ide and the ester functions on the hormone for strong interaction.[2,7] The geometry of the double bonds and the stereochemistry of the epoxide function are also important determinants of specific binding.[7,8] The carrier protein–hormone complex is stable and can be subjected to gel filtration chromatography and gel electrophoresis under native conditions.[2,4,6]

Stability. The carrier protein is not stable under prolonged storage at −20 or 4° for periods longer than 1 month. The most satisfactory procedure is storage at 4° in 5 m*M* Tris–HCl, pH 8.3.

Acknowledgments

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[7] R. C. Peterson, M. R. Reich, P. E. Dunn, J. H. Law, and J. A. Katzenellenbogen, *Biochemistry* **16,** 2305 (1977).

[8] D. A. Schooley, B. J. Bergot, W. Goodman, and L. I. Gilbert, *Biochem. Biophys. Res. Commun.* **81,** 743 (1978).

[32] Analysis of Juvenile Hormone Esterase Activity

By BRUCE D. HAMMOCK and RICHARD M. ROE

Background

The terpenoid juvenile hormones (JHs) of insects control a variety of functions including metamorphosis and reproduction, and insects appear unique in having such terpenes as hormones.[1] The hormones identified to date are homologs and/or isomers of the 10,11-epoxide of methyl farnesoate. Thus, there are many possible sites of metabolic attack with hydration of the 10,11-epoxide and hydrolysis of the conjugated methyl ester representing hydrolytic pathways.[2] In several insect species it appears that degradation in addition to biosynthesis serve to regulate hormone titer.[3,4] For this and other reasons it is important to monitor metabolism of

[1] C. A. D. de Kort and N. A. Granger, *Annu. Rev. Entomol.* **26,** 1 (1981).

[2] M. Slade and C. H. Zibitt, *in* "Insect Juvenile Hormones: Chemistry and Action" (J. J. Menn and M. Beroza, eds.), p. 155. Academic Press, New York, 1972.

[3] B. D. Hammock and G. B. Quistad, *Prog. Pestic. Biochem.* **1,** 1 (1981).

[4] B. D. Hammock, *in* "Comprehensive Insect Physiology, Biochemistry and Pharmacology" (G. A. Kerkut and L. I. Gilbert, eds.), Vol. 7, Chapter 13. Pergamon, Oxford, 1984 (in press).

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the JHs. This chapter will emphasize methods for monitoring the ester cleavage of the juvenile hormones.

Three of the known juvenile hormones are commercially available. Both the labeled and unlabeled materials have a high degree of isomeric purity *(E, E, cis)*, but are racemic at C-10 or C-10 and C-11. The radiolabeled materials have a tritium at C-10 introduced by reductive tritiation of a haloketone followed by separation of the erythro and threo compounds and base catalyzed cyclization to the epoxide. There are several published methods leading to radiolabeled hormones,[3–5] and of note are biosynthetic methods yielding optically active hormones. Assay methods involving determination of radioactive methanol produced from JH methyl esters have been discussed elsewhere.[3,4] A caution is that radioactive methanol is readily lost during the assay procedure, even from plastic scintilliation vials.

Metabolite Identification

In performing the esterase assays, it is important to confirm that ester hydrolysis is the only pathway of importance in the assay used. Confirmation of metabolite identity by spectral means is seldom warranted. Tentative identification can be provided by a chromatographic method (thin-layer or high-performance liquid chromatography, TLC or HPLC) with careful attention to recovery of radioactivity and precise cochromatography with authentic standards. TLC is adequate to separate the major metabolites of JH from most organisms (JH, JH acid, JH diol, JH diol acid, polar conjugates),[2,6] but there has been some debate regarding the relative positions of JH acid and diol on TLC and HPLC. Since the acid can exist in a protonated or anion form, the developing solvent, the type or lot of TLC plate used, and even the method of extraction used can cause JH acid to change its position relative to the diol. Since the epoxide of JH is very stable to acid, a trace of acetic acid added before extraction will improve the extraction efficiency of acidic metabolites and reduce trailing on TLC. There are a variety of procedures to lend support to the structural assignment of JH acid by simple chromatography. If the R_f values of the metabolite and standard are significantly reduced on a plate predeveloped with 5% triethylamine in ether, or on a plate developed in the usual solvent with a trace of ammonium hydroxide added, the metabo-

[5] R. C. Jennings and A. F. Hamnett, *in* "Juvenile Hormone Biochemistry" (G. E. Pratt and G. T. Brooks, eds.), p. 375. Elsevier/North-Holland Biomedical Press, Amsterdam, 1981.

[6] B. D. Hammock, J. Nowock, W. Goodman, V. Stamoudis, and L. I. Gilbert, *Mol. Cell. Endocrinol.* **3**, 167 (1975).

lite is likely to be an acid. On a 10×10-cm TLC plate one can develop the plate in the first dimension with a solvent such as hexane : ethyl acetate (2 : 1), then in the second dimension with the same solvent containing a few drops of ammonium hydroxide after exposing the plate to ammonia vapors. JH acid should show a tight spot of $R_f \sim 0.4$ in the first dimension but a low R_f and possible a trailing spot in the second dimension. JH acid can be reesterified with diazomethane or ethane,[7] and it should fail to react with *n*-butylboronic acid (a few drops of a 0.1% solution in ethyl acetate added just before spotting), while JH diol does react, and the resulting adduct has a high R_f.

Thus to evaluate the structure of a metabolite suspected to be JH acid, incubate the enzyme system (0.1–1 ml) with the substrate concentration to be used in the routine assay, using ~0.01–1 μCi of ^3H per incubation, terminate the reaction by adding sodium chloride to saturation and authentic standards, then extract three times with equal volumes of peroxide-free ethyl ether. Alternatively, ether : ethanol or ethyl acetate can be used to remove more polar materials and a drop of acetic acid can be added if the acid spot trails on TLC. Following drying over anhydrous sodium sulfate, the solvent is removed under a blanket of nitrogen and the residue spotted as a narrow horizontal band 2 cm wide, 1.5 cm from the bottom, and 1.5 cm from the left-hand side of a silica gel TLC (such as Brinkman Silica Gel 60, F_{254}, 250 μm). The plate is then developed in hexane : ethyl acetate (2 : 1). If a large amount of material is present, the solvent may be passed repeatedly through the origin and developed to a distance of ~5 cm, or a more polar solvent such as ethyl acetate or methanol may be used to develop the plate just past the origin. If the presence of lipids causes diffuse spots, the plate then may be developed 20 cm in toluene before using the hexane : ethyl acetate system. After developing in the first dimension the plate is rotated 90° and developed in the second dimension. A polar solvent such as toluene : propanol (4 : 1) is used to bring the original band into a tight spot by developing 5 cm. Then the plate is developed in toluene : propanol (10 : 1) for 20 cm.[6]

If oxidative processes are expected a nonpolar solvent such as hexane : ether (10 : 1) or petroleum ether : ether (5 : 1) can be used to separate JH from the corresponding diepoxide and triene. The radioactive materials can then be qualitatively evaluated by fluorography and the appropriate spots to be scrapped can be identified by placing the developed film under the TLC plate over a light box.[8] For subsequent assays, one-dimensional development normally can be used.

[7] H. M. Fales, T. M. Jaouni, and J. F. Babashak, *Anal. Chem.* **45,** 2302 (1973).
[8] K. Randerath, *Anal. Biochem.* **34,** 188 (1970).

Selection of Analytical Method

Once JH acid is established as the sole or major metabolite under the conditions to be routinely evaluated, many analytical methods can be employed. A convenient, continuous assay using model substrates offers many advantages, but the numerous controls which must be run to ensure that the same esterases which metabolize JH are metabolizing the model substrate dictate that the radioactive, natural JHs offer the most unambiguous methods. One can monitor metabolism by gas–liquid chromatography using either flame ionization detection or electron capture, by utilizing the limited ability of the conjugated ester to capture electrons. HPLC can be employed using the weak chromophore of JH ($\lambda_{max} \cong 217$ nm, $\varepsilon \cong 14770$)[9] or radiochemical detection.[3,4] By far the two most rapid techniques yet developed are the partition and TLC methods described below.

The TLC method offers the advantage of having a lower background, requiring less radioactivity, allowing one to monitor several metabolic reactions on a single plate and to scrape the plates for liquid scintillation counting (LSC) at leisure. With improvements in TLC scanners, this method could replace the partition method in speed and economy, but at the present time it is more laborious and expensive and the partition method is suggested.

The assays suggested were optimized for the esterase activities for *Manduca sexta* and *Trichoplusia ni*. The conditions of assay should be adjusted for the experiment in hand. Partition methods can also be adjusted to optimize conditions for higher speed, lower cost, lower background, etc. usually at the expense of another factor.

The Partition Assay Method

Principle. Esterase activity is assayed by the conversion of radiolabeled JH (^{3}H at C-10) to the JH acid and subsequent partitioning of the substrate and product by simple solvent extraction. Under the conditions of the assay >99% of the radiolabeled JH is extracted into a hydrocarbon phase while >99% of the radiolabeled JH acid remains in a basic aqueous–methanol phase.[10,11]

Enzyme Preparation. Insect whole hemolymph is collected and diluted for assay immediately (before clotting occurs) in 4° sodium phos-

[9] W. Goodman, D. A. Schooley, and L. I. Gilbert, *Proc. Natl. Acad. Sci. U.S.A.* **75,** 185 (1978).

[10] B. D. Hammock and T. C. Sparks, *Anal. Biochem.* **82,** 573 (1977).

[11] T. C. Sparks, B. D. Hammock, and L. M. Riddiford, *Insect Biochem.* **13,** 529 (1983).

phate buffer (pH 7.4, ionic strength 0.2 *M* with 0.01% phenylthiourea to inhibit tyrosinases). Insect plasma is obtained only in those specific cases where clotting is slow enough to allow collection and centrifugation at 1000 *g* for 5 min to selectively remove only insect hemocytes. The plasma (supernatant) is then immediately diluted in sodium phosphate buffer as above. Serum is obtained by allowing the hemolymph to thoroughly clot (the time varies with insect species and temperature) and then is centrifuged at 1000 *g* for 5 min. The serum (supernatant) is then immediately diluted for assay. Whole tissue or whole body preparations are obtained by homogenization in sodium phosphate buffer, centrifugation at 20,000 *g* for 15 min, and filtration of the 4° supernatant through glass wool previously washed with pentane and dried. The filtrate should contain over 90% of the activity found in the crude homogenate.

Substrate Preparation. Labeled and unlabeled JH homologs are available from a number of commercial sources. It is preferable to have a source that provides JH in solution in a sealed glass ampoule as opposed to neat JH in a rubber septum, sealed serum vial which is available from some sources. Unlabeled JH is available from Calbiochem-Behring in nanograde hexane and [10-^{3}H]JH from New England Nuclear at 10–20 Ci/mmol in toluene : hexane (4 : 1). JH III is used as substrate for routine monitoring of JH esterase activity because it is the least expensive and most soluble of the available JHs, but differences in the rates of metabolism of the homologs may occur. The unlabeled JH received is diluted with nanograde hexane to 2.5×10^{-2} *M* and stored as a stock solution at −20°. Labeled JH is also diluted with nanograde hexane to ~80,000 cpm/μl and stored as a stock solution at −20°. A working substrate solution is formulated by transferring 20 μl of stock unlabeled and 80 μl of stock [^{3}H]JH to a 4 ml Teflon-sealed screw-capped vial. The toluene : hexane solvent is removed by gentle heat (~30 to 35°) while blanketing the vial with nitrogen. Extreme care should be taken to not use a strong flow of nitrogen. Once the solvent is evaporated just to dryness, 1 ml of absolute anhydrous ethanol is added to bring the JH to the final concentration of 5×10^{-4} *M*, with ~6400 cpm/μl. This substrate is stored at −20°, but is brought to room temperature before opening.

Assay Procedure

Step 1. Pipet 100 μl of phosphate buffer (blank, B), and 100 μl of each enzyme preparation (EP) into individual 10 × 75-mm disposable culture tubes in triplicate in an ice : water bath. *Caution:* Conical tubes are unacceptable since they reduce mixing during vortexing in step 5.

Step 2. One microliter of substrate solution is added to each tube with

a Hamilton repeating dispenser equipped with a 50-μl syringe. The standard deviation of the amount of label added by this method is $\pm 6\%$. It usually is unnecessary to clean the syringe needle between enzyme preparations. The final substrate concentration is 5×10^{-6} *M*.

Step 3. Once substrate is added to all tubes, they are immediately transferred to a 30° water bath, shaken vigorously for 30 sec, and incubated for various times (routinely 15 min). *Caution:* Vigorous shaking is essential for rapid temperature equilibration to 30°.

Step 4. After incubation at 30°, all tubes are transferred back to the ice : water bath, shaken vigorously for 30 sec, and then 50 μl of a methanol : water : concentrated ammonium hydroxide solution (10 : 9 : 1) is added. When this solution is added, all enzymatic JH esterase activity is irreversibly stopped. There obviously are many variations on this basic procedure. For instance one could initiate the reaction by addition of substrate or terminate the reaction by adding the basic methanol solution to the enzyme.

Step 5. Tubes can now be removed from the ice bath, 250 μl of isooctane or another hydrocarbon solvent added to each tube, and each tube vigorously mixed (Vortex) and centrifuged for 5 min at 1000 *g*. *Caution:* For an effective partition, it is necessary to vortex the tube several times until an emulsion forms.

Step 6. From each tube, remove 75 μl of the lower methanol : water phase with a Hamilton 100-μl syringe (a small air bubble should be extruded as the syringe passes through the organic layer) and count for 5 min in an aqueous counting solution. Also count by liquid scintillation in triplicate, aqueous counting solution alone (ACS_B) and aqueous counting solution plus 1 μl of working substrate (ACS_S). *Caution:* The assumption is made that there is no quenching due to the additon of 75 μl of the methanol : water phase from the enzyme preparations being assayed. If there is unequal quenching between samples and/or control vials, then controls (B, ACS_B, and ACS_S) must be redesigned to include the same quench factor or one must use dpm rather than cpm for the calculations. One can also determine maximum counts (ACS_S) by adding substrate to enzyme, adding basic methanol, and then counting a sample without prior extraction with isooctane.

Calculations. Percentage metabolism (*%M*) is

$$\%M = \left[\frac{(\bar{x}\text{cpm}_{EP} - \bar{x}\text{cpm}_B) \times 2}{\bar{x}\text{cpm}_{ACS_S} - \bar{x}\text{cpm}_{ACS_B}}\right] \times 100$$

In these equations $\bar{x}$ refers to the average counts per minute (cpm) indicated by the subscripts defined in the text. Enzyme dilutions and incubation times should be chosen so that data are accumulated in a region

where *%M* is linearly dependent upon incubation time and protein concentration. When lower substrate concentrations are used, the linear relationship between *%M* and incubation time occurs over a shorter range. It is critical that this relationship be determined at all working substrate concentrations. If graphically the line does not pass through the origin one may correct for this "zero time" hydrolysis (*d*) by the following relationship.

Corrected percentage metabolism =

$$\left[\left\{\frac{(\bar{x}\text{cpm}_{EP} - (\bar{x}\text{cpm}_{B}) \times 2}{\bar{x}\text{cpm}_{ACS_S} - \bar{x}\text{cpm}_{ACS_B}}\right] \times 100\right\} - d$$

Activity per unit time per unit volume is

$$n\textit{M} \text{ JH metabolized/min/ml} = (\textit{\%M/100})(\text{Dilution factor})(0.333)$$

The correction factor (0.333) is applicable for a final substrate concentration of 5×10^{-6} *M* with a 15 min incubation time.

TLC Assay Method

Principle. Esterase activity is assayed by conversion of radiolabeled JH to the corresponding acid and separation of substrate and product following direct spotting of the reaction mixture on the cellulose prelayer of a TLC plate.

Assay Procedure

Step 4. Follow steps 1–3 as above. To terminate reaction remove 25 μl of the incubation mixture and mix with 25 μl of methanol (or tetrahydrofuran) containing unlabeled standards in a 6 × 50-mm glass tube. This procedure is important because simply spotting the material on the prelayer may not stop the reaction immediately. If spotting the reaction mixture directly stops the reaction, one may go to step 5 (below) using plates to which standards have already been applied. The juvenoid, 1-(4′-ethylphenoxy)-3,7-dimethyl-6,7-epoxy-*trans*-2-octene and its corresponding diol are routinely used in this laboratory as unlabeled standards. Alternatively one can use one of the commercial JH homologs and prepare JH acid by base hydrolysis or esterase action. One must be cautious since base hydrolysis may cause migration of the 2,3 double bond with loss of UV absorbance. A more reasonable approach is to use commerically available, UV dense standards such as benzophenone and diphenyl acetic acid. In this system JH I chromatographs slightly below benzophenone and slightly above phenol while JH I acid runs with diphenyl acetic acid

and slightly above 2-chlorobenzoic acid. In one run one can determine the amount of standard to add to the methanol based on step 7 (10 mg/ml is a reasonable amount), and the relative positions of JH, JH acid, and the standards on a TLC plate using the TLC system peculiar to one's laboratory.

Step 5. In most cases it is possible to spot the aqueous methanol directly, but if streaking occurs one can agitate the tube and centrifuge out the protein before spotting. Spot the material in 25-μl aliquots on the cellulose prelayer of Whatman LK5DF silica gel plates (250 μm thickness). The aqueous material must neither contact the silica layer nor extend below the surface of the developing solvent when the TLC plate is placed in the tank.

Step 6. The TLC plate is air dried for at least 20 min and then developed using hexane : ethyl acetate (2 : 1) or toluene : propanol (20 : 1). If trailing occurs one can develop the plate through the cellulose layer using a more polar solvent, air dry, and then develop in the usual solvent.

Step 7. Remove the plate from the developing tank and air dry. Visualize the standard spots using a 254-nm lamp and mark them with a pencil. Spray the plate lightly with water to reduce radioactive dust, effects of static, and chemiluminescence and then scrape the appropriate regions corresponding to JH and JH acid into a scintillation vial. Counting solutions designed for aqueous samples effectively extract the moderately polar JH acid from the silica gel. Because of severe chemiluminescence problems with LK5DF plates, the vials should be held in the dark for several hours before counting. Silica gel exposed to UV light should also be counted to ensure absence of chemiluminescence.

Step 8. The amount of JH acid can be expressed as a percentage of the radioactivity recovered from the TLC plate and the enzyme activity calculated by slight modifications of the procedure discussed above.

[33] Cellular Juvenile Hormone Binding Proteins

By ERNEST S. CHANG

Juvenile hormones (JH) are unique sesquiterpene derivatives that regulate a number of developmental functions in insects.[1] Although the dramatic morphological effects of the hormone were documented many years

[1] N. A. Granger and W. E. Bollenbacher, *in* "Metamorphosis: A Problem in Developmental Biology" (L. I. Gilbert and E. Frieden, eds.), 2nd ed., p. 105. Plenum, New York, 1981.

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