1 Sasaki, M., Yoshida, M. C., Kagami, K., Takeuchi, N., Kobayashi, H., Dempo, K., and Mori, M., Rat News Lett. *14* (1985) 4.
2 Yoshida, M. G., Masuda, R., Sasaki, M., Takeuchi, N., Kobayashi, H., Dempo, K., and Mori, M., J. Hered. *78* (1987) 361.
3 Sawaki, M., Enomoto, K., Takahashi, H., Nakajima, Y., and Mori, M., Carcinogenesis *11* (1990) 1857.
4 Mori, M., Enomoto, K., and Takahashi, H., Taisha *27* (1990) 3 (in Japanese).
5 Li, Y., Togashi, Y., Sato, S., Emoto, T., Kang, J.-H., Takeichi, N., Kobayashi, H., Kojima, Y., Une, Y., and Uchino, J., J. clin. Invest. *87* (1991) 1858.
6 Bartsch, R., Klein, D., and Summer, K. H., Archs Toxic. *64* (1990) 177.
7 Schosinsky, H. H., Lehmann, H. P., and Beeler, M. F., Clin. Chem. *20* (1974) 1556.
8 Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. biol. Chem. *193* (1951) 265.
9 Scheinberg, I. H., and Sternlieb, I., A. Rev. Med. *16* (1965) 119.
10 Sass-Kortsak, A., Adv. clin. Chem. *8* (1965) 1.
11 Evans, G. W., Myron, D. R., Cornatzer, N. F., and Cornatzer, W. E., Am. J. Physiol. *218* (1970) 298.
12 Evans, G. W., Cornatzer, W. E., Dubois, R. S., and Hambidge, K. M., Fedn Proc. *30* (1971) 461.
13 Suzuki, K. T., and Yamamura, M., Toxic. Lett. *6* (1980) 301.
14 Lewis, K. O., Gut *14* (1973) 221.
15 Sugawara, N., Sugawara, C., and Miyake, H., Archs Toxic. *56* (1984) 25.
16 Sugawara, N., and Sugawara, C., Archs Toxic. *59* (1987) 432.
17 Sugawara, N., J. Toxic. Sci. *3* (1978) 153.
18 Evans, G. W., Physiol. Rev. *53* (1973) 535.
19 Hasegawa, H., and Tomoyasu, S., Showa Igaku Shi *40* (1980) 423 (in Japanese).

0014-4754/91/101060-04$1.50 + 0.20/0

Reversible juvenile hormone inhibition of ecdysteroid and juvenile hormone synthesis by the ring gland of *Drosophila melanogaster*

D. S. Richard and L. I. Gilbert*

Department of Biology, CB No. 3280 Coker Hall, University of North Carolina at Chapel Hill, Chapel Hill (North Carolina 27599-3280, USA)
Received 6 November 1990; accepted 23 January 1991

Abstract. Juvenile hormone bisepoxide (JHB_3) and juvenile hormone III (JH III) both inhibited the in vitro production of ecdysteroids by ring glands and brain-ring gland complexes from third instar post-feeding larvae of *Drosophila melanogaster* in a reversible manner, although JHB_3 had greater efficacy. The JH III and JHB_3 precursor, methyl farnesoate, did not affect ecdysteroid production. The in vitro synthesis of total detectable JH (JHB_3 + JH III + methyl farnesoate) by the corpus allatum portion of the isolated ring gland was also inhibited reversibly in the presence of exogenous JHB_3 and JH III, but not by methyl farnesoate. These data indicating negative feedback are in agreement with the accepted dogma of endocrine gland regulation.
Key words. Juvenile hormone; corpus allatum; prothoracic gland; dipteran endocrinology.

The interplay of ecdysteroids and juvenile hormones (JH) in larval insects serves to orchestrate the progression from one developmental stage to the next, with ecdysteroids regulating the onset and timing of the molting cycle and JH regulating the quality of the molt[1]. Ecdysteroids are produced by the prothoracic glands under the control of the brain via prothoracicotropic hormone (PTTH)[1]. In dipteran larvae, these glands are fused with the corpus allatum, the site of JH synthesis, and the corpus cardiacum, to form a unique composite endocrine organ, the ring gland. As part of an ongoing project on the endocrinology of *Drosophila melanogaster* that will provide a base for genetic analysis, we investigated the ability of juvenile hormone bisepoxide (JHB_3), the presumed major JH of higher flies, and its putative precursor JH III[2], to modulate the production of both ecdysteroids and JHs in vitro by brain-ring gland complexes and isolated ring glands from third instar post-feeding larvae.

Materials and methods

Animals. Wild type *Drosophila melanogaster* (Canton-S strain) were maintained at 25 °C under a 12L:12D photoperiod and fed on *Drosophila* medium (Carolina Biological Co.) supplemented with dried bakers yeast. They were staged according to both wandering behavior and salivary gland morphology[3]. Mid postfeeding stage larvae were used in all experiments.

Chemicals. Methyl farnesoate and JHB_3[2] were synthesized by Drs F. Baker and D. Schooley (Sandoz Crop Protection Co.) while JH III was purchased from Sigma. The JH esterase inhibitor octyl-1,1,1-trifluoropropanone (OTFP)[4] was a gift from Dr M. Roe (North Carolina State University) and was added to all incubations to a final concentration of 0.5 μM. The JHs were stored at −80 °C in either acetonitrile (JHB_3 and JH III) or hexane (methyl farnesoate) at a concentration of 1 mg · ml^{-1} and as required, aliquots were dried down under N_2 in siliconized microfuge tubes at room temper-

ature. MEM medium minus methionine (Lineberger Cancer Research Center, University of North Carolina) plus 20 mg · ml^{-1} Ficoll 400 (Sigma) and 25 µg · ml^{-1} ampicillin (United States Biochemical)[2] was added and the tubes sonicated and vortexed to dissolve the JH. The high concentrations of JHs utilized in these experiments may have resulted in the formation of micelles or non-specific interactions with macromolecules present in the incubation medium[5]. For each JH compound, the efficiency of recovery into distilled water at the 1 mM level was checked by UV spectrophotometry (JHB_3 = 78%, JH III = 93%, methyl farnesoate = 69%) and the dosages reported in each figure were adjusted accordingly.

Assay conditions. Isolated ring glands or brain-ring gland complexes from post-feeding third instar larvae were incubated for 2 h at 25 °C in 5-µl droplets of incubation medium placed in the center of the wells of 24-well microtitre plates (Falcon 3047). In the case of the ecdysteroid assays, the medium was supplemented prior to use with 20 µM unlabeled methionine. The in vitro assay for JH was based on that of Tobe and Pratt[6] and measures the incorporation of the methyl moiety of L-[^{3}H-methyl]-methionine into late JH precursor molecules, yielding JHB_3, JH III or methyl farnesoate. In this assay, the medium was supplemented with 20 µM L-[^{3}H-methyl]-methionine (New England Nuclear Corp., specific activity 80 Ci · $mmol^{-1}$) and the assay conducted as described previously[2]. Specific JHs were measured following silica gel thin layer chromatography (TLC) with hexane:ethyl acetate (60:40) solvent[2]. Ecdysteroids were measured by radioimmunoassay (RIA) with H-22 antiserum[7].

Results

Effects of JH on ecdysteroid synthesis. Figure 1 A shows that JHB_3 inhibits the production of ecdysteroids (H-22 immunoreactive material) by isolated *Drosophila melanogaster* brain-ring gland complexes in vitro in a dose-dependent manner. JH III affects a similar inhibition (fig. 1 B), although a slightly higher dose is required to bring about a 50% reduction in ecdysteroid biosynthesis (c. 0.9 mM vs 0.5 mM for JHB_3). The addition of the JH metabolic precursor, methyl farnesoate, to the medium failed to elicit a decrease in synthesis over the same concentration range. Similar results were obtained when isolated ring glands rather than complexes were incubated with JHB_3 (fig. 1 C), JH III or methyl farnesoate, suggesting that JH may act directly upon the ring gland, and probably on the prothoracic gland portion of that composite structure.

The possibility that high levels of JHB_3 may be having a toxic effect upon the brain-ring gland complexes resulting in lowered ecdysteroid synthesis is addressed by the data in figure 2. In this experiment complexes were incubated in the presence or absence of 1 mM JHB_3 for 2 h at 25 °C, and then transferred to fresh medium either

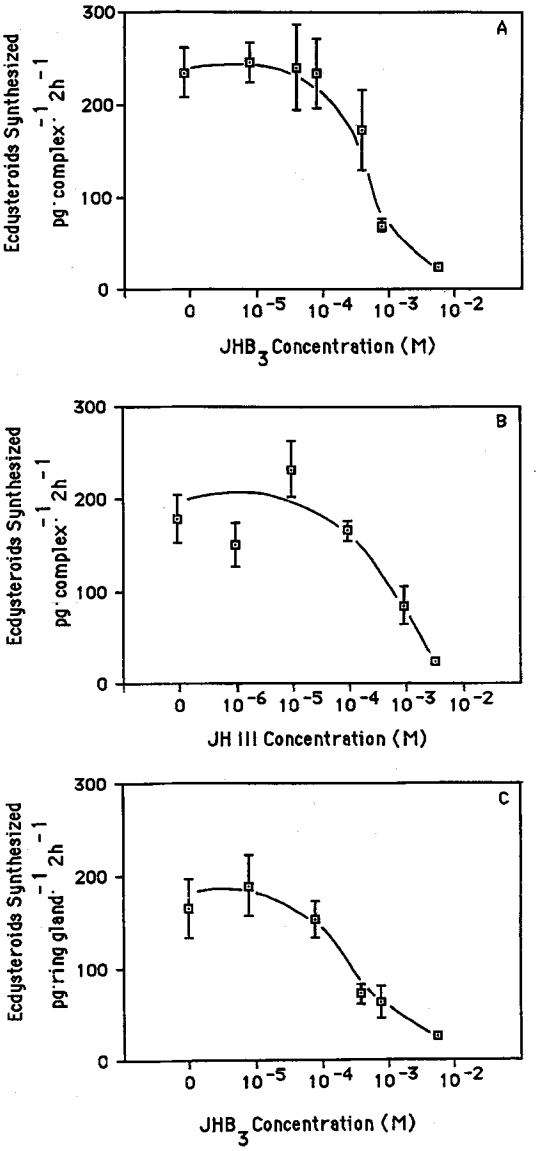

Figure 1. Effects of juvenile hormone on ecdysteroid synthesis by ring glands in vitro. *A* Dose response curve for the effects of JHB_3 on ecdysteroid production in vitro by brain-ring gland complexes from third instar post-feeding *Drosophila melanogaster*. *B* JH III effects on ecdysteroid production by brain-ring gland complexes in vitro. *C* JHB_3 effects on ecdysteroid synthesis by isolated ring glands in vitro. All points represent the mean ±SEM of 6–8 determinations.

with or without JHB_3. The data show that for a plus JHB_3 to minus JHB_3 transfer (3 to 7), the complexes recover the ability to produce ecdysteroids during the second incubation. Those incubated in JHB_3-containing medium for both periods (1 to 5) failed to produce normal levels of ecdysteroids during either period, and those transferred from JHB_3-free medium to JHB_3-supplemented medium (4 to 8) produced low levels during the second period of incubation. Those complexes incubated in the absence of JHB_3 during both periods (2 to 6) produced only about half as much ecdysteroid during the second period as they did during the first, possibly as a result of substrate depletion. The effect of 1 mM JH III was also found to be reversible in vitro by the use of a

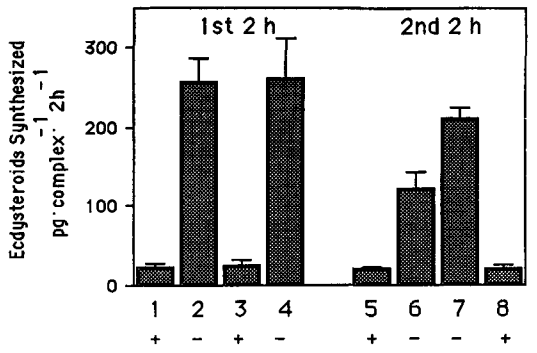

Figure 2. The effect of dual incubation periods in the presence (+) and absence (−) of 1 mM JHB_3 on ecdysteroid production by brain ring gland complexes from third instar post-feeding *Drosophila melanogaster* larvae. In each case, the complexes were incubated individually for 2 h, removed and the media assayed for ecdysteroid content by radioimmunoassay. Group 1 was incubated for 2 h in the presence of JHB_3 and then transferred for a further 2 h in the presence of the hormone as group 5; group 2 was transferred to become group 6; group 3 to become group 7; and 4 to become 8. Each point represents the mean ±SEM of 8 separate determinations.

similar protocol. Complexes removed from the JH III were able to resume the production of ecdysteroids at normal levels during the second incubation period.

Effects of JH on juvenile hormone synthesis. An analogous series of experiments was conducted to examine the effect of JH in the medium on JH production by isolated ring glands. Figure 3 reveals that JHB_3 was able to inhibit total JH production (JHB_3 and JH III) in vitro by isolated ring glands with an effective dose in the same range as that for JHB_3 inhibition of ecdysteroid production. JH III (at 1 mM) was also effective in reducing total JH production but methyl farnesoate was ineffective at this concentration. The experiments concerned with feedback effects on JH synthesis were only carried out on isolated ring glands because JH production by whole complexes is already depressed due to allatostatic control by the brain[8]. No changes were observed in the relative levels of production of JHB_3 and JH III by the ring glands in the presence of either JHB_3 or JH III as measured by TLC. As previously, ring glands were able to recover the ability to produce JH when removed from

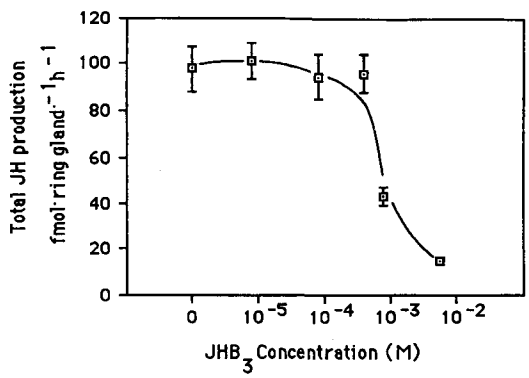

Figure 3. Dose response of JHB_3 on total juvenile hormone production (JHB_3, JH III and methyl farnesoate) by isolated ring glands from third instar post-feeding *Drosophila melanogaster* larvae. Each point represents the mean ±SEM of 8 separate determinations.

medium containing JHB_3 or JH III demonstrating that JH was not having a toxic effect upon the corpus allatum portion of the ring gland.

Discussion

The data presented here suggest that JH may act directly upon ring glands from *Drosophila melanogaster* to regulate ecdysteroid synthesis. This connection between JH and the stimulation[9-11] or inhibition[12,13] of prothoracic gland activity has been made many times on Lepidoptera although no direct in vitro effect has been noted. Another report showed that the prothoracic glands of *Bombyx mori* become active on day 3 of the final larval instar, coincident with a fall in the hemolymph JH titer[14]. Furthermore, the prothoracic glands become responsive to PTTH on the same day[15,16]. If the corpora allata are removed at the time of the molt to the last instar, PTTH responsivity and ecdysteroid production recover even earlier, strongly suggesting that JH may normally *inhibit* ecdysteroid production in vivo[17]. Indeed, if the JH analog methoprene is applied immediately after the allatectomy, the recovery of competence to respond to PTTH and the secretory activity of the prothoracic glands are suppressed further, suggesting that JH inhibits ecdysteroid synthesis. However, there was no attempt in that study to inhibit ecdysteroid synthesis in vitro with JH[17]. A similar study on final instar *Manduca sexta* prothoracic glands failed to show an in vitro effect of the JH analog hydroprene on ecdysteroid synthesis although it did demonstrate that JH may be involved in the inhibition of gland competence[18].

The present data suggest that JH can actually inhibit the production of both ecdysteroids and JHs in vitro by brain-ring gland complexes or isolated ring glands from mid-postfeeding stage *Drosophila* larvae. Dipteran prothoracic glands appear not to make 3-dehydroecdysone[19] and therefore the use of the H-22 antiserum likely results in the measurement of total ecdysteroid synthesized. The possibility that JH stimulates the synthesis of 3-dehydroecdysone is currently being investigated. Because of the difficulties of separating the ring gland into its component parts without incurring major damage to the dissociated endocrine glands during the delicate surgical procedures, we have not attempted to further localize the effective site(s) of JH action. The involvement of the brain in the transduction of this JH elicited inhibition seems unlikely since no differences were noted between the responses of brain-ring gland complexes or isolated ring glands alone. The presence of a high affinity, high molecular weight, JH III-specific binding protein was demonstrated in the hemolymph of post-feeding third instar *Drosophila melanogaster* larvae and its involvement in the transport of JH to the target sites was proposed[20]. For the in vitro *Drosophila* system, the possibility still exists therefore that the absence of binding protein may account for the high doses of JH required in order to elicit an effect.

That farnesoic acid, a precursor of JH III and JHB_3, is able to stimulate the *Drosophila* corpus allatum to produce maximal levels of JHB_3 at a lower concentration (20 μM) than that required for either JH III or JHB_3 inhibition of JH synthesis[2, 8], suggests that the cell membranes of the corpora allata may be more permeable to some members of this class of compounds than to others. However, the methylation and epoxidation of this molecule to methyl farnesoate, JH III or JHB_3 may affect the ability of these molecules to traverse the cell membrane. The addition of equivalent, or higher, doses of methyl farnesoate, the intermediate compound subsequent to farnesoic acid in the JH biosynthetic pathway, failed to elicit an inhibitory response, suggesting that either this compound did not enter the cells, or that it was not an inhibitory agent. JH III and JHB_3, however, were able to affect an inhibitory response and therefore, either entered the cells or exerted their effect at the cell surface. When radiolabelled JH III is incubated with intact ring glands, a small proportion is converted to JHB_3 and released back into the medium[2]. However, when it is incubated with extracts of ring glands in the presence of NADPH, most of it is converted to JHB_3. Thus, the high levels of both JH III and JHB_3 required to inhibit JH synthesis in vitro may also be the result of only low levels entering the cell. Once inside, most of the JH III is probably converted to JHB_3 which may act via a negative feedback mechanism for the regulation of JH production. This concept of negative feedback is in agreement with the general dogma of endocrine gland regulation.

Unfortunately, the role of JH in the larval development of the higher Diptera remains conjectural. It has not been possible to conduct classic insect endocrinological experiments involving the extirpation of the corpora allata, in part due to the size of these fly larvae and because ring gland extirpation would result in the removal of the prothoracic glands and corpus cardiacum as well. Recent studies with JH deficient *apterous* mutations have shown that third instar larval JH production in vitro by ring glands from homozygous *apterous*[4] animals is lower than by glands from wild type larvae (DSR, LIG and D. Segal, unpublished observations). Nevertheless, larval development does not seem to be adversely affected. This suggests that either JH has a subtle role to play in dipteran post-feeding larval development, or that the levels produced by wild type larvae are in excess of what is required for development to proceed normally or even that JH is metabolized more slowly in these mutants.

Acknowledgments. This work was supported by grants DK30118 and RR06627 from the National Institutes of Health and DCB-8802108 from the National Science Foundation.

* To whom correspondence should be addressed.

1 Gilbert, L. I., Bollenbacher, W. E., Goodman, W., Smith, S. L., Agui, N., Granger, N., and Sedlak, B. J., Rec. Prog. Hormone Res. *36* (1980) 401.
2 Richard, D. S., Applebaum, S. W., Sliter, T. J., Baker, F. C., Schooley, D. A., Reuter, C. C., Henrich, V. C., and Gilbert, L. I., Proc. natl Acad. Sci. USA *86* (1989) 1421.
3 Myohara, U., and Okada, U., Dev. Biol. *122* (1987) 396.
4 Hammock, B. D., Abdel-Aal, Y. A. I., Mullin, C. A., Hanzlik, T. N., and Roe, R. M., Pestic. Biochem. Physiol. *22* (1984) 209.
5 Goodman, W. S., and Chang, E. S., in: Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 7, p. 491. Eds G. A. Kerkut and L. I. Gilbert. Pergamon Press, Oxford 1985.
6 Tobe, S. S., and Pratt, G. E., Biochem. J. *144* (1974) 107.
7 Warren, J. T., Smith, W., and Gilbert, L. I., Experientia *40* (1984) 393.
8 Richard, D. S., Applebaum, S. W., and Gilbert, L. I., J. comp. Physiol. B *159* (1989) 383.
9 Gilbert, L. I., and Schneiderman, H. A., Nature *184* (1959) 171.
10 Ichikawa, M., and Nitshiitsutsuji-Uwo, J., Biol. Bull. mar. Biol. Lab., Woods Hole *116* (1959) 88.
11 Williams, C. M., Biol. Bull. mar. Biol. Lab., Woods Hole *116* (1959) 323.
12 Fain, M. J., and Riddiford, L. M., Gen. comp. Endocr. *30* (1976) 131.
13 Safranek, L., Cymborowski, B., and Williams, C. M., Biol. Bull. mar. Biol. Lab., Woods Hole *158* (1980) 248.
14 Plantevin, G., Bosquet, G., Calvez, B., and Nardon, C., Comp. Biochem. Physiol. B *86* (1987) 501.
15 Sakurai, S., J. Insect Physiol. *30* (1984) 657.
16 Okuda, M., Sakurai, S., and Ohtaki, T., J. Insect Physiol. *31* (1985) 455.
17 Sakurai, S., Okuda, M., and Ohtaki, T., Gen. comp. Endocr. *75* (1989) 222.
18 Watson, R. D., and Bollenbacher, W. E., Molec. cell. Endocr. *57* (1988) 251.
19 Kiriishi, S., Rountree, D. B., Sakurai, S., and Gilbert, L. I., Experientia *46* (1990) 716.
20 Shemshedini, L., and Wilson, T. G., Insect Biochem. *18* (1988) 681.

0014-4754/91/101063-04$1.50 + 0.20/0