Purification and Characterization of the Carrier Protein for Juvenile Hormone from the Hemolymph of the Tobacco Hornworm * Manduca sexta Johannson (Lepidoptera: Sphingidae)

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The larval hemolymph of the tobacco hornworm, Manduca sexta, contains a carrier protein that binds specifically and with high affinity the juvenile hormone, an important regulator of insect development. This protein serves to transport the hormone and to protect it from the action of degradative enzymes during early larval stages. Using hemolymph from the last larval stage, we have isolated a pure carrier protein using acetone precipitation, gel filtration, ion exchange chromatography, and preparative isoelectric focusing. Gel filtration, polyacrylamide gel electrophoresis in sodium dodecyl sulfate, and equilibrium ultracentrifugation established that the carrier protein is a single chain polypeptide of approximately 28,000 daltons. The amino acid composition is unexceptional, and no evidence for hexosamine has been obtained.

An ion exchange filter disc assay method was used to determine the formation of the complex between the carrier protein and isotopically labeled juvenile hormone. With this technique it was shown that each carrier protein binds one hormone molecule with a dissociation constant of 4.4 ± 0.2 x 10^-7 M at 0°.

During larval growth in insects, the expression of genetic information necessary for metamorphosis is suppressed by the presence of juvenile hormone (JH). Chemically, the juvenile hormones are a homologous series of epoxy acid methyl esters derived from a sesquiterpene carbon chain. In the larval hemolymph of the tobacco hornworm, Manduca sexta, JH is present in the form of a noncovalent stoichiometric complex with a binding protein (1). The binding protein acts as a carrier; it transports the hormone from the site of synthesis, the corpus allatum, through the hemolymph to target tissues, protecting it from general carboxyl esterases present in the hemolymph and other tissues. These enzymes hydrolyze the uncomplexed hormone to the inactive epoxy acid (2-4). In addition, the carrier protein exerts a synergistic effect with JH on target tissue (5) because of its protective function, and possibly, also by facilitation of hormone penetration into target cells. In addition to general esterases, Manduca hemolymph contains hormone-specific esterases than can hydrolyze equally well free and carrier-bound hormone (2, 5). The level of these latter enzymes increases dramatically in the hemolymph of the larva shortly before the larval-pupal molt, and probably contributes significantly to the abrupt decline in JH titer that occurs preceding pupation.

Our goal has been to study the interaction of the hormone carrier protein complex with specific JH esterases and with target tissues and also to examine the specificity of the interaction of hormone and protein at the hormone binding site. Because of the presence of degradative enzymes and proteins that bind JH nonspecifically in the larval hemolymph (1, 6), we recognized that detailed studies of hormone-protein, protein-protein, and protein-tissue interactions were possible only with purified carrier protein. In this paper, we report the isolation and some of the properties of the hemolymph JH carrier protein from M. sexta larvae.

**EXPERIMENTAL PROCEDURE**

**Materials**

Pure synthetic methyl-trans,trans,cis-3,11-dimethyl-7-ethyl-10,11-epoxytrideca-2,6-diene (methyl-7,11-dihomojuvenile) was purchased from Eco Control and Regis Chemical Co. Methyl-trans,trans,cis-3,11-dimethyl-7-[1,2-3H]ethyl-10,11-epoxytrideca-2,6-diene and methyl-trans,trans,cis-3,11-dimethyl-7-ethyl-10,11-epoxy-[10-3H]trideca-2,6-diene (11.7 Ci/mmol and 13.5 Ci/mmol, respectively) were from New England Nuclear. Disopropylphosphorofluoridate (DFP) was from Pierce Chemical, and Sephadex G-100 and G-50 (superfine) were from Pharmacia. 1-Phenyl-2-thiourea, acrylamide, N,N'-methylenebisacrylamide, tryptamine, and p-toluenesulfonic acid were obtained from Sigma Chemical Co.
acid were from Eastman Kodak Co. Iodoacetic acid from Matheson was recrystallized twice from ethyl ether/petroleum ether (30-60°) mixtures. Diethyl-p-nitrophenyl phosphate (Paroxyne) was from Sigma. Fluorescamine (FLURAM) was obtained from Roche Diagnostics, Hoffmann-LaRoche, Inc. Guanidine hydrochloride (Ultrapure) was purchased from Schwarz/Mann. The Ampholine used for the isoelectric focusing experiments was from LKB Instruments, Inc. Sodium dodecyl sulfate was from Triton X-100 and DEAE-Bio-Gel A from Bio-Rad laboratories. DEAE-filter paper was obtained from REEVE Angel. Other materials used were the same as described previously (1) or of the highest purity commercially available.

**Manduca sexta** eggs were a gift from Drs. R. A. Bell and J. P. Reincke, USDA, Fargo, N.D., and larvae were reared as described previously (1).

**Protein Concentration**

For routine determinations during purification, the absorption at 280 nm was used as a measure of protein, except in crude hemolymph and acetone powders, when the biuret method (7) was employed. For more accurate determination, protein samples were either hydrolyzed in 6 n HCl for 24 hours and subjected to amino acid analysis (see below) or assayed by the Folin procedure (8). For determination of protein at very low concentrations, fluorimetry was used as follows: 50 µl of each sample was added to 3.5 ml of 0.2 M sodium borate buffer, pH 8.5. Fluorescamine (50 µl of a 0.5 mg/ml acetone solution) was added with vigorous mixing and the fluorescence was recorded on a C. R. Turner fluorimeter equipped with a Wratten No. 8 filter. An appropriate buffer blank was run for each measurement. By this method the limit of detection was 10^-9 M carrier protein.

**Amino Acid Analyses**

Amino acid compositional data were obtained according to the procedures of Spackman et al. (9) by automated ion exchange chromatography using a single column in a Durrum 500 analyzer. Desalted and lyophilized protein samples were hydrolyzed in vacuo at 110°C in 6 n HCl for 24, 48, and 72 hours. Recoveries of serine and threonine were corrected for decomposition by extrapolation to zero time. Tryptophan was measured after hydrolysis of a protein sample in vacuo at 110°C for 24 hours in 5 M p-toluene sulfonic acid containing trypthamine (0.0125 M) (10).

**Reduction, Carboxymethylation, and Perf orm imi c Acid Oxidation**

The disulfide bonds of the carrier protein were reduced with 2-mercaptoethanol and the cysteinyl residues were S-alkylated with iodoacetic acid (11). The lyophilized protein was dissolved in 6 M guanidine hydrochloride/0.5 M Tris-HCl/0.003 M disodium EDTA, pH 8.6 (0.1% w/v) and heated to 95°C for 10 min. The solution was incubated for 2 hours at 50°C under nitrogen in 2-mercaptoethanol (0.1 M). After cooling to room temperature, iodoacetic acid (0.17 M) was added. Fifteen minutes later, the sample was exhaustively dialyzed against 0.1 M Tris-HCl. The salt-free S-carboxymethylated carrier protein was lyophilized and stored at -20°C. Alternatively, the protein was oxidized with performic acid according to the procedure of Hirs (12).

**Polyacrylamide Gel Electrophoresis**

Polyacrylamide gel electrophoresis was carried out in 7.5% (w/v) acrylamide gels at pH 8.4 according to the procedures of Ornstein (13) and Davis (14). Samples (10 to 50 µl) were applied in 50 µl aliquots made 10% (v/v) in glycerol and electrophoresis was carried out at 3 mA per gel (6 mm diameter) at 4°C. Proteins were visualized by staining with Coomassie brilliant blue R followed by destaining in a solution of 75 ml of acetic acid, 250 ml of methanol, and 675 ml of water. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate (10% (w/v) acrylamide and 0.1% (w/v) SDS at pH 7.2) was performed according to Weber and Osborn (15).

**Determination of Molecular Weight**

The molecular weight of the carrier protein was determined by ultracentrifugation, polyacrylamide gel electrophoresis in sodium dodecyl sulfate, and gel filtration. Mercapto-depletion sedimentation equilibrium was carried out at 17.7°C as described by Yphantis (16). The protein samples were dissolved in 5 mM Tris-Cl buffer, pH 7.3, containing 0.1 M NaCl and these solutions (0.5 and 1 mg of protein/ml) were dialyzed against the same buffer. The dialysates were used for the reference solutions. Sedimentation equilibrium was performed at approximately 32,000 rpm in a Beckman model E ultracentrifuge equipped with an electronic speed control. The density of the solution was determined by pycnometry and corrected to 17.7°C, giving a value of 1.0029 g/ml. A partial specific volume of 0.738 cc/g was calculated for the carrier protein from the amino acid content (17).

For molecular weight determination by SDS-polyacrylamide gel electrophoresis (15), the molecular weight of the S-carboxymethylated carrier protein was compared with the mobilities of standards of known molecular weight: bovine serum albumin (Metrix), 66,000; pepsin (Workington), 35,000; chymotrypsinogen A (Workington), 25,000; and cytochrome c (Workington), 11,700. Calibrated gel filtration was carried out at 4°C in a column of Sephadex G-100 (2.5 x 110 cm) equilibrated with 0.02 M Tris-Cl, pH 7.3, containing 0.1 M NaCl by the methods of Whistler (18) and Fish et al. (19), using the following standards: blue dextran 2000 (Pharmacia); bovine serum albumin (Metrix), 68,000; ovalbumin (Workington), 43,000; pepsin (Workington), 35,000; chymotrypsinogen A (Workington), 25,000, and u-lactalbumin (Workington), 15,500.

**Polyethylene Glycol Coating**

Both the juvenile hormone and carrier protein readily adsorb to hydrophobic surfaces. To decrease this adsorption and allow more quantitative recoveries, all glassware coming into contact with the hormone or carrier protein was precoated with a 1% (w/v) solution of polyethylene glycol (PEG). Fisher, M. = 20,000) rinsed extensively with ionized water and dried at 105°C.

**Aqueous Solutions of JH**

Stock solutions of [3H]JH mixed with unlabeled carrier JH were prepared by transferring benzene/hexane (4/1) solutions of the labeled and unlabeled hormone to a PEG-coated test tube and evaporating to dryness at 0°C under N2. An appropriate volume of 5 mM Tris-Cl buffer, pH 8.3, was added to give a final concentration of 10^-4 to 10^-6 M JH of known specific activity and the tube was mixed repeatedly on a Vortex mixer over a 1-hour period to aid in solubilization of the hormone. The final concentration was ascertained by measurement of the radioactive JH in the solution.

**JH Binding Assay**

For the rapid determination of JH binding protein, we have used a DEAE-filter disc assay in which binding protein and hormone-protein complex are adsorbed to ion exchange paper under conditions in which the free hormone is not adsorbed. All JH binding assays were performed in PEG-coated glass microcentrifuge tubes (Misco) at 0°C. For routine analysis, the binding assay mixture was composed of 10 µl of [3H]JH (10^-4 M) in 5 mM Tris-Cl buffer, pH 8.3, and 10 µl of binding protein solution. Assay mixtures were incubated for 30 min before measurement of complex formation by the DEAE-filter assay. Filtration experiments were performed as follows: DEAE-cellulose filter discs were washed with 0.5 ml of HCl, distilled water, 0.5 ml of NaOH, distilled water, and finally, exhaustively washed with 5 mM Tris-Cl, pH 8.3. Equilibrated filters were then placed on a filter manifold (Millipore) and washed with 1 ml of ice-cold 1% (w/v) Triton X-100 (Research Products International Corp.) in 5 mM Tris-Cl, pH 8.3. Samples to be assayed (5 to 50 µl) were transferred to the washed filter disc using PEG-coated glass capillary tubes. After a 1-min incubation, the filters were washed with 0.5 ml of 1% (w/v) Triton X-100 in 5 mM Tris-Cl, pH 8.3, using sufficient vacuum to allow a flow rate of 2.5 ml/min. The washed filters were then rapidly dried by suction and the adsorbed radioactivity measured in a liquid scintillation counter (Nuclear Chicago Isocap 300) using a liquid scintillation fluid prepared from 1 liter of Triton X-100, 2 liters of toluene, and 12 g of Omnifluor (New England Nuclear Corp.). A counting efficiency of 40 to 45% for tritium was routinely observed.

**Binding Unit**

A binding unit (BU) has been defined as that amount of carrier protein required to bind 1 nmol of JH when equilibrated with 1 x 10^-6 M [3H]JH and incubated with assay using the DEAE-filter method.

**Purification of Carrier Protein**

**Acetone Powder—Larvae** (5th day of the 5th instar) were cooled to 4°C in ice, and hemolymph was obtained by cutting off the abdominal
horn at its base. The hemolymph (approximately 0.5 ml per larva) was collected directly into a 20-fold excess of cold (0°C) acetone. The resulting suspension was stirred for an additional hour and the precipitated protein was recovered by suction filtration. This procedure yielded a fine light blue powder which was stored at 4°C in a desiccator over CaSO₄ (Drierite).

**Gel Filtration**—Acetone powder from 40 to 50 larvae (approximately 2 g) was reconstituted in 11 ml of cold 0.01 M Tris-HCl, pH 7.3, containing 0.1 M NaCl and 10⁻⁴ M phenylthiourea (to inhibit phenol oxidase). After centrifugation (3,000 x g for 10 min), 10 ml of the supernatant was applied to a column of Sephadex G-100 (2.5 x 110 cm) equilibrated with the same buffer at 4°C and fractions (5 ml) of effluent were collected.

**Ion Exchange Chromatography**—Fractions from Sephadex G-100 chromatography that contained JH-binding protein were pooled and mixed with 10⁻³ M DFP or paraoxon in order to inhibit any proteases or esterases present. The pooled fractions were concentrated and equilibrated with 5 mM Tris-HCl buffer, pH 8.3. By ultrafiltration using a UM-2 membrane (Amicon). The concentrate was applied to a column of DEAE-Bio-Gel A (1.5 x 40 cm) equilibrated with 5 mM Tris-HCl/0.02 M NaCl, pH 8.3, at 4°C and fractions (3.25 ml) of effluent were collected. After washing with 3 column volumes of buffer, a shallow gradient from 0.02 to 0.15 M NaCl was applied to elute the carrier protein.

**Isoelectric Focusing**—The fractions from ion exchange chromatography that contained JH-binding protein were concentrated by ultrafiltration (as above) and subjected to isoelectric focusing in a 110-ml preparative column (LKB Instruments, Inc.) using a sucrose gradient and 1% (w/v) Ampholine, pH 4 to 6, at 600 V. After 48 to 72 hours, the column was drained into a fraction collector, in which fractions (2 ml) were collected at a rate of 0.4 ml/min. The fractions that contained JH-binding protein were pooled and the Ampholine and sucrose were removed either by gel filtration on a column of Sephadex G-100 (2.5 x 110 cm) or by extensive dialysis against glass-distilled water.

**JH Esterase Assay**

Samples (50 µl) were preincubated for 15 min at room temperature with DFP (2 x 10⁻⁴ M), and JH hydrolysis was measured by the method of Sanburg et al. (2).

**RESULTS**

**Binding Protein Assay**—During initial purification attempts, JH-binding protein was assayed using a charcoal adsorption assay (6) modified from the estrogen binding assay of Korenman (20). The relatively low exposure of the hormone-protein complex to charcoal, however, resulted in erratic and inaccurate assays and, therefore, a more quantitative and reliable assay method was sought. The fact that the carrier protein could be adsorbed on DEAE ion exchange material at pH 8.3 and low ionic strength was used to develop a rapid assay procedure using DEAE-cellulose filter discs. It was found that, in the absence of any protein, variable amounts of labeled hormone were retained on the filter. Incorporation of the nonionic detergent Triton X-100 into the washing buffer (21) reduced this protein-independent hormone adsorption to a low and reproducible level and allowed for satisfactory determination of hormone-carrier protein complex formation. After washing with 1 to 6 ml of Triton-containing buffer, the amount of hormone retained on the filters in the presence or absence of carrier protein remained constant (Fig. 1), and after subtraction of the small no-protein control value, the amount of radioactive hormone retained on the filter was an accurate measure of complex formation. The amount of hormone-carrier protein complex retained on the filter is linearly related to the amount of complex applied to the filter (Fig. 2).

Addition of bovine serum albumin (0.25 mg/ml) to the assay mixture did not interfere with the quantitative measurement of hormone-protein complex formation. In some experiments, bovine serum albumin was used to decrease adsorption of JH and carrier protein on glass surfaces.

**Purification**—The need to process relatively large volumes of Manduca sexta hemolymph necessitated suitable concentration methods. Two techniques have been used to reduce the volume of the hemolymph: lyophilization and acetone dehydration. However, during storage of lyophilized hemolymph, some degradation of the carrier protein was observed, and so we have adopted the acetone powder procedure for collection and storage.

Reconstituted hemolymph was first fractionated by gel filtration on a column of Sephadex G-100 (Fig. 3). The high affinity JH carrier protein was eluted with an apparent molecular weight of 3 x 10⁶ as a symmetrical peak. This peak slightly overlapped a hemolymph esterase fraction which was eluted with an apparent molecular weight of 6 x 10⁴. JH carrier protein was totally resolved from the high molecular weight lipoproteins (Mₚ > 10⁶) which also bind JH, but with lower affinity (not shown).

The single component with high affinity for JH obtained from gel filtration was further fractionated by ion exchange chromatography. Carrier protein was adsorbed on DEAE-Bio-Gel A from 5 mM Tris-HCl buffer, pH 8.3, and eluted with a rapid assay using DEAE-cellulose filter discs. It was found that, in the absence of any protein, variable amounts of labeled hormone were retained on the filter. Incorporation of the nonionic detergent Triton X-100 into the washing buffer (21) reduced this protein-independent hormone adsorption to a low and reproducible level and allowed for satisfactory determination of hormone-carrier protein complex formation. After washing with 1 to 6 ml of Triton-containing buffer, the amount of hormone retained on the filters in the presence or absence of carrier protein remained constant (Fig. 1), and after subtraction of the small no-protein control value, the amount of radioactive hormone retained on the filter was an accurate measure of complex formation. The amount of hormone-carrier protein complex retained on the filter is linearly related to the amount of complex applied to the filter (Fig. 2).

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**FIG. 1** (left). [3H]Juvenile hormone retained in the DEAE-cellulose filter disc in the absence (O) and presence (O) of 1.3 x 10⁻⁶ M carrier protein. [3H]Juvenile hormone concentration was 1.2 x 10⁻⁶ M (1 Ci/mmol) in both cases. Aliquots of the assay mixtures were applied to prewashed filters and washed with varying amounts of 1% (w/v) Triton X-100 in 5 mM Tris, pH 8.3.

**FIG. 2** (right). [3H]Juvenile hormone retained on DEAE-filters as a function of the amount of hormone-binding protein complex applied. Varying volumes (5 to 40 µl) of a single incubation mixture were applied to prewashed DEAE-cellulose discs and washed with 5 ml of 1% Triton X-100 in 5 mM Tris, pH 8.3. Final concentrations in the incubation mixture were 1.2 x 10⁻⁶ M [H-labeled juvenile hormone (1 Ci/mmol) and 1.5 x 10⁻⁴ M binding protein.

**FIG. 3**. Gel permeation chromatography of reconstituted hemolymph acetone powder on a column of Sephadex G-100. Fractions (5.0 ml) were collected and analyzed for protein (--), JH-carrier protein (- - -), and DFP-resistant JH esterase (O--O). For further details, see "Experimental Procedures."
Juvenile Hormone Carrier Protein

Starting with an acetone powder prepared from fresh hemolymph, only one peak of JH-binding protein was eluted. However, when lyophilized hemolymph was used as the source of carrier protein, a second JH-binding component was eluted at higher salt concentration. This second component was less abundant and generally comprised 20% of the total recovered JH1-binding activity. This protein may be a derivative of native carrier protein produced during storage.

Final purification of the carrier protein was accomplished by preparative isoelectric focusing, using a pH gradient from 4 to 6 (Fig. 5). Two fractions with JH-binding activity, designated CP-α and CP-β, were obtained, with isoelectric points (pI) of 4.95 and 5.25, respectively. The relationship between these two components is not clear. When carrier protein CP-α was subjected to a second isoelectric focusing, a single JH-binding component with pI = 4.95, was obtained. However, when carrier protein CP-β was similarly treated, two binding components were again observed with pI = 4.95 and 5.25, respectively. To determine whether both components were originally present in the insect, fresh hemolymph was collected, treated with 10 mM DFP overnight at 4°, and chromatographed on Sephadex G-100. The high affinity JH-binding fraction from this column was then subjected to isoelectric focusing. Only one JH-binding component was observed with pI = 4.95. Thus, we feel that carrier protein CP-α is the native JH-carrier protein of M. sexta and that carrier protein CP-β may be an artifact generated during our isolation procedure. The purification of the M. sexta carrier protein is summarized in Table I. The cumulative yield of JH binding activity was 30% with a purification factor of about 300-fold.

**Purity**—When carrier protein CP-α was subjected to electrophoresis in 10% (w/v) polyacrylamide gels at pH 7.2 in the presence of sodium dodecyl sulfate and 2-mercaptoethanol, and in 7.5% (w/v) polyacrylamide gels at pH 8.4, more than 95% of the total protein migrated as a single component with mobilities of 0.46 and 0.60, respectively. To test for the presence of minor contaminants that bind JH, carrier protein CP-α was incubated with methyl-7,11-[3H]dihomojuvenile and subjected to electrophoresis in 7.5% (w/v) polyacrylamide gels at pH 8.4. A single peak of [3H]JH was observed which co-migrated with the protein (Fig. 6). Analysis of carrier protein CP-β by polyacrylamide gel electrophoresis at pH 8.4 in 7.5% (w/v) gels showed that it contained several proteins. The mobility of the major component was identical to that of carrier protein CP-α.

**Stability**—JH-carrier protein purified as described was not stable under prolonged storage. Loss of activity and the appearance of degradation products have been observed after storage of the purified protein at -20° as a desalted, lyophilized powder and as a solution in several buffers at -20° and 4° for periods longer than 1 month. The most satisfactory procedure is storage at 4° in distilled water and repurification when significant impurities accumulate as judged by polyacrylamide gel electrophoresis.

**Molecular Weight Determinations**—The molecular weight of purified carrier protein CP-α was determined by chromatography on a column of Sephadex G-100, using several proteins of known molecular weight as standards. An apparent molecular weight of 2.8 x 10^6 was estimated (Fig. 7a). Reduced and S-carboxymethylated carrier protein CP-α was subjected to electrophoresis in 0.1% (w/v) SDS with several protein standards. From the observed mobility of 0.46, an apparent molecular weight of 2.9 x 10^4 was obtained (Fig. 7b).

Meniscus-depletion sedimentation equilibrium analysis of purified JH-carrier protein CP-α indicated a molecular weight of 45,000 daltons. The effect of pH on the sedimentation constant of the carrier protein was determined at pH range of 7.0 to 8.0. The sedimentation constant was found to increase with increasing pH, reaching a maximum at pH 7.7. The concentration dependence of the sedimentation was determined at 20°. The sedimentation constant was found to be independent of concentration over the range of 0.05 to 0.6 mg/ml.

**TABLE I**

<table>
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<tr>
<th>Step</th>
<th>Total binding*</th>
<th>Overall yield</th>
<th>Total protein</th>
<th>Specific binding</th>
<th>Overall purification factor</th>
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<td>1580</td>
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<tr>
<td>Sephadex G-100</td>
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<td>100</td>
<td>48*</td>
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<td>23</td>
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<tr>
<td>DEAE-Bio-Gel A</td>
<td>21.8</td>
<td>35</td>
<td>4*</td>
<td>5.56</td>
<td>139</td>
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<tr>
<td>Isoelectric focusing</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>α</td>
<td>8.9</td>
<td>14</td>
<td>0.8*</td>
<td>11.72</td>
<td>283</td>
</tr>
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<td>β</td>
<td>9.3</td>
<td>15</td>
<td>0.7*</td>
<td>12.74</td>
<td>318</td>
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</table>

*Measured by DEAE-filter disc assay.
*5.5 g of acetone powder of hemolymph collected from 145 fifth day, fifth instar larvae was dissolved in 20 mM Tris pH 7.4. Reconstitution results in a solution of some insoluble material, however, the overall purification is minimal.
*Measured by the butanol method using bovine serum albumin as protein standard.
*Full recovery of binding units after gel filtration was assumed. The assay of crude hemolymph or reconstituted acetone powder was complicated by the presence of lipoproteins, esterases, etc.
*Measured by the Polin-Glucatene method using bovine serum albumin as protein standard. Total protein was also measured following hydrolysis (6 N HCl, 110°, 24 hr) by amino acid analysis and the amount determined was 83% of that reported.
Juvenile Hormone Carrier Protein

FIG. 6. Polyacrylamide gel, densitometer scan, and radioactivity profile from electrophoresis of JH-carrier protein (CP-α, 10 μg) after incubation with [3H]JH (10⁻⁴ M, 5 Ci/mmol). Electrophoresis (anode on right) was performed as described under “Experimental Procedures” in 7.5% gels, pH 8.4. Gel stained with Coomassie blue is shown in the upper panel. Middle panel is a densitometer scan of the Coomassie blue-stained gel (full scale deflection = 1.0 A) and, the lower panel shows radioactivity profile obtained when a duplicate gel was sliced (2 mm), and tritium in the slices determined.

carrier protein CP-α gave linear plots of the logarithm of the fringe displacement against the square of the radius of rotation for the entire length of the cell, indicating homogeneity and the absence of molecular association. Calculation from the plots obtained with protein concentrations of 0.53 and 1.24 mg of carrier protein CP-α per ml yielded molecular weights of 2.75 x 10⁴ and 2.68 x 10⁴, respectively. These two values can be extrapolated to a molecular weight of 2.8 x 10⁴ at zero protein concentration.

Amino Acid Composition—The amino acid composition of the M. sexta JH carrier protein is presented in Table II. The molar compositions were based upon the molecular weight determined by physical methods. The recovered amino acids accounted for approximately 90% of the mass of protein hydrolyzed. There was no evidence of hexosamine in the samples analyzed.

Equilibrium Binding Measurements—A dissociation constant for the JH-carrier protein complex of 3 x 10⁻⁷ M measured by gel filtration experiments using DFP-treated hemolymph has been reported (1). With the availability of pure carrier protein and a reliable assay method, we have confirmed this value and attempted to determine the number of hormone binding sites on the carrier protein. A series of binding experiments was performed in which the total concentration of carrier protein was maintained at a constant value and the total concentration of hormone was varied. Methyl-7,11-[3H]dihomojuvenile was again used as ligand and radioactive complex was measured by DEAE-filter disc assay. The data from these experiments were plotted according to Equation 1 (22), in which r is the ratio of moles of bound hormone to moles of total protein, [JH] is the concentration of free hormone, n is the number of binding sites and K_d is the dissociation constant for complex formation.

\[
\frac{1}{r} = \frac{K_d}{n} \frac{1}{[JH]} + \frac{1}{n}
\]

A straight line was obtained (Fig. 8) which yielded a dissociation constant of 4.4 ± 0.2 x 10⁻⁴ M for the complex and a value of one binding site per protein molecule.
TABLE II
Amino acid composition of M. sexta juvenile hormone carrier protein (CP-a)

<table>
<thead>
<tr>
<th>Gram residue per 100 g of protein</th>
<th>Amino acids per molecule*</th>
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<tr>
<td></td>
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<tr>
<td>Aspartic acid</td>
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<td>Glycine</td>
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</tbody>
</table>

* Mean values of 24-, 48-, and 72-hour hydrolysates. Labile amino acids extrapolated to zero time. Molar composition based upon the molecular weight determined by physical methods. Nearest integers given in parentheses.

Molecular weight determined by physical methods. Nearest integers given in parentheses.

Efforts to develop a rapid, reproducible assay for the juvenile hormone carrier-protein were hampered by the physical properties of both hormone and protein. In the very dilute solutions employed, both components adsorbed to glass surfaces. Much of the problem was obviated by pretreatment of all glassware with polyethylene glycol, but it was necessary to make routine measurements of the final concentration of total hormone in solution, rather than relying upon values calculated from dilutions of stock solutions.

When we used assay methods based upon separation of free ligand from complex by gel filtration, charcoal treatment or dialysis, we were plagued by poor reproducibility and apparent binding of more than one hormone molecule per protein molecule. These problems were solved by adopting a DEAE-filter paper assay method, a technique that has also been used for steroid hormone receptors (23, 24). We found the major problem in adapting a DEAE-filter paper assay to measurement of JH-carrier protein complex to be significant and variable binding of free hormone to the filters. Triton X-100 has been used to wash free and nonspecifically bound sterol from chromatin in a radioreceptor assay for 1α,25-dihydroxyvitamin D3 (21). Addition of this detergent to the wash buffer in our assay lowered the hormone blank to a small, reproducible value and still permitted a sensitive and reliable determination of hormone-protein complex. While other assay methods yielded hormone/protein binding stoichiometry larger than 1:1, the filter disc method indicated a single binding site. The higher values may be due to some sort of nonspecific binding, but we cannot rule out the possibility of a second hormone binding site on the carrier protein from which hormone can be removed by the detergent.

The most successful strategy for purification of the carrier protein started with an acetone powder of hemolymph. In addition to reducing the volume of material to be processed, this step appeared to inactivate some factor in crude hemolymph that gives rise to variable amounts of an artifactual form of binding protein. Hemolymph can be collected over a long period of time and safely stored as the acetone powder.

The carrier protein was extracted from the acetone powder by a buffer solution and subjected to gel permeation chromatography, ion exchange chromatography, and isoelectric focusing. Only the ion exchange step resulted in serious loss of activity. The isoelectric focusing step yielded two fractions containing binding protein, CP-α and CP-β, in approximately equal amounts. However, much of the material in the CP-β fraction behaved as CP-α upon repeated isoelectric focusing under the same conditions. Furthermore, while carrier protein CP-α showed a single band after polyacrylamide gel electrophoresis, the CP-β fraction displayed an identical band as its major component as well as other minor bands. It is possible that the CP-β fraction represents carrier protein CP-α with some additional compound noncovalently complexed to it. This is clearly not juvenile hormone, but may be a peptide or some other material acquired in the course of purification.

From the overall yield of carrier protein given in Table I, the average concentration of binding protein in crude hemolymph of 5th day, 5th instar M. sexta larvae can be calculated as about one-third of that determined earlier (1) using gel filtration methods on crude homolymph from 5th instar larvae.
of indeterminate age. The discrepancy may be due either to poor recovery of carrier protein from hemolymph acetone powder, or it may be due to changes in carrier protein titer during larval life. Since purified carrier protein can be precipitated with acetone and recovered in good yield, the latter possibility seems more probable.

The molecular weight of carrier protein CP-α was determined to be 2.8 × 10^6 by gel filtration, SDS-polyacrylamide electrophoresis, and sedimentation equilibrium ultracentrifugation. The dissociation constant for the hormone-protein complex was determined to be 3 to 4 × 10^{-7} M at 0°C by using equilibrium dialysis or the DEAE-filter assay method, and this value is in agreement with that determined earlier (1), using gel filtration methods on crude hemolymph (Kd = 3 × 10^{-7} M). This agreement verifies our initial assumption (1) that formation of the protein-hormone complex may be described as a simple thermodynamic equilibrium process. It also indicates that the position of the equilibrium is essentially the same in the crude hemolymph as it is with purified protein. We have found, however, that certain materials can shift dramatically the position of the equilibrium. Acetone, acetonitrile, isopropyl alcohol, and dimethylformamide at 15% (w/v), for example, will strongly inhibit complex formation.

Mammalian sera have yielded a variety of binding proteins for lipophilic molecules including corticoids (23), sex steroids (24, 25) and retinol (26). Most of these have higher affinity for the ligand than does the JH-carrier protein, and the size of the binding protein varies widely. Although little is known of the function of these mammalian protein-ligand complexes, they may serve a protective function and guide the complexed ligand to specific targets while preventing nonselective absorption by a variety of cells.

Carrier proteins for juvenile hormone have been reported recently from several insect species (6, 27). These proteins have very similar molecular weights, but differ in electrophoretic mobility at pH 8.4. Even though the JH carrier proteins from M. sexta and Plodia interpunctella (Indian meal moth) differ in charge, the Manduca protein exerts a synergistic effect with JH on Plodia wing imaginal discs in vitro (5). However, it is not yet possible to determine whether these carriers belong to a family of homologous proteins.

The availability of pure JH-carrier protein should be useful in defining the details of the interaction of this important hormone with target tissue cells. In addition, the way is now open for detailed studies on the interaction of the carrier protein with degradative enzymes in the hemolymph and in other tissues. Antibodies to the carrier protein will be useful in probing the physiological function of the protein-hormone complex and in establishing a radioimmune assay for the carrier protein.

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