

INTERACTION OF JUVENILE HORMONE WITH CARRIER PROTEINS AND HYDROLASES FROM INSECT HAEMOLYMPH

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Abstract—Haemolymph from six species of lepidopterous larvae was chromatographed on Sephadex G-100 and analyzed for juvenile hormone (JH) binding and hydrolytic activities. A single JH-binding protein with molecular weight of 3×10^4 daltons and two populations of JH-hydrolytic enzymes were identified in all species. The binding proteins displayed distinct hormone affinities ($K_d = 5-77 \times 10^6 \text{ M}^{-1}$), haemolymph titers ($3-50 \times 10^{-7} \text{ M}$) and ionic properties. The smaller hydro-lases EII (mol. wt = 5.5×10^4 daltons) had the higher specific activity toward JH and increased in haemolymph titer by more than 10-fold during the last larval instar. Organophosphate and sulfonyl-halide compounds, and also the carrier proteins, inhibit JH hydrolysis by the larger hydrolases EI (mol. wt $\geq 10^5$ daltons), but they are less effective against EII enzymes. These results support the hypotheses that JH-specific carrier proteins and esterases are widespread in insects and that both play important physiological rôles in the regulation of hormone titer during larval development.

INTRODUCTION

ONE OF the many functions of the haemolymphatic system of insects is the transport of morphogenetic hormones from the site of synthesis to peripheral tissue. In the tobacco hornworm, *Manduca sexta* (L.), the juvenile hormone (JH) is distributed in the blood as a complex with a specific carrier protein (KRAMER *et al.*, 1974) that prevents hydrolytic enzymes from inactivating the hormone (SANBURG *et al.*, 1975a, b). The physical and chemical properties of the homogeneous carrier from *M. sexta* were recently reported (KRAMER *et al.*, 1976b). In order to extend these studies, we have examined several other insects for similar JH-transportation and inactivation systems in haemolymph, and we describe here JH-carrier proteins and hydrolases in five other Lepidoptera.

MATERIALS AND METHODS

Insects

M. sexta eggs were the gift of Dr. J. P. Reinecke, USDA, Fargo, North Dakota and larvae were reared according to BELL and JOACHIM (1976). *M. quinquemaculata* (Haworth) and *Sphinx chersis* (Hübner) were collected locally on tomato plants and ash trees, respectively. *Paratyelois transitella* (Walk.), *Cadra cautella*, *Anagasta kuehniella* and *Plodia interpunctella* were obtained from the U.S. Grain Marketing Research Center laboratory cultures reared on ground wheat media. Haemolymph was collected from young and mature fifth instar larvae according to KRAMER *et al.* (1974) or by cutting off one of the thoracic legs and collecting the blood with a micro-capillary pipet.

* Mention of a proprietary product does not constitute an endorsement by the USDA.

The sample was immediately transferred to a centrifuge tube and spun at 10,000 *g*. The resulting supernatant was frozen, lyophilized and stored at -20°C .

Chemicals

DL-Cecropia JH (methyl trans, trans, cis 3,11-dimethyl-7-ethyl-10,11-epoxytrideca-2,6-dienoate) was purchased from Regis Chemical* Labelled JH (7-ethyl-1,2- ^3H , 11.8 Ci/m-mole or $10\text{-}^3\text{H}$, 13.5 Ci/m M) was from New England Nuclear Corp. Stock solutions of ^3H -JH mixed with unlabeled carrier JH were prepared in 5 mM Tris buffer, pH 7.3 or 8.3. JH-acid was prepared by hydrolyzing JH using a general esterase preparation obtained from gel-filtered *M. sexta* haemolymph (SANBURG *et al.*, 1975a). Other materials used were the same as described previously (KRAMER *et al.*, 1974; SANBURG *et al.*, 1975b; KRAMER *et al.*, 1976b) or of the highest purity commercially available.

Protein concentration

Relative protein concentration was determined by absorbance at 280 nm with a Cary 118C spectrophotometer using the conversion factor of 1.0 absorbance unit equals 1 mg protein per ml.

Gel filtration

Gel permeation chromatography was carried out at 4°C on a 0.9×65 cm column of Sephadex G-100 (Pharmacia) equilibrated with 50 mM Tris, 0.1 M NaCl, pH 7.3. Lyophilized haemolymph (0.2 to 1 ml) was reconstituted in column buffer (0.04 to 0.2 ml) containing 10^{-4} M 1-phenyl-2-thiourea. One ml fractions were collected.

Molecular weight

The molecular weight was measured by calibrated gel filtration (WHITAKER, 1963; FISH *et al.*, 1969) using the following standards: Blue dextran 2000 (V_0 , Pharmacia), bovine serum albumin (6.8×10^4 , Sigma), ovalbumin

(4.3×10^4 , Schwarz-Mann), pepsin (3.5×10^4 , Worthington), chymotrypsinogen (2.5×10^4 , Worthington), α -lactalbumin (1.5×10^4 , Sigma) and ^{14}C -leucine (V_1 , New England Nuclear).

Electrophoresis

Fractions of gel-filtered haemolymph were subjected to electrophoresis in 3.8% (w/v) polyacrylamide gels (Eastman) at pH 8.4 (ORNSTEIN and DAVIS, 1964) and in 5% gels at pH 4.5 (REISFELD *et al.*, 1962). The sample was incubated with 10^{-7} M ^3H -JH before loading of the gel. For liquid scintillation counting, two mm gel slices were extracted with 0.4 ml of 2% sodium dodecyl sulfate (Pierce) at 37° overnight.

Binding activity

Binding of JH to protein was measured at 4° and pH 8.3 by the DEAE filter disk assay of KRAMER *et al.* (1976b), except that a teflon system was used to eliminate active surface binding by JH encountered in glass vessels. Incubations were performed in wells (0.6 cm diameter \times 1 cm deep) drilled in teflon blocks ($15 \times 4 \times 1.5$ cm, Bolab). All solutions containing JH were pipetted using heavy wall teflon tubing (0.027 in ID \times 0.063 in O.D. Merchromatographic) adapted for use with a 5 to 50 μl Finnpiptette. Binding assays were performed in duplicate as follows: while the protein concentration was held constant, the ligand concentration was varied 100-fold (10^{-8} - 10^{-6} M). A second assay was run in parallel in which the protein concentration was one-third that in the first. Bound (JH-BP) and unbound hormone (JH) were measured, and data were treated according to the method of KRAMER *et al.* (1974) using equation (1)

$$\text{JH} - \text{BP} = \text{BP} - \frac{1}{K_a} \cdot \frac{\text{JH} - \text{BP}}{\text{JH}} \quad (1)$$

to determine the association constant, K_a , and the total binding protein concentration in the assay, BP. As a check on the stability of JH at the end of each assay, an aliquot of the incubation mixture was analyzed with the silica gel chromatographic system described below. Binding of JH-acid to haemolymph components was done using the charcoal assay of KRAMER *et al.* (1976a).

Hydrolytic activities

1-Naphthyl acetate (1-NA) hydrolytic activity of gel filtered haemolymph was determined spectrophotometrically (KATZENELLENBOGEN and KAFATOS, 1970). Aliquots of column eluate (25 to 100 μl) were added at room temperature to 1 ml of 0.1 M phosphate buffer pH 7.0 containing 5×10^{-4} M 1-NA and fast red TR salt in acetone. The red color resulting from the conjugate of the product 1-naphthol with the diazonium compound was measured at 625 nm with a Cary 118C. 1-NA hydrolytic activity was expressed as the increase in absorbance at 625 nm in 30 min per mg protein. Activity in polyacrylamide gels was detected according to the procedure of KATZENELLENBOGEN and KAFATOS (1970).

JH hydrolytic activity was measured by a procedure similar to that of SLADE and ZIBITT (1972), except that a monomeric solution of JH was used instead of an emulsion. Hydrolase fractions (0.01 to 0.02 ml) from the gel filtration of haemolymph were incubated with ^3H -JH (10^{-7} - 10^{-6} M) dissolved in 25 mM Tris, pH 7.3 (0.05 ml) at 30°C in polyethylene glycol (PEG, Fisher) coated glass vessels. Treatment of glassware with PEG minimized JH

binding to glass. At various times (15, 30, and 60 min), an aliquot of the reaction mixture was applied directly to a silica gel chromatoplate (Eastman) for product analysis. A methanol wash of the pipet was also applied to the TLC plate. The strip was developed with ethyl acetate-hexane (3:7) and cut into one cm sections for scintillation counting in a Searle Isocap 300 spectrometer. The scintillation cocktail was composed of 500 ml toluene (Research Products), 500 ml methyl cellulose (Pierce), and 4 g Omnifluor (New England Nuclear). Tritium counting efficiency was 18% as determined by the samples channel ratio method. The retention factors (R_f) of JH and its polar derivatives were intact hormone, 0.85; diol ester, 0.60; epoxy acid, 0.15; and diol acid, 0.05. The diol ester and epoxy acid had similar R_f 's to those found by AJAMI and RIDDIFORD (1973) but opposite to those listed by SLADE and ZIBITT (1972) and SLADE and WILKINSON (1974). JH hydrolytic activity was expressed in picomoles JH-acid produced in 30 min per mg protein.

Inhibition of JH hydrolytic activity was tested using 10^{-4} to 10^{-3} M diisopropylphosphorofluoridate (DFP, Sigma), phenylmethanesulfonyl fluoride (PMSF, Sigma), paraoxon (E600, Sigma), ethylenedinitrilo tetraacetic acid (EDTA), mercuric chloride and phenylmercuric acetate (PMA) mixed with hydrolase fractions from gel filtered haemolymph at 4°C for 12 hr or at 30°C for 30 min. The effect of binding protein on hydrolytic activity was measured after incubation of the substrate with the binding protein fractions from gel filtered haemolymph (0.05 ml) for 10 min.

RESULTS

Gel filtration was used to separate the various haemolymph components that interact with JH. Figure 1A shows the Sephadex G-100 elution profiles of 280 nm absorbance, JH binding and 1-NA hydrolytic activity obtained from one ml of mature fifth instar haemolymph of the navel orange worm, *P. transitella*. Macromolecules that bind or hydrolyze JH (see below) were eluted as follows: a single peak of binding activity (BP, peak fraction 33) and two peaks of hydrolytic activity (EI, peak fraction 20 and EII, peak fraction 28). The molecular weights of two of these components were estimated by comparison of elution volumes with those of standard proteins (Fig. 2). BP and EII had apparent molecular weights of 3.1×10^4 and 5.5×10^4 daltons respectively. EI was eluted in the exclusion volume of the column and thus had a mol. wt of $\geq 10^5$ daltons.

Disc gel electrophoretic analysis of the *P. transitella* BP (fraction 33, Fig. 1A) preincubated with ^3H -JH revealed that a single protein was associated with the hormone (Fig. 3). The relative mobility of the protein-hormone complex was 0.40 and 0.70 at pH 8.4 and pH 4.5, respectively. The small peak of radioactivity with a mobility of 1.0 at pH 8.4 was the carboxylic acid derivative of JH generated under the alkaline conditions of the experiment. A similar analysis of the hydrolytic fractions from the Sephadex column showed that a multiplicity of enzymes were present in each peak, with EI consisting of two ($R_{m, \text{pH } 8.4} = 0.25$ and 0.56) and EII of five 1-NA hydrolyzing com-

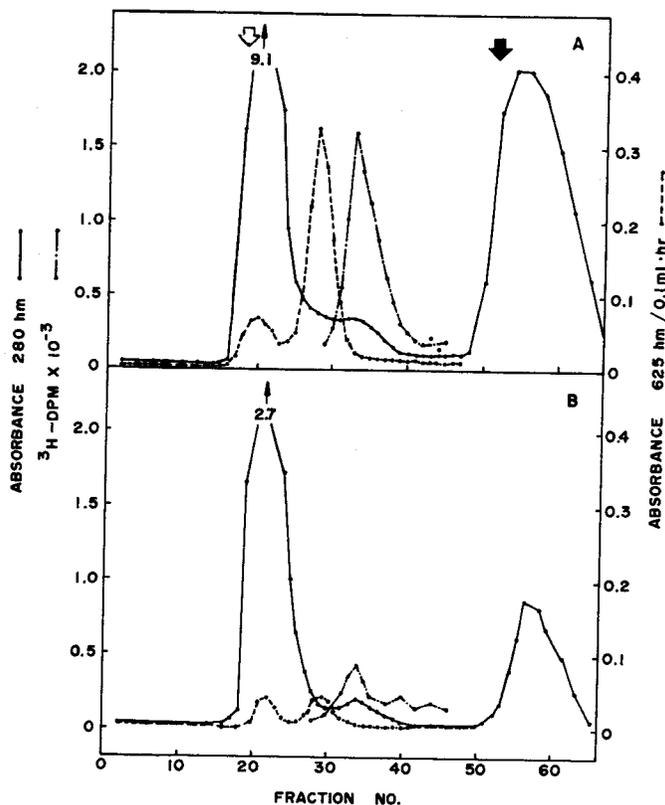


Fig. 1. Gel filtration of fifth instar *P. transitella* haemolymph on Sephadex G-100. Fraction size = 1 ml. Absorbance at 280 nm (protein, —), absorbance at 625 nm (1-NA hydrolytic activity, ·····), JH binding activity (---). A. Mature fifth instar, B. young fifth instar. Open and closed arrows, V_0 and V_b , respectively.

ponents ($R_{m, pH 8.4} = 0.15, 0.27, 0.37, 0.66$ and 0.82). In mature larvae the total 1-NA hydrolytic activity in EII was approximately 40 times greater than the activity in EI (Fig. 1A). Such was not the case for immature larvae where both levels of activity in the haemolymph were nearly the same (Fig. 1B). The total binding protein present at this time was about two-thirds of the concentration found in mature larva.

A survey for JH-binding proteins and hydrolytic enzymes in the haemolymph of other insects using the above techniques demonstrated that similar proteins were also present in *P. interpunctella*, *C. cautella*, *A. kuehniella*, *M. quinquemaculata*, and *S. chersis*. The JH-binding protein had been detected previously in *P. interpunctella* (FERKOVICH *et al.*, 1975). All of these insects had a single JH-binding protein (BP) and two populations of hydrolases (EI and EII), all with molecular weights virtually identical to those determined for the *P. transitella* macromolecules. Also, the EII activities were significantly lower in the young fifth instar larvae than those in the mature larvae. These proteins were found to differ, however, in their overall ionic charge at pH 8.4. The relative electrophoretic

mobilities of the BP's in 3.8% gels were *P. transitella*, 0.40; *P. interpunctella*, 0.45; *C. cautella*, 0.61; *A. kuehniella*, 0.72; *M. quinquemaculata*, 0.80; *M. sexta*, 0.82, and *S. chersis*, 0.85. There was a multiplicity of hydrolytic enzymes as well, each species displaying many enzymes (6–10) with widely ranging electrophoretic mobilities.

In order to ascertain whether the JH-binding proteins were able to act as hormone carriers under physiological conditions, the affinity and specificity of these macromolecules were studied. Figure 4 shows the results of experiments that determined the association constant, K_a , for the JH-protein complex using binding protein from gel filtered haemolymph (fraction 33, Fig. 1A) of *C. cautella*. From the slope and intercept of the upper curve, an association constant of $6.0 \times 10^7 M^{-1}$ and a binding protein concentration of $1.40 \times 10^{-8} M$ were calculated. With the protein concentration diluted by a factor of three, the lower curve was obtained with $K_a = 8.2 \times 10^7 M^{-1}$ and BP = $6.2 \times 10^{-9} M$. These values are within the error of the experimental method ($\pm 25\%$). Association constants of the JH-binding protein complexes

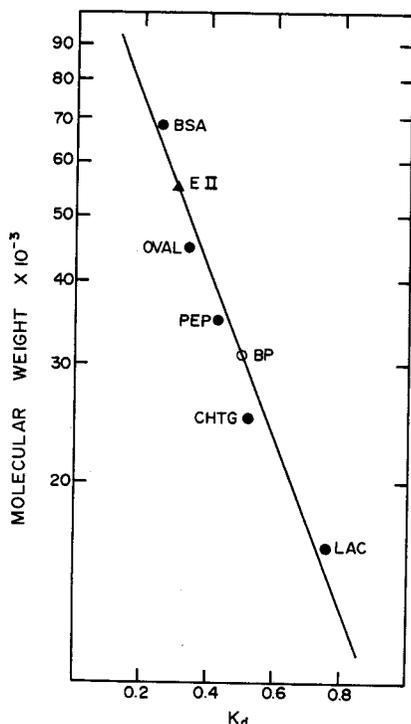


Fig. 2. Determination of the molecular weights of JH-carrier protein (BP) and JH-hydrolase (EII) by gel filtration on Sephadex G-100. Standards were blue dextran 2000 (V_0), bovine serum albumin (BSA), ovalbumin (OVAL), pepsin (PEP), chymotrysinogen A (CHTC), α -lactalbumin (LAC), and ^{14}C -leucine (V_i).

from other insects were determined similarly and are listed in Table 1. The K_a 's varied by a factor of 15, from $4.38 \times 10^6 \text{ M}^{-1}$ for *P. transitella* to $76.70 \times 10^6 \text{ M}^{-1}$ for *P. interpunctella*. By taking into account the appropriate dilution and summing the total JH-binding eluted from the Sephadex G-100 column, the haemolymph concentrations of BP were also estimated (Table 1). These values also differed by greater than an order of magnitude, from $3.1 \times 10^{-7} \text{ M}$ for *A. kuehniella* to $49.7 \times 10^{-7} \text{ M}$ for *M. sexta*. Physiological levels of JH in other insects at this stage of development are usually of the order of 10^{-8} M (SCHOOLEY *et al.*, 1976). If the concentration of JH were similar in the animals used for this study, then hormone in the haemolymph would be associated with the carrier protein to the extent of 80 to 99%, depending on the species. It should be noted that the haemolymph from all insects except the *Manduca* and *Sphinx* species were composite samples from many animals (20 to 200) and results thereupon reflect average values.

To examine whether the binding proteins displayed specificity for JH, the ability of the various haemolymph fractions to associate with the JH-acid metabolite was measured using the charcoal binding assay (KRAMER *et al.*, 1976a). At 100 times the concentration at which JH was bound approximately 50% by gel filtered fractions of binding protein, the acid was complexed less than 10% for all insects studied here. The association constants of the protein for the acid must be at least two orders of magnitude lower than those of the intact hormone. The specificity and affinity for JH, as well as the haemolymph titer of these binding

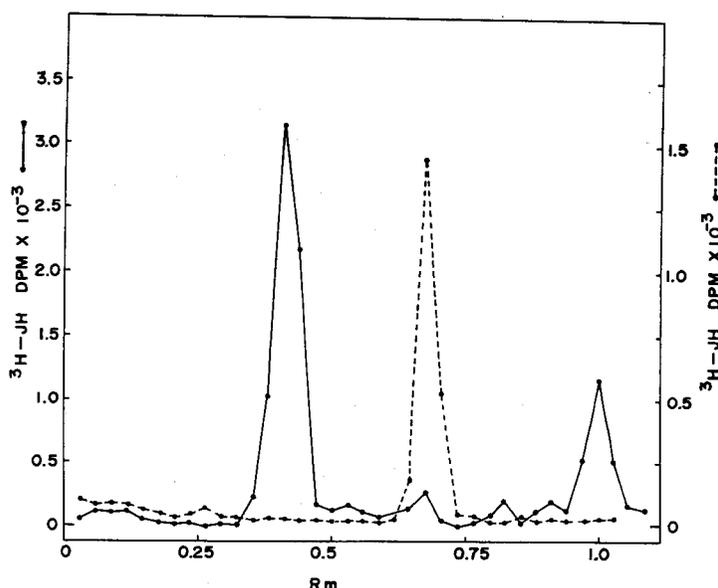


Fig. 3. Radioactivity profile from electrophoresis of JH-carrier protein after incubation of fraction 33 (Fig. 1) with ^3H -JH ($5 \times 10^{-7} \text{ M}$, 13.5 Ci/mM) at pH 7.3. Electrophoresis was performed at pH 8.4 in 3.8% gels (---) and at pH 4.5 in 5% gels (—).

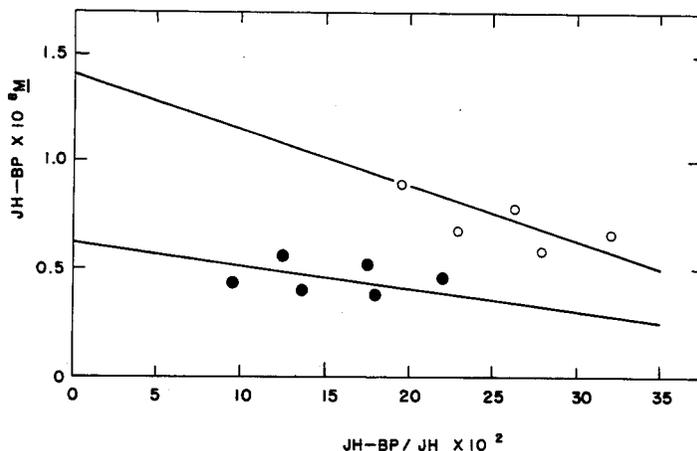


Fig. 4. Plot of binding data according to Eqn. 1 for the interaction of JH with binding protein from gel filtration of *C. cautella* haemolymph. Undiluted (○) and 3 × diluted (●) fraction.

Table 1. Association constants and haemolymph concentrations of JH-binding protein from six Lepidoptera

Species	$K_a \times 10^{-6} M^*$	Haemolymph concentration $\times 10^7 M^{-1}$
<i>P. transitella</i>	4.38 ± 0.12	23.1 ± 1.9
<i>M. sexta</i>	5.02 ± 1.70	49.7 ± 13.3
<i>M. quinquemaculata</i>	11.14 ± 6.10	19.3 ± 1.8
<i>A. kuehniella</i>	18.27 ± 3.56	3.1 ± 0.7
<i>C. cautella</i>	70.32 ± 31.81	8.2 ± 1.6
<i>P. interpunctella</i>	76.70 ± 21.87	30.3 ± 5.8

* 4°C and pH 8.3.

proteins are indeed adequate for a physiological function as hormone carrier in the haemolymph.

With the hydrolases separated from the carrier protein, it was then possible to determine the effect of potential inhibitors and carrier protein on the hy-

drolisis of JH by the hydrolases *in vitro*. Table 2 lists the results of JH hydrolytic assays using fractions obtained from the gel filtration of *P. interpunctella* haemolymph. Part A shows the hydrolytic activities of fractions 22 (EI) and 28 (EII) toward 1-NA and JH. Both groups of enzymes were quite active toward each substrate under our assay conditions, with EII showing the higher specific activity. An examination of the inhibitor specificities showed that chemicals which specifically inhibit metallo (EDTA) or sulfhydryl (Hg^{2+} , phenylmercuric acetate) hydrolases were not effective toward the esterases at a concentration of 10^{-4} – 10^{-3} M, but compounds usually specific for hydrolases possessing a serine residue at the catalytic site were. Quantitative inhibition of both EI and EII occurred with DFP (10^{-3} M), PMSF (10^{-3} M) and paraoxon (10^{-4} M). A closer examination of the effect of DFP revealed that enzymes in EI were more susceptible to inhibition (<5% activity at 10^{-4} M, Table 2, part B), while EII was only par-

Table 2. Activities of haemolymph hydrolases from *Plodia* toward JH and 1-NA.* Effect of DFP and BP

Fraction	Specific activity $\times 10^6$ (JH) (units/mg)	Specific activity $\times 10^3$ (1-NA) (units/mg)	% Inhibition of 1-NA activity	
			JH activity	activity
A. 22 (EI)	8.6	1.2		
28 (EII)	320.2	72.9		
B. 22 + DFP	0	0	100	100
28 + DFP	153.2	4.0	48	95
C. 22 + 32 (BP)	0.8		91	
28 + 32	207.5		61	
D. 22 young	5.7			
28 young	30.6			
E. 22 young + DFP	0		100	
28 young + DFP	0		100	

* Concentration of JH, 1-NA and DFP = 3×10^{-7} M, 5×10^{-4} M and 10^{-4} M, respectively. Reaction time = 30 min, pH = 7.3, and 30°.

tially affected under comparable conditions (50% activity at 10^{-4} M). These data suggest that approximately half of the activity in EII is due to DFP-resistant enzymes that are similar to the JH-specific esterases found in *M. sexta* (SANBURG *et al.*, 1975b), *Schistocerca gregaria* (PRATT, 1975) and *Leptinotarsa decemlineata* Say (KRAMER and DE KORT, 1976b).

Results of experiments listed in Table 1, part C show the effect of carrier protein fractions on JH hydrolysis by the esterases. Like DFP, BP was a potent antagonist of the EI enzymes, affording >90% protection of the hormone under our assay conditions. In contrast, the carrier protein was only 60% effective against the EII hydrolases. These results indicate that the carrier prevents or at least retards the enzymatic hydrolysis of JH in the haemolymph and also that the EII enzymes play the more active rôle in JH degradation. This is in accord with the observation that the titer of the EII hydrolases was very low in the young larval instar (Table 2, part D and Fig. 1B), a time when the JH concentration should be relatively high. No DFP-resistant enzymes were present in the young instar (Table 2, part E).

The interaction of JH with haemolymph *in vivo* was studied using *P. transitella* larvae. One μ l of 10^{-6} M 3 H-JH was injected into the haemocoel of several larvae of approximately equal weight (10 mg) and haemolymph was collected after one, two and four hour incubations at room temperature. A gel filtration profile of radioactivity similar to that in Fig. 1 was obtained. After 1 hr, 45% of the radiolabel in the haemolymph was recovered as hormone associated with the carrier protein. The remaining radioactivity was eluted in the inclusion volume and analyzed on silica gel chromatoplates identically as marker JH-acid. Carrier protein bound 3 H-JH was also detected in larvae after 2 hr (approximately 20% of the total radioactivity), but less than 10% was found after 4 hr incubation. These results show that binding and hydrolysis of exogenous JH occur *in vivo* and that the half-life of added JH is approximately 1 hr under these conditions.

DISCUSSION

The interaction of JH with haemolymph was analyzed by separating the various components on the basis of molecular size and then measuring the binding and hydrolytic activities of individual fractions. This approach has been used previously with the tobacco hornworm (KRAMER *et al.*, 1974; SANBURG *et al.*, 1975b) for the detection of activities that may otherwise mask each other when present in the same solution. In each of the seven insect species examined, single JH-carrier proteins with apparent molecular weights of approximately 3×10^4 daltons were identified in blood. Results of *in vitro* experiments showed that these proteins bound the methyl ester hormone at least 100 times better than the carboxylic acid metabolite and that each had a

distinct ionic nature, as well as affinity for hormone and titer in the haemolymph. The latter two properties may differ by more than an order of magnitude from species to species. Despite this variability, all of the properties of the carrier proteins are in harmony with a physiological rôle in the translocation and protection of the hormone. It seems probable that these hormone carriers belong to a family of homologous proteins which evolved to distribute JH throughout the organism and to maintain the critical hormone titers required for proper growth and differentiation. Results of experiments in which labelled hormone was injected into the haemocoel of larvae indicated that the hormone-carrier protein interaction does occur *in vivo*.

The hydrolytic enzymes present in larval haemolymph of the Lepidoptera can be placed in two classes. Both classes have wide specificity for substrates as indicated by their ability to hydrolyze 1-NA and JH. In terms of physical and chemical properties, they were either moderate in size (mol. wt_{app} = 5.5×10^4 daltons), resistant to inactivation by serine hydrolase inhibitors, and active in hydrolyzing carrier-bound hormone, or they were larger (mol. wt_{app} $\geq 10^5$ daltons), more susceptible to inhibition, and unable to degrade complexed hormone. The property of organophosphate-resistance exhibited by the EII enzymes is reminiscent of that observed with the surface-active lipid esterase, pancreatic lipase (MAYLIE *et al.*, 1972). Like lipase (BROCKMAN *et al.*, 1973), these enzymes may be efficient at hydrolyzing substrates at hydrophobic surfaces (carrier protein binding site) where the hormone accumulates. The EI enzymes were present throughout the duration of the terminal larval instar, but the titer of the EII hydrolases was developmentally time dependent. As was the case with *M. sexta* (SANBURG *et al.*, 1975b), it also appears that JH-specific hydrolases arise in the haemolymph at a specific time during the last larval instar of the insects examined here. Apparently these latter enzymes function to lower hormone titer in the haemolymph in order for metamorphosis to occur in endapterygote insects. When JH was injected into larvae, hydrolysis of the hormone did occur, which suggests that such an enzymatic mechanism does function *in vivo*.

To our knowledge, all lepidopterous larvae examined carefully for JH-binding proteins or hydrolytic enzymes in the haemolymph have been found to contain these kinds of proteins. These species include *M. sexta* (WEIRICH *et al.*, 1973; KRAMER *et al.*, 1974; SANBURG *et al.*, 1975b; GODDMAN *et al.*, 1976), *P. interpunctella* (FERKOVICH *et al.*, 1975; KRAMER *et al.*, 1976a; this report), *P. transitella*, *C. cautella*, *S. chersis* (KRAMER *et al.*, 1976a, this report), *A. kuehniella*, *M. quinquemaculata* (this report), *Spodoptera exigua*, *Estigmene acraea*, *Heliothis virescens*, *Trichoplusia ni* and *Pectinophora gossypiella* (S. MUMBY and B. HAMMOCK, personal communication). Similar methods have not been used as extensively to demonstrate the same macromolecules in haemolymph of other orders

of Insecta. Nevertheless, JH-binding proteins of moderate molecular weight have been identified in the larval haemolymph of the coleopteran, *Tenebrio obscurus* (KRAMER *et al.*, 1976a) and the hemipteran *Oncopeltus fasciatus* (S. BASSI and K. KRAMER, unpublished), whereas JH-hydrolytic enzymes have been detected in haemolymph from the above two insects (K. KRAMER and C. CHILDS, unpublished; S. BASSI and K. KRAMER, unpublished) as well as from the orthopteran *Schistocerca gregaria* (PRATT, 1975) and coleopteran *Leptinotarsa decemlineata* (KRAMER and DE KORT, 1976a, b). However, the exact interplay between binding protein, hydrolases, and the juvenile hormone in non-Lepidoptera has not been examined in detail.

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