lation. Phosphorylation of factor III^{Lac} causes an increase in hydrophobicity as shown by detergent binding (Deutscher et al., 1982). The same could be expected if a segment of the protein composed of hydrophobic amino acids is turned to the surface. It is interesting that the N-terminal peptide, residues 4–38, interferes with factor III^{Lac} for binding to membrane-bound enzyme II^{Lac}. Defective factor III^{Lac} isolated from strain S 714G could still be phosphorylated by PEP, enzyme I, and HPr at about normal rate but was no longer able to transfer the phosphoryl group via enzyme II^{Lac} to the sugar (Sobek et al., 1984). The mutation was found to affect position 18 of the sequence: glycine was changed to glutamic acid. Thus, the binding site for enzyme II^{Lac} as well as the change in hydrophobicity and α-helical content upon phosphorylation could be related to the same part of the protein, the N-terminal region. The other functional domain of factor III^{Lac} carrying the histidine residue responsible for phosphoryl group transfer could be identified on the basis of earlier work and the sequence presented in Figure 1 (Deutscher et al., 1982). The phosphorylated nonapeptide, which was isolated and sequenced earlier, corresponds to residues 82–90 in Figure 1 and includes histidine-82 as 3-phosphohistidine according to Kalbitzer et al. (1981). Thus, the active site histidine residue 82 is identical with histidine B of the above-mentioned paper.

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Purification and Characterization of a Biliverdin-Associated Protein from the Hemolymph of *Manduca sexta*†

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ABSTRACT: A biliverdin binding protein, insecticyanin, has been isolated from the hemolymph of the fourth instar tobacco hornworm *Manduca sexta*. The protein has been purified to apparent homogeneity by conventional chromatography with a cumulative yield of 40–50%. The protein (M_w 71 600) is composed of three subunits (M_r 23 000). Each subunit binds one biliverdin molecule. Proton magnetic resonance spectroscopy and absorption spectroscopy demonstrate that the bilin is the biliverdin IX γ isomer.

The hemolymph of many invertebrates is blue due either to copper-containing proteins such as the hemocyanins or to biliverdin-associated proteins. A survey of the invertebrate hemolymph chromoproteins indicates that the majority of biliproteins are limited to the class Insecta, a diverse group that has evolved an oxygen transport system independent of circulating respiratory pigments. Although the functions of the biliproteins are not well understood, the tissue distribution of the proteins suggests that the blue chromophore, in conjunction with yellow pigments such as the carotenoids, gives rise to the cryptic green color observed in many phytophagous insects. A particularly rich source of biliproteins is found in the hemolymph and epidermis of the hornworm *Manduca sexta* (Dahlman, 1969; Cherbas, 1973; Goodman et al., 1982).

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Earlier studies indicated that the biliprotein represented a significant portion of the total hemolymph protein and that virtually all of the blue pigmentation can be attributed to a single protein. This particular protein (M_r 72000) was given the name insecticyanin (Cherbas, 1973).

More recently it has been demonstrated that epidermis not only contains INS[1] but synthesizes and secretes it into the hemolymph (Riddiford, 1981, 1982). Synthesis and release of INS from the epidermal cell are unusual since most hemolymph proteins are synthesized by the fat body (Wyatt & Pan, 1978). The presence of the biliprotein in the epidermis appears to be larval specific and is dependent upon the titers of juvenile hormone. During most of larval life, JH titers are high, and the protein is sequestered as a granule as well as secreted into the hemolymph. In the last larval instar, a decline in JH titers signals not only the onset of metamorphosis but a complete degranularization of the epidermal cell, which in turn leads to a significant rise in hemolymph INS content (J. T. Trost and W. G. Goodman, unpublished experiments). In a mutant with abnormally low JH titers, the epidermal cell appears to lack the ability to sequester the protein into storage granules (Goodman et al., 1982). Exogenous JH in physiological concentrations can rescue this particular lesion and restore epidermal INS levels to that observed in the wild type. Considering the potential importance of INS as a probe to study the mode of action of JH, we here report the purification and characterization of the protein from the hemolymph.

Materials and Methods

Materials. Chemicals were obtained from the following sources: amino acid, carbohydrate, glycoprotein and molecular weight standards, BF_3, biliverdin, complete and incomplete Freund's adjuvant, and hemin were obtained from Sigma; DEAE-Bio-Gel, CM-Bio-Gel, hydroxylapatite (Bio-Gel HTP powder), Tris, SDS, and electrophoresis chemicals were obtained from Bio-Rad; ampholytes and Sephacryl S-200 were from Pharmacia; DNS-Cl, $CDCl_3$, and organic solvents were from Aldrich; thin-layer plates and silica gel G powder were obtained from E. Merck; spectral-grade organic solvents were purchased from Burdick and Jackson. All other chemicals obtained were of the highest purity commercially available.

Purification of Hemolymph Insecticyanin. Manduca sexta were reared as described by Bell & Joachim (1976) as modified by Goodman et al. (1985) with a 16-h light to 8-h dark photoperiod at 25 °C with 60% relative humidity. Hemolymph samples from late fourth instar insects were obtained as previously described (Goodman et al., 1978) and frozen until sufficient hemolymph was collected. The pooled samples (100 mL) were thawed, dialyzed against a 50 mM potassium phosphate buffer containing 15 mM KCl, pH 6.7, centrifuged (10000*g*) for 10 min, and concentrated to 31.5 mL with a Diaflo ultrafiltration apparatus (PM10 membrane, Amicon).

Step 1. The concentrated hemolymph was applied to a Sephacryl S-200 column (135 × 2.5 cm) equilibrated with a 50 mM phosphate buffer containing 150 mM KCl, pH 6.7. Proteins were eluted with the same buffer at a flow rate of 0.5 mL/min. Eluate fractions of 5 mL were collected and monitored at 277 and 674 nm. Fractions absorbing at 674 nm were pooled and dialyzed against a 10 mM Tris buffer containing 10 mM KCl, pH 8.6.

Step 2. The pooled fraction (approximately 100 mL) was rapidly concentrated to 10 mL with a Diaflo ultrafiltration apparatus (PM30 membrane) and applied to a DEAE-Bio-Gel column (85 × 1.5 cm) equilibrated with 10 mM Tris and 10 mM KCl, pH 8.6. Protein was eluted at a flow rate of 0.25 mL/min, first with the equilibration buffer (300 mL) and then with a linear KCl gradient ranging from 10 to 125 mM KCl (400 mL). Eluate fractions of 2.5 mL were collected, and fractions with absorbance at 674 nm were pooled.

Step 3. With use of the Diaflo ultrafiltration apparatus (PM30 membrane), the pooled fraction (50 mL) was concentrated and dialyzed against 10 mM potassium phosphate buffer containing 10 mM KCl, pH 5.0. The dialyzate (5 mL) was then applied to a CM-Bio-Gel column (25 × 0.9 cm) equilibrated with the dialysis buffer. Protein was eluted at a flow rate of 0.25 mL/min, first with equilibration buffer (100 mL) and then with a linear KCl gradient ranging from 10 to 150 mM KCl (350 mL). Eluate fractions of 2.5 mL were collected, and fractions with absorbance at 674 nm were pooled. The pH of the pooled sample was quickly brought to 6.5.

Step 4. With use of the Diaflo ultrafiltration apparatus (PM30 membrane), the pooled fraction (50 mL) was concentrated and dialyzed against 10 mM potassium phosphate buffer containing 10 mM KCl, pH 6.5. The dialyzate (10 mL) was then applied to a hydroxylapatite column (15 × 0.9 cm) equilibrated with the dialysis buffer. The column was washed with 50 mL of the equilibration buffer and then with 50 mL of a 50 mM phosphate buffer containing 10 mM KCl, pH 8.0. The protein eluting with the step-off buffer was concentrated on the Diaflo ultrafiltration apparatus with an XM50 filter.

Analytical Procedures. (*A*) *Electrophoresis.* Polyacrylamide gel electrophoresis was performed in a continuous buffering system as previously described (Goodman et al., 1978). SDS–polyacrylamide gel electrophoresis was performed under reducing conditions according to the method of Laemmli (1970). Prior to application to the gel, the sample was mixed with an equal volume of sample buffer containing 1.33 M Tris, 4.4% SDS, 16.6% sucrose, and 20% 2-mercaptoethanol, pH 6.6. After incubation at 100 °C for 5 min, the sample was layered onto the stacking gel. Upon completion, the gel was stained with Coomassie Brilliant Blue R-250. Urea–SDS gels (10 M urea) were performed as previously described (Branch et al., 1972). Immunoelectrophoresis was performed on agarose Pol-E-Film (Pfizer, Inc.) in 65 mM barbital buffer, pH 8.6, at 90 V for 65 min. Anti-INS was added to the plate and the plate incubated at 4 °C for 24 h. After development, precipitin arcs were visualized by washing the plates in 300 mM NaCl for 24 h and then staining with Coomassie Blue.

(*B*) *Polyclonal Antiserum Production.* Purified INS was dissolved in water and then emulsified with complete Freund's adjuvant. The emulsion (2 mL), containing 250 μg of INS, was injected subcutaneously at 20–25 sites on the back of a female New Zealand white rabbit. Five weeks later a boost was administered in incomplete Freund's adjuvant. Several boosts were given at biweekly intervals. Blood was drawn from the ear starting approximately 12 weeks after the initial injection. The blood was allowed to clot and the serum harvested.

(*C*) *Protein Determination.* Protein concentrations were determined with the Bradford assay (Bradford, 1976), using bovine serum albumin as a standard. Prior to the assay, TCA (10 μL, 12% w/v) was added followed by ethyl acetate (100 μL) to remove the chromophore and so avoid spectral overlap

[1] Abbreviations: INS, insecticyanin; JH, juvenile hormone; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; DNS-Cl, 5-(dimethylamino)naphthalene-1-sulfonyl chloride; TLC, thin-layer chromatography; BF_3, boron trifluoride; ^1H NMR, proton magnetic resonance; Me_4Si, tetramethylsilane; $CDCl_3$, deuterated chloroform.

between biliverdin and the dye. After addition of ethyl acetate, the samples were mixed vigorously and then centrifuged (1000*g*) for 10 min. The procedure was performed twice and the sample then solubilized in 1 M NaOH and reprecipitated with TCA. The sample was then centrifuged (2000*g*) for 15 min, the precipitate redissolved in 1 M NaOH, and protein determination performed.

Crystallization. INS was crystallized by dialyzing the protein solution (4.5 mg/mL) against ammonium sulfate solution (30 g in 100 mL of water) adjusted to pH 7.0 with ammonium hyroxide (Cherbas, 1973). The crystallization procedure was allowed to proceed slowly at 4 °C for several days. Crystals were harvested by passing the mother liquor through a porous glass filter.

Molecular Weight Determinations. The molecular weight of INS was estimated by molecular sizing on a calibrated column of Sephacryl S-200 (105 × 0.90 cm) with a 50 mM phosphate buffer containing 150 mM KCl, pH 6.8. The molecular weight of INS was also determined by the high-speed equilibrium sedimentation technique of Yphantis (1964) on a Beckman-Spinco Model E analytical ultracentrifuge equipped with Raleigh interference optics. Insecticyanin (0.15 mg/mL) was dialyzed for 48 h against several changes of a 10 mM phosphate buffer, pH 6.8. Centrifugation was performed at 20 000 rpm for 48 h at 20 °C and the weight-average molecular weight calculated as described by Yphantis (1964). The partial specific volume was calculated from the amino acid composition (Cohn & Edsall, 1950); the partial specific volume of biliverdin was not included in the final calculation. The molecular weight of insecticyanin subunits was estimated by SDS–polyacrylamide gel electrophoresis (Laemmli, 1970) on calibrated gels. Subunit molecular weights were also estimated by the method of Weber et al. (1972). INS and molecular weight standards were first denatured in guanidine hydrochloride (100 °C) and then alkylated with iodoacetic acid. Both procedures for estimating molecular weight were in excellent agreement.

Subunit cross-linking was performed on varying concentrations of INS (0.67–6.7 mg/mL) and dimethyl suberimidate (Sigma) (0–6 mg/mL) in 0.2 M triethanolamine, pH 8.5 (Davies & Stark, 1970). After incubation (3 h, 25 °C), the reaction was stopped, and the samples were dissociated in SDS–mercaptoethanol and electrophoretically separated on a 7% acrylamide gel (Laemmli, 1970). Estimation of cross-linked protomer molecular weights was performed on calibrated gels.

Amino Acid Analysis. Insecticyanin was exhaustively dialyzed against water and then hydrolyzed under reduced pressure at 110 °C in 6 N HCl containing 0.2% phenol. Amino acid analyses were performed on a Durrum D500 automatic amino acid analyzer. Decomposition of serine and threonine was corrected for by examining decomposition at three time points and extrapolating to zero time. Cystine plus cysteine were determined as cysteic acid after performic acid oxidation. Tryptophan content was determined by ultraviolet spectral analysis (Edelhoch, 1967).

Spectroscopy. Proton magnetic resonance spectra of the biliverdin chromophore were obtained on a Bruker WP-200 SY spectrometer, while extinction coefficients were determined on a Perkin-Elmer Model 552 spectrophotometer.

N-Terminal Amino Acid Analysis. Analysis of the N-terminal amino acid residues of INS was performed as described by Morse & Horecker (1966) with DNS-Cl. INS and several proteins with known N-terminals were prepared and then compared with standard DNS-amino acid standards.

Isoelectric Point Determinations. Purified INS (780 μg) was focused by using a sucrose gradient and 1% (w/v) ampholytes, pH 5–8, in a 110-mL preparative isoelectric focusing column (LKB). After the protein was focused (72 h, 4 °C, 500 V), the column was fractionated into 1-mL aliquots, the pH checked, and the INS concentration measured.

Determination of Optical Absorptivity. INS was exhaustively dialyzed against water at 4 °C and lyophilized. Extinction coefficients were determined on triplicate protein solutions (1.04 mg of INS/mL in water) at 277, 378, and 674 nm. Water content (6.5%) was determined by drying triplicate samples to a constant weight at 110 °C.

Chromophore Analysis. (A) Development of Standards. With the method of Bonnett & McDonagh (1973), hemin (25 mg) was oxidized to crude amorphous verdohemochrome, and the resulting oxidation products were esterified with BF_3 (14%). The extract was applied to a pad of silica gel G and eluted with chloroform–acetone (4:1 v/v). The eluate was dried, taken up in chloroform–acetone (95:5 v/v), and separated into two major blue bands on preparative thin-layer plates (2.5 mm) in a chloroform–acetone (95:5 v/v) solvent system. The bands were eluted as described above, and a portion of each fraction was further separated on analytical TLC plates (0.25 mm) run under continuous development conditions (4 h, 25 °C) in a chloroform–acetone (97:3 v/v) solvent system. The four intensely blue bands were scraped from the plates and eluted from the silicic acid with ethanol.

The eluates were pooled from a number of plates to be used as biliverdin IX standards. These standards were first compared to a commercially available biliverdin IX α, which was esterified and extracted (McDonagh & Palma, 1980). The properties of the derivatized biliverdin IX α agreed with those of one of the four standards generated, suggesting that the products of the hemin oxidation were indeed biliverdin IX isomers. To tentatively identify the biliverdin isomers, the spectral peaks and the mobility of the standards on an analytical TLC plate were compared to those reported for each isomer. Upon consideration of the chromatographic and spectroscopic data, the following assignments were made: (1) biliverdin IX α dimethyl ester, R_f 0.44, $\lambda_{max}(CHCl_3)$ 379, 656–664 nm; (2) biliverdin IX β dimethyl ester, R_f 0.51, $\lambda_{max}(CHCl_3)$ 381, 648–654 nm; (3) biliverdin IX γ dimethyl ester, R_f 0.35, $\lambda_{max}(CHCl_3)$ 376, 639 nm; (4) biliverdin IX δ dimethyl ester, R_f 0.19, $\lambda_{max}(CHCl_3)$ 379, 651–657 nm. All procedures were performed in semidarkness.

(B) Analysis of INS Chromophore. To conclusively identify the biliverdin IX isomer found on INS, the purified protein from approximately 100 mL of hemolymph was dialyzed against 50 mM phosphate buffer containing 10 mM KCl, pH 8.0. The dialyzate was extracted with an equal volume of ethyl acetate and centrifuged at 1000*g* for 10 min to promote phase separation. The ethyl acetate layer was discarded and the aqueous phase brought to pH 3.00 by addition of 1 M HCl. The chromophore released upon acidification was extracted 3 times with ethyl acetate, then pooled, and evaporated to dryness under N_2. The extracted chromophore was esterified by refluxing it in 14% BF_3 (15 mL) for 15 min and then gently shaking it overnight. The esterified product was then worked up as described above (Bonnett & McDonagh, 1973). The identity of the extracted chromophore was confirmed by ^1H NMR spectroscopy.

RESULTS

Purification. Hemolymph collected from fourth instar animals at head capsule slippage stage contained between 250 and 320 μg of INS/mL. Although this represents only 40%

Table I: Purification of Insecticyanin from *M. sexta* Hemolymph

procedure	vol (mL)	tot protein (mg)[a]	tot act. (units)[b]	sp act. (units/mg)	purification (*x*-fold)	yield (%)
hemolymph (concd)	31.5	516.6	86.0	0.17	0	100
Sephacryl S-200	10.0	80.0	56.0	0.71	4.4	66
DEAE-Bio-Gel	5.0	25.3	41.7	1.65	9.7	48
CM-Bio-Gel	10.0	17.8	36.7	2.06	12.1	43
hydroxylapatite	11.0	17.6	36.6	2.08	12.2	42

[a] Determined by Bradford assay. [b] One unit = 1.0 absorbance unit at A_{674}.

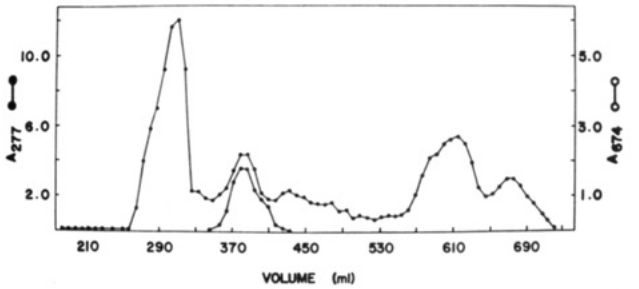


FIGURE 1: Gel filtration chromatography of fourth instar hemolymph (purification step 1). Hemolymph concentrated to one-third of its original volume was applied to a Sephacryl S-200 column (135 × 2.5 cm) equilibrated with 50 mM phosphate buffer containing 150 mM KCl, pH 6.7. Fractions of 5 mL were collected and monitored at 277 and 674 nm.

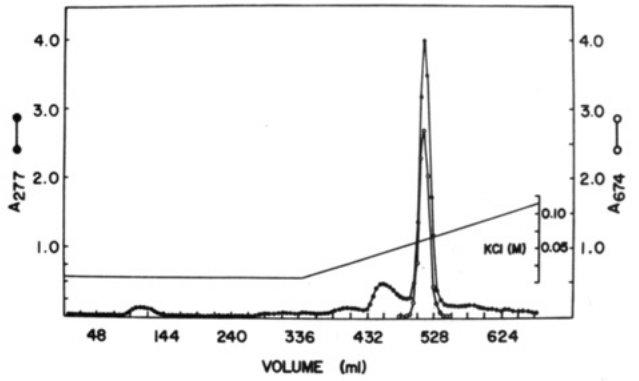


FIGURE 2: DEAE-Bio-Gel chromatography (purification step 2). The INS fractions from step 1 were simultaneously dialyzed and concentrated to 10 mL in a 10 mM Tris buffer containing 10 mM KCl, pH 8.6. The sample was applied to a column (85 × 1.5 cm) equilibrated with the same buffer. Protein was first eluted with 300 mL of the equilibration buffer and then with 400 mL of Tris buffer containing a linear KCl gradient from 10 to 125 mM KCl. Fractions of 2.5 mL were collected and monitored at 277 and 674 nm.

of the concentration found in the earlier part of the instar (J. T. Trost and W. G. Goodman, unpublished experiments), the amount of blood collection from older individuals (200–250 μL) far exceeds that which can be collected from younger animals. At fourth instar head capsule slippage stage, INS represents about 4–5% of the total hemolymph protein. Hemolymph samples stored at −30 °C up to 3 years have shown no INS degradation.

Molecular sizing on Sephacryl S-200 is a rapid and reliable first step in the large-scale purification of INS (Figure 1). High yields were sacrificed in taking a heart cut of the blue peak to eliminate several protein peaks eluting close to INS (see Table I). Since the hemolymph is particularly rich in INS, reduced recoveries do not pose serious problems. DEAE-Bio-Gel chromatography (Figure 2) served as an effective second step to remove all but trace amounts of the low molecular weight proteins; however, the storage protein, manducin (subunit M_r 78 000 and 72 000) (Kramer et al., 1980), remained a major contaminant (Figure 3). CM-Bio-Gel chromatography (Figure 4) appeared to yield a homogeneous protein preparation as determined by SDS–polyacrylamide gel electrophoresis (Figure 3). Nevertheless, hydroxylapatite chromatography was necessary to remove a minor contaminant that was detectable by immunochemical methods only (Figure 5). The cumulative yield of pure INS was 17.6 mg from 100 mL of hemolymph, which represented a 12-fold purification. Recoveries of pure INS from hemolymph varied from 42 to 54% (Table I).

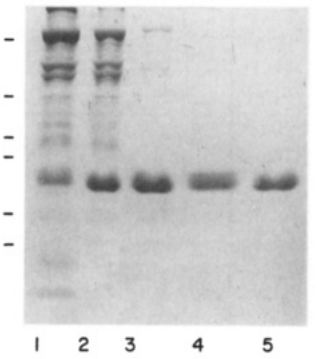


FIGURE 3: SDS–polyacrylamide gel electrophoresis of chromatographic fractions during the purification of INS: (lane 1) whole hemolymph (35 μg); (lane 2) INS fraction eluted from Sephacryl S-200 column (20 μg); (lane 3) INS fraction eluted from DEAE-Bio-Gel column (10 μg); (lane 4) INS fraction eluted from CM-Bio-Gel column (13 μg); (lane 5) INS fraction eluted from hydroxylapatite column (8 μg). The gel, 1-mm thick, was a 12.5% SDS–polyacrylamide slab with a 1-cm stacking gel. Migration of molecular weight standards are shown on the left side of the gel: bovine serum albumin (67 000), glyceraldehyde-3-phosphate dehydrogenase (36 000); carbonic anhydrase (29 000); trypsinogen (24 000); soybean trypsin inhibitor (20 000), myoglobin (17 500).

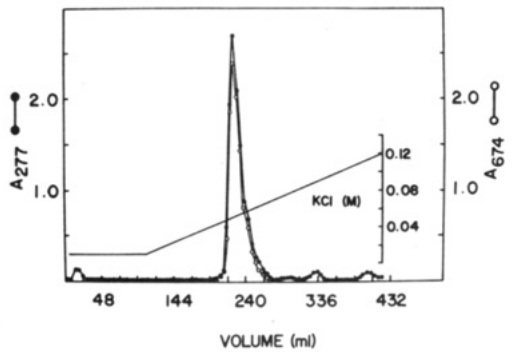


FIGURE 4: CM-Bio-Gel chromatography (purification step 3). The INS fractions from step 2 were simultaneously dialyzed and concentrated to 5 mL in a 10 mM phosphate buffer containing 10 mM KCl, pH 5.0. The sample was applied to a column (25 × 0.9 cm) equilibrated with the dialysis buffer. Protein was first eluted with 100 mL of equilibration buffer and then with 350 mL of phosphate buffer containing a linear KCl gradient from 10 to 150 mM. Fractions of 2.5 mL were collected and monitored at 277 and 674 nm.

Criteria for Homogeneity. INS appeared to be homogeneous when analyzed by native polyacrylamide gels or SDS gels in the presence or absence of 10 M urea (Figure 3). N-Terminal analysis yielded only one dansylated amino acid, glycine, indicating a single homogeneous species. Homogeneity was also indicated by the stoichiometric relationship of 3 mol

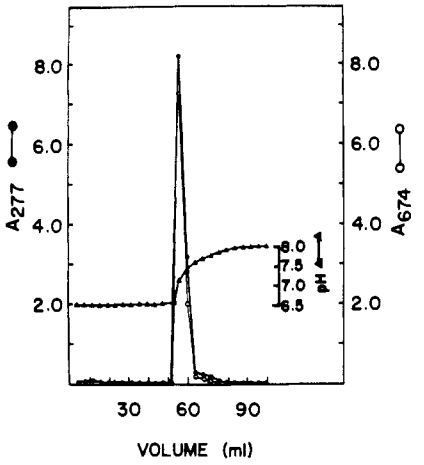


FIGURE 5: Hydroxylapatite chromatography (purification step 4). The INS fractions from step 3 were simultaneously dialyzed and concentrated to 10 mL in a 10 mM phosphate buffer containing 10 mM KCl, pH 6.5. The sample was applied to a column (15 × 0.9 cm) equilibrated with the dialysis buffer. Protein was eluted with 50 mL of the equilibration buffer and then with 50 mL of a 50 mM phosphate buffer containing 10 mM KCl, pH 8.0. Fractions of 2.5 mL were collected and monitored at 277 and 674 nm.

Table II: Physicochemical Properties of Insecticyanin

M_r	
native	71 600
subunit	23 000
no. of subunits	3
pI	6.25
$E^{0.1\%}_{1cm}$, 10 mM phosphate buffer, pH 6.5	
277 nm	2.90
378 nm	3.08
674 nm	2.56
carbohydrate content (%)	<0.5
N-terminal amino acid	glycine
chromophore	biliverdin IX γ

of biliverdin/mol of INS. Equilibrium sedimentation ultracentrifugation indicated a homogeneous species with a M_w of 71 600. Anti-INS serum, when exposed to either pure INS or hemolymph, yielded one precipitin arc upon immunoelectrophoresis.

Molecular Weight Determinations. The apparent molecular weight of INS was estimated by molecular sizing on a calibrated Sephacryl S-200 column by calibrating the elution volumes of molecular weight standards and interpolating that of INS. The molecular weight was determined to be 75 000. Equilibrium sedimentation studies demonstrated that the protein had a M_w of 71 600. SDS gel electrophoresis of INS resolved subunits of M_r 23 000. The trimeric structure of the native protein was further confirmed by cross-linking the monomers with dimethyl suberimidate. Electrophoresis on calibrated SDS gels revealed three major bands corresponding to the monomer, dimer, and trimer. Since N-terminal analysis yielded only one dansylated product and the ratio of biliverdin to protein = 3, it was concluded that INS is a trimeric protein composed of three identical subunits each containing one molecule of biliverdin (Table II).

Chemical Composition. Amino acid composition is presented in Table III. Values expressed are standardized to the molecular weight of the subunit. Determination of the N-terminal by derivatization with dansyl chloride indicated that glycine is the N-terminal amino acid.

Although INS contains no carbohydrate, the protein does stain positively on acrylamide gels and in the colorimetric assay for sialic acid and hexoses (White & Kennedy, 1981). The removal of the chromophore via acidified ethyl acetate extraction leads to loss of the carbohydrate-positive response, suggesting that the biliverdin chromophore was responsible.

Table III: Amino Acid Composition of Hemolymph Insecticyanin

amino acid	residues/ peptide chain[a]	amino acid	residues/ peptide chain[a]
aspartic acid	28	methionine	2
threonine[b]	11	isoleucine[d]	9
serine[b]	14	leucine[d]	12
glutamic acid	16	tyrosine	16
proline	9	phenylalanine	10
glycine	13	histidine	6
alanine	17	lysine	21
half-cystine[c]	4	arginine	2
valine	16	tryptophan[e]	4

[a] All values were standardized to a M_r of 23 000 and were an average of nine determinations except where noted. Total was 210. [b] Calculated from the average of three determinations at 24, 48, and 72 h extrapolated to zero time of hydrolysis. [c] Determined as cysteic acid. [d] Calculated from the average of three determinations at 72 h. [e] Determined by the method of Edelhoch (1967).

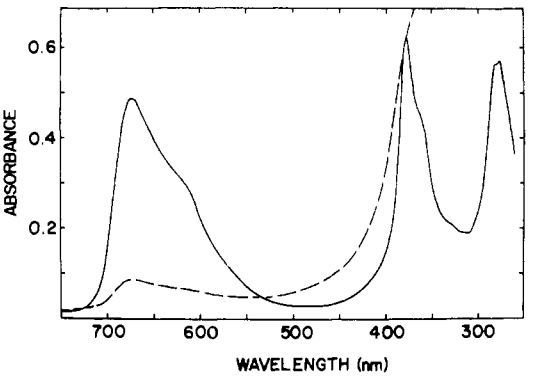


FIGURE 6: Absorption spectra of purified INS and hemolymph in 10 mM phosphate buffer, pH 6.5. The solid line represents INS while the broken line represents hemolymph diluted with phosphate buffer.

To establish the biliverdin content of the protein, a predetermined concentration of INS was stripped of its chromophore with acidified ethyl acetate. The resulting extracts were pooled and dried under vacuum. The material was then solubilized in chloroform–ethanol (99:1 v/v) and the concentration determined from the extinction coefficient established for biliverdin IX γ dimethyl ester (E = 54 400) (McDonagh, 1979). It was assumed that, as with bilverdin IX α, E is similar for both the esterified and nonesterified forms. The analysis indicated a biliverdin to protein ratio of 5.28×10^{-7} M to 4.93×10^{-7} M or approximately 1.07 mol of biliverdin/mol of INS subunit.

Identification of Chromophore. The absorption spectra for hemolymph and purified INS are shown in Figure 6. The purified protein dissolved in 10 mM phosphate buffer, pH 6.5, has three characteristic absorption peaks at 277, 378, and 672–675 nm, while under the same conditions hemolymph displays a broad peak at 668–677 nm.

Biliverdin was extracted from the purified protein and examined in both the esterified and unesterified form (Table IV). A comparison of the absorbance peaks observed for the INS chromophore with those of the biliverdin IX standards indicates that the isomer is biliverdin IX γ. However, on the basis of the spectral data, the possibility that the chromophore is a mesobiliverdin cannot be ruled out. Thin-layer chromatography provided further evidence that the chromophore was biliverdin IX γ. TLC of the esterified product resulted in the separation of two blue fractions of equal intensity, one of which comigrated with the γ isomer (R_f 0.35) and one with the δ isomer (R_f 0.19). Comparison of absorption spectra virtually

Table IV: Absorption Characteristics of Biliverdin Isomers

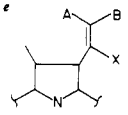
isomer	λ_{max} (nm)[a]	solvent
biliverdin IX α dimethyl ester	379, 656–664; 380, 656–664	$CHCl_3$
	375, 695; 376, 688–691	MeOH–HCl (95:5 v/v)
biliverdin IX β dimethyl ester	381, 648–654; 382, 648–654	$CHCl_3$
	NA; 376, 698	MeOH–HCl
biliverdin IX γ dimethyl ester	376, 639; 376, 639	$CHCl_3$
	360, 700; 367, 688–691	MeOH–HCl
biliverdin IX δ dimethyl ester	379, 651–657; 378, 651–657	$CHCl_3$
	NA; 356–358, 682–686	MeOH–HCl
dimethyl ester chromophore from INS	375, 635–638	$CHCl_3$
	357–359, 688–695	MeOH–HCl
nonesterified chromophore from INS	358, 600	$CHCl_3$
	357–358, 692–696	MeOH–HCl

[a] Where there are two sets of values, the first represents data taken from McDonagh (1979) and the second represents data from the present study. NA, not available.

Table V: Chemical Shifts of Resonances[a]

	biliverdin IX γ dimethyl ester		methoxylated biliverdin IX γ dimethyl ester	
resonance	obsd	McDonagh[b]	obsd	Choussy & Barbier[c]
5, 15	6.00, 5.88	5.92, 5.80	6.19, 5.90	6.10, 5.84
10	6.81	6.72	6.78	6.85
$-CO_2CH_3$	3.61	3.62	3.67	3.62
$-CH_2CH_2-$	2.52	2.51	2.59	2.54
$-CH_3$	2.07 (2)[d]	2.10	2.17	2.09
	2.10	2.13	2.18	2.14
	2.19	2.23	2.19	2.18
			2.20	
vinyl[e]				
A′	~5.4, 5.51	5.3–5.5	5.49	5.40
B′	~5.4, 5.46	5.3–5.5	5.45	5.36
X′	6.61, 6.74	6.4–6.7	6.47	6.62
$-OCH_3$			3.27	3.27
—CH			4.46	4.45
$-CH_3$			1.47	1.46

[a] All shifts in ppm relative to Me_4Si = 0. [b] McDonagh (1979). [c] Choussy & Barbier (1975). [d] The resonance of 2.07 was for two methyl groups. [e]



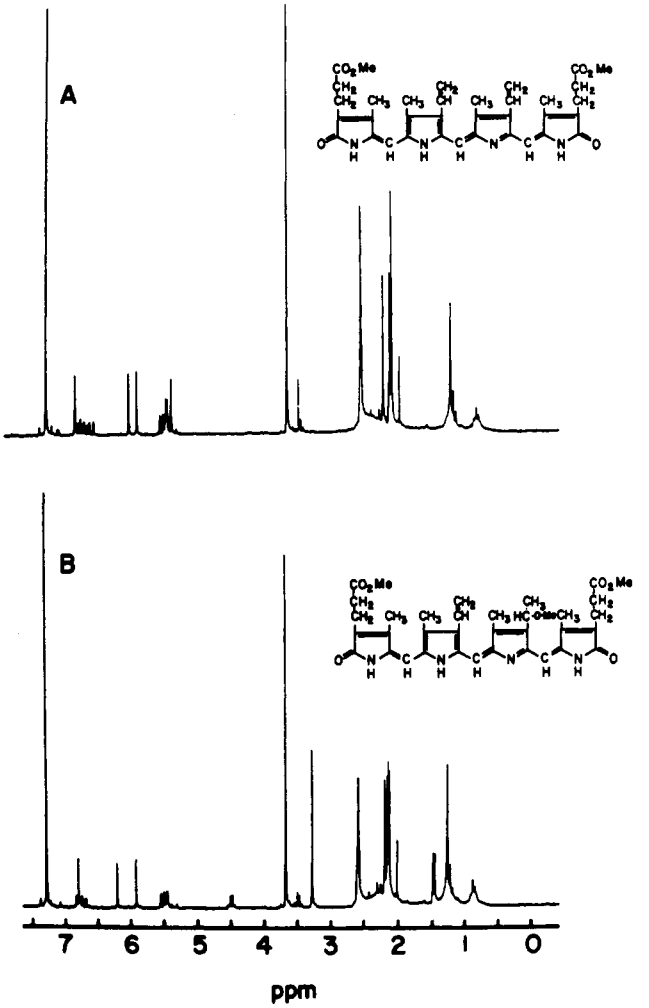


FIGURE 7: ^1H NMR of the esterified biliverdin extracted from INS. Spectra were taken at room temperature, 200 MHz, in $CDCl_3$. Panel A is the spectrum of biliverdin IX γ dimethyl ester; panel B is the spectrum of the methoxylated biliverdin IX γ dimethyl ester.

eliminated the possibility that the chromophore was the δ isomer. It was presumed that the products were the mono- and diesterified forms of the γ isomer; therefore, both fractions were eluted from TLC plates and analyzed by ^1H NMR spectroscopy. The ^1H NMR spectrum conclusively established the identity of the biliverdin as the γ form (Figure 7A). By virtue of all methylene protons on the propionic side chain appearing as a broad singlet (δ 2.52), this isomer was distinguished from the others, which displayed a pair of triplets. The rest of the resonances were consistent with values reported in the literature (McDonagh, 1979) (Table V). The spectrum of the slower migrating compound showed a considerably simplified olefinic region and new resonances at δ 3.27 (s, 3 H), 4.46 (q, 1 H), and 1.47 (d, 3 H) (Figure 7B). This suggested that methanol had been added across one of the vinyl groups. Decoupling experiments further established that the doublet and quartet were indeed coupled to one another (J = 7 Hz). At this time, no assignment can be made as to which vinyl group was modified, although Choussy & Barbier (1975) have indicated that methanol is added across the C13-vinyl group. It should be noted that Choussy & Barbier (1975) used sulfuric acid as the esterification catalyst and form a dimethoxylated biliverdin with methanol across the C8- as well as the C13-vinyl group. The absence of methyl resonances at δ 1.7–1.9 showed that the addition of methanol occurs without other isomerization of biliverdin. It was concluded that the slower migrating compound was an artifact of the esterification procedure.

Isoelectric Focusing. Purified INS focused in a pH gradient from 5 to 8 yielded a distinct blue band at pH 6.26. Multiple bands were not observed.

Crystal Formation. Crystals were grown as described by Cherbas (1973) in an ammonium sulfate solution. The in-

tensely blue crystals formed overnight at 4 °C and continued to grow in size over a 2–4-day period. Crystals varied in size depending upon the concentration of the protein. The best results were obtained when the initial concentration of INS was >5 mg/mL. The resulting crystals were bipyramidal in shape, with the long axis of the larger crystals reaching nearly 1 mm and the base approximately one-third to half the length. The pyramids were hexagonal, thus forming a 12-sided crystal.

Discussion

The larval hemolymph of *M. sexta* appears to contain three major proteins: lipophorin, a lipid transporting protein (Pattniak et al., 1979; Shapiro et al., 1984), manducin, a storage protein (Kramer et al., 1980), and insecticyanin. Although hemolymph concentrations of insecticyanin do not approach those observed for lipophorin or manducin, INS concentrations do exceed 0.6 mg/mL at ecdysis from the third to fourth instar. Despite the rapid decline of INS levels during the fourth instar, the concentrations remain sufficiently high at the time of hemolymph collection to employ a simple four-step procedure to yield purified INS. With recoveries ranging from 42 to 54%, the method presented here is a significant improvement over a previously cited but unpublished method for which recoveries ranged from 18 to 20% (Cherbas, 1973).

A comparison of the properties of INS with the only other biliverdin-associated protein purified from insect hemolymph indicates that the proteins are distinctly different. The *Locusta* hemolymph cyanoprotein is considerably larger (M_r 350 000) and is composed of four identical subunits (M_r 83 000), each of which binds 2 mol of biliverdin/mol of subunit (Chino et al., 1983). In contrast to INS, in which no carbohydrate was detected, *Locusta* cyanoprotein contains a small amount of mannose (3.5%). A comparison of the amino acid composition indicates no homology.

While the major biliverdin isomer found in nearly all organisms, including nonlepidopterous insects, is the IX α isomer (McDonagh, 1979), the IX γ form predominates in Lepidoptera. It was originally suggested that the chromophore is a mesobiliverdin (Dahlman, 1969), and although there is some spectral overlap between the two bilins, absorption and ^{1}H NMR spectroscopy as well as chromatographic analysis confirms that the chromophore is the biliverdin IX γ isomer. This assignment is consistent with those for biliverdin found in other species of Lepidoptera (Rüdiger et al., 1969; Barbier, 1981). From TLC analysis of the dimethyl esters, it was initially thought that two types of biliverdin might be associated with the protein. However, analysis by ^{1}H NMR spectroscopy of the more slowly migrating bilin identified it as an artifact generated during esterification, a methoxylated biliverdin IX γ. Choussy & Barbier (1975) first reported this derivative in their study on biliverdin IX γ of the bufferfly wing and indicated that one or two methanols could be added across the vinyl groups. The present study could establish the identity of only one addition, presumably at C13.

At present, the interaction between the chromophore and the protein is thought to be noncovalent in nature. In *Locusta*, the chromophore can be removed simply by the addition of methanol under neutral conditions (Chino et al., 1983); however, in *Manduca* the chromophore can be removed only after the protein solution is acidified and extracted with a moderately polar organic solvent. Mildly basic conditions (pH <11) do not appear to promote chromophore loss upon extraction.

Although several biological functions have been ascribed to the biliverdin-containing proteins in insects, the most important and obvious is that of cryptic coloration. The bilins, in conjunction with carotenoids of the epidermis, produce a green hue, which provides camouflage for the plant feeding insect. Studies on INS tissue distribution confirm that 70–80% of the total INS in a late fourth instar animal is sequestered in the epidermis, where it is clearly visible through the transparent exoskeleton. The light-sensitive nature of biliverdin IX γ has suggested another function. Barbier (1981) has proposed that in the presence of visible light biliverdin IX γ will undergo a phototransformation and that its synthesis and degradation under the influence of light allow it to function as a biological clock. Although the biliverdins are indeed light sensitive in the unbound state, they appear remarkably stable when associated with INS. Other novel functions for the biliverdins have been proposed by Barbier (1981, 1983) and by Bois-Choussy & Barbier (1981).

One of the most intriguing aspects of INS is its regulation in the epidermis by the juvenile hormones. At wandering stage, when JH titers drop and the animal undergoes physiological changes that initiate pupation, the epidermis loses its characteristic blue color. Histological studies (Sedlak & Gilbert, 1979) have correlated this bleaching with loss of pigmented granules from the epidermis. In the black larval mutant, a mutant with abnormally low JH titers (Bergot et al., 1981; Kramer & Kalish, 1984), the epidermal cell does not sequester the protein in the apical storage granules typical of wild-type epidermal cells. This aberrant condition is reversed when physiological concentrations of JH are topically applied; apical granules appear, and the epidermal INS titers nearly double (Goodman et al., 1982; W. G. Goodman unpublished experiments). Thus, the INS concentration in the hemolymph and epidermis appears to be under the direct control of JH, and by employment of INS as a marker, insight may be afforded into the mode of action of juvenile hormone.

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Registry No. Biliverdin IX γ, 20890-42-0; biliverdin IX γ dimethyl ester, 26280-00-2; methoxylated biliverdin IX γ dimethyl ester, 58416-09-4.

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Methionyl-tRNA Synthetase from *Escherichia coli*: Primary Structure at the Binding Site for the 3′-End of $tRNA_f^{Met}$†

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ABSTRACT: It was previously shown that when the tryptic fragment of methionyl-tRNA synthetase from *Escherichia coli* is incubated with periodate-treated initiator tRNA, it is inactivated due to the formation of a covalent 1:1 complex that could be stabilized by reduction with cyanoborohydride [Hountondji, C., Fayat, G., & Blanquet, S. (1979) *Eur. J. Biochem. 102*, 247–250]. In this work, the residues labeled in the trypsin-modified enzyme have been identified. After chymotryptic digestion of the protein–tRNA complex, two major labeled peptides (A and B) and a minor one (C) were isolated and identified by sequencing. The radioactivity associated with peptides A–C represented 65–75, 20–25, and 2–4%, respectively, of the radioactivity eluted from the peptide maps. Peptides A and B encompassed lysines-335 and -61, respectively. Both these lysines were fully labeled. Peptide C encompassed lysines-142, -147, and -149, each of which was incompletely labeled. The significance of these results is discussed in light of the known crystallographic structure of the enzyme.

Among bacterial aminoacyl-tRNA synthetases, *Escherichia coli* methionyl-tRNA synthetase has been the object of many studies aimed at probing structure–activity relationships [reviewed in Blanquet et al. (1979)]. In particular, a fully active proteolyzed fragment of M_r 64K derived from native methionyl-tRNA synthetase (2 × 76K) was crystallized (Cassio & Waller, 1971a; Waller et al., 1971). The crystallographic structure of the fragment (MTS_T),[1] presently solved at 2.5-Å resolution (Zelwer et al., 1982), indicates an elongated molecule (90 Å × 52 Å × 44 Å) composed of three domains organized in a biglobular structure. The N-terminal globule contains two domains, one of which presents a "mononucleotide binding fold" similar to the cofactor binding site of dehydrogenases (Zelwer et al., 1982; Blow et al., 1983). The primary structure of the enzyme has also been determined (Barker et al., 1982; Dardel et al., 1984), and efforts are being made in order to identify the side chains that belong to the substrate binding sites.

Several affinity labeling methods have been applied to aminoacyl-tRNA synthetases (Schimmel, 1977; Bruton &

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[1] Abbreviations: MTS_T, active crystallized tryptic fragment of methionyl-tRNA synthetase; $tRNA_{ox}$ or tRNA dialdehyde, tRNA oxidized by periodate at its 3′-terminal ribose; ATP_{ox}, ATP oxidized by periodate at the ribose; $ATP_{ox\text{-}red}$, ATP_{ox} reduced with sodium borohydride; N^{α}-acetyl-L-lysyl-ATP_{ox}, α-acetylated lysine derivative in which the ε-NH_2 group has reacted with the dialdehyde group of oxidized ATP; N^{α}-acetyl-L-lysyl-$ATP_{ox\text{-}red}$, N^{α}-acetyl-L-lysyl-ATP_{ox} reduced with sodium borohydride; PMSF, phenylmethanesulfonyl fluoride; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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