# Differences in Hydrolysis and Binding of Homologous Juvenile Hormones in *Locusta migratoria* Hemolymph

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Enantioselectivity of JH Carriers, Enantioselectivity of JH Esterases, *Locusta*, Juvenile Hormone, I and III, Protective Function of Carriers

Male *Locusta* hemolymph contains JH-esterase(s) and a JH-carrier protein with high affinity and low capacity that both interact enantioselectively with juvenile hormones. Exposure of racemic JH-I and JH-III results in preferential hydrolysis of the naturally configurated enantiomer of JH-I but the unnaturally configurated enantiomer of JH-III. The JH-carrier protein has a remarkable specificity for the natural enantiomer of JH-III but discriminates only weakly between enantiomers of JH-I. This observation indicates a protective function of the JH-specific carrier protein for JH-III but not for JH-I. A third major protein, the diglyceride carrier lipoprotein (DGCL), shows no stereoselectivity at all.

Juvenile hormones are synthesized by the corpora allata of insects as endocrine substances important in the regulation of growth and development. Many efforts have concentrated in the past on the metabolic fate of the hormones after their release from the endocrine glands. Their transportation in hemolymph is mediated by one or several binding proteins, differing in binding capacity and specificity (Goodman *et al.* [1, 2]; Hartmann [3]; Schooley *et al.* [4]; Kramer and de Kort [5]; Peterson *et al.* [6]; for citation of the earlier literature see Gilbert *et al.* [7]; Kramer *et al.* [8]). One of the key steps in metabolism leading to inactivation of juvenile hormones is the enzyme catalyzed hydrolysis of the ester function (Klages and Emmerich [9]; Vince and Gilbert [10]; Kramer *et al.* [11]; Kramer and Childs [12]; Kramer and de Kort [13]; Weirich and Wren [14, 15]; Weirich *et al.* [16]; Sanburg *et al.* [17]; Ajami and Riddiford [18]; White [19]; Slade and Zibitt [20]). With only one exception [4] investigations have always been performed utilizing optically inactive (*i. e.* racemic) juvenile hormone preparations for determination of metabolic rates and protein characteristics, with no special mention of the product stereochemistry. It is, however, well established that the JH-specific binding protein of *Manduca sexta* shows different affinities for geometrical isomers of JH-I [1, 2, 6] as well as for the optical isomers of JH-III [4]. Furthermore, the protein discriminates between hormone homologs (Goodman *et al.* [21, 8]). Geometrical and optical isomers of juvenile hormones differ significantly in their morphogenetic activity as determined by the *Tenebrio* and *Galleria* bioassays, respectively (Dahm *et al.* [22]; Loew and Johnson [23]; Henrick *et al.* [24, 4]). It therefore appeared as a prerequisite for a more comprehensive understanding of data concerning juvenile hormone biochemistry and mode of action to investigate the interaction with proteins, especially focusing on the stereochemistry of the processes involved. As object for our studies we chose the adult male locust *Locusta migratoria* and report on the enantioselectivity of JH-binding and hydrolysis in hemolymph of this insect.

Results were taken in part from the Diploma Thesis of S. G., University of Bonn, 1979.

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## Materials and Methods

### *Chemicals*

All juvenile hormone (JH) preparations used were racemates: JH-I (≧ 96% E, E-*cis*) was from ECO-Chemical Intermediates; a mixture of stereoisomers,

containing approx. 10% of E, E-*cis*-JH-I was from Fluka; JH-III (60% E,E, 40% E,Z) was synthesized from a commercially available mixture of farnesol-stereoisomers by the application of oxidative procedures described by Corey *et al.* [25]. Chemical composition was determined by gas liquid chromatography in a Carlo Erba Fractovap GI gas chromatograph, equipped with 0.32 mm × 50 FFAP or SF 96 coated glass capillary columns and flame ionization detector; carrier gas was nitrogen. JH-I-acid and JH-III-acid were prepared by base catalyzed hydrolysis of stereoisomeric mixtures of JH-I (Fluka) and E,E/E,Z-JH-III, respectively.

E,E-*cis*-[7-ethyl-$^3$H]JH-I (13.5 Ci/mmol), E,E-*cis*-[10-$^3$H]JH-I (13.5 Ci/mmol), and E,E-[10-$^3$H]JH-III (11.5 Ci/mmol) were products of NEN-chemicals. A stereoisomeric mixture of [methoxy-$^3$H]JH-I (4.3 Ci/mmol) was obtained through the courtesy of Mr. K. H. Trautmann (Dr. Maag A.G., Dielsdorf, Switzerland). Other chemicals used were at least analytical grade quality.

*Liquid scintillation counting*

Aqueous samples were dissolved either in 2 ml of Unisolve 100 (Zinsser, Frankfurt) or in 4 ml of a cocktail made up from 830 ml dioxane, 170 ml ethyleneglycolmonoethylether, 50 g naphtalene, 10 g PPO and 0.5 g POPOP. Counting efficiency in unquenched samples was 43%. Other samples were counted in 10 ml of a solution of 6 g PPO and 75 mg POPOP in 1,000 ml toluene with an efficiency of 54%. Counting equipment were a BF 5000 and a BF 8020 (Berthold laboratories, Wildbad) using external standardization and automatic dpm calculation; calibration curves were obtained from samples similar in composition to the analytical probes. An accuracy of at least ± 6% (2 $\sigma$) was regarded sufficient for discontinous monitoring of chromatography eluates, otherwise samples were counted with a statistical error of less than ± 2% (2 $\sigma$) with frequent control of background and sample reproducibility.

*Collection of hemolymph*

*Locusta migratoria* were reared under crowded conditions as described previously (Gellissen *et al.* [26]). Hemolymph was collected by draining the blood of an insect from an incision in the neck membrane into a glass capillary, followed by immediate mixing with an equal volume of ice cold Tris-HCl buffer pH 7.2 or 8.2, containing 0.5 M sodium chloride. Hemocytes were removed by centrifugation at 10,000 × *g* and 4 °C for 15 min.

*Hemolymph protein fractionation*

Hemolymph samples (between 0.4 and 0.5 ml) were chromatographed on a 1.2 × 60 cm Sepharose 6-B (Pharmacia) column with 0.05 M Tris-HCl buffer pH 8.2 containing 0.5 M sodium chloride and 0.003 M sodium azide to avoid bacterial contamination. The eluate was monitored continuously at 280 nm. If applicable, radioactivity was measured discontinuously by counting 100 µl aliquots of 1.2 ml or 5 ml size fractions. Appropriate fractions containing high molecular weight material were combined and concentrated by ultrafiltration through collodium bags (Sartorius No. SM 13200; Göttingen) at 4 °C. Diglyceride carrier lipoprotein (DGCL) was separated by dialysis against deionized water for 50 h with frequent changes of the water. Precipitated material was removed by centrifugation at 15,000 × *g* and 4 °C for 20 min and the supernatant concentrated by ultrafiltration.

*Interaction of JH with hemolymph proteins*

Analytical determinations of esterase activity were performed with 1-naphtyl acetate by the method of Katzenellenbogen and Kafatos [27] and by the charcoal assay described by Sanburg *et al.* [17] using a stereoisomeric mixture of [methoxy-$^3$H]JH-I. JH-binding was determined by the modified charcoal assay described by Kramer *et al.* [8]. Preparative separation of JH associated with proteins (called "bound hormone" in the following) from unbound components (called "free hormone" in the following) was achieved by chromatography on Sepharose 6-B as described in the foregoing section. Inhibition of esterase activity was performed by exposure to $10^{-3}$ M diisopropylfluorophosphate (DFP) for 30 min at 22 °C prior to incubation with juvenile hormone. In a typical experiment 0.5 ml hemolymph from adult males and 150,000 dpm of tritium labelled JH were used. Recovery of radioactivity from Sepharose 6-B was generally 80–90%.

*Stereochemical analysis*

Appropriate radioactivity containing fractions from Sepharose 6-B chromatography were combined

and, after addition of 20–50 µg E,E-*cis*-JH-I extracted four times with 1–2 ml ethyl acetate. Resolution of the extract by TLC (0.25 mm silicagel PF-60 (Merck) on 10 × 20 cm glass plates) with benzene/5% ethyl acetate yielded a JH-fraction and a zone of polar metabolites remaining at the start, which were eluted with ethyl acetate. The JH-fraction was further diluted with 200 µg E,E-*cis*-JH-I and/or 200 µg E,E/E,Z-JH-III and resolved by a second TLC separation. The fraction containing polar metabolites was diluted with 200 µg of the JH-acids and treated with a diethylether solution with an excess diazomethane for 3 min at 22 °C followed by immediate TLC separation and further dilution with unlabelled E,E-*cis*-JH-I and/or E,E/E,Z-JH-III as above. Samples of JH thus obtained were converted into the corresponding diols by acid catalyzed hydration of the epoxide (Peter and Dahm, [28]). Acylation with 25 µl (+)-α-methoxy-α-trifluoromethylphenylacetic acid chloride ((+)-MTPA; Dale *et al.*[29]) in 100 µl pyridine, followed by evaporation and TLC (benzene/15% ethyl acetate) yielded the diastereomeric (+)-MTPA derivatives which were resolved by HPLC on a 0.4 × 31 cm µ-porasil column (Waters Assoc.) with *n*-hexane/8% ethyl acetate/0.05% 2-propanol. The derivative of the natural (10 R, 11 S)-enantiomer of JH-I and the (10 R)-enantiomer of JH-III elute faster in this system than the derivatives of the unnatural enantiomers (Nakanishi [30], Judy *et al.* [31], Jennings *et al.* [32]).

## Results

### *Hydrolysis of (7-ethyl-$^3$H)-JH-I and (10-$^3$H)-JH-III in hemolymph*

Hemolymph from male and female *Locusta* (for this experiment obtained through the courtesy of Dr. Gäde, University of Bonn) was diluted 1 : 1 with 0.05 M Tris-HCl buffer pH 7.2 and hemocytes removed as described above. 50 µl aliquots were incubated with 50 µl of a solution of radiolabelled JH-I ($1.03 \times 10^5$ dpm, initial concentration: $3 \times 10^{-8}$ M) in the same buffer and extracted after the time intervals given in Fig. 1. The relative amount of JH-I-acid formed was determined by counting aliquots of the remaining JH-I and the JH-I-acid after TLC-separation, using benzene/15% ethyl acetate/1% acetic acid as a solvent. From Fig. 1 it can be seen that after an incubation period of 120 min. 30 ± 5% of the recovered total radioactivity was present in the JH-I-acid, considering the observed difference between male and female hemolymph as not being significant. Stereochemical analysis following reconversion of the JH-I-acid to JH-I revealed that the metabolite generated after 120 min was derived to 78.5 ± 0.5% from the natural (10 R, 11 S)- and to only 21.5 ± 5% from the unnatural (10 S, 11 R)-enantiomer of JH-I regardless the sex of the animals (Table I, experiments 1 and 2).

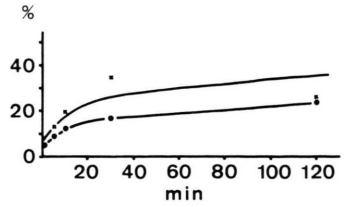


Fig. 1. Time dependence of JH-I-hydrolysis in *Locusta* male (×) and female (●) hemolymph. Percent values are expressed in relative amount of radioactivity present in JH-I-acid recovered from TLC.

When in a similar approach (Table I, experiments 3 and 4) equimolar amounts of JH-I and JH-III (initial concentrations: $1.1 \times 10^{-8}$ M of each hormone in experiment 3 and $0.7 \times 10^{-8}$ M in experiment 4) were subjected together to metabolism in cell-free male hemolymph, the relative amounts of unmetabolized JH-I and JH-III remaining after 120 min were 60% and 34% in experiment 3, respectively. The corresponding values in experiment 4 were 31% of the JH-I and 43% of the JH-III remaining unmetabolized. The amount of polar metabolites showed a linear increase over the reaction time rising up to 63% of the totally recovered radioactivity after 120 min incubation in experiment 4. Stereochemical analysis of the JH-acid from both experiments revealed that hydrolysis had occured preferentially on the natural (10 R, 11 S)-enantiomer of JH-I, reaching a maximum enantiomeric excess of 56.2% (78.1% (10 R, 11 S)-*versus* 21.9% (10 S, 11 R)-JH-I-acid) after 120 min. Most surprisingly, hydrolysis of the lower homologue JH-III followed a completely different pattern in the same incubation mixtures. The acids formed after 120 min were derived to 66.8% or to 74.0% from the unnatural (10 S)-enantiomer of JH-III. Generally, the enantioselectivity of hydrolysis was less pronounced after shorter incubation times when the substrates were still present at higher concentration.

Table I. Enantioselectivity of JH-hydrolysis in cell-free *Locusta* hemolymph.

| Expt. No. incubation time [min] | JH-I or JH-I-acid dpm | Configuration of JH-I or JH-I-acid | | JH-III or JH-III-acid dpm | Configuration of JH-III or JH-III-acid | |
|---|---|---|---|---|---|---|
| | | % 10R, 11S | % 10S, 11R | | % 10R | % 10S |
| 1 | | | | | | |
| ♀ (30) | | | | | | |
| JH | 94,400 | – | – | | | |
| JH-acid | 18,500 | 68.6 | 31.4 | | | |
| % JH-acid | 16.4 | | | | | |
| ♀ (120) | | | | | | |
| JH | 60,100 | – | – | | | |
| JH-acid | 19,100 | 78.1 | 21.9 | | | |
| % JH-acid | 24.1 | | | | | |
| 2 | | | | | | |
| ♂ (30) | | | | | | |
| JH | 62,600 | – | – | | | |
| JH-acid | (35,000) | 61.8 | 38.2 | | | |
| % JH-acid | (35.9) | | | | | |
| ♂ (120) | | | | | | |
| JH | 53,600 | – | – | | | |
| JH-acid | 18,800 | 78.8 | 21.2 | | | |
| % JH-acid | 26.0 | | | | | |
| 3 | | | | | | |
| ♂ (30) | | | | | | |
| JH | 101,000 | – | – | 91,200 | 53.2 | 46.8 |
| JH-acid | 161,400 | – | – | a | – | – |
| % JH-acid | 33.9 [a] | | | | | |
| ♂ (60) | | | | | | |
| JH | 226,000 | 32.0 | 68.0 | 173,600 | 58.5 | 41.5 |
| JH-acid | 274,200 | 74.0 | 26.0 | a | 34.6 | 65.4 |
| % JH-acid | 40.5 [a] | | | | | |
| ♂ (120) | | | | | | |
| JH | 181,600 | – | – | 102,100 | 73.6 | 26.4 |
| JH-acid | 317,300 | 76.7 | 23.3 | a | 33.2 | 66.8 |
| % JH-acid | 52.8 [a] | | | | | |
| 4 | | | | | | |
| ♂ (30) | | | | | | |
| JH | 84,960 | – | – | 101,900 | 53.4 | 46.6 |
| JH-acid | 71,580 | – | – | a | – | – |
| % JH-acid | 36.5 [a] | | | | | |
| ♂ (120) | | | | | | |
| JH | 39,100 | – | – | 53,800 | 64.6 | 35.4 |
| JH-acid | 156,100 | 78.1 | 21.9 | a | 26.0 | 74.0 |
| % JH-acid | 62.7 [a] | | | | | |

[a] Polar metabolites eluted together from the TLC-plate. Percentage of JH-acid is calculated from total activity present in both JH-zones plus polar metabolites.

Since *Locusta* hemolymph contains several proteins that are candidates for enantioselective interaction with juvenile hormones, it was indicated to study their effects separately as described in the following sections.

*Separation of hemolymph proteins*

The separation profile of male *Locusta* hemolymph that had been incubated with $2 \times 10^5$ dpm [7-ethyl-$^3$H]JH-I prior to gel filtration on Sepharose

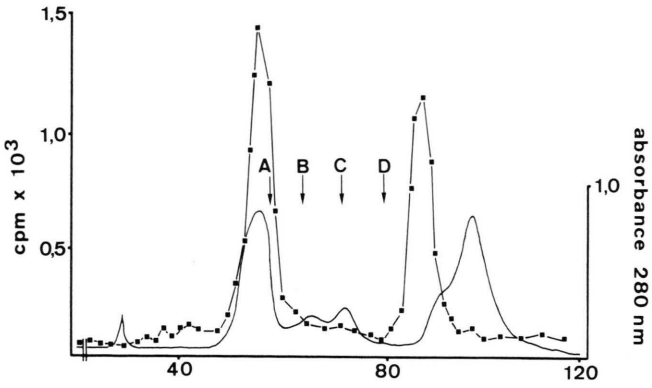


Fig. 2. Chromatography of 0.5 ml male *Locusta* hemolymph, containing $2 \times 10^5$ dpm [7-ethyl-$^3$H]JH-I on 1.2 × 60 cm Sepharose 6-B. Fraction size: 1.2 ml. ■ – ■: Radioactivity profile; ——: Optical density at 280 nm. Molecular weight markers: A: aldolase (140,000), B: bovine serum albumin (67,000), C: ovalbumin (45,000), D: cytochrome c (12,400).

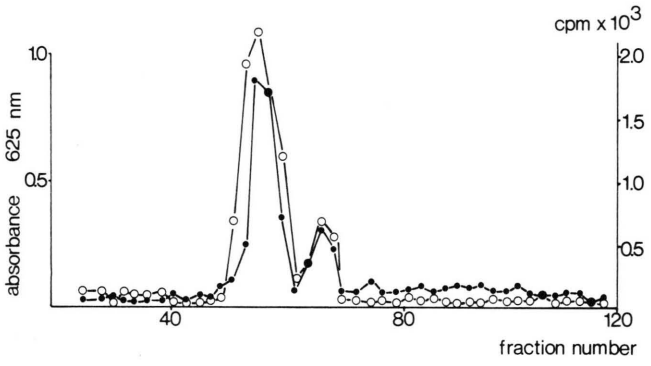


Fig. 3. Determination of esterase activity in chromatography of 0.5 ml male *Locusta* hemolymph on 1.2 × 60 cm Sepharose 6-B. Fraction size: 1.2 ml. ○ – ○ – ○: Activity towards 1-naphtyl acetate; ● – ● – ●: Activity towards [methoxy-$^3$H]JH-I (mixture of stereoisomers).

6-B is shown in Fig. 2. The major part of the radioactivity elutes together with the macromolecules, peak center in fraction 55, which contain the DGCL in addition to another large protein that possesses a high affinity but low capacity for racemic JH-I, and a population of high molecular weight esterases (MW 150,000). Another esterase population exists in *Locusta* hemolymph that elutes with peak center in fraction 67 (Fig. 3), indicating a molecular weight of approx. 50,000 Daltons. Both groups of esterases show esterolytic activity towards 1-naphtyl acetate and [methoxy-$^3$H]JH-I; both are readily inhibited by $10^{-3}$ M DFP.

*Stereochemical analysis of JH-I after Sepharose 6-B gel filtration*

*Experiment 5:* 150,000 dpm [$10^3$H]JH-I were incubated with 0.5 ml male hemolymph for 30 min at 4 °C and subsequently chromatographed on Sepharose 6-B as usual. The elution profiles shown in Fig. 4 A and 4 B are analogous to that in Fig. 3. Extraction of both bound and free hormone with ethyl acetate and removal of the polar metabolites by TLC was followed by stereochemical analysis (Table II, experiment 5). The result shows that 81.3% of the formerly bound hormone consisted out of the unnatural (10 S, 11 R)-JH-I, whereas the free hormone in fact is 74.1% (10 R, 11 S)-JH-I acid derived from the natural (10 R, 11 S)-enantiomer of JH-I.

*Experiment 6:* Fig. 4 C (black bars) shows the radioactivity profile after Sepharose 6-B gel filtration under otherwise identical conditions as in experiment 5 but inhibition of the esterases with $10^{-3}$ M DFP prior to incubation with [10-$^3$H]JH-I. Nearly all the radioactivity now eluted with the macromolecular fraction. Stereochemical analysis of the bound hormone revealed that both enantiomers were associated with the macromolecular material in equal amounts (Table II, experiment 6). The elution profile does not change qualitatively when the radiolabelled JH-I is diluted with a stereoisomeric mixture of unlabelled JH-I to a final concentration of $10^{-5}$ M in the incubation mixture (Fig. 4 C, white bars).

*Experiment 7:* A sample of male *Locusta* hemolymph was chromatographed on Sepharose 6-B with

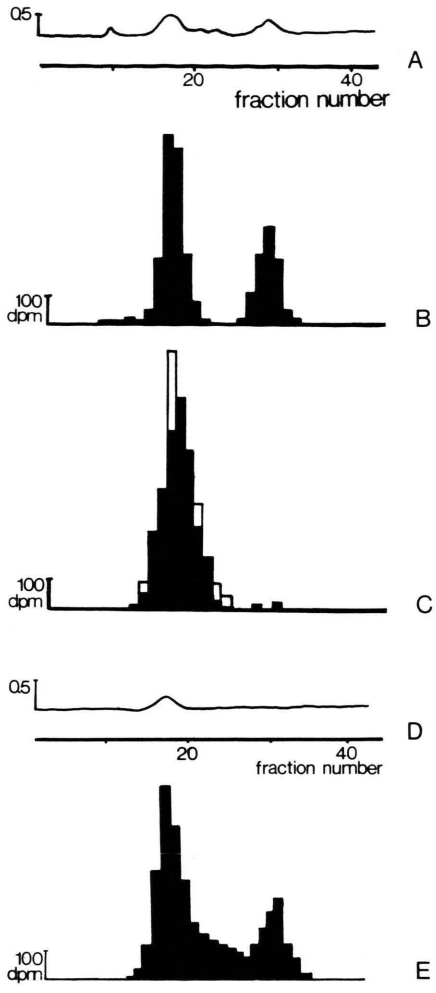


Fig. 4. Elution profiles of male *Locusta* hemolymph from experiments 5–7 on Sepharose 6-B. Fraction size: 5 ml. A: hemolymph incubated with [10-$^3$H]JH-I, optical density at 280 nm; B: Same as A, corresponding $^3$H-elution profile; C: Black bars: Esterase inhibition with DFP prior to incubation with [10-$^3$H]JH-I, radioactivity profile. White bars: Same conditions, except incubation with $10^{-5}$ M JH-I (stereoisometric mixture); D: Removal of DGCL prior to esterase inhibition and subsequent incubation with $10^{-8}$ M [10-$^3$H] JH-I, optical density at 280 nm; E: Same as D, corresponding radioactivity profile.

0.05 M Tris-HCl buffer pH 8.2, containing 0.5 M sodium chloride. The fractions containing high molecular weight proteins (*cf.* Fig. 2, fractions 51–60) were combined and subjected to dialysis. Removal of the precipitated DGCL by centrifugation left the JH-carrier protein and the esterases in the supernatant, which were concentrated by ultrafiltration and treated with $10^{-3}$ M DFP. Incubation with $2 \times 10^5$ dpm [10-$^3$H]JH-I was followed by a second chromatography on Sepharose 6-B as usual. Fig. 4 D and 4 E show the corresponding elution profiles. The results of the stereochemical analysis (Table II, experiment 7) showed that the bound hormone was 65.3% natural (10 R, 11 S)-enantiomer of JH-I. The free hormone contained a slight excess of the unnatural enantiomer. Analysis of the polar metabolites was not performed due to the low yield of JH-I upon treatment with diazomethane.

Table II. Stereochemical analysis of JH-I after interaction with different protein fractions from male *Locusta* hemolymph (n. a. means not analyzed).

| Experiment No. | Configuration of JH-I or JH-I-acid | |
|---|---|---|
| | % 10R, 11S | % 10S, 11R |
| 5, bound, JH | 18.7 | 81.3 |
| free, JH | n. a. | |
| JH-acid | 74.1 | 25.9 |
| 6, bound | 49.7 | 50.3 |
| free | n. a. | |
| 7, bound, JH | 65.3 | 34.7 |
| free, JH | 41.5 | 58.5 |
| JH-acid | n. a. | |

*Enantioselectivity of the JH-carrier protein: comparison of JH-I and JH-III*

The results of the stereochemical analysis of these experiments, the conditions of which were analogous to experiment 7 (*i.e.* removal of the DGCL prior to esterase inhibition and subsequent incubation with radiolabelled JH-I and/or JH-III, respectively) are documented in table 3. $2 \times 10^5$ dpm [10-$^3$H]JH-I were used for experiment 8 which is a duplicate of experiment 7. In each of the experiments 9 and 10 the analysis was performed with $2 \times 10^5$ dpm [10-$^3$H]JH-III. In experiment 11, a mixture of $1.0 \times 10^5$ dpm each [10-$^3$H]JH-I and [10-$^3$H]JH-III, and, finally, in experiment 12, $1.5 \times 10^5$ dpm of both hormones were used. The extracted bound and free hormones were first separated by TLC from polar degradation products. The JH was further resolved by a second TLC to yield JH-I and JH-III. In a few cases the polar metabolites were treated with diazomethane to yield generally small amounts of JH-I and JH-III which were resolved by TLC as usual. Table III clearly shows that there is a remarkable

Table III. Stereochemical analysis of JH-I and JH-III and corresponding JH-acids after interaction with JH-carrier protein from *Locusta* male hemolymph; radioactivity of JH derived from the acids is given after TLC resolution of the diazomethane treated polar metabolites.

| Experiment No. | JH-I [dpm] | JH-I configuration | | JH-III [dpm] | JH-III configuration | |
|---|---|---|---|---|---|---|
| | | % 10R, 11S | % 10S, 11R | | % 10R | % 10S |
| 8, *bound* JH | 109,400 | 54.8 | 45.2 | | | |
| JH from acid | 6,410 | 55.5 | 44.5 | | | |
| *free* JH | 8,380 | 35.9 | 64.1 | | | |
| JH from acid | 2,070 | – | – | | | |
| 9, *bound* JH | | | | 44,740 | 85.7 | 14.3 |
| JH from acid | | | | 5,000 | 58.8 | 41.2 |
| *free* JH | | | | 29,320 | 20.9 | 79.1 |
| JH from acid | | | | 2,300 | – | – |
| 10, *bound* JH | | | | 45,800 | 82.5 | 17.5 |
| JH from acid | | | | 2,530 | – | – |
| *free* JH | | | | 25,700 | 11.6 | 88.4 |
| JH from acid | | | | 2,070 | – | – |
| 11, *bound* JH | 87,600 | 55.1 | 44.9 | 78,780 | 78.6 | 21.4 |
| JH from acid | 17,000 | 56.9 | 43.1 | 7,700 | 33.8 | 66.2 |
| *free* JH | 16,000 | 35.6 | 64.4 | 28,400 | 12.7 | 87.3 |
| JH from acid | 2,730 | – | – | 3,290 | – | – |
| 12, *bound* JH | 93,600 | 47.6 | 52.4 | 116,100 | 77.4 | 22.6 |
| JH from acid | 3,580 | – | – | 1,190 | – | – |
| *free* JH | 12,440 | 51.2 | 48.8 | 34,400 | 8.9 | 91.1 |
| JH from acid | 3,700 | – | – | 2,160 | – | – |

difference in the enantioselectivity of the JH-carrier protein with respect to JH-I and JH-III. The bound JH-III contains up to 86% of the natural (10R)-enantiomer, whereas the corresponding values for bound JH-I indicate the presence of practically racemic material. As an example for the separation of diastereomers the elution profile of the (+)-MTPA derivatives obtained from JH-III in experiment 12 is shown in Fig. 5.

## Discussion

For determination of the enantioselectivity of processes involved in JH-metabolism in *Locusta* hemolymph we have applied the methodology of analyzing diastereomeric (+)-MTPA derivatives as described in refs. [30, 31, and 32]. Our interest focused on the enzyme catalyzed JH-hydrolysis and on the binding of JH by two proteins differing widely in their capacities and affinities for juvenile hormones. From the data presented here, it becomes evident that the proteins act with varying degrees of substrate specificity, discriminating not only between JH-homologues but also between JH-enantiomers. For the data concerning the specificity of JH hydrolysis in other insect species the reader is refered especially to the papers cited in the introduction [11, 12, 13, 15, 17] and the article of Nowock *et al.* [33].

The specific JH binding protein of *Manduca sexta* hemolymph has been purified and analyzed [12, 7] in detail, only for this protein the substrate specificity towards a number of JH analogues and homologues has been investigated [2, 4, 6, 8]. In summary, binding affinity decreases in the order JH-I > JH-II > JH-III and is highest for the natural geometrical isomer of JH-I. Enantiospecificity was studied with respect to JH-III, resulting in preferential binding of the natural (10R) optical isomer [4]. Data on the enantioselectivity of the JH-carrier from *Manduca* towards JH-I are not avalable presently, it might, however, possibly not be less for JH-I than for JH-III, if the specificity of the protein otherwise is taken into a speculative account.

In the first series of our experiments (Fig. 1 and Table I), we have studied the combined action of proteins mediating JH hydrolysis and binding. In cell-free adult locust hemolymph, JH hydrolysis occurs relatively slowly; even after two hours the amount of degraded JH does not exceed 70%. Sub-

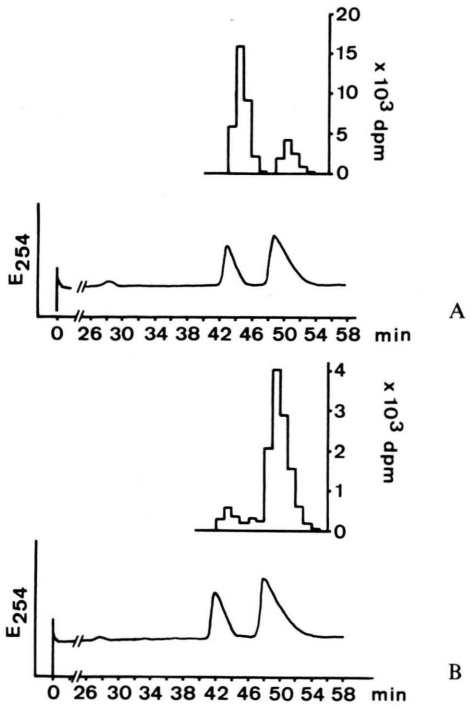


Fig. 5. Elution profiles of (+)-MTPA derivatives of JH-III from experiment 12 (Table III). A: Bound JH-III(+)-MTPA derivative; B: Free JH-III-(+)-MTPA derivative (the larger integral of the optical density in the (10 S)-derivative is caused by the presence of the 2 E, 6 Z-isomer in this elution volume).

strate saturation is at least given for the first 30–60 minutes, since the hydrolysis increases linearly during this period. Separation of the hemolymph components by molecular weight (Fig. 2) shows that the esterase activity is associated with two groups of proteins having molecular weights of $1.5 \times 10^5$ and $6.7 \times 10^4$, respectively, that both hydrolyze 1-naphtyl acetate and JH (Fig. 3), and that both are inhibited completely by $10^{-3}$ M DFP. Based on the criteria discussed above (see [11, 12, 13, 15, 17, 33]), namely slow reaction rate, hydrolytic activity toward 1-naphtyl acetate, and susceptibility to inhibition, both groups of proteins may be classified as "general esterases". They therefore should exhibit low specificity and be not effective in hydrolyzing JH bound to carrier protein. The results of stereochemical analysis (Table I) clearly show, that these criteria do not hold for the esterases of *Locusta* adult hemolymph.

When racemic JH-I is incubated with cell-free male or female hemolymph, the natural enantiomer is hydrolyzed with a high degree of preference, leading to JH-I-acid which contains 78.5% of the natural (10 R, 11 S)-enantiomer. A similar degree of enantioselectivity is observed when a sample of male hemolymph containing racemic JH-I is subjected to gel filtration (Fig. 4 A, B) in order to separate JH-I associated with macromolecular components from unbound hormone or its metabolites. The analysis (Table II, experiment 5) reveals that the unbound material in fact is JH-I-acid which, as expected, is separated from the binding proteins (Emmerich and Hartmann [34]). Its enantiomeric composition shows 74.0% of the naturally configurated (10 R, 11 S)-JH-I-acid, corresponding closely to that of JH-I-acid extracted after hydrolysis in hemolymph. 70% of the radioactivity present in the bound hormone fraction actually is unhydrolysed JH-I, which now consists to 81.3% of the unnatural enantiomer. The slight differences in the enantiomeric excess between bound JH-I and free JH-I-acid is understood by the fact that the macromolecular fraction contains esterase activity (Fig. 3) which preferentially hydrolyses the natural enantiomer of JH-I.

Completely different results were obtained from the stereochemical analysis of JH-III hydrolysis in cell-free hemolymph from adult male locusts (Table I). The enantiomeric composition of the JH-III-acid shows an excess of the unnaturally configurated (10 S)-enantiomer, indicating preferential hydrolysis of the unnatural (10 S)-JH-III which, in a strict sense, has to be regarded as a JH-analogue. These results may be explained by two different models. The first one would have to describe the interaction of a JH-carrier protein distinguished by a high affinity for the unnatural enantiomer of JH-I but the natural enantiomer of JH-III. A general esterase then may hydrolyse only those enantiomers of the hormones that are not protectively bound to the carrier protein. In the second model, the JH-carrier may have a low degree of enantioselectivity for JH-I which is hydrolysed by a highly enantioselective specific esterase. The same carrier protein in contrast binds selectively the natural enantiomer of JH-III, leaving the unnatural antipode free for hydrolysis by an esterase that has low enantioselectivity for JH-III.

By inhibiting the esterase with $10^{-3}$ M DFP it is possible to study the specificity of the binding components separately. When a sample of hemolymph is subjected to gel filtration after DFP treatment, practically the total amount of previously added JH-I

now migrates with the bound hormone fraction (Fig. 4 C, black bars). The racemic nature of this material is confirmed by the stereochemical analysis (Table II, experiment 6). This type of binding is caused by a high capacity – low specificity protein, identical with the DGCL (Gellissen [35], Gellissen and Emmerich [36, 37]). The elution pattern of radioactivity does not change even if, after esterase inhibition, the hemolymph is loaded with $10^{-5}$ M JH-I (mixture of stereoisomers) as shown in Fig. 4 C, white bars.

The presence of a JH-specific carrier protein becomes apparent after removal of the DGCL, achieved by dialysis of the protein fraction from gel chromatography. Inhibition of the esterases in the absence of the DGCL now leads to separation of added JH-I into fractions containing bound and free hormone, as shown in Fig. 4 D and 4 E. The results of the stereochemical analysis are given in Table II, experiment 7. The part of the radioactivity bound to the macromolecular fraction is associated with JH-I that has a significant excess of the (10 R, 11 S)-enantiomer, thus indicating a relatively low degree of enantioselectivity of the JH carrier protein for the natural isomer of JH-I. The small excess of the unnatural (10 S, 11 R)-JH-I present in the free hormone does not correspond exactly to the enantiomeric composition of the bound hormone. Fig. 4 E shows a relatively low resolution between bound and free hormone, indicative of a continous dissociation of bound JH-I from the protein during migration on the column. Even if the preference for the binding of (10 R, 11 S)-JH-I is low, a slightly faster dissociation of the unnatural enantiomer will result in a relatively larger part of naturally (10 R, 11 S)-configurated JH-I in the bound hormone fraction, whereas the enantiomeric composition of the free hormone more likely reflects that one in the original incubation mixture.

In marked contrast the enantioselectivity of the JH-carrier protein is much higher for JH-III than for JH-I. Table III shows that after interaction with JH-III the bound hormone contains up to 86% of the natural (10 R)-enantiomer. Qualitatively the same results are obtained after application of JH-I and JH-III as a mixture. The differences in the absolute values of corresponding enantiomeric compositions in bound and free hormones have to be explained by the interference with dissociation processes, incomplete removal of DGCL, residual esterase activity, and chemical decomposition of JH in the slightly alkaline aqueous buffer medium.

The results taken together indicate that the second model discussed above, describes the interaction of JH metabolizing proteins more accurately than the first one. Since the JH-carrier protein has a low degree of enantioselectivity for the natural (10 R, 11 S)-JH-I, the esterase must have a high degree of substrate specificity, leading to enantioselective hydrolysis of the natural antipode. On the other hand, since the JH-carrier protein has a high specificity for the natural enantiomer of JH-III resulting in protective binding, the esterase may attack preferentially only the unbound unnatural enantiomer.

The esterase of *Locusta* seems to differ in its properties from JH esterases described in other insects so far. It appears to recognize the configuration of the epoxide group when racemic JH-I is offered as a substrate, but it is not able to hydrolyse carrier bound (10 R)-JH-III effectively. Thus the enzyme shows characteristics of a general and also of a specific esterase. It will, however, be necessary to confirm the validity of this conclusion with a more rigorously purified protein preparation. The possibility that the observed overall pattern of JH hydrolysis reflects the action of a family of esterases differing individually in their specific properties, cannot be excluded presently. The only other known example for the substrate specificity of a protein involved in reaction at the carboxylic site of the JH basic skeleton, is the S-adenosyl-L-methionine: JH-acid methyl transferase from accessory sex glands of the silkmoth *Hyalophora cecropia:* This enzyme discriminates between homologous carbon skeletons and methylates only (10 R, 11 S)-JH-I-acid to the natural enantiomer of JH-I (Dahm *et al.* [38], Weirich, Culver and Röller, in preparation; Peter, Shirk, Dahm and Röller, in preparation; Peter *et al.* [39]).

Two proteins with different capacities and specificities for the binding of JH occur in *Locusta* hemolymph. The DGCL acts by unspecific lipophilic interactions with JH-I, resulting in high binding capacity but low specificity and a lack of enantioselectivity. Its poor properties with respect to the binding of JH evoke some doubts on its physiological function in connection with JH stability and transport *in vivo.* On the other hand, the JH-specific carrier protein has a high affinity but low capacity (Gellissen and Emmerich, in preparation). Similar two component systems have been described to be involved in JH transport and stability in other insects [7]. In contrast to the reported findings, the JH-specific carrier

of *Locusta* adults has a much higher molecular weight than that of lepidopterous larvae. The fact that the enantioselectivity of the JH-carrier protein in *Locusta* is much more expressed with JH-III than with JH-I was quite unexpected.

It opens the question for the physiological significance of protein specificity for the mode of action of juvenile hormones known presently (Dahm *et al.* [38]): in *Manduca sexta* immature stages the most abundant hormones are JH-I and JH-II, but in adult females, however, JH-III and JH-II are the main components (Peter *et al.* [40], Schooley *et al.* [41]). JH-I is the biologically most active hormone in *Manduca* as determined by the black mutant assay, differing from JH-III by a factor of about 200 (Fain and Riddiford, [42]). In *Locusta,* too, JH-I seems to be the most active morphogenetic compound (Roussell [43]). On the other hand, the closely related *Schistocerca gregaria,* which may be considered representative also for *Locusta,* contains JH-III as the major, if not only JH compound in immature and adult stages (Blight and Wenham [44]). It may cautiously be speculated that evolution of three different hormone structures was accompanied by evolution of proteins catalyzing not only biosynthesis but also metabolism with a high degree of specificity. It is hoped that a more detailed picture of insect hormone evolution will emerge from elucidation of their precise mechanism of metabolism, including substrate specificity with regard to homologous JH structures and their stereochemistry.

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