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The Juvenile Hormone Binding Protein in the Hemolymph of *Manduca sexta* Johannson (*Lepidoptera : Sphingidae*)

(tobacco hornworm/dissociation constant/specificity/esterases/diisopropylphosphofluoridate)

KARL J. KRAMER, LARRY L. SANBURG, FERENC J. KÉZDY, AND JOHN H. LAW

Department of Biochemistry, University of Chicago, Chicago, Ill. 60637

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ABSTRACT **C_{18}:juvenile hormone is quite soluble in water, yielding a monomeric solution greater than 10^{-5} M. *In vivo* injection or addition of aqueous juvenile hormone to the hemolymph *in vitro* shows the complexation of juvenile hormone to a protein, as demonstrated by gel permeation chromatography and disc-gel electrophoresis. The protein has an apparent molecular weight of 3.4×10^4 and is present in the hemolymph at a concentration in the micromolar range. The binding of the hormone to the protein can be described as a simple thermodynamic equilibrium with a dissociation constant of 3×10^{-7} M, and the protein has a much higher affinity for the hormone than for the hydrolysis products.**

Juvenile hormone (JH) plays a key role in the development of insects from the embryo to the adult (1). It is synthesized by the *corpus allatum* and secreted into the hemocoel. To reach the target cells, it must be transported by the hemolymph. Recent reports suggest the presence of lipoproteins or proteins in the hemolymph which are capable of binding JH and its analogs in the concentration range of 10^{-6}–10^{-5} M (2, 3). In order to establish that these macromolecules are able to fulfill the role of a hormone carrier at physiological concentrations of JH, the chemical nature, affinity, and specificity of these molecules must be demonstrated. With this goal in mind, we investigated the interaction of JH with the hemolymph of the tobacco hornworm, *Manduca sexta*. In this paper we demonstrate the presence of a protein (molecular weight, 3.4×10^4) which binds JH and its geometric isomers with a high degree of specificity.

MATERIALS AND METHODS

Animals. M. sexta eggs were a gift from Dr. R. A. Bell, USDA, Fargo, N.D. The larvae were reared at 27° in a 15-hr light-9-hr dark photoperiod using the standard diet (4), as modified by R. A. Bell.

Chemicals. Pure synthetic *Hyalophora cecropia* juvenile hormone (methyl *trans, trans, cis* 3,11-dimethyl-7-ethyl-10, 11-epoxytrideca-2,6-dienoate) was purchased from Eco Control. An isomeric mixture of *H. cecropia* JH (containing 17% of the natural compound) was a gift from Drs. A. J. Manson, R. Deghenghi, and F. Herr of Ayerst Laboratories Ltd., Montreal. Labeled *H. cecropia* JH (7-ethyl-1,2-[^{3}H], 67 mCi/mg) was purchased from New England Nuclear Corp. The diol ester was prepared by incubating the JH at pH 5 in universal buffer (5) for 1 week. This treatment yielded approximately 90% diol ester, 5% epoxy ester, and 5% diol acid and (or) epoxy acid, as identified by thin-layer chromatography (TLC) (7). The solution containing the reaction products was added directly to the hemolymph for binding experiments. The epoxy acid was prepared by incubating the JH with fifth instar larval hemolymph according to the procedure of Metzler *et al.* (6).

Determination of JH Solubility in Aqueous Buffer. Stock solutions of JH were prepared in petroleum ether containing 1.22×10^{-3} M and 6.5×10^5 dpm/μmol of JH. Aliquots were added to test tubes and the solvent was removed under N_2. To each tube was added 1 ml of 0.02 M Tris·HCl (pH 7.3), and the tubes were stoppered tightly and shaken in a water bath at 24°. After 48 hr, the tubes were centrifuged at $27{,}000 \times g$ for 15 min to remove any emulsion which might be present. Aliquots were then counted in the Nuclear Chicago ISOCAP 300 instrument to determine the amounts of solubilized JH. The hormone was found to be reasonably stable in aqueous solution at pH 7.0: after 2 weeks at room temperature, TLC analysis (see below) of this solution showed 40% intact JH, 54% diol ester, and 6% epoxy acid and (or) diol acid. To detect the presence of high-molecular-weight aggregates, a 0.9 × 23-cm column of Sephadex G-15 (Pharmacia) was calibrated using Blue Dextran and NH_4Cl to determine the exclusion and inclusion volume, respectively. A 0.2-ml aliquot of [^{3}H]JH (3.7×10^{-7} M), solubilized in aqueous solution, was eluted in 0.02 M Tris·HCl buffer and the fractions were counted in the ISOCAP 300.

Rapid Solubilization of JH. JH in organic solvent was transferred to a test tube and evaporated to dryness under N_2. An appropriate amount of aqueous buffer was added to give a final concentration of from 10^{-5} to 10^{-6} M JH. The solution was sonified three times at 25 W for 1 min in an ice bath. This allowed complete solubilization of the JH.

Collection of Hemolymph for In Vitro and In Vivo Studies. Hemolymph was collected from larvae cooled to 4° in ice by cutting off the abdominal horn at its base. The hemolymph was drained into a centrifuge tube containing approximately 50 μg of 1-phenyl-2-thiourea (PTU) to inhibit phenol oxidases. Routinely, 0.5–0.8 ml of hemolymph per animal was collected and mixed with PTU and with a 1/10 volume of 0.2 M Tris·HCl (pH 7.3), and this mixture was centrifuged at $27{,}000 \times g$. For *in vitro* studies, the aqueous JH solution was immediately mixed with the centrifuged hemolymph (10^{-7}–10^{-9} M final JH concentration), and this mixture was incubated for 15–30

Abbreviations: JH, juvenile hormone; TLC, thin-layer chromatography; PTU, 1-phenyl-2-thiourea; DFP, diisopropylphosphorofluoridate; BSA, bovine serum albumin.

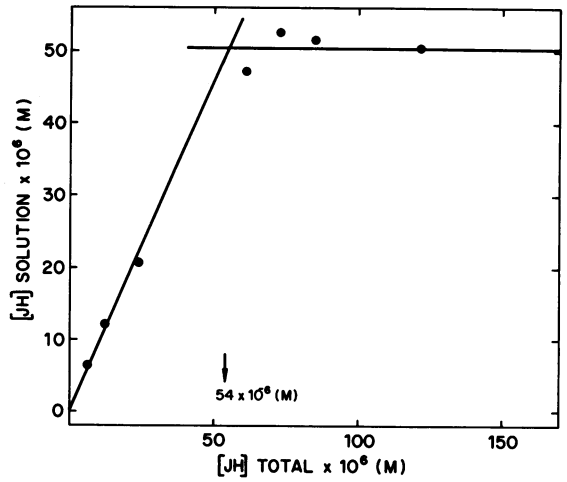

FIG. 1. The solubility of [^{3}H]JH in water containing 0.02 M Tris·HCl (pH 7.3).

min and applied to a Sephadex G-100 column. To inhibit JH esterases, the PTU-treated, centrifuged hemolymph was incubated at 4° for 12 hr with diisopropylphosphorofluoridate (DFP) from Pierce, at a final concentration 10^{-3} M.

For *in vivo* studies, fifth instar larvae, 2–3 days after molting, were cooled in ice prior to injection, and 10 μl of aqueous JH (1×10^{-6} M, 3.7×10^4 dpm/pmol) was injected into one of the terminal prolegs. Hemolymph was later collected as described above. After centrifugation, the supernatant was used for gel filtration studies and an aliquot was immediately counted in the ISOCAP 300; no radioactivity remained in the sediment.

Extraction and Analysis of JH from Hemolymph. Hemolymph (from both *in vitro* and *in vivo* studies) was extracted and analyzed by TLC according to the procedure of Slade and Zibitt (7). The chromatograms (Eastman) were cut lengthwise into 1-cm strips and each 1-cm section of the strip was measured for radioactivity by liquid scintillation counting. The extracts were also analyzed by gas chromatography on OV-17 (Reibstein, D. and Law, J.H., unpublished). Occasionally some diol was generated during the extraction procedure, and for this reason a control extraction was performed routinely to determine if the epoxide had survived the work-up.

Gel Filtration and Binding Experiments. Hemolymph was collected, treated as previously described, and diluted, if necessary, with 0.02 M Tris·HCl (pH 7.3). After incubation with JH (see above) the hemolymph was placed on a 1×46-cm column of Sephadex G-100 at 4° and eluted with 0.02 M Tris·HCl (pH 7.3). The flow rate was 0.5 ml/min and the effluent was collected in 1.0 ± 0.05-ml fractions. The absorbance of the eluate at 280 nm was measured and aliquots were analyzed for radioactivity. Blue Dextran and $CuSO_4$ were used to determine exclusion and inclusion volumes, respectively. For the bovine-serum albumin (BSA) binding experiments, 0.2 ml of 1 mg/ml of delipidated BSA in 0.02 M Tris·HCl (pH 7.3) containing 2×10^{-7} M [^{3}H]JH was chromatographed. For the human serum (gift of Professor A. Scanu) binding experiments, 0.2 ml of unfractionated serum was mixed with 2.5×10^{-8} M [^{3}H]JH before analysis. The gel filtration column was calibrated according to the procedure of Fish *et al.* (8).

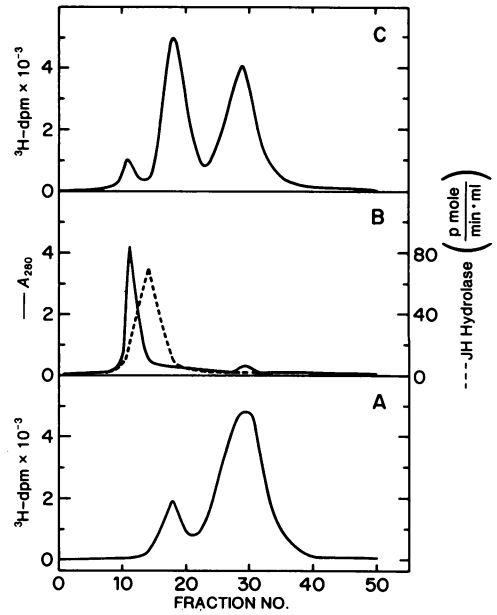

FIG. 2. Gel filtration patterns obtained by chromatography of 0.2 ml of *M. sexta* hemolymph containing [^{3}H]C_{18}:juvenile hormone on Sephadex G-100 in 0.02 M Tris buffer (pH 7.3), 1×45-cm column, 1 ml per fraction. (*A*) *In vivo* injected [^{3}H]JH. (*B*) 280-nm absorbance of the *in vivo* injected [^{3}H]JH hemolymph and JH hydrolase activity (7) using aqueous JH (10^{-7} M). (*C*) DFP-hemolymph + [^{3}H]JH.

Binding experiments were done with DFP-treated hemolymph. The dissociation constant of JH with the binding protein was estimated by gel filtration. For a mol to mol association of JH with the macromolecule, the dissociation constant (K) may be expressed as

$$K = (H \cdot P)/C \qquad [1]$$

where H is the concentration of JH (i.e., the sum of the molarities of all isomers), P is the concentration of the binding protein, and C is the concentration of the hormone-protein complex. Eq. 1 can be rearranged with the use of auxillary equations to give

$$nC = P_h - K(nC)/H \qquad [2]$$

where P_h is the analytical concentration of the binding protein in the hemolymph and n is the dilution factor for the hemolymph. A plot of nC *versus* nC/H yields the dissociation constant from the slope and the concentration of binding protein from the intercept at the ordinate axis. The hemolymph dilution range was from 1- to 40-fold and the initial JH concentration varied from 10^{-7} to 10^{-6} M.

Polyacrylamide Gel Electrophoresis. M. sexta DFP-hemolymph (5 μl) containing 2×10^{-8} M [^{3}H]JH was subjected to electrophoresis at pH 8.4 in 3.8 and 7.5% polyacrylamide (9). Disc electrophoresis at pH 4.5 in 5% polyacrylamide was also run (10). Gels containing [^{3}H]JH alone were subjected to electrophoresis as controls. Protein was visualized by staining with Coomassie Brilliant Blue (Bio-Rad), followed by removal of unbound dye in methanol:acetic acid:water (27:7.5:67.5). The gels were scanned at 560 nm with a Schoeffel Spectrodensitometer. Lipid Crimson (Pfaltz and Bauer) was used to stain for lipid binding proteins (11). For liquid scin-

tillation counting, 2-mm gel slices were minced and eluted with 2% periodic acid using a Gilson gel fractionator.

Pronase Digestion of DFP-Treated Hemolymph. DFP-treated hemolymph (0.2 ml) was mixed with 10 μg of pronase (Calbiochem) and incubated for 10 hr at 4°. [^{3}H]JH (2×10^{-7} M) was added to the pronase-treated DFP-hemolymph and binding was determined by gel filtration on Sephadex G-100. Initial reaction mixture with pronase and DFP-hemolymph incubated for 10 hr without pronase were also chromatographed as controls.

RESULTS

Solubility. Whitmore and Gilbert (2) observed that when hemolymph containing emulsified JH was subjected to gel filtration chromatography, the JH was eluted close to the inclusion volume. This suggested to us that a large portion of the hormone forms a true solution and that the monomeric species might be the predominant state of the unbound hormone in the hemolymph. We therefore determined the solubility of the C_{18}:JH in aqueous solution. As shown in Fig. 1, solutions of concentration as high as 5×10^{-5} M can be prepared in Tris·HCl buffer. The solubility curve of a pure compound should show a slope of one at concentrations below the limit and a sharp discontinuity at the limit. Within experimental error, the curve in Fig. 1 is consistent with these criteria and the discontinuity at 5×10^{-5} M is reasonably sharp, indicating the purity of the labeled hormone. Gel filtration behavior on Sephadex G-15 of JH solubilized in buffer showed the absence of high molecular weight aggregates of the hormone. At least 90% of the radioactive material after elution from Sephadex G-15 cochromatographed on silica gel TLC with authentic JH and thus it did not undergo chemical change during these manipulations.

The solubility curve was determined after lengthy incubation in order to achieve complete equilibration, but the rate of dissolution can be greatly accelerated by brief periods of sonication without changing the solubility characteristics.

In Vivo Studies. Injection of a hexane solution of JH into a larva or pupa resulted in rapid degradation of the hormone (2), presumably by the action of enzymes present in the hemolymph and fat body (12–15). Gel filtration of the hemolymph collected shortly after injection showed, however, that part of the intact hormone was bound to several high-molecular-weight species, most of them probably lipoproteins (2). When an aqueous solution of JH is injected into a fifth instar larval *M. sexta* and the hemolymph collected after 90 min, the presence of a high-molecular-weight species binding JH was also revealed by gel filtration in Sephadex G-100 (Fig. 2*A*). The figure also shows the 280 nm absorbance and the JH esterase activity (ref. 7, and Sanburg, L., Kramer, K., and Law, J.H., unpublished) which was eluted with $K_d = 0.21$ (Fig. 2*B*). No detectable amount of the hormone eluted in the exclusion volume, in spite of the presence of a large amount of lipoproteins in this region. Of the radioactivity in the hemolymph, 20% was eluted in fractions ($K_d = 0.42$), indicating a molecular weight around 3 to 4 $\times 10^4$. These fractions were extracted and shown by TLC and gas-liquid chromatography analyses to contain intact JH. The molecular weight range in which the JH is eluted, together with the high solubility of JH in water, suggests the strong binding of the hormone to a protein carrier in the hemolymph. The remaining 80% of the radioactivity was eluted in the inclusion volume, and TLC analysis showed this material to consist of the epoxy acid, indicating extensive degradation by esterases. These esterases had to be inactivated before we could determine the chemical nature and the physical properties of the binding protein. DFP inactivates the esterases (12), and we therefore turned our attention to an *in vitro* system.

In Vitro Studies. Gel filtration of hemolymph incubated *in vitro* with an aqueous solution of JH resulted in an elution pattern virtually identical to that obtained in the *in vivo* incubation. Preincubation of hemolymph with millimolar DFP (DFP-hemolymph) before adding JH and analysis by gel filtration, showed the major labeled product to be a macromolecular complex, $K_d = 0.42$ (Fig. 2*C*). In contrast to the *in vivo* experiment, a small fraction of labeled hormone was eluted in the exclusion volume, possibly associated with lipoproteins. Furthermore, the material which was eluted in the inclusion volume was the intact hormone rather than its hydrolysis product. Again, the radioactive material recovered by extraction of $K_d = 0.42$ peak was the intact hormone.

Several experiments established the protein nature of the macromolecular JH complex. If DFP-hemolymph was preincubated with pronase before addition of JH, all of the JH was eluted in the inclusion volume of the Sephadex column. Also, the crude hemolymph could be fractionated by ammonium sulfate precipitation and the binding protein was recovered in the precipitate formed between 20 and 60% saturation. Finally, disc-gel electrophoresis of the DFP-hemolymph with added JH showed the presence of distinct protein bands associated with radioactive JH. In Fig. 3*A* is shown a 3.8% polyacrylamide gel obtained by electrophoresis of the DFP-hemolymph and [^{3}H]JH mixture at pH 8.4, together with the 560-nm absorbance due to the Coomassie Blue stain and the radioactivity profile. There were at least 11 anionic components, most of which had mobilities between 0.1 and 0.75: four of these protein bands ($R_F = 0.38$, 0.48, 0.52, and 0.82) also bound Lipid Crimson, and three contained [^{3}H]JH [$R_F = 0.38$ (III), 0.82 (II), and 1.0 (I)]. Band II contained 64% of the radioactivity; band I and II contained 18% each. The radioactivity profile of a 7.5% polyacrylamide gel (Fig. 3*B*) showed two bands: $R_F = 0.55$ (II) and 1.0 (I) containing 83% and 17% of the counts, respectively. Band I is the epoxy acid and diol acid which are present in the JH stock solution and generated under conditions of the electrophoresis at pH 8.4; band II, the JH binding protein; and band III, a high-molecular-weight species (perhaps lipoprotein) which is unable to penetrate the 7.5% polyacrylamide gel. When the DFP-hemolymph and [^{3}H]JH mixture was subjected to electrophoresis at pH 4.5 in 5% polyacrylamide (Fig. 3*C*), a single labeled band was obtained with $R_F = 0.45$. These results indicate that the binding protein is a single species with an isoelectric point between pH 4.5 and 8.4.

Binding Specificity. We studied the interaction of the binding protein with JH metabolites in DFP-hemolymph. As measured by gel filtration, no binding occurred with the epoxy acid at a concentration of 2.5×10^{-6} M or of the diol ester at 7×10^{-6} M. Under identical conditions, 65% of the JH at a concentration of 2.6×10^{-7} M was associated with the binding protein.

In order to assess the ability of lipophilic molecules to bind JH nonspecifically, the interaction of delipidated BSA and

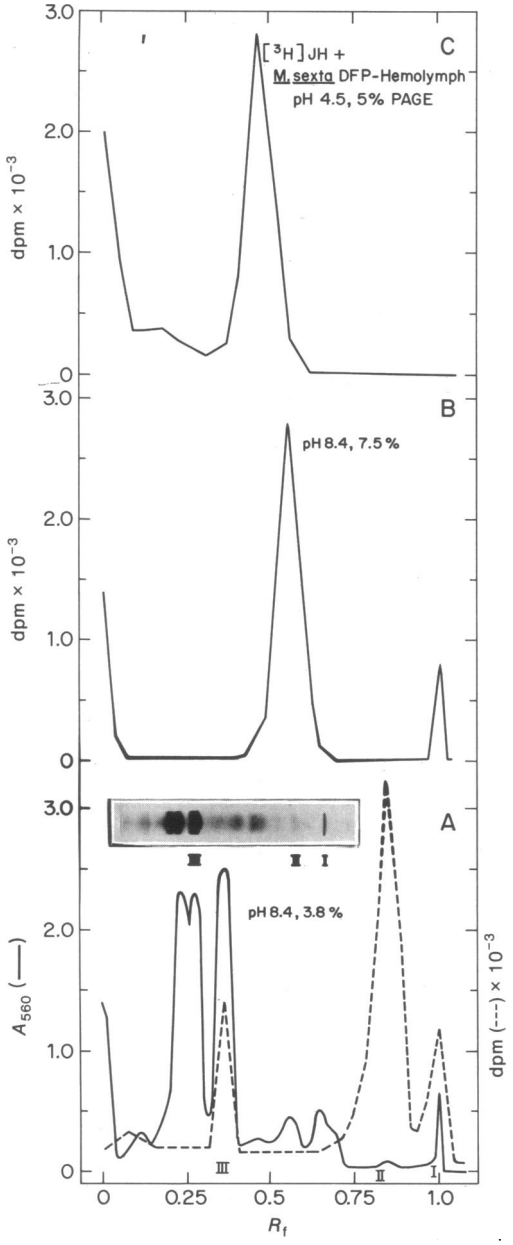

FIG. 3. Polyacrylamide gel, densitometer scan, and radioactivity elution profile from electrophoresis of *M. sexta* larval DFP-hemolymph (2.5 μl) containing 2.5×10^{-8} M [³H]JH. (*A*) pH 8.4; 3.8% polyacrylamide gel electrophoresis; A_{560}, ——; DPM, – – –; anode on right. (*B*) pH 8.4; 7.5% polyacrylamide gel electrophoresis; dpm; anode on right. (*C*) pH 4.5; 5% polyacrylamide gel electrophoresis; dpm; cathode on right.

JH was tested by the gel filtration technique on Sephadex G-100. At a concentration where BSA strongly binds fatty acids or steroids, no binding of JH was detectable. However, when unfractionated human serum was used, 75% of the radioactive JH was eluted in the exclusion volume, suggesting binding to lipoproteins.

Molecular Weight. The molecular weight of the *M. sexta* JH binding protein was determined by gel filtration on a column of Sephadex G-100 calibrated with standard proteins of known molecular weights. By this technique, the binding protein-hormone complex was eluted with a $K_d = 0.42$, corresponding to an apparent molecular weight of 34,000.

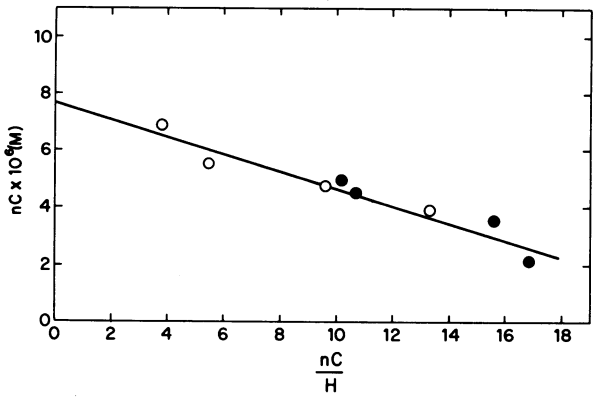

FIG. 4. Plot of binding data according to Eq. 2 for the interaction of *H. cecropia* juvenile hormone isomers with binding protein from *M. sexta* hemolymph. Sephadex G-100 chromatography in 0.02 M Tris·HCl (pH 7.3) at 4°; 10 × (●) and 40 × (○) diluted DFP-hemolymph.

Dissociation Constant. The dissociation constant of the JH-binding protein complex was measured by gel filtration experiments, using varying initial concentrations of JH and DFP-hemolymph. The experimentally measured quantities, bound and unbound hormone, allow one to determine K, the dissociation constant, and P_h, the molarity of the binding protein in the DFP-hemolymph, with the use of Eq. 2. A plot of the data according to Eq. 2 yielded the same straight line (Fig. 4) for both 10- and 40-fold diluted DFP-hemolymph. Thus, the binding of JH to the binding protein can be described as a simple thermodynamic equilibrium. From the slope and the intercept, one obtains $K = 2.99 \pm 0.03 \times 10^{-7}$ M and $P_h = 7.7 \pm 0.4 \times 10^{-6}$ M. Similar plots were obtained for the binding of JH to undiluted and 5-fold diluted DFP-hemolymph to yield the same dissociation constant. The intercepts of these plots, however, were lower by as much as 40% than that for the higher dilutions. Gel filtration of the more concentrated DFP-hemolymph:JH mixtures showed that an appreciable fraction of JH was bound to lipoprotein. This additional equilibrium might account for the apparent decrease in the molarity of the binding protein. The rate of dissociation of the complex appears to be slow on the time scale of the chromatographic experiments as indicated by the small percentage of radioactivity found in the inclusion volume after rechromatography of the complex.

Substitution of a large part of the radioactive hormone by its nonradioactive isomers did not change the degree of association of the radioactive hormone at a binding protein concentration comparable to that of the total isomeric mixture. Therefore, all geometric isomers must bind with approximately the same affinity.

DISCUSSION

The degree of water solubility of JH was surprising. The structure is that of a modified fatty-acid methyl ester, and indeed, most previous workers have treated it as a virtually water-insoluble lipoidal material and have resorted to vegetable oils (13, 16, 17), emulsifiers (14, 16, 17), or organic solvents (2, 7, 12, 14, 17, 18) as a vehicle for its administration. Undoubtedly the polar epoxide function contributes to the relatively high degree of water-solubility. Our studies show that true aqueous solutions can be prepared which should permit

injection of small volumes that have JH levels in excess of those needed to produce physiological effects.

One can imagine that the binding protein might receive JH molecules as soon as they are secreted by the *corpus allatum*, and that it might also interact with membranes in target cells. On the other hand, for practical reasons, one might want to create an artificial situation whereby JH dissolved in an injected oil droplet would slowly be leached out by the binding protein. In some insects, such as the adult male *cecropia* moth, where huge amounts of JH are sequestered (19), the same principle has probably been adopted by the insect, and the fat body can serve as the reservoir. Similarly, the hemolymph lipoproteins may serve to dissolve JH when large amounts are injected in a lipid or solvent carrier. In the hemolymph of *M. sexta* we find JH associated with what appeared to be a lipoprotein fraction only when very large doses of aqueous hormone are administered and after the binding protein has been saturated.

The JH binding protein has a strong affinity for JH, indicated by the low value of the dissociation constant, 3×10^{-7} M. It does, however, bind to several JH isomers that have different optical and geometrical configurations. On the other hand, it shows no affinity for two JH metabolites: the epoxy acid and the diol ester. Thus, the protein appears to have a binding site which recognizes the expoxide and ester functions, and perhaps the hydrocarbon backbone as a hydrophobic moiety.

The concentration of the binding protein in the hemolymph is equal to 7.7×10^{-6} M. If the protein is in large excess with respect to the concentration of JH, then this amount of protein is sufficient to maintain 96% of the hormone in the bound form since $K = 3 \times 10^{-7}$ M. Thus, the physiological concentration of the binding protein appears to be optimal for almost quantitative complexation of the hormone.

It is clear, therefore, that *M. sexta* hemolymph contains a binding protein of high affinity and high specificity for JH and related isomeric molecules. What can be its function? An attractive hypothesis is that it would carry the JH molecule from the secretory organ to the target site and protect it from the action of degradative enzymes which are widely distributed in the hemolymph and tissues (7).

The *in vivo* concentration of the *M. sexta* binding protein, its specificity and its physical properties certainly satisfy the requirements for a carrier of juvenile hormone. Further experiments will be required to establish whether this binding protein is the unique carrier in the tobacco hornworm. The techniques presented in this paper will be helpful in the purification of the binding protein. On the other hand, the specificity of the binding protein for juvenile hormone can be utilized to develop a radioactive affinity technique for the determination of endogenous JH. The unique properties of the binding protein clearly indicate that it has important physiological functions. The way is now open for the investigation of these functions and their role in insect development.

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