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Juvenile Hormone Induction of Esterases: A Mechanism for the Regulation of Juvenile Hormone Titer

(*Hyalophora gloveri*/pupa/hemolymph)

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ABSTRACT Within a few hours after injection of juvenile hormone into *Hyalophora gloveri* pupae, several fast-migrating carboxylesterases (EC 3.1.1.1) that are sensitive to di*iso*propylfluorophosphate appear in the hemolymph. Treatment of the pupae with puromycin or actinomycin D prevents the appearance of these hemolymph enzymes, suggesting *de novo* synthesis of the carboxylesterases. Of the several other compounds investigated, only a potent mimic of the juvenile hormone is able to induce these enzymes. When the induced enzymes are incubated *in vitro* with ^{14}C-labeled juvenile hormone, the hormone is rapidly and efficiently degraded. It is suggested that these induced carboxylesterases play an important role in the regulation of juvenile hormone titer.

The juvenile hormone of insects can be envisaged as a modulator that determines the quality of the molt, while molting itself is initiated by the molting hormone. The concentration of juvenile hormone is high at a larval–larval molt, low at the larval–pupal molt, and must be absent if the molt from pupa to adult is to be initiated normally (1). On the basis of indirect data, it was postulated more than a decade ago that last instar larvae have a powerful inactivation mechanism to decrease the circulating titer of juvenile hormone and that pupae are also quite efficient in ridding themselves of endogenous juvenile hormone, so that metamorphosis can proceed normally (2). It has recently been shown that hemolymph of the larval tobacco hornworm contains one or more enzymes (carboxylesterases; EC 3.1.1.1) that cleave the methyl group from the ester linkage of juvenile hormone (methyl *trans, trans, cis* 10-epoxy-7-ethyl-3,11-dimethyl-2,6-tridecadienoate) to yield the free carboxylic acid and that this is the probable first step in juvenile hormone catabolism (3, 4). We have investigated this phenomenon in saturniid moths and found that juvenile hormone is indeed broken down in the hemolymph (5). The present report describes the esterases in pupal hemolymph and reveals that juvenile hormone can induce the appearance of specific multiple molecular forms of carboxylesterase that degrade the juvenile hormone, and suggests that this is one mechanism an insect uses in regulating the titer of circulating juvenile hormone to insure normal development.

MATERIAL AND METHODS

The animals used in these experiments were male *Hyalophora gloveri* pupae stored at 6° for 2–4 months. The unlabeled juvenile hormone was a mixture of the eight possible isomers of the *H. cecropia* C_{18}-juvenile hormone, and was diluted in peanut oil to give a final concentration of 1 μg/μl. 10 μg of juvenile hormone were usually injected per gram of body weight. It should be noted that the *H. gloveri* C_{18}-juvenile hormone is identical to the *H. cecropia* C_{18}-juvenile hormone (6). The radioactive juvenile hormone was labeled at carbon 2 with ^{14}C (25.3 Ci/mol) (7) and will be designated here as [2-^{14}C]-juvenile hormone. Juvabione, hydrochlorination product (8), and 9,10-epoxyhexadecanoic acid methyl ester were diluted in peanut oil at concentrations of 1 or 50 μg/μl. Two dosages of these compounds were used; 10 and 50 μg/g.

Polyacrylamide gel electrophoresis used an Ortec slab gel apparatus (Ortec Inc; Oak Ridge Tenn.); gels were prepared with a discontinuous gradient of 7% (3.8 cm), 5% (2.5 cm), and 3.5% (1.3 cm) from bottom to top. Routinely, 10 or 15 μl of hemolymph were used for analytical studies; 0.3 ml of hemolymph was used for preparative studies. The buffer was either 1.5 M Tris·sulfate gel buffer–65 mM Tris·borate tank buffer (both pH 9.0), or 0.75 M Tris·sulfate gel buffer–0.65 M Tris·borate tank buffer (both pH 8.4). After electrophoresis, the gels were incubated in 0.1 M sodium phosphate buffer (pH 6.7) for 15 min at 25° and, in the esterase inhibition studies, the inhibitor was part of the incubation medium. The inhibitors used were di*iso*propyl fluorophosphate (0.8 mM), eserine (0.1 mM), and *p*-chloromercuribenzoate (1 mM). The gels were subsequently stained for general esterase activity in a solution containing Fast Blue RR and α-naphthyl acetate. After incubation for 30–45 min at 25°, the gels were destained and fixed in methanol–water–acetic acid 45:45:10.

Hemolymph was removed through an incision in the pupal wing. Fat body was dissected out, washed three times, homogenized in cold distilled water, and centrifuged at 27,000 × *g* for 15 min at 4°. Supernatants were removed and centrifuged again, care being taken to exclude the floating lipid layer.

Enzymes were removed from gels by carefully slicing the preparative slabs and grinding the gel in cold distilled water with a Sorvall Omnimixer. After centrifugation of the slurry at 27,000 × *g* for 15 min at 4°, the supernatant was removed and used as enzyme source. It contained about 2.2 mg/ml of hemolymph protein or 2.5 mg/ml of fat body protein.

To study the breakdown of juvenile hormone *in vitro*, 1 ml of the esterase extract was incubated with [2-^{14}C]juvenile hormone in 20 μl of ethyl alcohol in a shaken water bath at 30° for 40 min. The control vial contained an equal quantity of nonesterase protein from the polyacrylamide gel, prepared in the same manner as the esterase extract. Incubation was terminated by the addition of an excess of chloroform–methanol 3:1; the vial was thoroughly agitated on a Vortex mixer. The solvents were evaporated under N_2 to a volume of about 40 μl and subjected to thin-layer chromatography. Samples were mixed with cold juvenile hormone in hexane and chromatographed on silica-gel chromatoplates (15 or 25%

ethyl acetate in hexane). The plates were divided in relation to the cold juvenile hormone (visualized under ultraviolet irradiation) as follows: Region A, origin to slightly before juvenile hormone; B, juvenile hormone; and C, slightly ahead of juvenile hormone to front. The marked regions of the plate were scraped into scintillation vials containing 10 ml of scintillation fluid (5 g 2,5-diphenyloxazole (PPO) and 0.1 g dimethyl-1,4-bis-2-(4-methyl-5-phenoxazolyl)-benzene (POPOP)/liter toluene) and radioassayed (Packard 3380 Tri-carb Scintillation Spectrometer). In some cases the entire plate was radioscanned (Packard Radiochromatogram Scanner, model 7201/565).

RESULTS

Although there is some physiological variation, eight esterase bands are generally resolved in hemolymph of male pupal *H. gloveri* that are capable of hydrolyzing α-naphthyl acetate. In addition, three very faint (barely detectable) fast-migrating bands are seen after *prolonged* staining. Both the fat body and gut extracts also possess what appear to be the same eight bands of esterase activity, as well as very active, fast-migrating esterases. It should be noted that the use of α-naphthyl proprionate as substrate yielded the same results but β-naphthyl acetate appeared to be a poor substrate, particularly for the fast-migrating esterases.

In an attempt to generally define the nature of the eleven esterases in pupal hemolymph, a series of inhibition studies was conducted with di*iso*propyl fluorophosphate, *p*-chloromercuribenzoate, and eserine. Only the slowest moving and four fastest moving esterases were sensitive to di*iso*propyl fluorophosphate (although not completely inhibited), and in accord with accepted practice (9), we classify them as carboxylesterases.

Preliminary studies with the fast-migrating esterases from fat body revealed that they were capable of degrading [2-^{14}C]-juvenile hormone. However, since juvenile hormone is transported in the hemolymph (5) and since the hemolymph of a larval tobacco hornworm could break down juvenile hormone (3, 4), we wished to determine the degradative capacity of *H. gloveri* hemolymph. Since the pupa normally harbors no juvenile hormone (10), perhaps hemolymph carboxylesterases would be induced if juvenile hormone was injected. Cold juvenile hormone was injected (10 μg/g), and the hemolymph was removed at intervals of 3, 5, 12, 18, and 24 hr after injection. Fig. 1 (1*a;* wells 1,2,3: 1*b;* wells 1,2) reveals that the rapidly migrating carboxylesterases show greatly enhanced activity and stain relatively intensely under conditions of normal incubation. It appears that this induction of carboxylesterase activity in the hemolymph is complete between 5 and 12 hr after injection of juvenile hormone, since we could never induce carboxylesterase formation in all of the pupae 3 and 5 hr after injection, but we always could 12 hr after injection.

Since juvenile hormone is known to stimulate the prothoracic glands to initiate development by increasing the titer of molting hormone, the possibility existed that this enzyme induction was only an indirect result of juvenile hormone. However, when β-ecdysone (5 μg/g) was injected into the pupae, there was only a slight stimulation of carboxylesterase activity over the basal level within 24 hr. In addition, the juvenile hormone is capable of stimulating the brain neurosecretory cells (K. Davey, personal communication), but injection of juvenile hormone into brainless *H. gloveri* pupae yielded the same results as injection into ani-

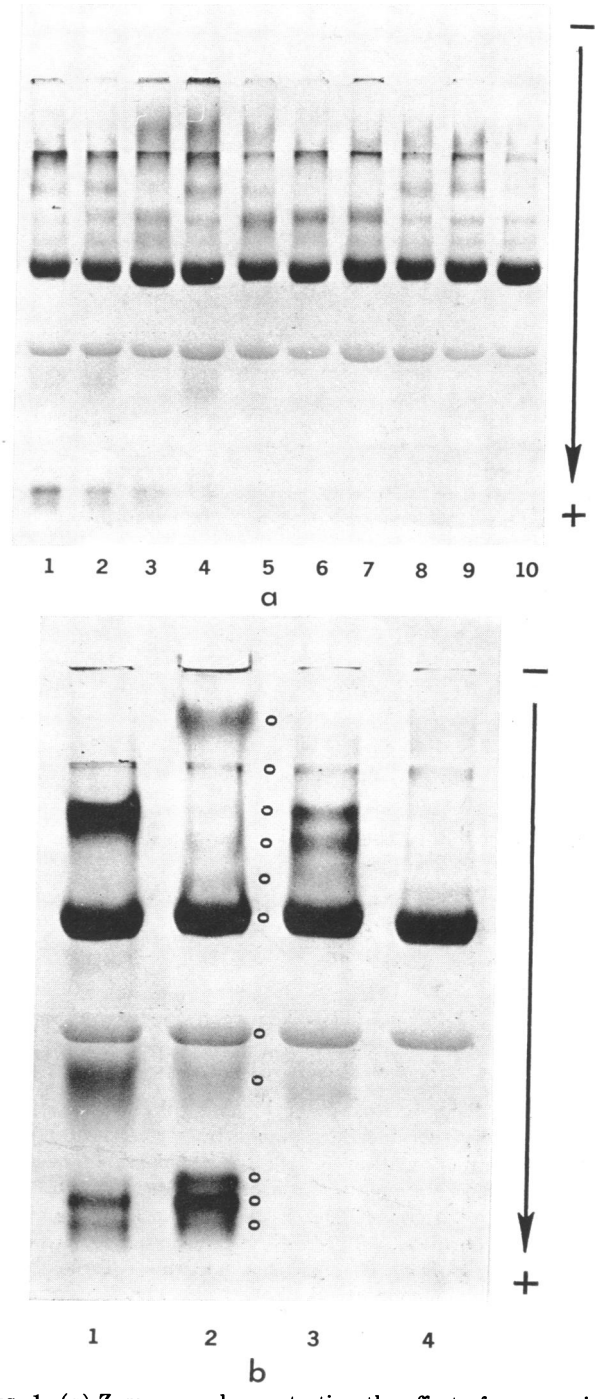

FIG. 1. (*a*) Zymogram demonstrating the effect of puromycin on the induction of hemolymph carboxylesterases by juvenile hormone. **1–3** show the fast migrating carboxylesterases induced by injection of 10 μg/g juvenile hormone. **4–7** reveal the inhibitory effect on the induction process when puromycin (0.2 mg/g) is injected simultaneously with juvenile hormone. **8–10** are from animals that received puromycin alone. Hemolymph was collected 14 hr after injection.

(*b*) Zymogram demonstrating the effect of actinomycin D on the induction of hemolymph carboxylesterases by juvenile hormone. **1** and **2** equivalent to **1–3** in Fig. 1*a*, **3–4** show the inhibitory effect on the induction process when actinomycin D (3 μg/g) is followed 6 hr later by a simultaneous injection of more actinomycin D (2 μg/g) and juvenile hormone. Hemolymph was collected 14 hr after injection. *0,* denotes the eleven esterases in well 2.

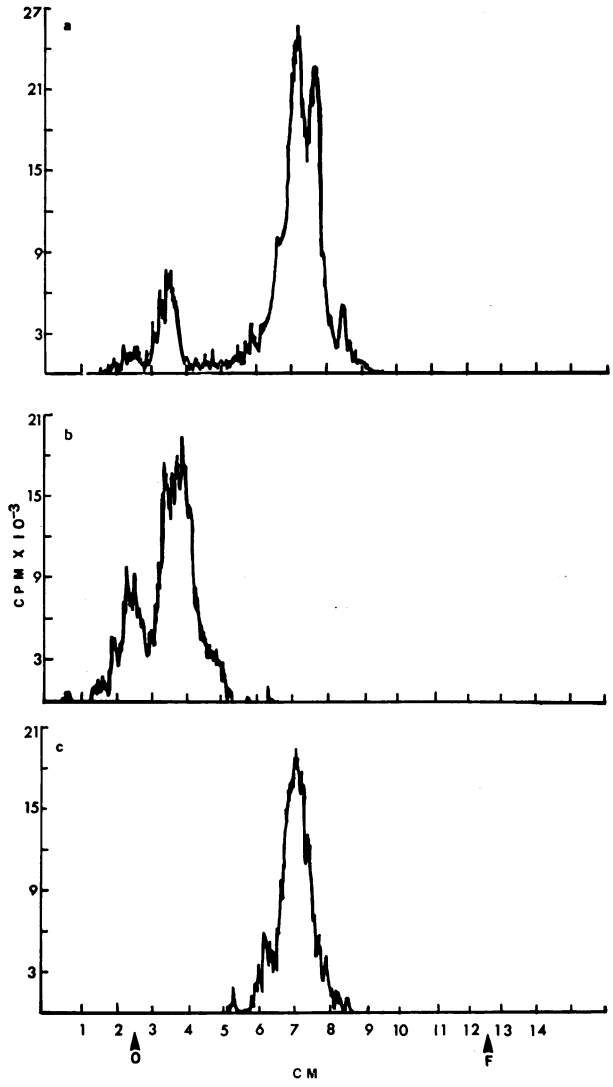

FIG. 2. Radiochromatoscans of thin-layer plates illustrating the breakdown of [^{14}C]juvenile hormone by carboxylesterases. (*a*) From incubation mixture of fat body carboxylesterases and [2-^{14}C]juvenile hormone. (*b*) From incubation mixture of hemolymph carboxylesterases induced by juvenile hormone and [2-^{14}C]juvenile hormone. (*c*) [2-^{14}C]juvenile hormone. *O* = point of application. *F* = front. Conditions as in legend to Table 1, except that the protein concentration of the fat body enzyme was 1.3 mg/ml, while that of the hemolymph was 2.2 mg/ml.

mals with brains intact. The above suggests, but does not conclusively prove, that juvenile hormone is acting directly and not secondarily through another hormone.

Although we have used the term induction, it is possible that injection of juvenile hormone increased the activity of existing enzyme, or caused its selective release from another tissue. Fig. 1*a* reveals that when pupae are injected with juvenile hormone and 0.2 mg/g of puromycin simultaneously, the carboxylesterases do not appear in the hemolymph. Therefore, protein synthesis appears to be essential for induction of hemolymph carboxylesterases by juvenile hormone. To determine whether new mRNA is also essential, a series of studies was conducted with juvenile hormone and actinomycin D. When actinomycin D (2 μg/g) was injected with juvenile hormone, or (3 μg/g) was injected 2 hr before injection of the hormone, the carboxylesterases were induced to about 50% of the amount attained without the inhibitor. When actinomycin D was injected (6 μg/g) 1 hr before injection of juvenile hormone, no induction of carboxylesterases was noted in 75% of the animals. A complete suppression of induction occurred when pupae were injected first with 3 μg/g of actinomycin D and 6 hr later with 2 μg/g of actinomycin D, along with juvenile hormone (Fig. 1*b*). These data suggest that juvenile hormone may be acting at the transcriptional level to induce these enzymes. The next question to be examined is the function of these induced hemolymph carboxylesterases.

When the induced hemolymph carboxylesterases were eluted from the preparative gels and incubated with [2-^{14}C]-juvenile hormone for 40 min at 30° and the incubation mixture was analyzed, it was readily seen that the juvenile hormone was rapidly and completely degraded (Fig. 2). The radiochromatogram (Fig. 2*b*) indicates the presence of two major degradation products, the slower-migrating one possibly being the 10,11-dihydroxy acid (3, 4). If so, one or more of the induced enzymes may have epoxide hydrolase activity. The fast-migrating fat body carboxylesterases are also capable of breakdown of juvenile hormone, but are not nearly as efficient as the induced enzymes. Table 1 is from a similar experiment, but presents the data in more quantitative terms. Although the metabolite(s) was not chemically identified, the standard free carboxylic acid (3, 4) (juvenile hormone minus methyl group) migrates to the A region.

Finally, to obtain some idea of the specificity of this induction, pupae were injected with 9,10-epoxyhexadecanoic acid methyl ester, which is chemically similar to juvenile hormone but without hormone activity (11); or with juvabione, which has juvenile hormone activity in bugs but not in *H. gloveri;* or with the hydrochlorination product (8), which has potent juvenile hormone activity in *H. gloveri,* but is chemically very different from juvenile hormone of *H. gloveri.* The results demonstrated that 9,10-epoxyhexadecanoic acid methyl ester stimulated the appearance of carboxylesterases only slightly, even at the maximum dose of 50 μg/g of fresh weight.

TABLE 1. *Degradation of juvenile hormone by carboxylesterase*

Chromatoplate region	Control (cpm)	Induced hemolymph carboxylesterases (cpm)	Fat body carboxylesterases (cpm)	Protein control (cpm)
A	0	4890 (100%)	3060 (76.4%)	0
B	3570 (100%)	0	942 (23.6%)	3620 (100%)
C	0	0	0	0

[2-^{14}C]Juvenile hormone was incubated with the appropriate carboxylesterases eluted from preparative gels or controls (see *Methods*) for 40 min at 30°. The resulting material soluble in chloroform–methanol was analyzed by thin-layer chromatography and appropriate regions were radioassayed (see *Methods*). The percentage of the total cpm recovered is given in parentheses. The free carboxylic acid derived from juvenile hormone migrates to region A, while the unadulterated juvenile hormone spot is designated as region B.

10 μg/g of the hydrochlorination product stimulated the appearance of the carboxylesterases to some extent, but 50 μg/g was comparable to the juvenile hormone in inducing the appearance of the enzymes. Juvabione, on the other hand, was completely ineffective.

DISCUSSION

These studies have demonstrated that a class of carboxylesterases appear in the hemolymph of *H. gloveri* pupae (a stage normally devoid of juvenile hormone) when juvenile hormone is injected, and that inhibition of protein synthesis and mRNA synthesis prevents this induction. To our knowledge, this is the first example of an animal hormone that induces the synthesis of an enzyme whose function is mediating the breakdown of the hormone. An analogous situation may be the induction of isozymes of isoperoxidase (EC 1.11.1.7) by indole acetic acid in plants (12), although the specificity of this enzyme has been questioned. In fibroblasts, adenosine 3:5′-cyclic monophosphate may induce the synthesis of the phosphodiesterase (EC 3.1.4.C) (13). The only other well studied induction of synthesis of a specific protein by juvenile hormone is that concerning the vitellogenic protein synthesized in the fat body of some adult female insects and that ultimately finds its way to the maturing oocyte (14). That the synthesis of the carboxylesterases is occurring *de novo* has not been conclusively proven and is a function of the sensitivity of techniques used. We are currently attempting to obtain antibodies against these carboxylesterases and the present case for induction rests to a great extent on the inhibitor studies. Since we still do not know the site of synthesis of these enzymes, the possibility exists that new mRNA and protein must be synthesized for the carboxylesterases to be selectively released from some storage tissue or to be converted from a proenzyme to the active form. It should be noted that we have also been able to determine spectrophotometrically (*p*-nitrophenyl acetate: A_{400} nm) that pupae treated with juvenile hormone yield hemolymph possessing 20% greater *total* esterase activity than controls, corroborating the electrophoretic data. The fact that the only material tested that approximated juvenile hormone in its ability to induce the carboxylesterases is also a potent mimic of juvenile hormone in *H. gloveri* suggests that carboxylesterase synthesis is a result of juvenile hormone action.

Further studies have revealed that hemolymph from other saturniid pupae that have been kept at low temperature (6°) for 5 months (male and female *Philosamia cynthia;* male and female *Hyalophora cecropia*) already possess marked carboxylesterase activity at about the same R_f as that induced by juvenile hormone in *H. gloveri*, and that several of the slow-moving esterases in larval tobacco hornworm hemolymph can degrade juvenile hormone *in vitro* (unpublished results). The former observations imply that when the pupa is on the verge of initiating adult development, these fast-migrating carboxylesterases appear, and they may have catalytic functions, in addition to destroying circulating juvenile hormone.

How insects regulate their hormone titer is not really known, although they must possess sensitive mechanisms since excess hormone results in abnormal and inviable insects (2, 15). Recent work has revealed that injection of juvenile hormone results not only in activation of the prothoracic gland, but in the activation of the corpora allata (source of juvenile hormone) as well (16). This finding suggests positive feedback; we do not know how the glands are turned off in larval insects. The induction of enzymes capable of degrading the hormone may be one mechanism that insects use to insure normal metamorphosis. These data may also have important implications for those interested in the use of juvenile hormone and analogs of juvenile hormone as insect control agents, for they suggest that insects possess a biochemical repertoire that not only allows them to break down foreign molecules (e.g., DDT), but in a sense allows them to resist their own hormones when necessary.

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