from isotope dilution and from gas chromatography with electron capture detection. The procedure is sensitive enough to isolate the juvenile hormone out of 1–4 grams insect material for qualitative and quantitative analysis. Only juvenile hormone 3 (methyl 10.11-epoxy-2-*trans*, 6-*trans*-farnesoate) could be detected as the predominant hormone homolog of all the three developmental stages. Juvenile hormone 1 (methyl 3.11-dimethyl-10.11-*cis*-epoxy-7-ethyl-2-*trans*, 6-*trans*-tridecadienoate) could, if at all, only be identified in trace amounts, and juvenile hormone 2 was completely absent in all stages. This first systematic analysis of a hymenopteran insect shows a modulation of JH3 titre between zero and 2 ng per gram body weight during imaginal development and a rather constant JH3 titre near 10 ng per gram in the adult.

Juvenile hormone (JH) has a basic function in insect morphogenesis. It inhibits metamorphosis in imaginal development and it seems to stimulate ovarian activity in the adult. Since the discovery by Wigglesworth [1], the physiological action of the corpora allata is suggested as to produce the juvenile hormone. Changes in the volume have been correlated with activity of the glands during larval development [2]. Three types of biologically active homosesquiterpenoids, named JH1, -2, and -3, have been isolated from insects:



JH1: $R^1=R^2=C_2H_5$
JH2: $R^1=C_2H_5$, $R^2=CH_3$
JH3: $R^1=R^2=CH_3$

In the *Galleria* wax test the biological activity of JH3 is lower than that of JH1 and JH2 by a factor 100 and in the *Tenebrio* assay even of 10^4 as was shown by Dahm *et al.* [3]. A spectrum of functions in the life cycle of insects has been attributed to these three hormones [4]. It has been suggested that JH1 and JH2 have a juvenilizing effect whereas JH3 is the gonadotropic hormone in the cockroach *Nauphoeta* as well as in other insects [5]. With the few sources in mind from which juvenile hormones have been identified, and from the fact that adult insects have mostly been used as starting material, it is also possible that at least some insect species use different JH homologs as morphogenetic and as gonadotropic hormones [5].

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As a first example from the order of Hymenoptera, adult worker honey bees have been analyzed by Trautmann *et al.* [6]. Using radioactive dilution, only JH3 could be detected in a concentration of 2.8 ng per gram in the adult honey bees. Absence of JH1 or JH2 was also controlled by gas chromatography, GC-MS analysis, and by the *Galleria* wax test. On the assumption that only JH3 is present, Rutz *et al.* [7] determined the JH activity in extracts from worker haemolymph with the *Galleria* wax test. They calculated a specific activity of 16 to 190 ng JH3 per ml haemolymph (equivalent to 300–3800 *Galleria* units) depending from age of workers. In a similar way, Wirtz [8] found JH titres which were on the boundary of detection in the haemolymph from worker larvae less than five days old and of 600–1300 *Galleria* units per ml for spinning larvae and prepupae. Prerequisite for a better understanding of the mechanisms by which the endocrine system of the honey bee [9] controls development and caste differentiation [10] is to know the quality and quantity of hormones present in the different developmental stages. For juvenile hormone this is only possible by use of a highly sensitive and specific chemical method which allows to separate all the three JH homologs in the nanogram range.

For a chemical analysis of JH, either a direct chromatographic detection [5, 6] or an identification after chemical derivatization [11–13] can be used. By use of a halogenated derivative and an electron capture detector, subnanogram amounts of juvenile hormone have been detected. Adaptation of these methods to the isolation of juvenile hormone

from about one gram of insect material, identification of the JH homologs in different developmental stages, and estimation of JH titres will be described in the following.

Materials and Methods

All solvents and chemicals were purchased from Merck, Darmstadt, and were of analytical grade. NaCl and Na_2SO_4 were kept at 300 °C overnight before use. All glass vessels were rinsed with methanol and ethyl acetate. Solvents of small volume were usually blown off with nitrogen and larger amounts were concentrated under reduced pressure in a rotary evaporator. For TLC, precoated silica gel plates (Merck Si 60 F_{254}) were used. [^{3}H-C_{10}] JH1, spec. act. 13.5 Ci/mmol, was purchased from NEN, Dreieichenhain.

Insect material was collected from Carnica bee colonies which are maintained near the institute. Immediately after removal from the comb, the animals were frozen in liquid nitrogen and then stored at −20 °C. For collecting a large amount (20 g batches) of honey bee stages, a homogenous brood frame was selected and after sealing a rather defined larval stage (L_5, spinning larva) and the pupal stage P_w (white eyed pupa with white cuticle) were pooled. For collecting newly hatched adults (A_1), a brood frame was kept in the incubator overnight and all bees which had hatched during 15 h were pooled. By that way, a homogenous experimental material could be used for extraction of the juvenile hormone. Only in case of nurse bees, such worker bees of an age of 8 ± 5 days were collected.

Extraction procedure. A defined amount (usually 2.9 ng) of radioactive JH1 was put into a glass homogenizer (Braun, Melsungen), filled up to 5 ml with methanol: ethyl acetate (1 : 1), and 1 – 4 grams of frozen insects were added. By cooling with ice these were homogenized, using a Teflon piston, and after centrifugation the precipitate was again extracted in the same way with about 5 ml methanol : ethyl acetate (1 : 1). Depending from the insect material, extraction had to be repeated for another 2 – 3 times. The pooled supernatants were finally concentrated in a rotary evaporator under reduced pressure to a volume of 15 ml. To this concentrated extract 20 ml of 1.7% NaCl solution were added and then the lipids were extracted first with 20 ml and then five times with each 10 ml hexane. After drying with Na_2SO_4, filtration, concentration of hexane and transfer of the concentrated solution into a small centrifuge tube, the solvent was evaporated under nitrogen and the residue was dissolved in 6 ml methanol : ether (1 : 1). The solution was kept for 0.5 h at −78 °C (dry ice) and the tube was then packed in dry ice and centrifuged. The precipitate was washed with 4 ml cold solvent for another three times and the four extracts were pooled, concentrated under N_2 to dryness, redissolved in ethyl acetate and applied to a 0.5 mm silica gel plate which was run in hexane : ethyl acetate (65 : 35). Dibutyl phthalate was used as a reference with similar R_f value (0.51) like JH1. The silica gel was scraped off between R_f 0.38 and 0.57, extracted with ethyl acetate, and samples isolated from larvae and pupae were used as such for preparing the derivative, whereas extracts from old pupae and from adults had to be rerun on a second TLC, using chloroform : carbon tetrachloride (1 : 1) for separation. In this case, the range from R_f 0.22 – 0.43 was extracted.

Derivatization and further purification. Material from preparative TLC was dissolved in 50 µl methanol which contained 0.014% perchloric acid. After a minimum of 30 min, 500 µl of a 2% NaCl solution in water were added and the methanol was 5 times extracted with 50 µl ethyl acetate each. After concentration under N_2 the solution was applied to a 0.25 mm silica gel plate which was developed in hexane : ethyl acetate (65 : 35). The methanol addition product was eluted between R_f 0.21 – 0.40 with ethyl acetate in the usual way. After further purification by HPLC (see below), the dry substance was warmed to ca. 40 °C, 10 – 12 µl N-heptafluorobutyrylimidazole (Pierce, Rockford) were added, and the reaction mixture was kept at 60 °C for 30 min under N_2. Volatile material was evaporated by a streem of N_2 and the residue was dissolved in ether. As described for the methanol addition product, this extract was purified by TLC. The 11-methoxy-10-heptafluorobutyrate esters of the juvenile hormones were extracted from silica gel with ether in the range R_f 0.48 – 0.67, further purified by HPLC, and then analyzed by GC-ECD. From an aliquot of each sample radioactivity was measured in a Packard Tri Carb 3380 Scintillation Counter and from this the total yield was calculated which was usually between 20 – 50% of the starting radioactivity (1.5×10^5 cpm). Only such samples were used for quantitative estimation of juvenile

hormone titres. JH1 as calculated from radioactivity was found in the same amount by GC-ECD and was subtracted from the total JH1 peak. For calculation of JH3, the yield of JH1 was used.

High pressure liquid chromatography (HPLC) was performed in a modified Siemens S 200. UV was recorded at 218 nm with a slit of 20 nm. For purification of the methanol addition product (JH-methoxyhydrine), a silicic acid column (4.3 mm i. d., 25 mm length, Whatman partisil 5 μm) was used. As external standard served triphenylcarbinol with a V_R of 11.3 ml. V_R values of the methoxy hydrines of JH1 = 9.8, JH2 = 10.4, JH3 = 12.7 ml. The heptafluorobutyrates of the JH-methoxyhydrines were chromatographed on a packed column of silicic acid (Merck Lichrosorb Si 60, 5 μm) with 3 mm inside diameter and 300 mm length, using *n*-hexane with 8% ether as eluent and a flow rate of 1.5 ml · min^{-1}. External standard dibutyl phthalate with $V_R = 8.4$ ml. V_R values of the derivatives of JH1 = 6.9, JH2 = 7.7, and JH3 = 11.1 ml. It was essential to check all such solvents which were used for the second HPLC-purification step for impurities, which could interfere with JH peaks during GC-ECD analysis. Due to relatively high enrichment of such impurities during the final evaporation of the HPLC-eluent, it is not advisable to purify such solvents.

Gas chromatography of the JH derivatives was performed in a Hewlett-Packard gas chromatograph of the series 5730, equipped with a ^{63}Ni electron capture detector. For integration of peaks and for quantitative estimation of JH signals a Minigrator (Spectra Physics) was used. Packed columns with 3 mm i.d., 2 m length and Gaschrom Q as carrier were used. Liquid phases with 3% load were SE 30, OV 1, OV 17, and OV 225 (Serva, Heidelberg). For more details see the legend of Fig. 1.

Results and Discussion

Electron capture detection after separation by gas-liquid chromatography of the heptafluorobutyrates was chosen as one of the most sensitive methods for qualitative identification of the juvenile hormones. Signals of sufficient intensity for quantitative measurements were obtained in the concentration range of 5 picograms after direct injection of 0.8 – 2 μl solution. The insect material must therefore contain about 0.3 nanograms of juvenile hormone originally. A general advantage of derivatization lies in additional purification steps which change the molecule's polarity: juvenile hormone was first chromatographed with the crude extract by TLC, then the more polar methoxyhydrine by TLC and HPLC, and finally the heptafluorobutyrate again by TLC and HPLC. By this alternating approach most of the apolar and polar compounds are removed which normally interfere with the ECD signals of juvenile hormones. The heptafluorobutyrate (HFB) derivatives from different developmental stages were then fractionated by HPLC and pooled according to their V_R values. After concentration, the combined fractions could finally be separated by GC-TLC.

In Fig. 1 A, the three synthetic HFB-derivatives have been separated on an OV-1 column with retention times of 131 ± 2 sec for JH3, 180 ± 2 sec for JH2, and 210 ± 2 sec for JH1. Also for the other three columns retention times were clearly defined and could therefore be used for identification of the hormones from biological material. As is clear from Fig. 1 B – 1 D, for an optimum analytical discrimination it is necessary to separate the natural hormone samples on different gas chromatographic columns. Only by that way accompanying ECD active compounds can be removed. For quantitative calculation that GC peak was used, the retention time of which was in its peak maximum within the deviation of the synthetic HFB-hormone, and the signal intensity of which was lowest after separation on all the four columns. As an additional control the amount of radioactive JH1 was used: the peak of JH1 corresponded exactly to its amount of radioactivity, if no natural JH1 was present. From the fact, that JH1 concentration could be calculated from the JH3 standard, it further follows that the sensitivity of ECD is the same for JH3 and JH1 and consequently also for JH2.

Worker larvae in the fifth larval instar (see Fig. 1 B) have a JH3-titre of about 1.5 ng/g. The signal for JH1 originates from added ^3H-JH1 primarily and, after substraction of the marker, corresponds to about 0.2 ng/g of JH1 contained in the L_5 larva. No signal for JH2 can be detected. The pupal stage P_w (Fig. 1 C) exhibits a JH3-titre of 1.3 ng/g and, after subtraction of ^3H-JH1, neither this hormone nor JH2. A similar result was obtained for the newly hatched worker bee with about 0.7 ng JH3 per gram and neither JH1 nor JH2. The young nurse bees with age of 3 – 13 days (Fig. 1 D) have a high JH3-titre of about 10 ng/g. Neither JH1 nor JH2 are detectable in the extract.

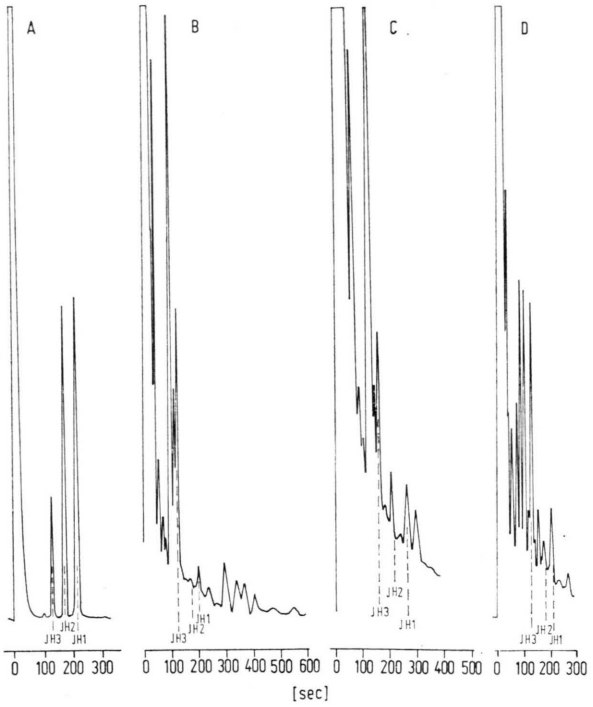

Fig. 1. Gas chromatograms of juvenile hormone-methoxyhydrine-heptafluorobutyrates (JH-HFB). A: Synthetic juvenile hormones. The signal for JH3-HFB corresponds to 1.07 pmol at an attenuation of 128. B: Chromatogram from an extraction of L_5-worker larvae as described in materials and methods. From a final volume of 50 μl, 1.45 μl were injected; attenuation 64. The JH3-peak corresponds to 0.78 pmol. C: A corresponding chromatogram from worker pupae, stage P_W. From 55 μl final volume, 0.5 μl were injected; attenuation 16. The JH3-peak is equivalent to 0.05 pmol, that of JH1 to 0.027 pmol. D: Chromatogram from nurse bees, conditions like under B. Out of 75 μl final volume, 0.90 μl were injected. The JH3-peak corresponds to 0.78 pmol. For GC a glass column of 2 mm i. d. and 193 cm length was used, packed with 3% OV-1 on GasChrom Q. Injector temperature 200 °C, detector temperature 300 °C. For A, B, D, 10% methane/90% argon with a flow rate of 28 ml/min was used as carrier gas; oven temperature 200 °C. Conditions for C: nitrogen, flow rate 28 ml/min, oven temperature 195 °C.

The fact that JH3 is the predominant juvenile hormone of the honey bee larvae and is the only JH homolog in the pupal and adult stages investigated, is highly remarkable. No JH2 was found with our highly sensitive analytical method, and only a trace of JH1 could be detected in the larvae of stage L_5. One must conclude from this result that in the honey bee JH3 controls all the hormonal functions which are attributed to juvenile hormone, *i. e.* regulation of metamorphosis during larval development and control of ovarian function or accessory glands in the adult. The hormone titre is rather low in juvenile developmental stages with zero to about two nanograms per gram in the larva, young pupa and newly hatched adult and with about ten nanograms per gram in the nurse bee. The data for total extracts from adult honey bees coincide fairly well with haemolymph JH3 titres published by Rutz *et al.* [7] from their *Galleria* test values. The authors calculate an average of 16 ng JH3 per ml haemolymph from 12 to 24 hours old worker bees and 90 ng/ml for the 12 days old worker bee. The data as given by Wirtz [8] for the spinning worker larva (600 Galleria units per gram haemolymph) can be calculated with 30 ng JH3 per ml. If chemical data are compared with such calculated from a bioassay, it must be kept in mind that only a chemical estimation combined with isotope dilution allows a quantitative statement for the titre of the three naturally occuring juvenile hormone homologs. A bioassay can only give relative units depending from the test animal, hormone quality or such compounds which interfere with the test and last not least from the laboratory.

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