

A simplified method for synthesizing juvenile hormone-protein conjugates

Walter G. Goodman

Department of Entomology, University of Wisconsin, Madison, WI 53706

Summary A convenient and simple method is presented for the synthesis of juvenile hormone-thyroglobulin conjugates. Methods previously described for this hapten linkage have used a two-step process in which a reactive intermediate was isolated and then used for conjugation. With the approach described here, an efficient, single-step conjugation reaction is effected between the carboxyl moiety of juvenile hormone III and the carrier protein, bovine thyroglobulin. Isolation of the reactive intermediate is eliminated. Isotopic dilution indicates that between 100 and 115 moles of hapten are conjugated per mole of carrier protein. — **Goodman, W. G.** A simplified method for synthesizing juvenile hormone-protein conjugates. *J. Lipid Res.* 1990. 31: 354-357.

Supplementary key words juvenile hormone III • insect hormones • radioimmunoassay • haptenic linkage • hapten conjugation

The juvenile hormones, a series of sesquiterpenoids, play a central role in the development and reproduction of insects. Despite extensive qualitative information about the hormones' biochemical actions, accumulation of quantitative data has been slow due to the lack of a convenient quantitative assay. Although RIAs are extensively used to quantify other insect hormones, radioimmunoassays for the JHs have been slower to be accepted (1). A major problem has been the development of the immunogen. Although JH has two sites readily available for covalent attachment, the epoxide moiety at C-10, 11 and the methyl ester at C-1, published procedures for linkage at either site (2, 3) are difficult for the nonchemist to perform. The attachment procedure described here makes hapten generation amenable to most biological laboratories. In addition, this synthesis is efficient and yields a highly substituted carrier protein.

EXPERIMENTAL PROCEDURE

Materials

JH III (racemic) was purchased from Sigma and repurified by HPLC (4). [³H]JH III (sp act 15.5 Ci/mmol) was purchased from NEN Research Products. THF (HPLC grade, inhibitor-free, stored under N₂), NHS, and DMAP were purchased from Aldrich. Water-soluble carbodiimide (EDAC) was obtained from Calbiochem while BTG (type 1) was purchased from Sigma. All other solvents were HPLC grade and were purchased from Burdick and Jackson Laboratories.

Procedures

JH III (10 mg radioinert plus 0.5 μCi radiolabeled) was converted to JH acid by base hydrolysis, purified, and quantified spectrophotometrically ($E = 11,840$, 211 nm in acetonitrile) (4). The newly synthesized (less than 48 h old) JH III acid (5 mg) was transferred to a round-bottom flask, the solvent was evaporated under a gentle stream of N₂, and the acid was then redissolved in 6 ml of THF (hereafter termed reagent A). EDAC (172 mg) was dissolved in 3.6 ml double-distilled water and NHS (11.5 mg) was dissolved in 5 ml THF. A portion (4.5 ml) of the NHS solution was added to the EDAC solution (hereafter termed reagent B). Reagent B (2.7 ml) was then added to reagent A and the solution was adjusted to pH 6.0 with HCl. The flask was capped, wrapped in aluminum foil to exclude light, and shaken gently for 6 h at 25°C. The product of this reaction was the JH-NHS intermediate.

Reagent C was composed of 6 mg of BTG dissolved in 8.7 ml double-distilled water. Reagent D was composed of 6 mg of DMAP dissolved in 0.5 ml of THF; it was added (0.3 ml) to reagent C and the pH was carefully brought to 9 with NaOH. The JH-NHS was then added to the BTG-DMAP solution slowly to avoid precipitating BTG. The reaction vessel was rewrapped in aluminum foil and incubated 18 h at 25°C. The product of this reaction was the conjugate.

To remove spent reactants, the conjugate was dialyzed for at least 72 h against several changes of double-distilled water adjusted to pH 8-8.5. At this point, samples from inside and outside the dialysis bag were radioassayed and the extent of conjugation was determined. Since JH readily interacts with hydrophobic domains, attachment was further confirmed by ethyl acetate extraction of a portion of the protein-hapten complex. The remaining conjugate was concentrated to 3 ml using a pressure ultrafiltration unit (Amicon, PM 30) and dispensed into microfuge tubes (100 μg/tube) for storage (-80°C).

TLC was used to monitor JH-NHS synthesis under various conditions. Samples were spotted on glass-backed silica gel G plates (E. Merck) and developed in chloroform-acetone 9:1 (v/v) (R_f JH acid = 0.24-0.28, JH-NHS = 0.35-0.39).

RESULTS AND DISCUSSION

The JHs are a family of at least six naturally occurring acyclic sesquiterpenoid hormones, but to date only JH III

Abbreviations: BTG, bovine thyroglobulin; DCC, dicyclohexylcarbodiimide; DMAP, 4-dimethylaminopyridine; EDAC [1-ethyl-3-(3'-dimethylaminopropyl)-carbodiimide HCl, HPLC, high performance liquid chromatography; JH, juvenile hormone; JH-NHS, ester of juvenile hormone III acid and N-hydroxysuccinimide; NHS, N-hydroxysuccinimide; RIA, radioimmunoassay; THF, tetrahydrofuran; TLC, thin-layer chromatography.

and JH III derivatives have been unequivocally identified in insect orders outside Lepidoptera (5, 6). Thus, considering its widespread distribution across the higher taxa, JH III is the hapten of choice. Attachment of the hormone to the carrier molecule through the C-1 position rather than the epoxide was chosen for several reasons. First, studies performed by Lauer et al. (3) demonstrated that an antibody with reasonably high specificity and affinity could be derived from an immunogen linked through the carboxyl moiety. Second, attachment via the epoxide group (2), although very useful in generating alternative haptens, is more difficult to perform, especially given the labile nature of the carboxyl ester function.

DCC alone can promote the covalent attachment of haptens to protein (7). However, Lauer et al. (3) noted that JH acid was unreactive under standard linkage conditions and introduced NHS to form the reactive intermediate, JH-NHS. The present study confirms the requirement for NHS, as less than 1% of the JH is attached using DCC in the absence of NHS. In contrast to previous studies (3, 8), NHS in conjunction with DCC leads to very low incorporation of the hapten (Fig. 1a). The presence of dicyclohexyl urea, a byproduct of the reaction, may inhibit JH-NHS synthesis under the present conditions. However, using both NHS and the water-soluble carbodiimide, EDAC, leads to a high yield of the JH-NHS intermediate (Fig. 1a).

Formation of JH-NHS is concentration- and time-dependent. Fig. 1b indicates that the reaction is essentially complete after 4 h. In addition, the reaction is pH-sensitive with optimal synthesis occurring between pH 5 and 6. To minimize acid-catalyzed epoxide hydration, the reaction is carried out at pH 6 (Fig. 1b). Table 1 indicates that the ratio between EDAC and NHS is important in the optimization of the reaction. Equimolar concentrations of EDAC and NHS lead to poor derivatization, while a ratio of 10:1, EDAC:NHS, appears best. The amount of JH acid present in the reaction mixture does not influence the rate of JH-NHS synthesis at pH 6.

Attachment of the JH-NHS derivative to the carrier protein must be carried out under conditions different

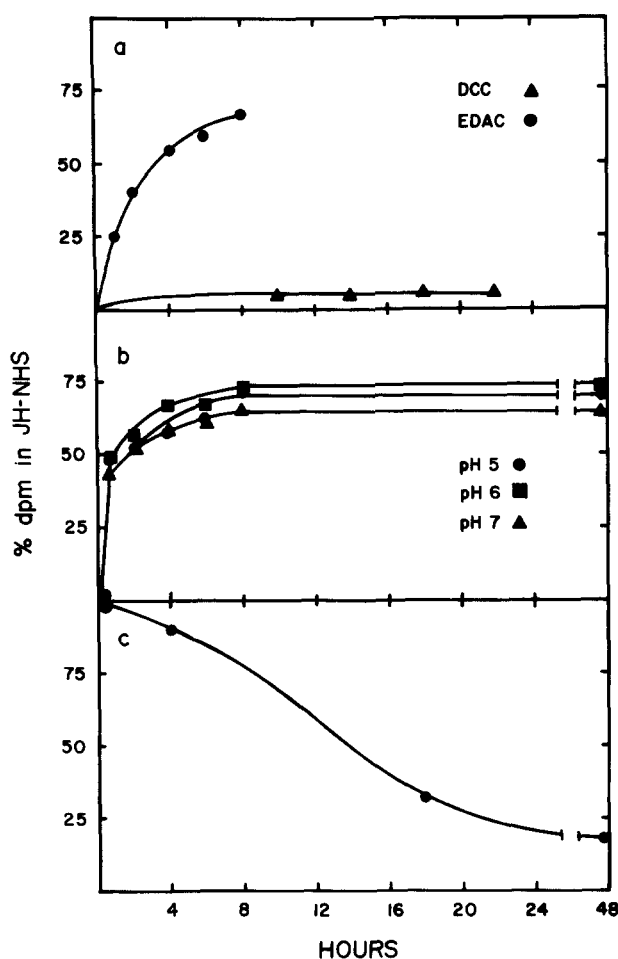


Fig. 1. Conditions for generating the JH-NHS intermediate. (a) Time course comparing the reactants DCC and EDAC in synthesis of JH-NHS. Reaction conditions and reactant concentrations were as described in Table 1, reaction 1. (b) Effect of pH on the synthesis of JH-NHS. Reaction conditions and reactant concentrations were as described in Fig. 1a using EDAC. (c) Rate of JH-NHS decomposition. JH-NHS was synthesized, then incubated under protein-hapten linkage conditions without BTG.

than those for its synthesis. Lauer et al. (3) purified the derivative before introducing the carrier molecule. This study demonstrates that hapten linkage does not require purification of the intermediate. Sequential addition of

TABLE 1. Conditions for JH-NHS synthesis

Reactants ^a	Reaction 1	Reaction 2	Reaction 3	Reaction 4
	<i>μmol</i>			
EDAC	60.0	60.0	600.0	600.0
NHS	6.0	60.0	6.0	60.0
JH acid	0.4	0.4	0.4	0.4
% Incorporation into JH-NHS ^b	54	29	43	63

^aJH acid was composed of radiolabeled and unlabeled hormone. Reactants were dissolved in the appropriate solvents and incubated for 6 h at room temperature. Samples of the reaction mixture were developed on TLC, then radioassayed. The area corresponding to JH-NHS was counted and expressed as a percentage of the counts from the entire plate.

^bAverage of two determinations.

TABLE 2. Conditions for JH-NHS covalent attachment to BTG

Procedures ^a	nmol Attached to BTG ^b
1. Incubate EDAC, NHS, and JH acid for 6 h at pH 6. Add BTG and DMAP and adjust pH to 6. Incubate 42 h at 25°C.	1.27
2. Incubate EDAC, NHS, and JH acid for 6 h at pH 6. Add BTG and DMAP and adjust pH to 6. Incubate for 1 h then bring pH to 9. Incubate 41 h at 25°C.	5.40
3. Incubate EDAC, NHS, and JH acid for 6 h at pH 6. Add BTG and DMAP and do not adjust pH (final pH = 7.6). Incubate 42 h at 25°C.	5.08

^aReactant proportions were identical to those in Table 1; however, concentrations were 10-fold less.

^bAverage of two determinations. Upon completion of incubation, incorporation was determined by exhaustively dialyzing the reaction mixtures, then radioassaying samples from inside and outside the dialysis bag. Counts outside the bag were considered noncovalently attached and subtracted from counts inside the bag to obtain the amount of hormone covalently attached.

the reactants, coupled with a shift in pH, effects a highly efficient attachment. Reaction conditions outlined in **Table 2** indicate that maintaining the pH at 6 during the coupling reaction leads to very low incorporation, while elevating the pH to 9 increases the incorporation fourfold. Addition of BTG and DMAP without pH adjustment results in a final pH of 7.6 in the reaction vessel and supports the hypothesis that higher pH favors the coupling reaction. In preliminary studies, the pH was adjusted 1 h after mixing JH-NHS with the protein carrier (Table 2); however, recent studies indicate that adjustment of pH at the time of addition of JH-NHS to the carrier protein is equally effective, thus avoiding further manipulation. Assuming epsilon amino group modification of lysine residues, attachment conditions at pH 9 suggest that the reaction favors the dissociated ammonium group (7). However, adjusting the pH to 11 results in no incorporation (data not shown).

In further modification of the original synthesis (3), DMAP was used. The addition of this commonly used catalyst nearly doubles the amount of JH-NHS covalently attached to the protein. In determining the optimal incubation period for attachment, the reactive intermediate, JH-NHS, was incubated under hapten-protein linkage conditions (Fig. 1c). The instability of the intermediate at pH 9 argues against significant incorporation when the incubation exceeds 18 h.

The use of BTG as a carrier protein was prompted by the report that antisera raised to a homologous series of prostaglandins with BTG as the carrier displayed greater specificity than antisera generated with serum albumin as the carrier (9). This point was not confirmed for the present study. The number of available attachment sites (lysine and arginine) per mole of BTG is reported to be approximately 500 (10); however, at pH 9, only the lysine residues, numbering between 125 and 177 (10), would be

reactive. It is unclear how many of these residues are actually available under the reaction conditions described here; nevertheless, incorporation studies indicate that between 100 and 115 moles of JH-NHS are attached per mole of BTG. A molar ratio of 5 JH-NHS: 1 lysine is sufficient for generating this relatively high level of incorporation. Although decreasing the amount of BTG while holding the JH-NHS concentration constant will further increase the level of incorporation (data not shown), studies with bovine serum albumin suggest that very high levels of hapten conjugation are not desirable as they exhibit poor antigenicity (11).

The integrity of the epoxide group after covalent attachment could not be directly determined. Nevertheless, the antiserum subsequently generated from the conjugate displayed no cross-reactivity with JH diol, indicating that the epoxide remained intact during the attachment reaction (12).

The conjugation reaction offers several attractive features. First is its simplicity and relatively high yield. Second, the conditions are sufficiently mild to avoid unwanted modification of the hapten. This is a particularly important point considering the lability of the epoxide moiety and double bonds. The JH-BTG conjugate developed by this method has now been demonstrated to generate a specific, high affinity polyclonal antiserum against the JHs (12). Moreover, the procedure may provide a general method of conjugation for other haptens with carboxyl moieties. For example, biliverdins (A. Jones, personal communication) and other JH acid homologs (N. Granger, personal communication) have been covalently attached to BTG using this method. ■

This work was supported in part by grants from the NIH (GM 34257) and the College of Agricultural and Life Sciences, University of Wisconsin-Madison. I thank Drs. N. Granger and K. Ritter

for their useful comments and suggestions on the manuscript and Ms. Hedda Goodman for her editorial assistance.

Manuscript received 4 August 1989 and in revised form 10 November 1989.

REFERENCES

1. Granger, N. A., and W. G. Goodman. 1988. Radioimmunoassays: juvenile hormones. In *Immunological Techniques in Insect Biology*. L. I. Gilbert, and T. A. Miller, editors. Springer-Verlag, New York. 215-251.
2. Strambi, C., A. Strambi, M. L. De Reggi, M. H. Hirn, and M. A. DeLagge. 1981. Radioimmunoassay of insect juvenile hormones and of their diol derivatives. *Eur. J. Biochem.* **118**: 401-406.
3. Lauer, R. C., P. H. Solomon, K. Nakanishi, and B. F. Erlanger. 1974. Antibodies to insect C16-juvenile hormone. *Experientia*. **30**: 558-560.
4. Goodman, W. G., and B. Adams. 1984. Semipreparative synthesis and purification of juvenile hormone acids by high-performance liquid chromatography. *J. Chromatogr.* **294**: 447-451.
5. Schooley, D. A., F. C. Baker, L. W. Tsai, C. A. Miller, and G. C. Jamieson. 1984. Juvenile hormones 0, I, II exist only in Lepidoptera. In *Biosynthesis, Metabolism and Mode of Action of Invertebrate Hormones*. J. Hoffman, and M. Porchet, editors. Springer-Verlag, Heidelberg. 373-383.
6. Richard, D. S., S. W. Applebaum, T. J. Sliter, F. C. Baker, D. A. Schooley, C. C. Reuter, V. C. Henrich, and L. I. Gilbert. 1989. Juvenile hormone bisepoxide biosynthesis in vitro by the ring gland of *Drosophila melanogaster*—a putative juvenile hormone in the higher diptera. *Proc. Natl. Acad. Sci. USA.* **86**: 1421-1425.
7. Erlanger, B. F. 1980. The preparation of antigenic hapten-carrier conjugates: a survey. *Methods Enzymol.* **70**: 85-104.
8. Baehner, J.-C., P. Pradelles, C. Lebreux, P. Cassier, and F. Dray. 1976. A simple and sensitive radioimmunoassay of insect juvenile hormone using an iodinated tracer. *FEBS Lett.* **69**: 123-128.
9. Raz, A., M. Schwartzmann, R. Kenig-Wakshal, and E. Perl. 1975. The specificity of antisera to conjugates of prostaglandins E with bovine serum albumin and thyroglobulin. *Eur. J. Biochem.* **53**: 145-149.
10. Skowsky, W. R., and D. A. Fisher. 1972. The use of thyroglobulin to induce antigenicity to small molecules. *J. Lab. Clin. Med.* **80**: 134-144.
11. Carraway, R. E. 1979. Neurotensin and related substances. In *Methods of Hormone Radioimmunoassay*. B. M. Jaffe and H. R. Behrman, editors. Academic Press, New York. 139-169.
12. Goodman, W. G., D. C. Coy, F. C. Baker, L. Xu, and Y. C. Toong. 1990. Development and application of a radioimmunoassay for the juvenile hormones. *Insect Biochem.* In press.