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Action of Juvenile Hormone (JH) Esterase on the JH–JH Binding Protein Complex. An *In Vitro* Model of JH Metabolism in a Caterpillar

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A decline in juvenile hormone (JH) titer early in the last larval instar leads to the metamorphosis of lepidopterous larvae into pupae. Clearance of JH is associated with an increase in the level of JH-specific esterase (JHE) that hydrolyzes JH into the biologically inactive JH-acid. Experiments with purified recombinant JHBP (rJHBP) and with both natural and recombinant JHE (rJHE) were designed to test the hypothesis that JHBP "protects" JH from JHE degradation in insect hemolymph. First, at low JHE concentrations, the rate of hydrolysis of JHBP-bound JH by JHE was determined by the rate of dissociation of JH from JHBP. Second, the rate of hydrolysis of JH increased with increasing JHE concentrations even in the presence of excess JHBP. In contrast, JH was not hydrolyzed by a general carboxylesterase in the presence of JHBP. Third, the rate of JH hydrolysis when the JHBP–JH complex was added to JHBP-depleted hemolymph was essentially identical to that in undiluted hemolymph. Taken together, these results suggest that JHBP protects JH from nonspecific hydrolytic processes, while still allowing highly specific JHE to hydrolyze JH by recognizing the JH–JHBP complex. Finally, JHBP efficiently extracted JH from lipophilic depots (the so-called "refractory pool"), resulting in *more rapid* degradation of JH by JHE. Thus, JHBP is crucial for maintaining and distributing JH in hemolymph in the *absence* of JHE, and may assist in the rapid clearance of JH in the *presence* of JHE.

Insect Metamorphosis JH binding protein Metabolism Protective role

INTRODUCTION

The onset of the metamorphosis of lepidopterous larvae into pupae and eventually into adults is controlled by subtle and carefully orchestrated endocrine changes. Insect development, growth, and the formation of larval structures are largely regulated by juvenile hormones (JHs) (Riddiford, 1986, 1994). Larval molting is initiated by an ecdysteroid, molting hormone, in the presence of JH, while pupation occurs in the absence of JH (deKort and Granger, 1981). Thus, JH must be removed during the last larval stadium in order for insects to develop into pupae and adults.

The JH titers that control these morphological changes are regulated by both biosynthesis and degradation (Weirich *et al.*, 1973; Hammock, 1985). The reduction of JH titer in the last larval stadium is associated with a dramatic increase of a JH-specific esterase (JHE) that efficiently hydrolyzes JH into biologically inactive JH-acid (Hammock, 1985). The crucial role played by JHE in insect metamorphosis has been demonstrated by two remarkable morphological effects. First, when JHE was inhibited, an elevated JH titer *in vivo* kept the larvae in the feeding stage, resulting in giant larvae (Sparks and Hammock, 1980). Second, an injection of JHE (or the *in vivo* expression of JHE in a baculovirus vector) showed an anti-JH effect, resulting in dose-dependent blackening and decreased feeding by early larval instars as a biological indication of low JH titers (Hammock *et al.*, 1990; Philpott and Hammock, 1990). The second major pathway for JH inactivation is

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the conversion of the 10,11-epoxide to 10,11-diol function by JH-epoxide hydrolase (JHEH). JHEH may therefore function as the ultimate scavenger for JH by hydrating JH-acid to JH-acid-diol, an effect recently documented in eggs of *Manduca sexta* (Touhara and Prestwich, 1993, 1994; Touhara *et al.*, 1994a), thus preventing recycling of JH and JH-acid via the JH acid methyl transferase route (Sparagana *et al.*, 1985).

Endocrine signaling pathways require transport systems for secreted hormones. In lepidopterans, a high-affinity JH-binding protein (JHBP) transports lipophilic JH in hemolymph to the target tissues (Kramer *et al.*, 1974; Goodman and Chang, 1985). It has been proposed that JHBP may protect JH from nonspecific adsorption to lipid depots (Hammock *et al.*, 1975) and nonspecific enzyme action (Kramer *et al.*, 1974; Sanburg *et al.*, 1975). JHBP may also modulate JH degradation at critical developmental stages (Hammock, 1985).

In this study, we have used homogeneous recombinant JHBP (rJHBP) (Touhara *et al.*, 1993) and JHE (Bonning *et al.*, 1992) obtained from baculovirus-infected insect cells to investigate the mechanisms by which JHBP influences JH degradation during the last larval stadium. Use of an *in vitro* model suggests how JH, JHBP, and JHE interact during the early last larval instar, a critical period for insect development (Prestwich *et al.*, 1994). While this study provides evidence in support of the mechanisms hypothesized earlier (Hammock *et al.*, 1975; Sanburg *et al.*, 1975), the key result is that JHBP *facilitates* clearance of JH by JHE by increasing the accessibility of JH to developmentally specific degradation to JH-acid.

MATERIALS AND METHODS

Chemicals and materials

Optically active [^{3}H](10*R*,11*S*)-JH I (58 Ci/mmol, >95% enantiomeric excess) (Prestwich and Wawrzeńczyk, 1985) and a spectrophotometric substrate [1-hexylthioacetothioate: $CH_3(CH_2)_5SCH_2C(O)SCH_3$] (McCutchen *et al.*, 1993) were synthesized at Stony Brook and Davis, respectively. Purified recombinant *Manduca sexta* JHBP (rJHBP) and recombinant *Heliothis virescens* JHE (rJHE) were obtained as described previously (Touhara *et al.*, 1993; Shiotsuki *et al.*, 1994). *Manduca sexta* eggs were obtained from Dr J. S. Buckner (USDA, Fargo, ND), and insects were raised (14 h L:10 h D; 27°C) on an artificial diet (Bio-Serv) (Goodman *et al.*, 1985). Hemolymph of *M. sexta* (fifth larval instar, day 3–day 4, body weight 6–8 g) was collected and processed as previously described (Lerro and Prestwich, 1990).

Enzyme assays for JH I

JHE assays were performed at 30°C in a glass tube (6 × 50 mm; Kimble) precoated with 1% polyethylene glycol 20,000. The TLC plate (silica gel LK6D; Whatman) was developed in hexane/EtOAc/HOAc (66:32:2) (Hammock and Roe, 1985), and analyzed by radio-TLC scanning using an Imaging Scanner (Bio-Scan, System 500) as described previously (Touhara and Prestwich, 1993).

Hemolymph of *M. sexta* (fifth larval instar, day 3–day 4) was diluted either 400- or 4000-fold with 100 mM phosphate buffer (pH 7.4), and then incubated with 300 nM [^{3}H]JH I for 2–15 min. In addition, 300 nM [^{3}H]JH I preincubated with 0.2, 0.4, or 1 μM rJHBP for 10 min at 30°C was added into 400-fold diluted hemolymph to initiate the enzyme reaction.

Pure rJHE or porcine liver carboxylesterase (Sigma) (75 nM) was incubated with 300 nM [^{3}H]JH I for 2–15 min. Porcine carboxylesterase (75 nM) was also incubated with 300 nM [^{3}H]JH I preincubated with 1 μM rJHBP. In addition, 300 nM [^{3}H]JH I preincubated with 1 μM rJHBP was added into 400-fold diluted hemolymph from *M. sexta* containing 10, 20, 75, or 750 nM rJHE or porcine carboxylesterase.

Enzyme assay for spectrophotometric substrate

Esterase activity for the spectrophotometric substrate was assayed by following the method described elsewhere (McCutchen *et al.*, 1993). Thus, the release of 5-thio-2-nitrobenzoic acid was measured for 3 min at 405 nm in a plastic cuvette using a spectrophotometer (Ultrospec II; LKB).

Steady-state kinetics for JH hydrolysis

Kinetic analysis of JH I hydrolysis by 400-fold diluted hemolymph was carried out using various concentrations of [^{3}H]JH I (ranging from 50 to 330 nM) with or without preincubation with 1 μM rJHBP. The incubation time was 1 min without rJHBP and 15 min with rJHBP at 30°C. The reaction was analyzed by TLC as described above. The assay was replicated twice, and kinetic parameters were derived from double reciprocal plots.

Removal of JHBP from hemolymph by immunoaffinity chromatography

The anti-rJHBP antibody (Touhara *et al.*, 1993, 1994b) (750 μl serum) was coupled to protein A beads (0.15 g) (Sepharose CL4B; Pharmacia) by following a general protocol (Harlow and Lane, 1988). Pre-immune serum was utilized to make a control resin. The antiserum-modified protein A beads were gently shaken with 800 μl *M. sexta* hemolymph (fifth larval instar, day 3–day 4) for 3 h at 4°C, and the supernatant was collected. The sample was again incubated with a resin to provide "JHBP-free" hemolymph. The residual JH-binding activity was determined using a modification of the dextran-coated charcoal (DCC) assay (Touhara *et al.*, 1993).

Assay in the presence of a "refractory pool"

A droplet of mineral oil (2 μl) (E. R. Squibb & Sons Inc.), preequilibrated with 1.3 μM [^{3}H]JH I, was carefully layered on to the surface of a 100 μl aliquot

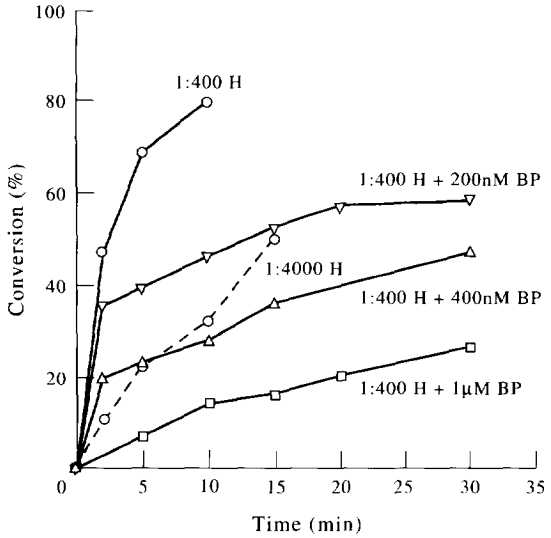

FIGURE 1. Rate of JH hydrolysis by diluted *M. sexta* hemolymph as a function of rJHBP concentration. [^{3}H]JH I (300 nM) that had been preincubated with exogenous rJHBP at 30°C for 10 min prior to enzyme reaction, was added to diluted hemolymph of fifth larval instar *M. sexta* (day 3–day 4) that contained endogenous JHE. "Conversion" denotes hydrolysis to JH-acid. Solid lines: 400-fold diluted hemolymph in the absence (○) or presence (▽, 200 nM; △, 400 nM; □, 1 μM) of rJHBP. Dotted line: 4000-fold diluted hemolymph without added rJHBP. Experiments were performed in triplicate, and the data were averaged (SD ≤8% of the reported values).

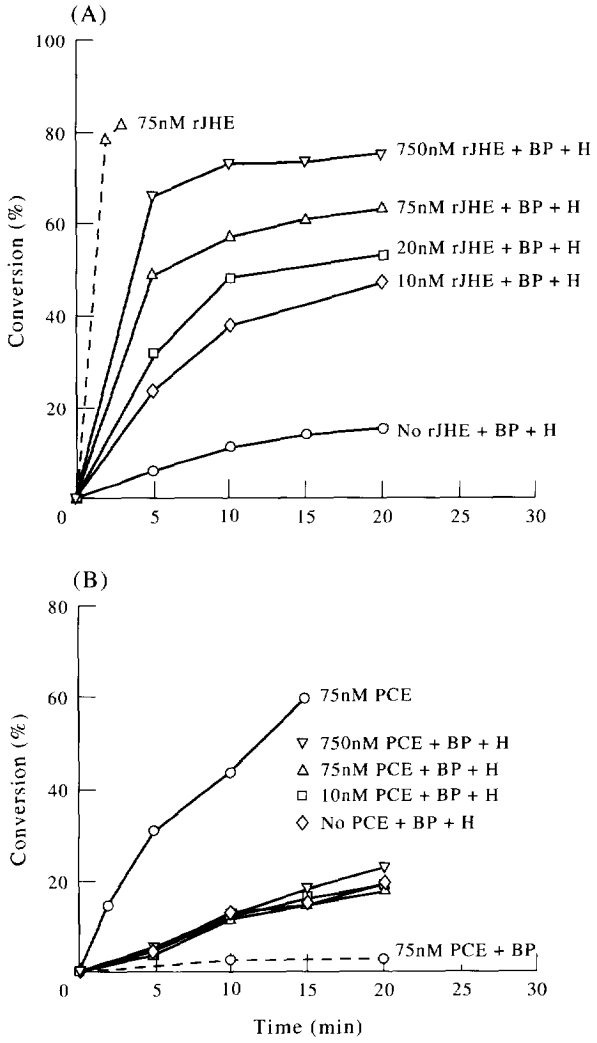

FIGURE 2. Effect of rJHE (A) or porcine liver carboxylesterase (B) on the rate of JH hydrolysis in the presence of rJHBP. (A) Diluted hemolymph (400-fold) containing rJHE (▽, 750 nM; △, 75 nM; □, 20 nM; ◇, 10 nM; ○, 0 nM; solid line) was incubated with 300 nM [^{3}H]JH I that had been preincubated with 1 μM rJHBP. As a control (△, dotted line), 75 nM rJHE in the absence of rJHBP. (B) Diluted hemolymph (400-fold) containing porcine liver carboxylesterase (▽, 750 nM; △, 75 nM; □, 10 nM; ◇, 0 nM) was incubated with 300 nM [^{3}H]JH I that had been preincubated with 1 μM rJHBP. "Conversion" denotes hydrolysis to JH-acid. ○, 75 nM carboxylesterase in the absence (solid line) or presence (dotted line) of 1 μM rJHBP. Experiments were performed in triplicate, and the data were averaged (SD ≤7% of the reported values).

(100 mM phosphate buffer, pH 7.4) containing 750 nM rJHE and 1 μM rJHBP, or only 750 nM rJHE without rJHBP present. After a period of incubation at 30°C without agitation, an aliquot was carefully collected from the aqueous phase without disturbing the oil layer, and the radioactivity was counted using a liquid scintillation counter (LKB RackBeta Liquid Scintillation Counter, model no. 1218) using Scintiverse II (Fisher). Another aliquot from the aqueous phase was analyzed by TLC as described above. A control experiment was performed using 750 nM bovine serum albumin (BSA) in the absence of both rJHE and rJHBP.

RESULTS

Clearance of JH could occur purely by mass action, i.e. distribution of JH to JHBP and JHE solely on the basis of bimolecular binding and catalytic constants.

TABLE 1. Apparent kinetic parameters at a low concentration of JHE

	K_m (nM)*	V_{max} (nmol/min/ml)†
−JHBP‡	290	78
+JHBP§	280	2.5

*Values are averages of duplicates.
†V_{max} values were obtained from Lineweaver–Burk plots.
‡Diluted L5D3 *M. sexta* hemolymph (1:400) was incubated with various concentrations of [^{3}H]JH I in the absence of rJHBP.
§[^{3}H]JH I was preincubated with 1 μM rJHBP prior to addition of diluted hemolymph.

Alternatively, selective protein–ligand–protein or protein–protein interactions might occur, which would add trimolecular complexity to the JH–JHBP–JHE system. Thus, the concentrations of JHBP and JHE were varied over a wide range, mimicking a variety of physiological and nonphysiological situations.

Effect of JHBP concentration on the rate of hydrolysis of JH

Hemolymph from *M. sexta* (fifth larval instar, day 3–day 4) was diluted 400- or 4000-fold to lower the endogenous JHBP concentration. In this case, the rate of JH hydrolysis was consistent with first-order kinetics for

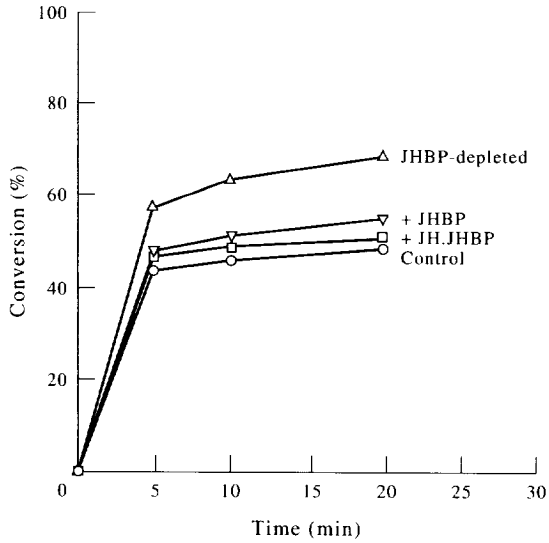

FIGURE 3. Time course of JH hydrolysis by undiluted hemolymph of *M. sexta*. Crude hemolymph of fifth larval instar *M. sexta* (day 3–day 4) was passed through immunoaffinity columns coupled to either anti-rJHBP containing rabbit serum (JHBP-depleted hemolymph) or preimmune serum (control), and then incubated with 15 nM [^{3}H]JH I at 30°C. Next JHBP-depleted hemolymph was augmented with 400 nM rJHBP, and the reaction was initiated by adding 15 nM [^{3}H]JH I. In parallel, the 15 nM [^{3}H]JH I that had been preincubated with 400 nM rJHBP was added to the JHBP-depleted hemolymph. "Conversion" denotes hydrolysis to JH-acid. △, JHBP-depleted hemolymph; ○, control; ▽, JHBP added *without* preincubation; □, JHBP added *with* preincubation. Experiments were performed in duplicate, and the data were averaged.

JHE concentration (Fig. 1). Next, 300 nM [^{3}H]JH I that had been pre-incubated with purified rJHBP was added to the 400-fold diluted hemolymph. In this case, the time course of the hydrolysis in the presence of 200 or 400 nM rJHBP was biphasic (Fig. 1), and a shift in the inflection point was observed with increasing JHBP concentration. The second slower phase of the rate of JH hydrolysis appeared to be limited by the rate of dissociation of the JH–JHBP complex, and the essentially parallel curves after 5 min show that metabolic rates are the same regardless of rJHBP concentrations. Thus, the rate in the second phase was independent of the amount of rJHBP added. Addition of 1 μM rJHBP, sufficient to bind virtually all of JH I applied (300 nM) based on the JH–JHBP binding affinity (Touhara *et al.*, 1993), gave a linear rate of JH hydrolysis (Fig. 1).

Apparent relative kinetic parameters for JH hydrolysis were obtained using 400-fold diluted hemolymph with or without preincubation of various concentrations of [^{3}H]JH I with 1 μM rJHBP (Table 1). The calculated specific activity for JHE in 400-fold diluted hemolymph was 3.9 nmol/min/mg based on the protein concentration of hemolymph at this stage (20 mg/ml), consistent with earlier data (Abdel-Aal and Hammock, 1986; Venkatesh *et al.*, 1990). The apparent V_{max} value was substantially lowered by the presence of rJHBP; in contrast, the apparent K_m value remained essentially unchanged. Thus, the JH–JHE binding and catalytic interactions were unchanged, but the reduced rate of hydrolysis was simply due to the decreased availability of substrate. For *low* JHE concentration in the presence of hemolymph components, it appears that JHE and JHBP only interact with free JH by mass action.

To test for a direct inhibitory effect of JHBP on JHE activity, we utilized a spectrophotometric substrate, 1-hexylthioacetothioate, that could be hydrolyzed by JHE, but did not bind JHBP. This surrogate substrate (McCutchen *et al.*, 1993) was designed to have high, selective turnover by JHE but negligible affinity for JHBP. Indeed, the rate of hydrolysis of 1-hexylthioacetothioate was unaffected by 1 μM rJHBP (Touhara, 1993), indicating the absence of a direct inhibition of JHE activity by JHBP. Thus, at a *low* JHE concentration, the rate-limiting step for hydrolysis of bound JH must be the dissociation of JH from JHBP.

Effect of esterase concentrations of hydrolysis of JH

The next step in mimicking the *in vivo* condition that occurs early in the last larval stadium is to increase rJHE concentration of the sample in the presence of rJHBP. Addition of an increasing amount of purified rJHE resulted in a concentration-dependent increase of the rate of hydrolysis for the bound JH [Fig. 2(A)]. Yet, the rate of hydrolysis of bound JH was slower than that of free JH [Fig. 2(A)]. In contrast, the porcine liver carboxylesterase (PCE, a "general esterase") did not affect the rate of hydrolysis (Fig. 2(B)]. The general esterase exhibited a lower catalytic activity for JH than did rJHE, and the hydrolytic activity was completely inhibited by JHBP [Fig. 2(B)]. These results show that while JHE

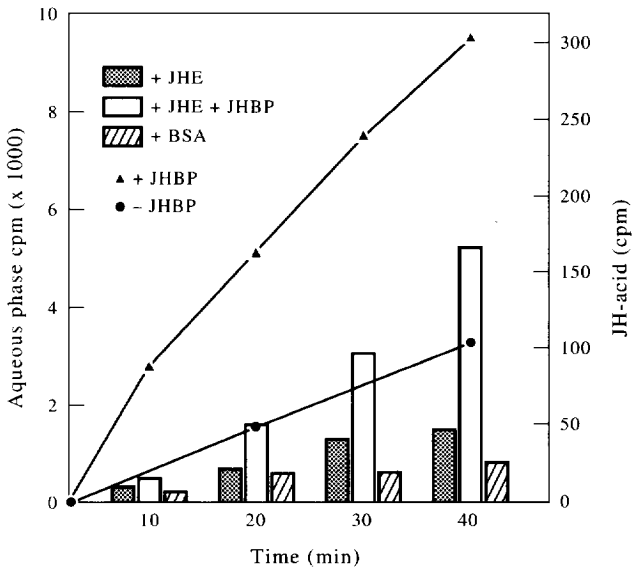

FIGURE 4. Effect of JHBP on the rate of JH-acid formation from JH in a "refractory pool." A droplet of mineral oil (2 μl) containing 1.3 μM [^{3}H]JH I was applied on to the surface of rJHE solution (750 nM) in the presence (open bars, △) or the absence (filled bars, ○) of rJHBP (1 μM). Radioactivity in aqueous phase (5 μl) was measured using a scintillation counter (bars). The amount of JH-acid in the aqueous phase was measured by a radio-TLC assay (lines). BSA (750 nM) was utilized as a control (shaded bars). Experiments were performed in triplicate, and the data were averaged (SD ≤21% of the reported values).

could hydrolyze JH even in the presence of JHBP, a general esterase could not effectively hydrolyze JH when JHBP was present.

Hydrolysis of free and bound JH by crude hemolymph

To further address the *in vivo* situation, undiluted crude hemolymph from fifth larval instar (day 3–day 4) was employed. The hemolymph was passed through an immunoaffinity resin containing anti-rJHBP antibodies to deplete the endogenous JHBP. As a control, a resin coupled to pre-immune antibodies was used. About 60% of the total JH-binding activity was removed as determined by a JH binding assay (Touhara *et al.*, 1993). The remaining binding activity originated from residual JHBP and from lower-affinity JH binding substances in hemolymph.

Partial removal of endogenous JHBP affected the time profile of hydrolysis of 15 nM [^{3}H]JH I (Fig. 3), albeit not dramatically. Although the initial rate of hydrolysis was increased, addition of 400 nM rJHBP to this JHBP-depleted hemolymph restored the profile found for the control resin-incubated hemolymph (Fig. 3). Thus, JHBP in hemolymph can affect the rate of hydrolysis in this model system, even though the presence of other low-affinity JH binders does not allow rigorous kinetic analysis. The rate profile for JH that had been preincubated with JHBP was essentially identical to that of JH added directly into the JHBP–JHE solution (Fig. 3). A biphasic rate profile, similar to that in Fig. 2(A), was also observed.

Effect of JHBP on the rate of hydrolysis on JH in a putative "refractory pool"

A more complex model related to the *in vivo* environment can be simulated by adding a "lipid refractory pool" that absorbs lipophilic JH. We used a mineral oil droplet preequilibrated with [^{3}H]JH I as the so-called "refractory pool." The oil droplet was carefully applied on to the surface of the samples containing rJHE (750 nM), BSA (750 nM), or both rJHE (750 nM) and rJHBP (1 μM). The presence of JHBP greatly improved the "solvation" of JH into the aqueous phase (Fig. 4). The formation of JH-acid in the presence of JHBP was also faster than in the absence of JHBP. JH-acid was the predominant [^{3}H]JH I derivative in the aqueous phase, indicating that JH extracted from the oil phase by JHBP was quickly hydrolyzed by JHE. Again, JHE effectively hydrolyzed JH in the presence of excess JHBP in this model system. This experiment showed that both JHBP and JHE are required to remove and inactivate JH efficiently from a lipophilic pool. These observations support the notion that JHBP serves to effect rapid hydrolysis of JH in the presence of high concentrations of JHE during critical periods in insect development. *In vivo*, JHBP could also *prevent* adsorption of JH into lipophilic tissues; thus, its combined action with JHE to remove JH is likely to be even more dramatic than that found *in vitro*.

DISCUSSION

Numerous studies have suggested transport and "protective" roles for hemolymph JHBP in Lepidoptera (Goodman and Chang, 1985). It has been also postulated that JHBP regulates JH titer by "protecting" the hormone from enzymatic degradation (Hammock *et al.*, 1975; Sanburg *et al.*, 1975). However, absolute "protection" from enzymatic degradation is not realistic in a dynamic situation. Indeed, JHE in the last larval instar is known to hydrolyze JH even in the presence of JHBP (Sanburg *et al.*, 1975). Thus, a number of questions arise. What is the mechanism of JH hydrolysis by JHE in the presence of JHBP? Can it be explained on the basis of simple bimolecular mass action as suggested by Abdel-Aal and Hammock (1988), or are specific trimolecular protein-ligand-protein interactions required? Does JHBP modulate JH degradation by JHE at critical developmental stages? This study addresses these questions experimentally in a model system using hemolymph and recombinant JHBP from *M. sexta* and recombinant JHE from *H. virescens*.

Although the use of hemolymph is physiologically relevant, it is too complicated to demonstrate the interactions among JHBP, JHE, and JH. Thus, we first set up an experiment using diluted hemolymph, in which the JHBP and JHE concentration is lowered by 400-fold dilution with buffer. With this system, exogenous pure JHBP and JHE were added over a wide range of concentrations and ratios. Our data for diluted hemolymph, e.g. conditions simulating low JHE concentration, were consistent with the previous kinetic analysis using diluted hemolymph of *Trichoplusia ni*, which argued in favor of the model based on mass action (Abdel-Aal and Hammock, 1988). Based on the previously determined K_D for the JH I–JHBP complex (Touhara *et al.*, 1993), the calculated amount of free JH in our experiments approximated the amount of JH degraded at an initial high rate by JHE.

In marked contrast, however, data obtained at high JHE concentrations demonstrated that JHE hydrolyzed JH present predominantly as the JH–JHBP complex at a *slower* rate than it hydrolyzed JH in the absence of added JHBP, as expected, but at a *faster* rate than would be predicted by the slow dissociation rate of JH from JHBP. Moreover, a general esterase that had moderate catalytic activity could not hydrolyze JH present predominantly as the JH–JHBP complex even at high esterase concentration, consistent with the previous report for the protective role of JHBP from nonspecific enzymes in the absence of JHE (Hammock *et al.*, 1975; Sanburg *et al.*, 1975). Since nonspecific esterases in lepidopteran hemolymph have very low catalytic activity for conjugated esters such as JH (Sparks and Hammock, 1979, 1980), the porcine liver carboxylase was selected as a control to test for a protective function of JHBP.

The data presented herein can best be accommodated by a system in which an interaction occurs either between JHBP and JHE or between the JH–JHBP complex

and JHE. Two models could explain the mechanism of hydrolysis of JHBP-bound JH. First, JHE could recognize the ester moiety of JH during partial dissociation from JHBP. It could then initiate dissociation of JH by ester hydrolysis since JH-acid has a 1000-fold lower affinity for JHBP. Alternatively, JHE could interact with JHBP via protein–protein interactions, leading to a conformational change and subsequent release and hydrolysis of JH. Indeed, preliminary circular dichroism experiments (Wojtasek H., Touhara K. and Prestwich G. D., unpublished results) with rJHBP and JHE provide evidence for the possibility of protein–protein interaction between the binding protein and esterase in the absence of added JH I. We suggested a model based on the clam-shell interpretation of JH binding by JHBP (Touhara and Prestwich, 1992) as reinforced by studies on *H. virescens* JHBP (Wojtasek and Prestwich, 1995 and protection of JH from JHEH action by JHBP (Touhara and Prestwich, 1994). In this model, JH would bind to JHBP with the epoxide moiety buried in the interior of the JHBP, placing the crucial methyl ester in a solvent-exposed location.

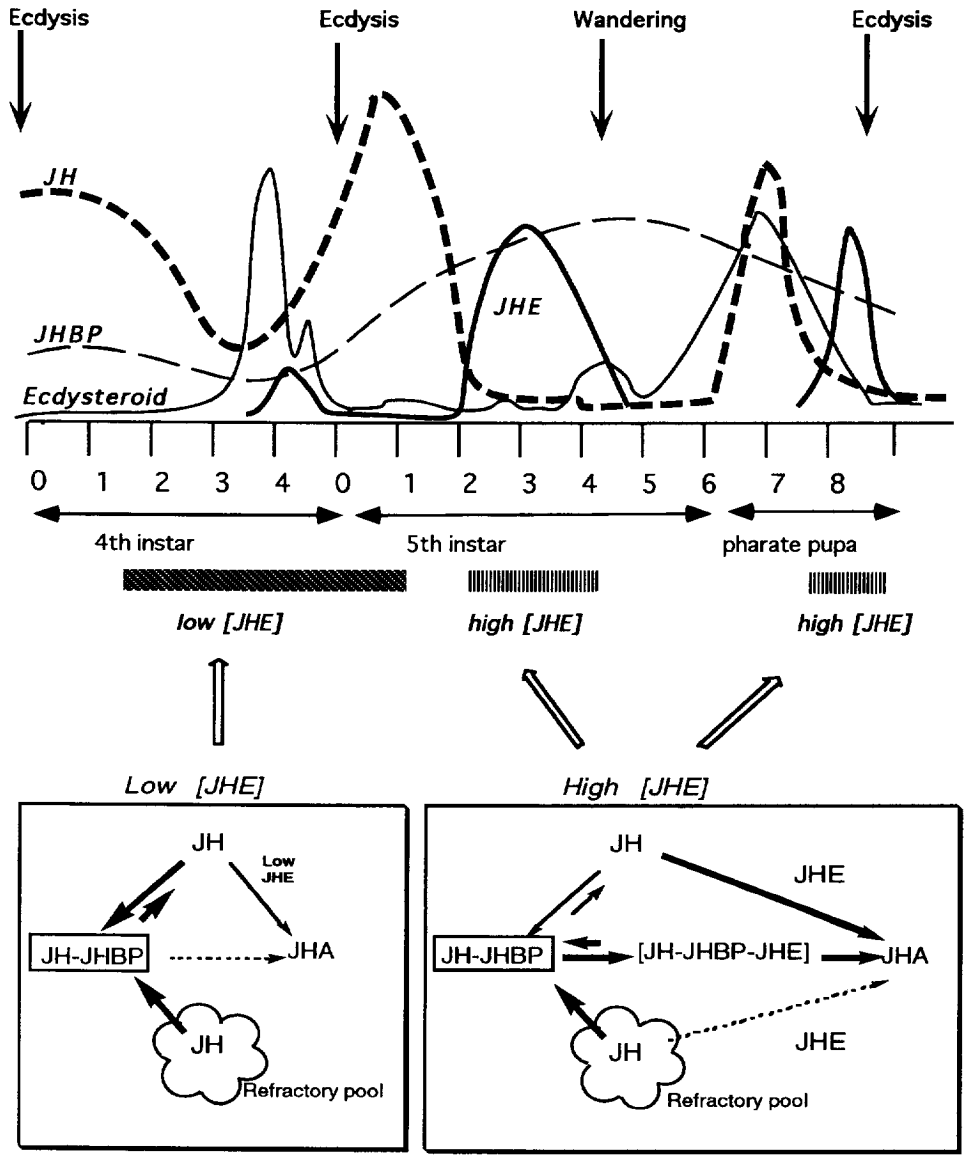

FIGURE 5. Schematic diagrams of proposed mechanism of JH metabolism in hemolymph of the fifth larval instar. Top: stylized composite of endocrine events occurring in a lepidopterous larva (adapted from Riddiford (Riddiford *et al.*, 1986)). Titers of JH (heavy dashed line) (Baker *et al.*, 1987), JHE (heavy solid line) (Weirich *et al.*, 1973; Hammock, 1985), ecdysteroids (light solid line) (Bollenbacher *et al.*, 1981), and JHBP (light dashed line) (Goodman, 1985) were from the last instar of *M. sexta*. The vertical scale for each titer is different. Bottom: protein–ligand interactions occurring at low and at high JHE concentrations. At low concentrations of JHE, hydrolysis of JH by JHE in the presence of JHBP can be explained by mass action. As the concentration of JHE increases, the hydrolysis of the bound-JH by JHE becomes apparent, in which mass action bimolecular kinetics do not account for all observed results. Virtually all JH is present in a JHBP-bound form, and JHBP prevents adsorption of JH into refractory pools. Bold arrows, predominant pathways; dotted arrows, minor pathways; "refractory pool," lipid depots or lipophilic tissues.

The model that leads to the more rapid metabolism of JH than could be predicted by simple kinetic dissociation from the rJHBP is of course, more complex than the simplistic notion of the ester moiety of JH exposed in solution. Based on analogy to the X-ray structure of lipases and other esterases, one assumes that the catalytic site of JHE is very deeply imbedded in the protein and that it will be unable to "clip off" an exposed ester end of JH while it is bound to the JHBP. A more realistic scenario is that both the JHE and JHBP offer a hydrophobic path channeling the JH to a catalytic site and high affinity binding site, respectively. Possibly, at the high levels of JHE used in these experiments, the proximity of the molecules allows JH to partially enter the catalytic cleft of JHE while partially bound to the JHBP. The very close proximity of JHE and JHBP either shifts the kinetic association constant by preventing JH from reattaching in the active site from a high local concentration or actually offers JH two thermodynamic minima while it is partially attached to both molecules. Either of these situations would increase the apparent dissociation rate of JH from the JHBP. Thus, one may view JHBP as a delivery mechanism for proper presentation of JH to JHE form efficient hydrolysis, but not to other enzymes such as general esterase or JH epoxide hydrolase.

The *in vivo* concentrations of JHBP in the last *Manduca* larval instar have been reported to be between 0.2 and 1 μM, depending on the age of the larva and assay method (Wing *et al.*, 1984; Goodman, 1985). The *in vivo* concentrations of JHE in the last stadium increase to 300–500 nM (Abdel-Aal and Hammock, 1986; Venkatesh *et al.*, 1990). Thus, the concentrations of JHBP and JHE in our experiments approximated the *in vivo* situation. However, the *in vivo* JH titer is much lower that that utilized in our *in vitro* experiments. Thus, we set up experiments using undiluted hemolymph and a lower JH concentration that allows a reliable quantification of ester hydrolysis. By the time the JHE concentration becomes elevated early in the last stadium, the JH concentration has already been lowered due to a reduction in the rate of biosynthesis. Thus, we may assume that essentially all JH will be in a JHBP-bound form at this stage *in vivo*. The experiments using crude hemolymph also supported the efficient hydrolysis of JHBP-bound JH in *M. sexta* hemolymph. However, we observed a biphasic rate profile. The slower degradation phase could be attributed to effects of other JH-binding proteins in hemolymph or to a "high affinity" form of JH–JHBP.

Lipophilicity of JH facilitates its nonspecific adsorption to lipid depots or tissues *in vivo*. Thus, a more complex model was designed by adding a mimic of the so-called "refractory pool," i.e. lipophilic tissues that may nonselectively adsorb JH. Apparently, JHBP serves to solvate lipophilic JH. This is consistent with the previous report that JHBP retarded both the uptake of JH by lipophilic tissues sites and the subsequent degradation of JH by enzymes within the tissues (Hammock *et al.*, 1975; Nowock *et al.*, 1976). Hemolymph JHBP both transports and protects JH in early larval instars regardless of the concentration of JHE. Then, in the last larval instar when JH must be rapidly and complete inactivated by a high level of JHE, JHBP appeared to assist in the rapid clearance of even low levels of JH in hemolymph and lipid depots. That is, without JHBP, the rapid and complete clearance of JH, which is crucial for the development of adult structures, would not be properly accomplished.

Figure 5 summarizes our model for JH–JHBP–JHE interactions. In the absence of JHE, JHBP keeps JH in the aqueous phase and prevents nonspecific adsorption to lipophilic tissues (Law, 1980) and possibly protects it from degradation by tissue-bound esterases and epoxide hydrolases (Nowock *et al.*, 1976). At low concentrations of JHE, the rate of degradation of JHBP-bound JH by JHE can be adequately predicted by the kinetic constants determined on the isolated protein. As the concentration of JHE increases during the prewandering state and again during the prepupation stage, efficient hydrolysis of the JHBP-bound JH by JHE becomes even faster than one would anticipate based on calculations from kinetic constants determined on isolated proteins. JHBP in the presence of rising JHE titers now assists in the even more efficient removal of JH from refractory pools to effect complete hydrolysis by JHE. Once the ester is cleaved, the JH no longer binds efficiently to JHBP and is free to enter tissues where epoxide hydrolases and conjugation reactions irreversibly inactivate the molecule. At high JHE concentration, the proximity effect and the putative protein–protein interaction could explain the faster rate of hydrolysis relative to the dissociation rate of the JH–JHBP complex.

These mechanisms of JH hydrolysis are consistent with the correlation between JH titer and JHE activity in hemolymph of prewandering larvae (Weirich *et al.*, 1973; Riddiford, 1986, 1994; Baker *et al.*, 1987). Thus, JH titer in hemolymph and its availability in the target tissues are delicately regulated not only by JHE but also by JHBP. The hemolymph is the model system addressed here, but JHE and JHBP also occur in many tissues. Thus, local concentrations of JH, JHBP, and JH receptor proteins all can influence local concentrations of JH. These experimental approaches may be applicable to elucidating the more complex interaction of JH, JHE, and JHBP in tissues (Prestwich *et al.*, 1994).

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