A SIMPLE AND SENSITIVE RADIOIMMUNOASSAY OF INSECT JUVENILE HORMONE USING AN IODINATED TRACER

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1. Introduction

Juvenile hormones $(JH)^+$ are acyclic sesquiterpenes involved in the growth and reproduction of insects. They are secreted by the 'corpus allatum', a small paired or fused gland located behind the brain. Ecdysone, a moulting hormone, initiates the moulting process and induces a larval molt with a high JH titer while the absence of JH is followed by an adult molt [1]. In the adult, JH is secreted again and acts as a gonadotropic hormone by stimulating vitellogenesis and activities of the accessory glands [2].

Bioassays [3] and physicochemical assays [4] have been developed to test for JH activity. The clear-cut success of the radioimmunological techniques should encourage the development of radioimmunoassay for juvenile hormone. The first tentative study using an homologous tritiated tracer did not permit a sensitive assay of JH_I [5].

We report the production of specific JH_I antibodies and the synthesis of iodinated tracer which allow a simple and sensitive determination of this hormone in biological fluids.

Abbreviations: $JH_{I, II, III}$ Juvenile hormone I or II or III; NHS N-hydroxysuccinimide; DCCI N,N'-dicyclohexylcarbo-diimide; HSA Human serum albumin; PEG Polyethylene glycol; TLC Thin-layer chromatography; PBS Phosphate buffer saline; BSA Bovine serum albumin; THF Tetrahydro-furan.

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2. Materials and methods

2.1. *Unlabelled compounds*

Structure and nomenclatures of natural JH hormones and their analogs are reported in table 1. Compounds (I) and (II, VIII, IX, X) were generous gifts of Roussel-Uclaf and Dr. Sehnal (Prague), respectively. Compound (III) was supplied by Calbiochem (USA).

2.2. *Radiolabelled compounds*

Tritiated hormone ($10\text{-}^3H\text{-}JH_I$) (I) at 13.5 Ci/mmol was purchased from New England Nuclear, and carrier free Na ^{125}I from the Radiochemical Center (Amersham).

2.3. *Chemicals and solvents*

N-hydroxysuccinimide (NHS), N,N'-dicyclohexyl-carbodiimide (DCCI), bovine gamma globulin (Cohn fraction II) and histamine were obtained from Sigma. Human serum albumin (HSA) was obtained from Behringwerke and polyethylene glycol (PEG) (6000) from Touzart et Matignon, France. All chemicals and solvents were used without further purification. Thin-layer chromatography (TLC) was performed on pre-coated silica gel plate (Merck) with fluorescent indicator.

2.4. *Apparatus*

Radiochromatograms and infrared spectra were performed on a Berthold scanner and a Perkin-Elmer 137 spectrometer, respectively.

Table 1
Structure and nomenclature of natural JH hormones (I-III) and their analogs (IV-X).
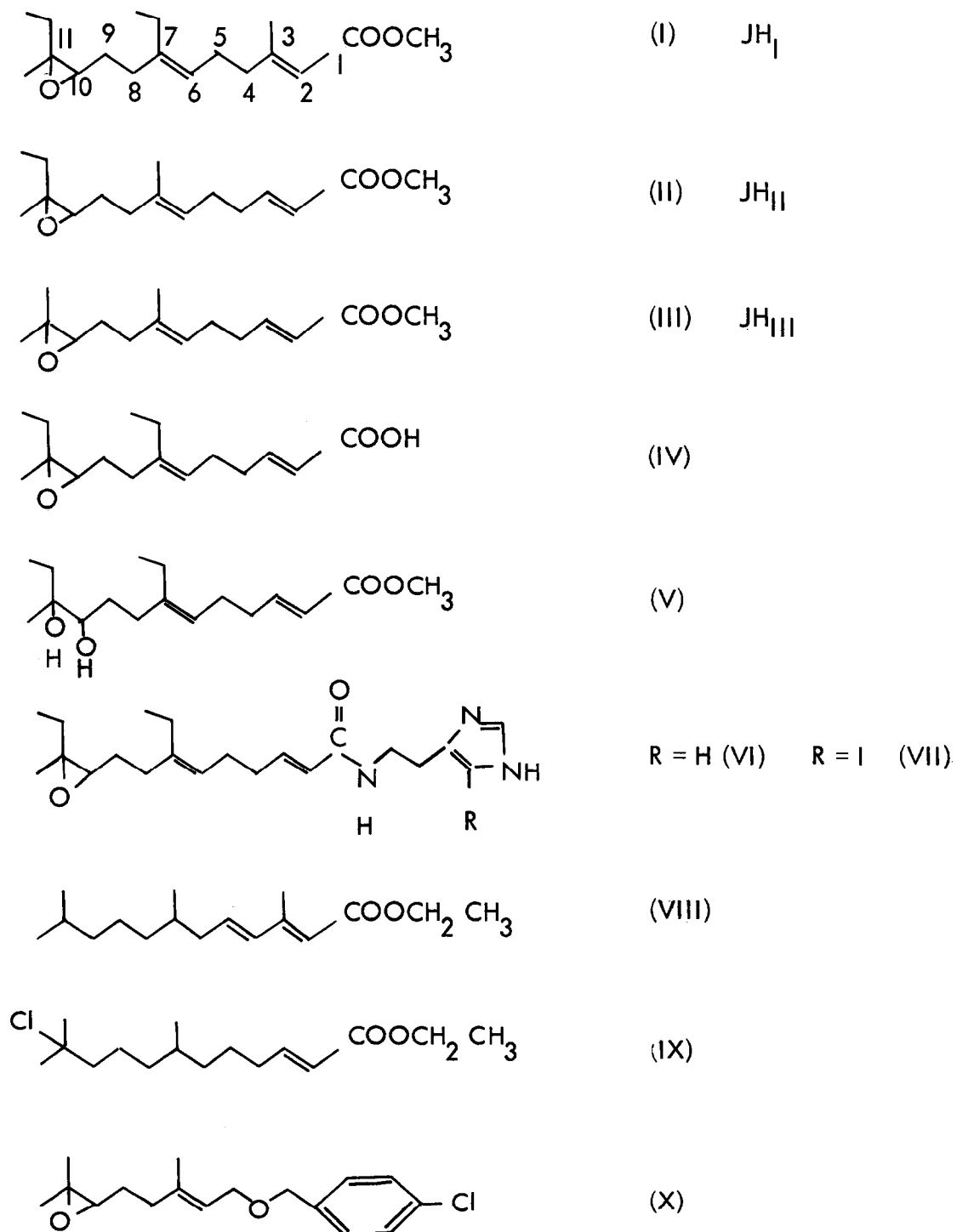
2.5. Alkaline and acid hydrolysis of JH_I for preparation of compounds (IV) and (V)

2.5.1. Preparation of compound (IV)

Juvenile hormone (I) (20 mg) dissolved in 0.25 ml of methanol was mixed with 15 μCi of tritiated hormone for isotopic dilution. Then 0.4 ml of methanol/NaOH (2 N) (v/v) was added. Kinetics of the ester hydrolysis were carried out by radiochromatograms of purified products on TLC in chloroform/methanol, 90 : 10 (v/v) solvent system. A new radioactive product was progressively formed (R_F = 0.65) whereas the JH_I spot (R_F = 0.97) disappeared. One day of reaction was necessary for complete hydrolysis. After a preparative TLC, the new radioactive product assumed to be the free acid derivative (IV) was extracted (10.45 mg) and stored at $-20°C$ until use.

2.5.2. Preparation of compound (V)

Preparation of the diol derivative was accomplished according to Broekhoven's procedure [4]. JH_I (1 μg) and ^3H-JH_I (0.1 μCi) were treated with 1 ml of THF/H_2SO_4, 0.125 N (3 : 2) for 4 h. The reaction mixture was purified on TLC in chloroform/acetone (9 : 1) solvent system. A new radioactive product (R_F = 0.87), compound (V), was isolated from unreacted JH_I (R_F = 0.96) with a yield of 86%.

2.6. N-hydroxysuccinimide ester preparation

Thirty-five μmol of JH_I acid (IV) in 500 μl of THF were mixed with 910 μmol of NHS and 525 μmol of DCCI at pH 4.5. After 18 h of reaction at room temperature the ester derivative was purified on TLC in ethyl acetate/hexane, 80 : 20 (R_F = 0.830), extracted with chloroform and evaporated to dryness. Twenty μmol of ester derivative (NHS-JH_I) was obtained and identified by infrared spectroscopy. Table 2 reports the principal absorbing bands of this product compared with those of JH_I, NHS and N-acetoxy NHS [6].

2.7. Immunogen preparation

2.7.1. JH_I-HSA coupling

Thirty mg of HSA were dissolved in 2 ml of THF/H_2O (10 : 8) and the pH adjusted to 9–10 with $NaHCO_3$. The solution was transferred into a glass tube containing 16.5 μmol of dry NHS-JH_I. The reaction mixture was left overnight at room temperature and the conjugate was dialysed against phosphate buffered saline (PBS), 0.05 M, pH 7.4 for one night. On the basis of isotopic dilution, the conjugate was estimated to contain 26 JH_I residues per molecule of albumin.

2.7.2. Immunization schedule

Five adult female white rabbits were immunized intradermally with 600 μg of the conjugate emulsified in complete Freund's adjuvant. At five week intervals, booster injections were given intradermally with the same amount of conjugate. Bleedings taken ten days after booster injection were tested for antibody binding parameters and the best one was used for the assay.

Table 2
IR absorbtion bands (cm^{-1}) of NHS-JH_I and their related compounds

Products	HO–N$<^{CO}$	–C=C–	–COOCH$_3$	R–N$<^{CO}$
NHS[a]	1709 (s)			
	1789 (s)			
N-acetoxy NHS[a]				1752 (s)[b]
				1779
				1802
				1832
JH_I		1655 (s)	1730 (s)	
NHS-JH_I		1650 (s)		1750 (s)
				1780 (m)
				1810 (w)

[a] Taken from Ames et al. [6].
[b] Acetate band.

2.8. *Iodinated tracer preparation*

2.8.1. JH$_I$-histamine (V) synthesis

JH$_I$ was linked to histamine by the following procedure: to 2 μmol of dry NHS-JH$_I$, 160 μl of THF/H$_2$O (v/v) pH 9–10, containing 5 μmol of histamine were added. The reaction was left to proceed for 16 h at room temperature. The reaction mixture was purified by TLC in chloroform/methanol 80 : 20 solvent system. A new Pauly positive and radio-active spot (R_F = 0.45) appeared, whereas unreacted histamine remained at the origin. This product was eluted with methanol (70%). On the basis of isotopic dilution the yield of coupling was 43%.

2.8.2. Preparation of radioactive ^{125}I-JH$_I$-histamine derivative (VII)

JH$_I$-histamine was labelled with ^{125}I using the chloramine T method [7]. Two μl (1.5 nmol) of JH$_I$-histamine derivative (VI) were evaporated to dryness. Successively 5 μl of PBS 0.5 M, pH 7.4, 2 μl of Na-^{125}I (320 μCi) and 4 μl of chloramine T (3 mg/ml in 0.05 M PBS, pH 7.4) were added. This mixture was stirred for 1 min and the reaction stopped by addition of 4 μl of sodium metabisulfite (15 mg/ml in 0.05 M PBS pH 7.4). After purification by TLC (chloroform/methanol/water 80 : 20 : 2), autoradiography and radioscanning, a labelled material was separated (R_F = 0.64) from unlabelled compound (VI) (R_f = 0.40) and free iodine (R_f = 0.03). The yield of iodination was estimated to be 30%. The radioactive material was extracted by methanol (70%) and stored at −20°C for further immunological tests.

2.9. *Standard radioimmunoassay procedure*

Phosphate buffer 0.1 M, pH 7.4, 0.1% BSA was routinely used to dilute the reagents of the assay. In polypropylene test tubes, standard hormone or buffer (0.1 ml), tritiated or iodinated tracer (0.1 ml) (17 000 dpm) and diluted antiserum (0.1 ml) were successively added. The mixture was stirred gently and incubated for 2 h at room temperature. A volume of 0.5 ml of polyethyleneglycol (50%) was added to each tube at 0°C followed by addition of 0.1 ml of gamma globulin solution (0.5% in PBS). The tubes were immediately centrifuged at 2200 × g for 20 min at 4°C. The supernatant or the precipitate was counted in β scintillation or gamma counter respectively.

2.10. *Scatchard analysis*

Scatchard analysis with tritiated and iodinated tracers, was performed using dextran-coated charcoal to separate bound from free as described previously [8]. One ml of dextran charcoal mixture (Norit A 2.5 g, Dextran T 70 0.25 mg in 100 ml buffer) was added to each tube. Calculations were performed according to Scatchard [9].

3. Results and discussion

3.1. *Binding parameters of tracers to antiserum*

The antiserum dilutions adjusted to obtain 40% binding (Bo) for the best bleed were 1/6000 and 1/120 000 for tritiated and iodinated tracers, respectively.

The complete separation of unlabelled product (VI) from iodinated derivative (VII) allowed us to approach the theoretical specific activity of the tracer ($\simeq$ 2000 Ci/mmol), since the excess of coupled substrate (1.5 nmol) related to the amount of iodide (0.16 nmol) did not allow the synthesis of diiodo-derivative. On this basis, association constants calculated from Scatchard analysis with tritiated and iodinated tracers were 1.8×10^9 M^{-1} and 1×10^{10} M^{-1}, respectively. The mean antibody concentration in undiluted anti-serum was estimated to be 6.5×10^{-6} M/litre.

3.2. *Sensitivity and specificity*

Figure 1 represents the standard curves obtained with tritiated or iodinated tracers and table 3 reports the cross reactivities of antiserum with different inhibitors using the iodinated tracer.

Only a small increase of sensitivity was obtained when the [^{125}I]JH$_I$-histamine derivative was used instead of [^{3}H]JH$_I$ tracer. This result could be expected since the iodinated tracer has a greater structural similarity to the immunogen, through the blocking of the carboxyl group in a peptidic linkage. In contrast the ester bond in the standard hormone seems less important in the recognition by antibody sites. So the association constant of iodinated tracer was found to be 5–6 times higher than that of tritiated hormone. As a confirmation, a 21-fold increase in sensitivity was obtained when the iodinated tracer was displaced by JH$_I$-histamine derivative (table 3) and a relatively high cross reaction was obtained with the JH$_I$ acid hormone.
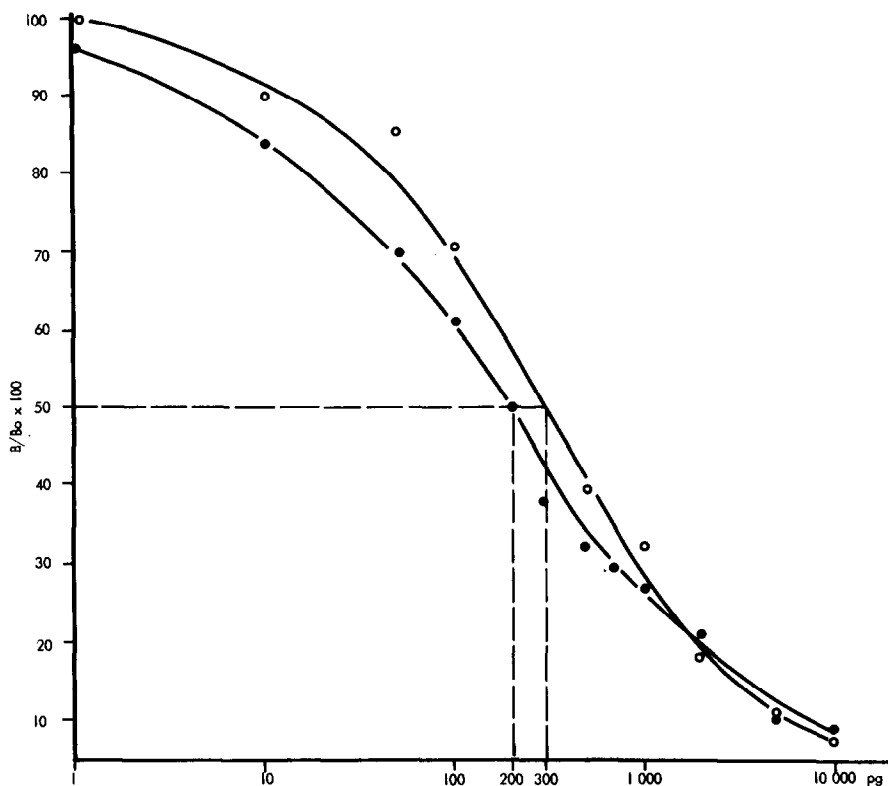
Fig.1. Inhibition of labelled JH_I binding to anti-JH_I in the presence of increasing amounts of unlabelled JH_I. (o——o) $[^3H]JH_I$. (●——●) $[^{125}I]JH_I$.

In vitro or in vivo transformation of the JH_I molecule linked to HSA into a diol derivative by

Table 3
Cross reactivities of anti JH_I antiserum with different inhibitors

Inhibitors	Crossing percentage[a]
(VI)	210
(I)	100
(IV)	50
(II)	37
(V)	14
(X)	0.8
(III)	0.4
(VIII)	0
(IX)	0

[a] Calculated on the basis of mass of inhibitor required to displace 50% of bound radioactivity.

chemical or enzymatic processes can explain the loss of recognition by the antibodies in the part of molecule (14% of cross reaction with inhibitor (V), since no OH band was detected in the infrared spectrum of NHS-JH_I product, we think that the possible transformation could occur during the conjugation process to HSA or in vivo, after injection of immunogen by an enzymatic degradation previously described in the mouse [10].

It is surprising to note the difference of cross reactions between JH_{II} and JH_{III}. Since JH_{III} which has two methyl groups in C_7 and C_{11} positions, gives 0.4% of cross reaction in comparison with 37% for JH_{II} which possess ethyl and methyl groups in the same positions, we assume that the hydrophobic area on C_{11} plays an important role in inducing the conformation which is involved in the interaction with the antibody sites.

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