Antibodies to Insect C_{16}-Juvenile Hormone

Since the discovery by WILLIAMS[1] in 1956 that male *Cecropia* moths were a rich source of the insect juvenile hormone (JH), 3 naturally occurring JH's have been characterized, i.e., the so called C_{18}-JH **1**[2], C_{17}-JH **2**[3], and C_{16}-JH **3**[4]. The JH's have attracted continuing interest, practical as well as academic, in view of the possible usage of JH mimics as pest growth regulators[5]. Research into the mode of action of JH would be greatly facilitated by rapid, sensitive quantification techniques more simple than those of bioassay[6] and gas-liquid chromatography[7], and we have sought to develop immunochemical methods for this purpose.

In the following we report the first successful preparation[8] of a JH antibody which binds specifically with the 3 naturally occurring JH's, distinguishing them from representative JH mimics with which binding occurs to a far lesser extent.

Methods and materials. The C_{16}-JH was coupled to human serum albumin (HSA) via the N-hydroxysuccinimide ester to make it immunogenic. The stability of the critical epoxide moiety during this process was ascertained by the chemical methods outlined below.

The N-hydroxysuccinimide ester was prepared by condensation of 45 mg of 10,11-epoxyfarnesic acid, obtained by hydrolysis of C_{16}-JH with 0.5 *N* NaOH in 50% aqueous MeOH, and 82 mg of N-hydroxysuccinimide in tetrahydrofuran (THF) containing 43 mg of dicyclohexylcarbodiimide, to give 20 mg of the active ester[9]. In order to check conditions for the linking of JH to protein, 21 mg of the JH N-hydroxysuccinimide ester was coupled to 25 mg of ε-aminocaproic acid in 75% aqueous THF at pH 8.5 (pH adjusted with 0.1 *N* NaOH) for 18 h at room temperature. The reaction proceeded cleanly, and the product, after methylation, was fully characterized as having structure **5b** by 100 MHz nuclear magnetic resonance and high-resolution mass spectroscopies.

Conjugation of 17 mg of C_{16}-JH N-hydroxysuccinimide ester to 60 mg of HSA was carried out in 50% aqueous THF at pH 9.5 giving a conjugate having 20 hormone molecules per molecule of protein as determined by spectrophotometric estimation of the unreacted lysine residues[10]. The conjugate was emulsified in complete Freund's adjuvant to give a final concentration of 1 mg/ml and injected once a week for 3 weeks into rabbit toepads (0.4 ml per rabbit per week). After an interval of 1 week, the rabbits were bled for 1 month, boosted, and bled again for a 2nd month. A pooled globulin fraction of the 2nd month's bleedings was used in this study.

The presence of antibody specific for the hormone was demonstrated by Ouchterlony gel diffusion[11], microprecipitin analysis[12] with a JH-rabbit serum albumin

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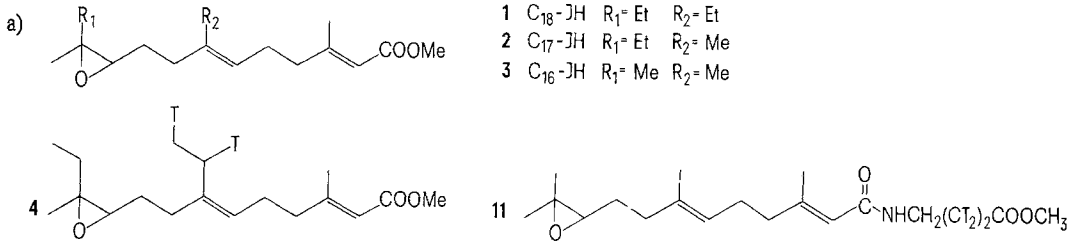
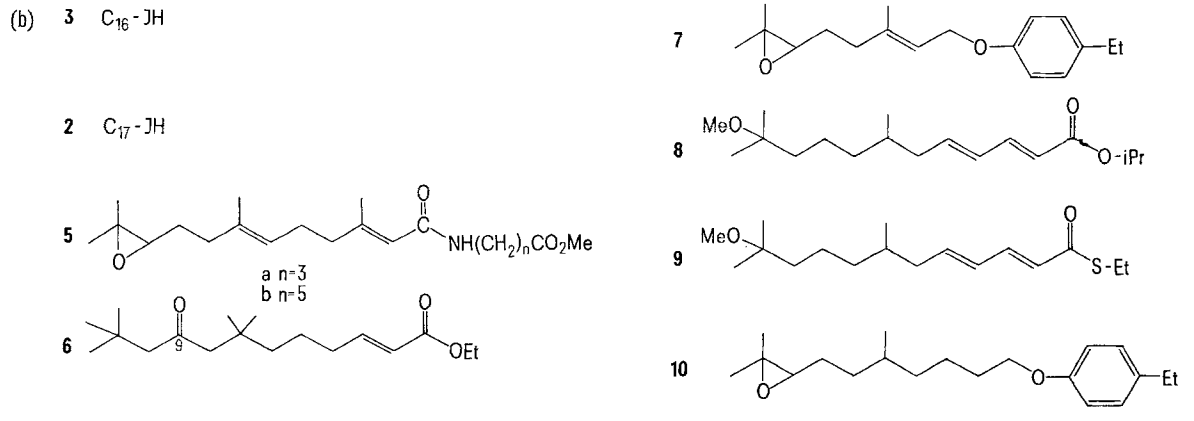
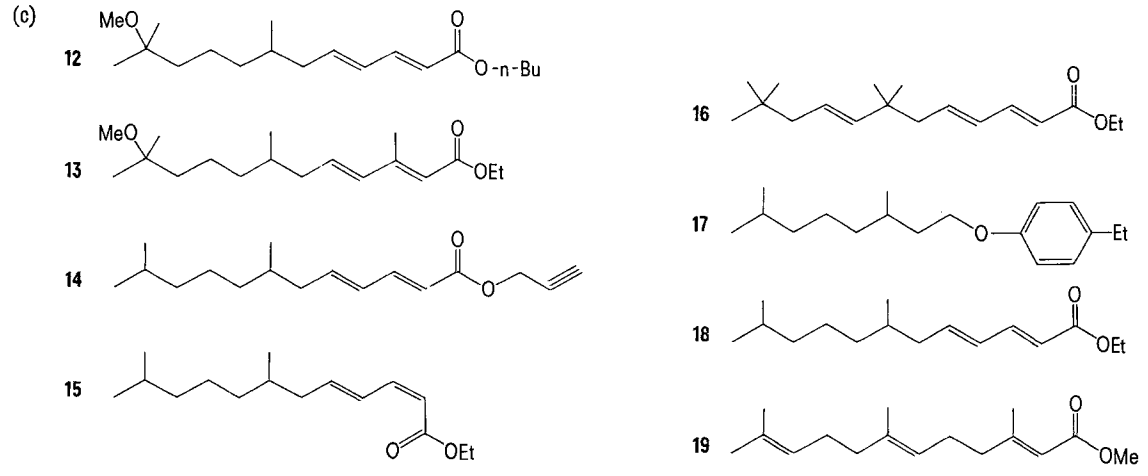
(a) The 3 naturally occurring juvenile hormones and radioligands employed



(b) Compounds (DL) which compete with C_{18} *Cecropia* Moth juvenile hormone for antibody



(c) Compounds (DL) which do not compete in the C_{18} and C_{16} systems



conjugate, and inhibition of precipitation in the presence of free hapten.

The specificity of the antibodies was studied by radioimmunoassay competition experiments[13] using commercially available C_{18}-JH[14] (14.1 Ci/mmole) and the radioactive C_{16} derivative **11**. This derivative was prepared by coupling the C_{16}-JH N-hydroxysuccinimide ester (1.2 mg) with tritiated γ-aminobutyric acid (250 μCi diluted to 1 Ci/m*M*) using the same conditions as for the previous coupling with ε-aminocaproic acid; the free carboxyl group was then esterified with diazomethane. The antibody hapten complex was precipitated with sheep anti-rabbit γ-globulin[13]. Figure b) and c) show the structures of the synthetic juvenile hormone analogs used in the competition experiments.

Results and discussion. Calculations based on microprecipitin analysis gave an antibody titer at equivalence of 1.8 mg/ml. The antibody when diluted 3-fold bound 45% of the counts, corresponding to 0.25 picomoles, of the radioactive C_{18} hormone, while a 6-fold dilution bound 45% of the C_{16} derivative **11**.

The results of competition experiments in which the radioactive C_{18}-JH was displaced with increasing molar concentrations of unlabeled C_{16}-JH and its analogs are shown in Table I. The only compounds that were highly active as competitors for the antibody combining site were derivatives of the C_{16} and C_{17}-JH's, i.e. compounds **2**, **3** and **5** (Figure). 50% inhibition occurred at concentrations between $1\text{–}5 \times 10^{-10}$ *M*. It was surprising to find that at all concentrations tested the C_{17} analog was a somewhat better competitor than the C_{16}, even though the C_{16} was the immunogen, presumably the structure for which the antibody was specific. It was concluded that by using the radioactive C_{18} ligand, we were selecting for a population of antibody molecules which had a greater affinity for the C_{18} and C_{17} compounds than for the C_{16} hormone. These results could be interpreted as an example of an antibody population more reactive with an alternative structure than with the immunizing hapten[15]. Subsequent experiments indicated that this was the case.

Table II shows the results of a typical radioimmunoassay competition experiment using labeled C_{16} hormone. Of all the compounds tested, only the C_{16} and C_{17} compounds showed significant activity. The C_{16}-JH was twice as active as the C_{17} analog in displacing 50% of the counts. Thus, in our earlier experiments with C_{18}-JH, we were indeed selecting for a small population of antibody molecules which had a higher affinity for C_{18}-JH than for the determinant group of the immunogen (C_{16}-JH). Assuming similar binding affinities, it could be calculated

Table I. Moles of hapten displacing 50% of ^3H-C_{18} *Cecropia* moth juvenile hormone bound to antibody

Compound[a]	Moles ($\times 10^{-10}$)[b]	Factor[c]
2	2.0	1.0
3	4.0	2.0
5a	15.0	7.5
6	115.0	57.5
7	182.0	91.0
8 (extrapolated)[d]	305.0	152.5
9 (extrapolated)[d]	365.0	182.5
10 (extrapolated)[d]	472.0	236.0

[a] See Figure for structures of various compounds. [b] The number of moles of hapten in 50 μl added to a tube containing 50 μl of radioactive hormone and 100 μl of antibody. [c] Ratio of concentration giving 50% displacement relative to that of best inhibitor. [d] 50% inhibition could not be reached with the available solutions since the amount of EtOH introduced would interfere with the assay. (Stock solutions were at 10^{-2} *M* in ethanol)

Table II. Moles of hapten displacing 50% of ^3H-C_{16} *Manduca sexta* juvenile hormone bound to antibody

Compound[a]	Moles ($\times 10^{-8}$)[b]	Factor[c]
3	2.5	1.0
5a	3.0	1.2
2	5.0	2.0

Footnotes as in Table I.

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that the antibody specific for the C_{18} compound constituted no more than 4% of the total population.

We have thus shown that the present JH antibodies are highly specific for the structure of the immunogen. High antibody specificity was demonstrated in the competition experiments, in that the only major competitors of either the radioactive C_{18}-JH or C_{16}-JH (derivative 11) were the C_{17} and C_{16} epoxy compounds. The antisera used in these experiments were of comparatively low binding affinities; however the affinity should increase with increasing time of immunization and this aspect is currently being investigated. With better sera and a C_{16} radioligand of higher specific activity, the sensitivity of the radioimmunoassay should increase well beyond that reported in this paper. It is obvious that these techniques will be useful in quantifying and screening for structurally related juvenile hormones in insects[16].

Résumé. Les auteurs ont produit des anticorps contre l'hormone juvenile du parasite du tabac *Manduca sexta* (C_{16} JH) par immunisation avec un conjugé haptène-protéine. Les antisérums ont montré une forte spécificité pour le C_{16} JH et le C_{17} JH et ils contiennent très vraisemblablement un groupe d'anticorps ayant une affinité plus grande pour le C_{17} JH que pour le C_{16} JH.

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Antibodies to the Insect Moulting Hormone β-Ecdysone

The moulting hormone of insects and crustaceae, β-ecdysone *1*, in conjunction with the juvenile hormone, regulates metamorphosis[1], and together with similar compounds is widely distributed in various insects, crustaceae and plant species[2]. A rapid and sensitive method of detection and quantification of β-ecdysone would be a valuable tool in further investigations concerning its physiological role. To this end we have sought to develop an immunochemical assay for β-ecdysone and here report the successful preparation of antibodies highly specific for β-ecdysone and capable of distinguishing it from closely related compounds.

Methods and materials. A hapten-protein conjugate of a hemisuccinate derivative of β-ecdysone with human serum albumin was used as the antigen. The hemisuccinate was prepared by treating 40 mg of β-ecdysone in 20 ml of tetrahydrofuran containing 25 µl of pyridine with 25 µl of succinyl chloride (freshly distilled) at 0 °C for 16 h. Excess succinyl chloride was decomposed with water and the material was purified by preparative layer chromatography on silica gel. The α, β-unsaturated carbonyl system showed a UV maximum at 242 nm (14,500) and NMR (d_5 pyridine, d_5 pyridine + conc. HCl) showed the product to be an equimolar mixture of 2, 3, 22-monohemisuccinates *2*.

Treatment of the hemisuccinates with N-hydroxysuccinimide and dicyclohexylcarbodiimide in tetrahydrofuran for 24 h at 4 °C followed by 48 h at room temperature yielded the N-hydroxysuccinimide ester[3] *3*.

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