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Isolation and characterization of a yellow-colored protein from the hemolymph of the tobacco hornworm, *Manduca sexta*

Martel, Ralph Roland, Ph.D.

The University of Arizona, 1991
ISOLATION AND CHARACTERIZATION OF A YELLOW-COLORED PROTEIN FROM THE HEMOLYMPH OF THE TOBACCO HORNWORM, MANDUCA SEXTA

by

Ralph Roland Martel

A Dissertation Submitted to the Faculty of the
DEPARTMENT OF BIOCHEMISTRY
In Partial Fulfillment of the Requirements For the Degree of
DOCTOR OF PHILOSOPHY
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THE UNIVERSITY OF ARIZONA

1991
As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Ralph Roland Martel entitled Isolation and Characterization of a Yellow-colored Protein from the Hemolymph of the Tobacco Hornworm, Manduca sexta and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

Date 06/24/91

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

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ABSTRACT

A yellow-colored protein (YCP) has been isolated from the hemolymph of fifth instar, wandering stage larvae of *Manduca sexta*. The molecular mass of reduced and denatured YCP was 31 kDa. Gel filtration chromatography suggested that native YCP was a monomer. The absorbance spectrum of YCP contained maxima at 278 nm and 405 nm.

The amino acid composition and the N-terminal sequence of YCP were determined. Circular dichroism indicated that YCP consisted of 68% B-pleated sheet and 32% random coil. The YCP polypeptide chain was found to be glycosylated. Carbohydrate analysis suggested that mannose and N-acetylglucosamine were present in a 3:1 ratio.

Chromophore was released from YCP through treatment with methanol and chloroform. In neutral solution and in acid, the released chromophore showed the absorbance characteristics of the ommochrome, ommatin D. In addition, the chromophore was sensitive to treatment with arylsulfatase as would be expected for ommatin D. The polypeptide chain of YCP was synthesized by the larval fat body and was detectable in hemolymph throughout the life cycle. However, only during the fifth instar did YCP polypeptide levels in the hemolymph increase significantly. The highest hemolymph concentration was observed on the first day of pupation, whereafter it gradually decreased.

The association of chromophore with the YCP polypeptide
was transient. In fifth instar wandering stage larvae and in female moths, YCP polypeptide and chromophore were detectable in the hemolymph. During the wandering stage, increasing amounts of chromophore became associated with the YCP polypeptide. However, in feeding fifth instar larvae and in male moths, the YCP polypeptide but not the chromophore was detectable.

No representatives of seven other insect orders contained hemolymph proteins that cross-reacted with anti-YCP antiserum. However, each of four other lepidopteran examined had an immunologically-related hemolymph protein of approximately 31 kDa.

Ommochromes arise in insects as end products of the metabolism of tryptophan. As such, ommochromes occur in both the tissues and the excreta of insects. We propose that in M. sexta, one such tryptophan metabolite is found in the hemolymph associated with a specific 31 kDa protein.
CHAPTER 1

INTRODUCTION
This dissertation describes the isolation and characterization of a novel yellow-colored protein (YCP) from the blood of *Manduca sexta*. The tobacco hornworm, *M. sexta*, is a sphinx or hawk moth (order Lepidoptera, family Sphingidae). It is a holometabolous insect: after larval life as a caterpillar, the animal undergoes a complete metamorphosis, ending life as a moth. YCP was purified from the blood (i.e. hemolymph) of larvae.

The isolation of YCP, described in Chapter 2, relied on the density, solubility, size, glycosylation and surface charge of this chromoprotein. There are two components to YCP: a glycosylated polypeptide chain and a chromophore. Chapter 3 presents the characterization of the polypeptide chain, including the compositional analysis of protein-linked carbohydrate. Chapter 4 deals with the characterization of the chromophore which appears to be ommatin D, a metabolite of tryptophan. Some aspects of the biology of YCP are discussed in Chapter 5. The site of synthesis of the YCP polypeptide was identified, titers in the hemolymph were measured, and the association of chromophore with polypeptide was monitored. Chapter 5 concludes with a hypothesis regarding the function of YCP.

This introduction will describe the life cycle of *M. sexta* and the use of this animal in insect biochemistry. The metabolism of tryptophan to ommochromes in insects will be reviewed. Finally, research on ommochrome-binding proteins
from other lepidoptera will be summarized.

**MANDUCA SEXTA**

The egg of *M. sexta* (formerly *Protoparce sexta*) is a light-green sphere, approximately 1.5 mm in diameter and weighing approximately 1 mg. Usually oviposited on the underside of tobacco leaves, eggs are occasionally found on the leaves of other *Solanaceae* such as tomatoes and peppers. Approximately four days after oviposition, a larva hatches and begins feeding on the host plant. Larvae have an exoskeleton which restricts their growth. To circumvent this restriction, the insect molts, that is, it will shed its exoskeleton and synthesize a new, larger one. The form the insect assumes between molts is called an instar (Chapman, R.F., 1969). Over the course of 11 to 12 days, *M. sexta* larvae go through four such instars, feeding voraciously whenever they are not molting.

The fifth and final instar is divided into two periods: a feeding stage followed by a wandering stage. The feeding stage lasts about four days. At the end of this period, the animal has reached its greatest weight of approximately 10 g. This represents a 10,000-fold increase from the 1 mg egg from which it hatched 16 days earlier. At the end of the feeding stage, all the nutritional requirements of the animal have been met. It will not need to feed or drink for the remainder of its life. The wandering stage begins: the larva stops feeding, purges its gut of all contents, and crawls off the
host plant. For approximately three days, the wandering larva searches for a suitable place to burrow and pupate.

Following pupation, metamorphosis proceeds over the course of three weeks before the adult moth emerges. A few days after emergence of the adult, the females will have mated and will have laid their eggs, thus starting the cycle anew. The duration of the cycle is 6 to 7 weeks.

Throughout larval life, *M. sexta* has a cryptic green coloration that matches a background of plant leaves (Kawooya et al., 1985). This coloration results from the blending of yellow and blue pigments. In *M. sexta*, yellow pigment is derived from dietary carotenes and blue coloration comes from a blue-colored biliprotein, insecticyanin (Kawooya et al., 1985). Both pigments are found in the integument and hemolymph of the insect. Lipophorin, the major hemolymph lipoprotein, transports dietary carotenes, and thus has an intense yellow color (Kawooya et al., 1985). Insecticyanin is synthesized by the larval epidermis (Riddiford, 1982) and derives its color from biliverdin IX-\(\gamma\) (Cherbas, 1973 and Goodman et al., 1985); its sequence (Riley et al., 1984) and crystal structure (Holden et al., 1987) have been reported.

**YCP in wandering stage larvae**

With the onset of the wandering stage, the behavior and the appearance of larvae change. The coloration of these active larvae becomes less intense as synthesis of insecticyanin ceases and as this biliprotein is mobilized from
the epidermis to the hemolymph (Kiely and Riddiford, 1985). Also, a pinkish stripe appears in the epidermis overlying the dorsal vessel; presumably, this stripe provides the larva with camouflage as it crawls on the ground in search of a suitable site to burrow and pupate (Hori and Riddiford, 1982). By the end of the wandering stage, most of the insecticyanin has been eliminated from the epidermis and the pinkish stripe has also disappeared. The mass of the larva is approximately 60% of what it was on the last day of feeding (Reinecke et al., 1980). At the end of wandering, the activity of the larva decreases as the prolegs retract in preparation for pupation.

To isolate YCP, hemolymph was obtained from laboratory-reared fifth instar larvae that were at the end of the wandering stage. When M. sexta are reared on an artificial diet that has only traces of carotene, larvae have a decided blue color, their hemolymph is greenish-blue, and lipophorin isolated by density gradient ultracentrifugation (Shapiro et al., 1984) is not as intensely yellow as it is in the natural state. However, the hemolymph from laboratory-reared animals became distinctly green during the wandering stage just before the metamorphosis of caterpillars to pupae. This change in color from blue-green to green was more pronounced when lipophorin was removed by density-gradient ultracentrifugation: the color of lipophorin-free hemolymph changed from blue during the feeding stage of the fifth instar to green during the wandering stage. Only a protein-bound
pigment, YCP, appeared to be responsible for this change.

**Hemolymph of wandering stage larvae**

*M. sexta* is frequently used in insect biochemistry since it is easily reared in the laboratory, and grows rapidly to a comparatively large size (Bell and Joachim, 1976). Fully developed larvae can yield more than 1 ml of hemolymph. As a consequence, many proteins from the hemolymph have been studied (reviewed by Kanost et al., 1990). Proteins from the hemolymph of wandering stage fifth instar larvae, subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) are shown in Fig. 1. In Fig. 1, the major protein bands are labelled. Arylphorin is the predominant protein found in the hemolymph at wandering (Kramer et al., 1980). This is a hexameric protein of approximately 540 kDa which comprises 92 kDa and 87 kDa subunits. Arylphorin is a storage protein: it serves as a reservoir for amino acids from which pupal and adult proteins are synthesized. It is rich in aromatic amino acid residues and the primary sequence of each subunit has been deduced (Willott et al., 1989). Two other storage proteins have been identified in wandering larvae: larval serum protein (LSP) 2 and LSP-3. LSP-2, a hexamer with 79 kDa subunits, contains 6.2 mol% methionine (Ryan et al., 1985, and X.Y. Wang, personal communication). LSP-3 is distinct from both arylphorin and the methionine-rich storage protein (Kanost et al., 1990). Proteins involved in the transport of lipids in the hemolymph are apolipoporphorins I,
Hemolymph collected from wandering stage fifth instar larvae served as the starting material from which YCP was purified. The left-most lane contains molecular weight marker proteins; their molecular masses (in kDa) are indicated on the left. The remaining lanes show hemolymph proteins. Bands due to characterized proteins are labelled on the right-hand side. The abbreviations are: ApoLP, apolipophorin; TFe, transferrin; LSP, larval-specific protein; PLP, post-larval protein; Serpin, serine-protease inhibitor.
II and III (for review, see Shapiro et al., 1988). While apolipophorin III does not become associated with the lipophorin particle of wandering larvae, this protein is present in the hemolymph.

Two binding proteins which occur in the hemolymph of wandering stage larvae are the monomeric 77 kDa, iron-binding transferrin (Bartfeld and Law, 1990) and the bilin-binding protein, insecticyanin (Cherbas, 1973). The latter is a tetramer comprised of identical 21.5 kDa subunits (which appear slightly larger in SDS-PAGE gels) (Riley et al., 1984). The hemolymph of wandering stage larvae also contains a post-larval protein (PLP) (Ryan et al., 1988) which appears to be a homodimer with 50 kDa subunits. Serpins are serine protease inhibitory proteins; in M. sexta larvae, four serpins of approximately 47 kDa and with differing specificities have been identified (Kanost, 1990). Finally, Fig. 1 shows the protein band which corresponds to YCP.

OMMOCHROMES

Since an ommochrome, ommatin D, appears to be responsible for the color of YCP, a overview of ommochromes and tryptophan metabolism in insects is appropriate. The overview that follows is based on the extensive review by Linzen (1974) and the more recent review by Kayser (1985).

Ommochromes are pigments that arise in insects as products of the metabolism of tryptophan. Specifically, ommochromes arise from the oxidative condensation of 3-
hydroxykynurenine (Butenandt and Schaefer, 1962). These pigments contain phenoxazine and quinoline heterocyclic ring systems.

The ommochromes were named by Becker in 1942 because of their occurrence in the ommatidia of the insect eye. Early knowledge of the structure, chemistry and biochemistry of the ommochromes was largely gained in the laboratory of Butenandt (see references in Butenandt and Schafer, 1962, and in Linzen, 1974).

As in vertebrates, tryptophan is an essential amino acid for insects. However, insects lack the glutarate pathway by which tryptophan is metabolized to acetate and carbon dioxide. Likewise, insects have a dietary requirement for nicotinic acid, since the pathway which utilizes tryptophan to synthesize nicotinamide dinucleotide (NAD'') is inoperative. However, excess tryptophan is harmful to the insects: elevated levels of dietary tryptophan cause retardation or complete failure of development in a number of species, including the butterfly Pieris brassicae (Kayser, 1979, and references therein). Thus, tryptophan levels must be regulated. This is accomplished via the ommochrome pathway.

The tryptophan to ommochrome pathway is presented in Fig. 2. First, the pyrrole ring of tryptophan is cleaved by tryptophan 2,3-dioxygenase to yield N-formylkynurenine. Tryptophan 2,3-dioxygenase is an heme-containing enzyme which requires molecular oxygen. N-formylkynurenine is subsequently
Fig. 2:

Metabolism of tryptophan in insects

The metabolic pathway from tryptophan to ommochromes is shown here (adapted from Linzen, 1974). The compounds shown are: (I), tryptophan; (II), N-formylkynurenine; (III), kynurenine; (IV), 3-hydroxykynurenine; (V), xanthommatin; (VI), dihydroxanthommatin; (VII), ommatin D; (VIII), rhodommatin; (IX), kynurenic acid; (X), kynurine; (XI), xanthurenic acid; (XII), 4,8-dihydroxyquinoline; (XIII), anthranilic acid; (XIV), 3-hydroxyanthranilic acid; (XV), cinnabarinic acid.
converted to kynurenine by kynurenine formamidase. Kynurenine-3-hydroxylase, a NADPH-dependent monooxygenase, is responsible for the conversion of kynurenine to 3-hydroxykynurenine. Finally, the oxidative condensation of 3-hydroxykynurenine yields ommochromes. Phenoxazinone synthase catalyzes the oxidative condensation.

Several side reactions divert intermediates from the ommochrome pathway. Both kynurenine and 3-hydroxykynurenine can undergo transamination to keto acids which then undergo spontaneous cyclization, yielding kynurenic acid and xanthurenic acid respectively. Decarboxylation of these compounds yields kynurine and 4,8-dihydroxyquinoline. Kynurenine and 3-hydroxykynurenine are also substrates for kynurenase which catalyzes their conversion to anthranilic acid and 3-hydroxyanthranilic acid, respectively. Through a condensation reaction, 3-hydroxyanthranilic acid can yield cinnabarinic acid, the simplest phenoxazine pigment occurring in insects.

On the basis of sulfur content and apparent molecular weight, the ommochromes have been divided into three subgroups: the ommatins, the ommins, and the ommidins. Both the ommins and the ommidins contain sulfur derived from cysteine or methionine, which is not present as sulfate. The ommins behave as high molecular weight compounds whereas the ommidins behave as low molecular weight compounds. Like the ommidins, the ommatins behave as low molecular weight
molecules. However, the ommatins contain no sulfur except in the form of an occasional sulfate moiety.

The simplest ommatin is xanthommatin, which is produced by the oxidative condensation of two molecules of 3-hydroxykynurenine. Xanthommatin can undergo a reversible reduction to dihydroxanthommatin. Both of these compounds are precursors to other ommochromes.

Rhodommatin and ommatin D are derived from dihydroxanthommatin. The first is the O-β-D-glucoside derivative and the second is the sulfate ester of dihydroxanthommatin. Whereas most ommochromes display a bathochromic shift on reduction and are scarcely soluble in water and in neutral organic solvents, rhodommatin and ommatin D are water-soluble and are maintained in a reduced form. These water-soluble ommochromes appear to be restricted to the lepidoptera.

Ommochromes are common in the Arthropoda. In insects, they appear to function as screening pigments in the eye, as pattern pigments in the integument and as excretory products used to eliminate excess tryptophan. Xanthommatin is found universally in the insect eye, sometimes in conjunction with other ommochromes. Ommochromes are also frequently present in the epidermis, where they contribute to the visible coloration of insects. As excretory products, ommochromes have been isolated from feces and meconia.

In holometabolous insects, the formation of ommochromes
is correlated with the massive break-down of larval proteins that occurs at the onset of metamorphosis. In lepidopteran larvae, xanthommatin is commonly found as the only ommochrome in epidermis. There, it accumulates within membrane-enclosed intracellular granules, in association with proteins. Kayser (1979) found that in the butterfly *Pieris brassicae*, xanthommatin accumulates in the integument of feeding larvae. At wandering, it is almost completely discharged from this site, presumably to be excreted in the form of the soluble conjugates, rhodommatin and ommatin D.

To date, rhodommatin and ommatin D have been isolated only from the wings and excreta of lepidoptera and never from the eyes or the larval epidermis. Thus, these compounds appear to serve solely as excretory products.

In *M. sexta*, the existence of the ommochrome pathway has been demonstrated (Hori and Riddiford, 1981 and 1982). During the feeding stage of the fifth instar, kynurenine and 3-hydroxykynurenine accumulate in the dorsal epidermis of larvae. At the onset of the wandering stage, these compounds are metabolized to dihydroxanthommatin which becomes visible as the pink stripe in epidermis overlying the dorsal vessel. At the end of the wandering stage, the stripe disappears as ommochrome is eliminated from the epidermis. Ommochrome synthesis in the dorsal epidermis of *M. sexta* is under hormonal control (Hori and Riddiford, 1982, reviewed in Riddiford, 1985). Juvenile hormone inhibits ommochrome
formation while ecdysteroid stimulates it.

At present, three ommochrome-binding proteins have been identified in lepidoptera. In the silkmoth, *Hyalophora cecropia*, a 24 kDa protein from the eyes contains covalently bound ommin and xanthommatin (Ajami and Riddiford, 1971). In the silkworm, *Bombyx mori*, two xanthommatin-binding proteins have been described: a red-colored 100 kDa protein from the hemolymph of the *rb* mutant (Ishiguro and Nagamura, 1971) and a 13 kDa protein from pigment granules within epidermal cells of the *quail* mutant (Sawada et al., 1990). YCP is distinct from these ommochrome-binding proteins.
CHAPTER 2

PURIFICATION OF THE YELLOW-COLORED PROTEIN
MATERIALS AND METHODS:

ANIMALS

Fertilized *M. sexta* eggs were supplied by Drs. J.P. Reinecke and J.S. Buckner (U.S. Department of Agriculture, Fargo, North Dakota). After hatching, the larvae were reared en masse until the end of the fourth instar after which they were reared individually (Bell and Joachim, 1976). Throughout, they were fed an artificial diet rich in wheat germ (Reinecke et al., 1980). Temperature was maintained at 26°C with a 16 h light/8 h dark photoperiod. On the first or second day of the wandering stage, the larvae were usually transferred to pupation chambers in wooden blocks.

PROTEIN PURIFICATION

Hemolymph was collected from fifth instar larvae that had ceased feeding and were in either the second or third day of the wandering stage; that is, one or two days before pupation. The animals were tranquilized by submerging them in a mixture of ice and water and were bled through an incision in the dorsal vessel. The hemolymph was collected at 0°C into a beaker which contained enough reduced glutathione (U.S. Biochemical Corp., Cleveland, OH) and diisopropyl fluorophosphosphate (Aldrich Chemical Co., Milwaukee, WI) to reach final concentrations of 50 mM and 10 mM respectively (Shapiro and Law, 1983). Typically, 110-120 animals were bled to obtain about 160 ml of hemolymph. The hemolymph was centrifuged (15,000 x g, 20 min., 4°C) to remove hemocytes and
insoluble materials. Next, lipoproteins were removed by ultracentrifugation in a KBr-density gradient (242,000 x g, 16 h, 4°C) using a vertical rotor (VTi 50, Beckman) in a Beckman L8-70 ultracentrifuge (Shapiro et al., 1984). After ultracentrifugation, the blue-green colored subphase was separated from the lower-density yellow-colored lipoproteins. The subphase was dialyzed against phosphate-buffered saline (PBS) (0.15 M NaCl, 0.10 M sodium phosphate, 0.02% (w/v) NaN₃, pH 7.0) and was brought to 65% ammonium sulfate saturation at 4°C and centrifuged (15,000 x g, 20 min., 4°C). The yellow-green colored supernatant was removed from the blue-colored pellet, brought to 90% ammonium sulfate saturation and centrifuged (15,000 x g, 20 min., 4°C). The resulting clear supernatant was discarded and the yellow-green pellets were resuspended and dialyzed in PBS.

The dialyzed material was concentrated to 15 ml by ultrafiltration (YM-30 membrane, Amicon) and applied to a Sephadex G-75 (Pharmacia) gel filtration column (250 x 2.5 cm, flow rate: 15 ml/h) equilibrated with PBS. Yellow-colored fractions collected from the column were pooled, concentrated by ultrafiltration and dialyzed against concanavalin A (Con A) starting buffer (0.5 M NaCl, 0.02 M Tris-HCl, 1 mM MgCl₂, 1 mM CaCl₂, 0.02% (w/v) NaN₃, pH 6.6). The sample was applied to a Con A-Sepharose (Pharmacia) lectin affinity column (7 x 2.5 cm, flow rate: 1 ml/min). Unretained colorless material was eluted with Con A starting buffer and bound, yellow-colored
material was released with 0.5 M methyl-\(\alpha\)-D-mannopyranoside added to Con A starting buffer. The yellow-colored fractions collected from the Con A-Sepharose column were pooled, concentrated and dialyzed against CM starting buffer (10 mM sodium succinate, 0.02% (w/v) \(\text{NaN}_3\), pH 5.4). The sample was applied to a carboxymethyl Biogel A (CM-Biogel A) (Bio-Rad, Richmond, CA) cationic exchange column (9 x 2.5 cm, flow rate: 1 ml/min). Yellow-colored unbound material was eluted with CM starting buffer and bound material was released with 1 M \(\text{NaCl}\) in starting buffer.

For all chromatographic steps, the eluted fractions were assayed for protein and monitored for absorbance at 405 nm. Protein determinations were made using the bicinchoninic acid (BCA) protein assay (Pierce Chemical Co., Rockford, IL) according to the method of Smith et al. (1985); bovine serum albumin served as the protein standard. Absorbance at 405 nm was measured in a Perkin-Elmer Lambda 3 spectrophotometer, using 1 cm pathlength quartz cuvettes.

**CHARACTERIZATION OF THE ULTRACENTRIFUGATION OF HEMOLYMPH**

To characterize the separation of lipophorin from proteins of higher density that was achieved by ultracentrifugation, the contents of an ultracentrifuge tube were fractionated from top to bottom into 18 equal-sized fractions of approximately 2 ml each. These fractions were dialyzed into PBS to remove KBr and were characterized by electrophoresis.
AMMONIUM SULFATE FRACTIONATION

Two samples were dialyzed against PBS. The first consisted of subphase obtained from the ultracentrifugation of hemolymph; the second was purified YCP. Aliquots of each sample were mixed with appropriate amounts of PBS and ammonium sulfate-saturated PBS to reach predetermined ammonium sulfate saturation levels. The mixtures were agitated overnight at 4°C and centrifuged. After centrifugation, the absorbance at 280 nm was measured for each supernatant derived from subphase and the absorbance at 405 nm was measured for the supernatants derived from the sample of purified YCP.

ALTERNATIVE BUFFER FOR CONCANAVALIN A-SEPHAROSE CHROMATOGRAPHY

As an alternative to the previously reported procedure for lectin-affinity chromatography, the Con A starting buffer recommended by the manufacturer (Pharmacia) was used: 0.5 M NaCl, 0.02 M Tris-HCl, 1 mM MgCl₂, 1 mM CaCl₂, 0.02% (w/v) NaN₃, pH 7.4.

ELECTROPHORESIS

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed under reducing conditions (Laemmli, 1970). Separating gels (17.5 cm x 12 cm x 1.5 mm) had a linear gradient from 5% to 18% acrylamide and were overlaid with 4% acrylamide stacking gel (17.5 cm x 1 cm x 1.5 mm). Protein molecular weight markers were purchased from BioRad (Richmond, CA). They were: lysozyme (14.4 kDa), soybean trypsin inhibitor (21.5 kDa), carbonic anhydrase (29 kDa),
ovalbumin (45 kDa), bovine serum albumin (66.2 kDa), phosphorylase B (92.5 kDa), β-galactosidase (116 kDa), and myosin (200 kDa). Electrophoresis was carried out at 35 mA until bromophenol blue tracking dye reached the bottom of the gels. The gels were stained with Coomassie Blue R-250.
RESULTS AND DISCUSSION:

For the tobacco hornworm, *M. sexta*, the wandering stage at the end of the fifth larval instar is a period of transition. Rather than feeding voraciously as it had before, the animal now searches for a suitable place to pupate. The wandering larva is substantially smaller and more mobile than it was at the end of the feeding stage. During the wandering stage, hemolymph proteins reach the maximal concentration of nearly 60 mg/ml. Various storage proteins are present at a total concentration of 40 mg/ml, with arylphorin being the most abundant storage protein by far (Kramer et al., 1980). It is from the hemolymph of such wandering larvae (Fig. 3, Lane 2) that a novel yellow-colored protein (YCP) has been purified.

The purification of YCP from the hemolymph of wandering stage larvae relied on five different properties of the chromoprotein: density, solubility, size, glycosylation, and surface charge. The progress of the purification, as monitored by SDS-PAGE, is summarized in Fig. 3.

Typically, about 160 ml of hemolymph served as the starting material. Adding reduced glutathione and diisopropyl fluorophosphate to hemolymph at 0°C prevented both clotting and melanization (Shapiro and Law, 1983).

During the KBr density gradient ultracentrifugation of hemolymph, the low-density yellow-colored lipophorin migrated to the upper portion of the ultracentrifuge tubes (Shapiro et
Fig. 3:

**SDS-PAGE illustrating the progress of the purification of YCP**

Lane 1 and Lane 8, molecular weight standards (described in Methods), molecular masses (in kDa) are indicated at the right; Lane 2, hemolymph from fifth instar, wandering stage larvae; Lane 3, subphase obtained from the ultracentrifugation of hemolymph; Lane 4, YCP-containing fraction precipitated from subphase with ammonium sulfate; Lane 5, YCP-containing fraction of material from previous lane eluted from gel filtration column; Lane 6, YCP-containing fraction of material from previous lane eluted from lectin affinity column; Lane 7, YCP-containing fraction of material from previous lane eluted from ion exchange column.
al., 1984), leaving lipid-free hemolymph proteins in the higher density subphase. Figure 4 shows an SDS-PAGE gel which illustrates the separation achieved by ultracentrifugation. The two protein subunits of lipophorin from wandering stage larvae, apolipophorin 1 (245 kDa) and apolipophorin 2 (78 kDa) (Shapiro et al., 1984) are visible in the left-hand lanes which correspond to the low-density region of the density gradient. All other hemolymph proteins accumulated in the subphase. There, arylphorin was the predominant species; its 92 kDa and 87 kDa subunits (Kramer et al., 1980) are visible in the right-hand lanes of Fig. 4. The subphase was blue-green in color due to the contributions of the yellow-colored YCP and the blue-colored insecticyanin.

A comparatively high concentration of ammonium sulfate was required to precipitate YCP (Fig. 5). Half of the total protein in the subphase (as determined by the absorbance at 280 nm) had precipitated at 55% ammonium sulfate saturation while in a parallel experiment, all of the YCP (as detected by the absorbance at 405 nm) was still in solution. More than half of the YCP precipitated at 85% ammonium sulfate saturation. For purification purposes, YCP-containing material precipitating between 65% and 90% ammonium sulfate saturation was used (Fig. 3, Lane 4). By salting out subphase proteins with ammonium sulfate, the protein content in the YCP-containing fraction was reduced by approximately 95%.
Fig. 4:

**SDS-PAGE of hemolymph proteins separated by ultracentrifugation**

Hemolymph collected from wandering stage fifth instar larvae was subjected to potassium bromide-density gradient ultracentrifugation. The contents of the ultracentrifuge tube were fractionated, dialyzed and subjected to SDS-PAGE. **Lanes A and B contain molecular weight marker proteins; their molecular masses (in kDa) are indicated on the left. The remaining lanes show from left to right, hemolymph proteins of increasing density. The subunits of lipophorin, apolipophorin 1 and apolipophorin 2 are visible in the left half of the gel. In the right half of the gel, the subunits of arylphorin form the largest protein bands.**
Fig. 5:

Ammonium sulfate fractionation of lipid-free hemolymph proteins

Proteins in the subphase obtained from the ultracentrifugation of hemolymph were fractionated with ammonium sulfate (●). At various levels of ammonium sulfate saturation, the absorbances at 280 nm of supernatants were expressed as percentages of the original absorbance at 280 nm. For purified YCP (○), the absorbance at 405 nm was used.
% Remaining in Solution vs. % Ammonium Sulfate Saturation
Most of the arylphorin and insecticyanin were separated from YCP. Indeed, the pellet which formed at 65% ammonium sulfate saturation was blue in color whereas the pellet that formed in the 65% to 90% saturation fraction was greenish yellow.

Ammonium sulfate-fractionated material was applied to a Sephadex G-75 column. After gel filtration, YCP could be detected by the yellow color of its chromophore or, more formally, by its absorbance maximum at 405 nm. The elution profile of the Sephadex G-75 column is shown in Fig. 6. Fractions 5 to 21 were examined by SDS-PAGE (Fig. 7) to assess the separation achieved by gel filtration chromatography. The major peak in protein concentration in the elution profile (Fig. 6), fractions 2 to 6, was due to arylphorin. The absorbance at 405 nm measured in fractions 9 to 12 was due to insecticyanin; in fractions 15 to 20, it was due to YCP. Sometimes, pooled YCP from the Sephadex G-75 column had to be reapplied to the same column in order to improve the separation of YCP from the proteins that appear in Fig. 7 as bands of approximately 45 kDa (presumably serpins; Kanost, 1990) and approximately 18 kDa (presumably apolipophorin 3; Shapiro et al., 1988).

After gel filtration chromatography, YCP was applied to a Con A-Sepharose lectin-affinity column. Here, the behavior of YCP was anomalous. At pH 7.4, when sugar-free buffers were used, most hemolymph proteins of M. sexta were either unretained or bound by the Con A-Sepharose matrix.
Fig. 6:

Elution profile of the gel filtration column

After ultracentrifugation and ammonium sulfate fractionation, YCP-containing sample was applied to a Sephadex G-75 column. Collected fractions were assayed for protein (●) and their absorbance at 405 nm was measured (○). Only protein-containing fractions are shown in this figure. The most abundant protein, arylphorin, was collected in fractions 2 through 7. The absorbance at 405 nm observed in fractions 8 through 13 was due to insecticyanin. YCP caused the absorbance at 405 nm of fractions 15 through 21.
Fig. 7:

SDS-PAGE of fractions from the gel filtration column

Lanes 2 and 20 contain molecular weight marker proteins; their molecular masses (in kDa) are indicated on the right. Lane 1 contains sample that was applied to the gel filtration column, i.e. proteins from the ultracentrifugation subphase fractionated with ammonium sulfate. Lanes 3 to 19 show the proteins in fractions 5 to 21 collected from the gel filtration column.
In contrast, YCP was retarded, indicating that there was only weak interaction between the protein and the chromatographic matrix. Although this characteristic of YCP set it apart from most other *M. sexta* hemolymph proteins, it was not useful in the purification of YCP. Indeed, YCP eluting from the Con A-Sepharose column in buffer at pH 7.4 was extremely dilute. Therefore, the pH of the Con A buffer was changed. At pH 6.6, YCP was retained by the Con A-Sepharose column; most remaining contaminants were not. The addition of 0.5 M methyl-α-D-mannopyranoside to the elution buffer caused YCP to be released. The elution profile of the lectin-affinity column is shown in Fig. 8, and an SDS-PAGE gel of relevant fractions collected from the column is shown in Fig. 9.

As a final purification step, the yellow-colored material collected from the Con A column was applied to a CM-Biogel A cationic exchange column in 10 mM sodium succinate, pH 5.4. Under these conditions, YCP eluted in the flow-through fractions (Fig. 10). The YCP thus obtained was pure as gauged by SDS-PAGE (Fig. 11), by immunoblotting and by the fact that a single amino acid residue (Ser) was identified at the N-terminus in the spinning-cup sequencer.
Fig. 8:

Elution profile of the concanavalin A column

A sample was enriched in YCP by ultracentrifugation, ammonium sulfate fractionation and gel filtration chromatography before being applied to a concanavalin A-Sepharose lectin affinity column. Fractions 1 to 18 are in buffer lacking methyl-α-D-mannopyranoside, whereas fraction 19 to 39 contain buffer with methyl-α-D-mannopyranoside. Collected fractions were assayed for protein (●) and their absorbance at 405 nm was measured (○).
Fig. 9:

**SDS-PAGE of fractions from the concanavalin A column**

Lanes 2, 5 and 7 contain molecular weight marker proteins; their molecular masses (in kDa) are indicated on the right. Lane 1 contains the sample that was applied to the concanavalin A column. For material that was not retained by the concanavalin A column, Lane 3 shows the proteins in Fraction 9 and Lane 4 shows the proteins in Fraction 13. The proteins in Lane 6 are from Fraction 22 which required sugar in the buffer to be released from the column.
Fig. 10:

Elution profile of the CM-Biogel A column

A sample was enriched in YCP by ultracentrifugation, ammonium sulfate fractionation, gel filtration chromatography and concanavalin A lectin affinity chromatography. That YCP-containing sample was applied to a CM-Biogel A cation-exchange column. Fractions 1 to 26 are in buffer that lacks NaCl whereas fractions 27 to 40 do contain NaCl. Collected fractions were assayed for protein (●) and their absorbance at 405 nm was measured (○).
Fig. 11:

SDS-PAGE of fractions from the CM-Biogel A column

Lane 2 contains molecular weight marker proteins; their molecular masses (in kDa) are indicated on the right. Lane 1 contains material that was not retained by the cation-exchange column and Lane 3 shows proteins that were eluted from the column with NaCl-containing buffer.
CHAPTER 3

CHARACTERIZATION OF THE POLYPEPTIDE CHAIN
MATERIALS AND METHODS:

CALIBRATED GEL FILTRATION COLUMNS

Two gel filtration columns were calibrated. The first column was packed with Sephadex G-75 Superfine (Pharmacia) (115 x 1 cm, flow rate: 2.3 ml/hr) and calibrated with blue dextran (>2,000 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), chymotrypsinogen A (25 kDa), ribonuclease A (13.7 kDa), and vitamin B₁₂. The second column was packed with Sephacryl S-200 HR (Pharmacia) (160 x 1.5 cm, flow rate: 2.3 ml/hr) and calibrated with blue dextran (>2,000 kDa), yeast alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), horse heart cytochrome C (12.4 kDa), and sucrose. Molecular weight markers were detected by monitoring the column eluate for absorbance at 280 nm. Sucrose was detected by the phenol-sulfuric acid assay for carbohydrates (Dubois et al., 1956). The elution volume of purified YCP on each of the two columns was measured as were the elution volumes of the standards.

MOLECULAR MASS DETERMINATIONS

The molecular mass of reduced and denatured YCP polypeptide in SDS-PAGE gels was determined graphically (Weber and Osborn, 1969). A plot of relative mobility (Rₚ) versus \( \log(\text{molecular mass}) \) was made. The equation used was:

\[
R_p = \frac{d_s}{d_f}
\]

where \( d_s \) was the distance migrated by sample and \( d_f \) was the distance migrated in the running gel by the bromophenol blue
tracking dye. The best-fitting straight line was determined which linked the points defined for each standard by $R_f$ and \log(molecular mass). Then, for YCP, \log(molecular mass) was determined, given $R_f$.

For the calibrated gel filtration columns, $v_o$ was the elution volume of material excluded by the chromatographic media. Likewise, $v_t$ was the elution volume of material eluting in the included volume of each column. For each molecular weight marker, $v_e$ was the elution volume and a corresponding $K_{av}$ was calculated where:

$$K_{av} = \frac{(v_e - v_o)}{(v_t - v_o)}$$

Graphic analysis of the $K_{av}$ versus \log(molecular mass) plots was as for the $R_f$ versus \log(molecular mass) plot.

**SPECTROSCOPY**

For all spectra, samples were placed in 1 cm pathlength quartz cuvettes. Absorbance spectra were obtained in a Beckman DU-50 spectrophotometer. For absorbance spectra of YCP at high and low pH values, 5 M NaOH or 5 M HCl was slowly added to a solution of YCP in PBS, pH 7.2.

The fluorescence spectrum of YCP illuminated at 405 nm was obtained in a Perkin-Elmer MPF-2A fluorescence spectrophotometer. The CD spectrum of 1 \mu M YCP in H$_2$O was obtained from a model 60DS spectropolarimeter (Aviv Associates Inc., Lakewood, NJ). During CD data collection, the sample was maintained under a constant stream of nitrogen, at 25.9°C. The CD spectrum was obtained by averaging 15 scans from 250 nm
to 195 nm. The data were analyzed with software from Aviv Associates Inc.

**AMINO ACID ANALYSIS**

Four 50 μg samples of YCP were hydrolyzed in 6 M HCl, in vacuo, at 110°C; phenol served as the antioxidant. Two samples were hydrolyzed for 24 h and two for 65 h. Analyses were performed with a Beckman 7300 Amino Acid Analyzer, at the Biotechnology Center of the University of Arizona.

**N-TERMINAL SEQUENCING**

The N-terminal sequence of purified YCP was determined by automated Edman degradation in a Beckman 890M spinning-cup sequencer; the phenylthiohydantoin derivatives of individual amino acids were identified by reversed-phase high pressure liquid chromatography as described (Kawooya et al., 1986). The N-terminal sequence of YCP electroblotted onto a polyvinylidene difluoride membrane was determined with a liquid pulse-gas phase sequencer (Model 477A, Applied Biosystems, Inc.) (LeGendre and Matsudaira, 1988). Sequencing was performed at the Biotechnology Center of the University of Arizona.

**FITC-CON A BINDING**

A modification (Ryan et al., 1985a) of the procedure of Furlan et al. (1979) was used to stain immobilized glycoprotein with fluorescent lectin. Purified YCP and pre-stained molecular weight marker proteins were subjected to SDS-PAGE in a 12% acrylamide minigel. The prestained protein
molecular weight markers (Amersham, Arlington Heights, IL 60005) were: lysozyme (14.4 kDa), soybean trypsin inhibitor (21.5 kDa), carbonic anhydrase (29 kDa), ovalbumin (45 kDa), bovine serum albumin (66.2 kDa), phosphorylase B (92.5 kDa), and myosin (200 kDa). After electrophoresis, proteins were electroblotted from the gel onto a nitrocellulose membrane (Towbin et al., 1979).

The nitrocellulose was incubated for one hour in 0.5% (v/v) Tween-20 in PBS to prevent subsequent non-specific binding of protein to the membrane. The nitrocellulose was washed in Con A buffer (pH 6.6) for five minutes and incubated for one hour in Con A buffer containing 0.04 mg/ml fluorescein isothiocyanate-labelled concanavalin A (FITC-Con A) (Miles Scientific, Naperville, IL 60566). After this incubation, the nitrocellulose was subjected to three 15 min washes in Con A buffer. The binding of FITC-Con A to glycoprotein immobilized on the nitrocellulose was visualized by the fluorescence of FITC under ultraviolet illumination. A double exposure photograph was made of the nitrocellulose sheet using a camera that was fitted with an ultraviolet filter. For the first exposure, the nitrocellulose was illuminated with ultraviolet light to detect fluorescing FITC-Con A; incandescent lighting was used for the second exposure to record the positions of the pre-stained molecular weight marker proteins.

CARBOHYDRATE ANALYSIS

The composition of the carbohydrates attached to the
polypeptide chain of YCP was determined by converting the carbohydrate moieties to their alditol acetate derivatives for identification by gas chromatography, according to a modification (Ryan et al., 1985) of the procedure of Grimes and Gregor (1976). Lyophilized YCP from which the chromophore had previously been extracted (see Chapter 4) served as starting material. Two samples were prepared; the first contained 2.0 mg of YCP and the second, 4.5 mg. Each sample was placed in a screw-cap test tube which could be sealed with a Teflon-lined cap. The samples were hydrolyzed for 140 min at 120°C with 1 ml 2 N trifluoracetic acid in sealed tubes (Ryan et al., 1985). The samples were dried in a Speed-Vac rotary evaporator (Savant Instruments) and 10 μg of 2-deoxyglucose in 0.5 ml H₂O and 0.5 ml 1N NH₄OH, 0.6 N NaBH₄ was added to each. The samples were incubated for one hour at room temperature and 200 μl of glacial acetic acid was added to each prior to drying. The samples were dissolved in 0.5 ml of methanol containing 0.5% (v/v) glacial acetic acid and dried. This was repeated five times. The dried samples were then stored overnight, in a desiccator, in vacuo, over P₂O₅ and KOH. Subsequently, one ml of acetic anhydride was added to each sample, the tubes were sealed, and incubated at 120°C for 30 min. One ml of toluene was added to each tube and the contents were dried. This step was repeated once. The samples were then dissolved in 0.5 ml H₂O and the alditol acetates were extracted twice into 0.5 ml CHCl₃. The pooled
CHCl₃ extracts were dried and dissolved in 50 μl acetone for the sample obtained from 2.0 mg of YCP, and 100 μl acetone for the 4.5 mg sample. One microliter samples were injected into a Hewlett-Packard 5700A gas chromatograph equipped with a flame ionization detector. The glass column (6 ft. x 1/8 in.) was packed with 3% OV-225 on Supelcoport (Supelco, Inc., Bellafonte, PA). The gas chromatograph was programmed to run at 180°C for 8 min. and then to rise to 230°C at a rate of 2°C/min. A Shimadzu C-R5A Chromatopac was used to integrate peak areas and measure retention times. The alditol acetate derivatives of mannose, glucose, and N-acetylglucosamine served as standards.
RESULTS AND DISCUSSION:

The molecular mass of reduced and denatured YCP polypeptide, determined by SDS-PAGE, was 31 kDa (Fig 12). The molecular mass of native YCP on calibrated gel filtration columns was 37 kDa using Sephadex G-75 Superfine (Fig. 13) and 30.2 kDa using Sephacryl S-200 HR (Fig. 14). Thus, the molecular mass of reduced and denatured YCP was in fairly good agreement with the molecular mass determined for the protein in the native state. This suggested then, that native YCP is a monomer.

The absorbance spectrum of YCP at pH 7.2 (Fig. 15) revealed maxima at 207 nm, 278 nm and 405 nm. There was an intervening minimum at 336 nm and a shoulder at approximately 445 nm. The following extinction coefficients were calculated: $\varepsilon=52,300 \text{ M}^{-1}\text{cm}^{-1}$ at 405 nm and $\varepsilon=45,400 \text{ M}^{-1}\text{cm}^{-1}$ at 278 nm, using 31,000 as the molecular weight of YCP. The absorbance maximum at 405 nm was due solely to the chromophore whereas it is likely that both the protein and the chromophore contributed to the maximum at 278 nm.

A spectrophotometric pH titration was also performed on YCP (Fig. 16). In going from pH 7.2 to pH 1.4, the absorbance at 278 nm remained virtually unchanged. There was however a significant change in the absorbance of the chromophore: the absorbance maximum shifted from 405 nm at pH 7.2 to 370 nm at pH 1.4 and was accompanied by a 61% decrease in the intensity of absorbance. A new absorbance maximum appeared at 675 nm,
Molecular weight determination of YCP by SDS-PAGE

The molecular mass of reduced and denatured YCP was 31 kDa. For each molecular weight marker protein, the mobility relative to bromophenol blue was plotted versus log (molecular mass). The best-fitting straight line defined by these points was found. For YCP, the relative mobility served to determine the molecular mass. The molecular weight marker proteins (and their molecular masses) were: lysozyme (14.4 kDa), soybean trypsin inhibitor (21.5 kDa), carbonic anhydrase (29 kDa), ovalbumin (45 kDa), bovine serum albumin (66.2 kDa), and phosphorylase B (92.5 kDa).
The molecular mass of native YCP, determined with a calibrated Sephadex G-75 gel filtration column, was 37 kDa. For each molecular weight marker, $K_{av}$ was calculated (see Materials and Methods) and was plotted versus log (molecular mass). The best-fitting straight line defined by these points was found. For YCP, $K_{av}$ served to determine the molecular mass. The molecular weight markers (and their molecular masses) were: blue dextran (>2,000 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), chymotrypsinogen A (25 kDa), ribonuclease A (13.7 kDa), and vitamin $B_{12}$ (1355 g/mol).
Fig. 14:

Molecular weight determination of YCP using Sephacryl S-200

The molecular mass of native YCP, determined with a calibrated Sephacryl S-200 gel filtration column, was 30.2 kDa. For each molecular weight marker, $K_v$ was calculated (see Materials and Methods) and was plotted versus log (molecular mass). The best-fitting straight line defined by these points was found. For YCP, $K_v$ served to determine the molecular mass. The molecular weight markers (and their molecular masses) were: blue dextran (>2,000 kDa), yeast alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), horse heart cytochrome C (12.4 kDa), and sucrose (342 g/mol).
The absorbance spectrum of purified YCP (0.166 mg/ml) in PBS, pH 7.2, showed maxima at 207 nm, 278 nm and 405 nm with a shoulder occurring at approximately 445 nm. The absorbance spectrum from 250 nm to 600 nm is shown enlarged in the inset. Using 31,000 as the molecular weight of YCP, the extinction coefficients were calculated: \( \varepsilon = 52,300 \text{ M}^{-1}\text{cm}^{-1} \) at 405 nm and \( \varepsilon = 45,400 \text{ M}^{-1}\text{cm}^{-1} \) at 278 nm.
**Fig. 16:**

Absorbance spectra of purified YCP at different pH levels

The absorbance spectrum of purified YCP in PBS, pH 7.2, is shown as a solid line. The absorbance spectra of YCP at pH 1.4 (dash-dotted line) and at pH 11.6 (dotted line) are also shown. The YCP in PBS was either acidified with 5 M HCl or made alkaline with 5 M NaOH. The absorbance maxima (and their corresponding intensities) were: at pH 7.2, 278 nm ($A_{278} = 0.240$) and 405 nm ($A_{405} = 0.250$); at pH 1.4, 276 nm ($A_{276} = 0.228$) and 370 nm ($A_{370} = 0.098$) and 675 nm ($A_{675} = 0.027$); at pH 11.6, 288 nm ($A_{288} = 0.396$) and 405 nm ($A_{405} = 0.146$).
but it was weak and very broad. In going from pH 7.2 to pH 11.6, there was no change in the position of the absorbance maximum of the chromophore. However, the intensity of absorbance at 405 nm was 42% lower at pH 11.6 than it had been at pH 7.2. The absorbance maximum that was at 278 nm at pH 7.2 shifted to 288 nm at pH 11.6 and increased in intensity by 58%. In all likelihood, deprotonation of the phenolic hydroxyl group from tyrosine residues was responsible for this change (Freifelder, 1982). No fluorescence was observed when YCP was illuminated with short-wave and long-wave ultraviolet light, nor when excited at 405 nm.

The CD spectrum of YCP had ellipticity maxima at 196 nm and 231 nm and a minimum at 211 nm; a shoulder was found at 203 nm (Fig. 17). The computer analysis of data obtained by averaging the results of 15 scans indicated that YCP contained no α-helix, 68% β-pleated sheet, and 32% random coil. This high percentage of β-pleated sheet coupled with the absence of α-helices makes it tempting to speculate that YCP forms a β-barrel where the interior might comprise a binding site for the chromophore (Richardson, 1985).

YCP was not unusual in its amino acid composition (Table 1). This is not always the case for M. sexta hemolymph proteins: 21% of the amino acid residues of arylphorin have aromatic side chains (Willott et al., 1989); the methionine-rich storage protein contains 6.4 mol % methionine (Ryan et al., 1985b). The first 31 N-terminal amino acid residues of
Fig. 17:

Circular dichroism spectrum of YCP

The spectrum shown was obtained by averaging 15 scans. Maxima are at 196 nm and 231 nm, with a minimum occurring at 211 nm and a shoulder at 203 nm. Computer analysis indicated that YCP consists of 68% β-pleated sheet and 32% random coil.
Table 1:
Amino acid composition of YCP (in mole percent)

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>24 hours (a)</th>
<th>65 hours (a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartate and asparagine</td>
<td>15.8</td>
<td>15.8</td>
</tr>
<tr>
<td>Threonine</td>
<td>5.5</td>
<td>5.3</td>
</tr>
<tr>
<td>Serine</td>
<td>7.7</td>
<td>6.8</td>
</tr>
<tr>
<td>Glutamate and glutamine</td>
<td>7.9</td>
<td>8.0</td>
</tr>
<tr>
<td>Proline</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Glycine</td>
<td>7.9</td>
<td>8.0</td>
</tr>
<tr>
<td>Alanine</td>
<td>5.9</td>
<td>5.9</td>
</tr>
<tr>
<td>Valine</td>
<td>10.7</td>
<td>11.3</td>
</tr>
<tr>
<td>Methionine</td>
<td>1.9</td>
<td>1.9</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>5.8</td>
<td>6.1</td>
</tr>
<tr>
<td>Leucine</td>
<td>6.8</td>
<td>6.8</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>5.6</td>
<td>5.6</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>6.6</td>
<td>6.6</td>
</tr>
<tr>
<td>Histidine</td>
<td>2.5</td>
<td>2.6</td>
</tr>
<tr>
<td>Lysine</td>
<td>8.4</td>
<td>7.9</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.9</td>
<td>1.3</td>
</tr>
<tr>
<td>Cysteine</td>
<td>N.D. (b)</td>
<td>N.D. (b)</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>N.D. (b)</td>
<td>N.D. (b)</td>
</tr>
<tr>
<td>TOTAL</td>
<td>99.9</td>
<td>99.9</td>
</tr>
</tbody>
</table>

(a): Duration of hydrolysis for duplicate samples
(b): N.D. = not determined
YCP were determined (Fig. 18). The sixth residue was identified as a mixture of serine and threonine. This may indicate allelic variations of the gene for YCP within the *M. sexta* population. A FASTA computer search found no sequences homologous to the N-terminus of YCP.

The polypeptide chain of YCP is glycosylated. During purification, YCP was retained by the Con A-Sepharose lectin affinity column at pH 6.6. At pH 7.4 however, the interaction of YCP with Con A-Sepharose was weak. Since the addition of methyl-α-D-mannopyranoside caused the release of YCP from the Con A-Sepharose column at pH 6.6, it appeared that the lectin was binding to carbohydrate associated with YCP. However, it was not known whether the lectin interacted with the chromophore of YCP or with the polypeptide chain. To resolve this question, purified YCP was subjected to SDS-PAGE. During electrophoresis, the chromophore was dissociated from the polypeptide chain. The chromophore migrated just behind the bromophenol blue tracking dye and well ahead of the YCP polypeptide. After SDS-PAGE, the YCP polypeptide was electroblotted onto nitrocellulose. No yellow color was visible at the position of the YCP polypeptide. The polypeptide chain did stain positively with FITC-Con A (Fig. 19). Thus, carbohydrate was associated with the polypeptide chain of YCP.

To determine the identity of carbohydrate associated with the YCP polypeptide, carbohydrate analysis was performed on
The N-terminal sequence was determined by automated Edman degradation. YCP was either in solution or immobilized on a membrane. At the sixth position, a mixture of serine and threonine was detected.
Ser-Lys-Asp-Ser-Val-Thr-Val-Asn-Gly-Lys-Ser

Asn-Tyr-Gly-Lys-Glu-Val-Leu-Lys-Asp-Asn-

Ile-His-Gln-Ala-Tyr-Gln-Leu-Ser-Phe-Asp-Glu
Fig. 19:

Binding of FITC-Con A to immobilized YCP polypeptide

Purified YCP was subjected to SDS-PAGE, electroblotted onto nitrocellulose and stained with FITC-Con A. During SDS-PAGE, chromophore of YCP was separated from the polypeptide chain. Glycoprotein-bound FITC-Con A was detected by its fluorescence under ultraviolet illumination at the position of the YCP polypeptide. Prestained molecular weight marker proteins are visible in the first, third and sixth lanes from the left. The second, fourth and fifth lanes from the left contained 25 μg, 50 μg and 75 μg of YCP, respectively.
YCP from which the chromophore had previously been extracted (see Chapter 4 for procedures). Carbohydrate which was associated with the YCP polypeptide, was hydrolyzed to monosaccharides which were identified by gas chromatography in the form of their volatile alditol acetate derivatives (Fig. 20). The average retention times for the alditol acetate derivatives of 2-deoxyglucose, mannose, glucose, and N-acetyl glucosamine were 6.89 min. (n=4), 10.21 min. (n=6), 11.93 min. (n=5) and 23.32 min. (n=5) respectively. The standard deviation in the retention time of 2-deoxyglucose alditol acetate was 0.08 min. (i.e. 1.2%). Within a single run however, if the retention time of 2-deoxyglucose was defined as zero and all the other retention times were determined accordingly, then the standard deviations of retention times of like compounds was less than 0.2% from one chromatogram to another.

Four chromatograms were obtained for the alditol acetates from YCP: two for the 2.0 mg sample and two for the 4.5 mg sample. In each of these, the mannose alditol acetate peak was the major carbohydrate-produced peak. A minor glucose alditol acetate peak followed, and N-acetylglucosamine alditol acetate was also present. For carbohydrate derived from YCP, the ratio of mannose alditol acetate to N-acetylglucosamine alditol acetate was 3:1. While the alditol acetate derivative of glucose could be detected, there was too little material to allow quantification.
Carbohydrate associated with the polypeptide chain of YCP was hydrolyzed to free monosaccharides which were in turn converted to their volatile alditol acetate derivatives. The three chromatograms at the top are of standards. They are, from top to bottom, the alditol acetate derivatives of mannose, glucose and N-acetylglucosamine. The chromatogram at the bottom is of material derived from YCP and to which 2-deoxyglucose had been added as an internal standard. For YCP, the ratio of the alditol acetate derivatives of mannose and N-acetylglucosamine was 3:1. A trace amount of glucose alditol acetate was also detected. Retention times (in minutes) are shown. The abbreviations used are for the alditol acetate derivatives of the compounds: 2dGlc, 2-deoxyglucose; Man, mannose; Glc, glucose; GlcNAc, N-acetylglucosamine.
For arylphorin (Ryan et al., 1985a) and vitellogenin (Osir et al., 1986a) from *M. sexta*, an asparagine-linked, triantenary Man$_x$GlcNAc$_2$ oligosaccharide structure was proposed, based on results from carbohydrate analyses and $^1$H-NMR spectroscopy. In both instances, trace amounts of Glc were detected during carbohydrate analysis and two unassigned doublets were found in each NMR spectrum. Nagao and Chino (1987) showed that for oligosaccharides derived from lipophorin of the migratory locust, *Locusta migratoria*, such NMR signals are in fact due to Glc linked α1-3 to the terminal Manα1-2Man residue of the Manα1-3Man arm of the oligosaccharide. Nagao et al. (1987) went on to show that lipophorin from the migratory locust, is glycosylated with six different high-mannose type oligosaccharides: Man$_x$GlcNAc$_2$ (x=5 to 9) and Glc$_2$Man$_x$GlcNAc$_2$. Compositional analysis of the oligosaccharide from YCP suggested Man$_x$GlcNAc$_2$ as an average composition with the results of Nagao et al. (1987) providing a possible explanation to account for the trace amount of Glc detected in the YCP oligosaccharide.
CHAPTER 4
IDENTIFICATION OF THE CHROMOPHORE
MATERIALS AND METHODS:

CHROMOPHORE EXTRACTION

Purified YCP was dialyzed extensively at 4°C, first against 5 mM nitrilotriacetic acid, 5 mM EDTA, 5 mM [ethylenebis(oxyethylenenitrilo)]tetraacetic acid in H₂O and then against H₂O alone. After being concentrated in a Speedvac (Savant Instruments, Farmingdale, NY), YCP was resuspended in a known volume of H₂O (usually between 0.5 ml and 2 ml). This YCP sample was extracted with CHCl₃ and methanol, according to the method of Bligh and Dyer (1959). First, two volumes of CH₃OH and and one volume of CHCl₃ were added to the YCP and the mixture was vortexed extensively. Then, an additional volume of CHCl₃ was added and the mixture was vortexed again. Finally, one volume of H₂O was added. After vortexing, the sample was centrifuged at low speed to separate the aqueous and organic phases. After centrifugation, the yellow-colored aqueous phase was carefully removed from the clear organic phase and from the white pellet that had formed at the interphase. The aqueous phase, the organic phase and the white pellet were dried in the Speedvac.

Material derived from the aqueous phase was resuspended in a small amount of H₂O (usually between 0.2 ml and 1 ml) and applied to a prepacked, 10 ml Econo-Pac 10DG desalting column (which contained prepacked Bio-Gel P-6) (Bio-Rad Laboratories, Richmond, CA 94804) that had previously been equilibrated with H₂O. Yellow-colored material eluted at the excluded volume of
the desalting column while orange-colored material eluted at the included volume. Both fractions were concentrated to dryness and lyophilized overnight. Absorbance spectra were measured for samples dissolved in water. For the orange-colored material, the absorbance spectra in 5 M HCl were also obtained.

ENZYME TREATMENT OF CHROMOPHORE

Chromophore from YCP was incubated in 67 mM phosphate, pH 7, at room temperature, in the presence of β-glucosidase (two units, from almonds, purchased from Sigma) or arylsulfatase (one unit of Type IV from limpets and one unit of Type VII from abalone entrails, purchased from Sigma). Absorbance spectra were obtained at the beginning of the incubation and after 72 hours.
RESULTS AND DISCUSSION:

A survey (Kayser, 1985) reports the occurrence in insects of the following eight classes of chromogenic compounds: carotenoids, tetrapyrroles, ommochromes, melanins, papiliochromes, pteridines, quinones, and flavonoids. The chromophore of YCP was thought to be a member of any one of these classes of compounds.

To gauge the solubility of the chromophore, the lipid-extraction procedure of Bligh and Dyer (1959) using methanol and chloroform, was performed on YCP. At the end of the extraction procedure, a white-colored pellet had formed at the interphase between the aqueous and the organic layers. The underlying organic layer was colorless while the overlying aqueous layer was yellow colored. The white-colored pellet was shown by SDS-PAGE, to consist of the YCP polypeptide. The white color of this pellet was an indication that it contained little if any chromophore. The clear organic layer left no visible residue when evaporated to dryness. However, when the yellow-colored aqueous layer was evaporated to dryness, an intensely-colored orange-yellow residue remained. Since this residue contained protein which was detected by the BCA protein assay, the sample was applied to a desalting column. Yellow-colored material eluted at the excluded volume of the column while orange-colored material eluted at the included volume. The yellow-colored material was YCP, as determined by the BCA protein assay, the absorbance spectrum and SDS-PAGE.
For the orange-colored material which eluted at the included volume of the desalting column, the BCA protein assay and SDS-PAGE indicated that no protein was present. The absorbance spectrum of this material was distinct from that of YCP (Fig. 21). Hence, this material contained released chromophore.

The chromophore was hydrophilic in nature since it remained in the aqueous phase during the lipid extraction procedure. Since it eluted at the included volume of the desalting column, its molecular mass was probably under 1,000 Da. Furthermore, released chromophore produced no visible fluorescence under ultraviolet illumination.

On the basis of these criteria, the chromophore did not belong to several of the classes of insect pigments. The carotenoids, which insects absorb through the diet, are lipophilic compounds which are readily soluble in chloroform (Kayser, 1985). Likewise, the chromophore did not display the characteristics of a tetrapyrrole. The tetrapyrroles comprise biliverdins, bilirubins, porphyrins and heme. At the end of the Bligh-Dyer lipid extraction procedure, both biliverdin and bilirubin are found in the organic phase. Porphyrins produce red fluorescence under ultraviolet illumination and heme has a characteristic absorbance spectrum (Kayser, 1985) distinct from that of the chromophore from YCP. The melanins are generally black and insoluble with the exception of phaeomelanins which are yellow to reddish-brown colored, alkali-soluble and acid-precipitable (Kayser, 1985). Most
Fig. 21:

Absorbance spectra of released chromophore and of purified YCP

The absorbance spectrum of released chromophore in water (dotted line) is distinct from that of YCP (solid line). Released chromophore displayed absorbance maxima at 370 nm ($A_{370\, \text{nm}} = 0.088$) and 490 nm ($A_{490\, \text{nm}} = 0.190$) whereas the maxima for YCP were at 278 nm and 405 nm.
pteridines (including riboflavin) fluoresce under ultraviolet illumination (Kayser, 1985). The papiliochromes are compounds which incorporate kynurenine, β-alanine and a phenolic compound (such as norepinephrine); their water-solubility and fluorescence vary (Kayser, 1985). However, this class of pigments appears to be restricted to the papilionid butterflies. Likewise, in insects, the quinones appear to be restricted to scale insects (anthraquinones) and aphids (aphins); there, the quinones are probably synthesized by microbial endosymbionts (Kayser, 1985). Finally, the flavonoids are only weakly colored and have been found only in certain butterflies (Kayser, 1985).

The absorbance spectrum of released chromophore in H$_2$O showed maxima at 370 nm and 490 nm (Fig. 21). These were the maxima reported for the ommochrome: ommatin D, in 67 mM phosphate, pH 7.4 (Table 2) (Linzen, 1974). While most ommochromes are scarcely soluble in water, there are two notable exceptions: rhodommatin, the O-β-glucoside of dihydroxanthommatin, and ommatin D, the sulfate ester of dihydroxanthommatin (Linzen, 1974). Thus, the partitioning of chromophore into the aqueous phase during the lipid extraction procedure is consistent with what would be expected for substituted ommochromes. Linzen (1974) also reported that in 5 M HCl, ommatin D displays absorbance maxima at 305 nm and 440 nm. When the absorbance spectrum of chromophore in 5 M HCl was obtained, it contained maxima at 305 nm and 438 nm.
### Table 2:

**Absorbance characteristics of ommochromes and of chromophore from YCP**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Solvent</th>
<th>$\lambda_{\text{Max}}$ 1</th>
<th>$\lambda_{\text{Max}}$ 2</th>
<th>Ratio$^{(a)}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ommatin $D^{(b)}$</td>
<td>67 mM phosphate pH 7.4</td>
<td>370 nm ($\varepsilon_{0.14}=3,160$)</td>
<td>490 nm ($\varepsilon_{0.14}=7,500$)</td>
<td>0.42</td>
</tr>
<tr>
<td></td>
<td>5 M HCl</td>
<td>305 nm ($\varepsilon_{0.14}=13,000$)</td>
<td>440 nm ($\varepsilon_{0.14}=6,620$)</td>
<td>1.96</td>
</tr>
<tr>
<td>Xanthommatin$^{(b)}$</td>
<td>67 mM phosphate pH 7</td>
<td>---</td>
<td>440 nm ($\varepsilon_{0.14}=13,200$)</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>5 M HCl</td>
<td>375 nm ($\varepsilon_{0.14}=7,350$)</td>
<td>475 nm ($\varepsilon_{0.14}=11,730$)</td>
<td>0.63</td>
</tr>
<tr>
<td>Chromophore from YCP</td>
<td>H$_2$O</td>
<td>370 nm ($\varepsilon_{0.14}=0.088$)</td>
<td>490 nm ($\varepsilon_{0.14}=0.190$)</td>
<td>0.46</td>
</tr>
<tr>
<td></td>
<td>5 M HCl (initially)</td>
<td>305 nm ($\varepsilon_{0.14}=0.388$)</td>
<td>438 nm ($\varepsilon_{0.14}=0.192$)</td>
<td>2.02</td>
</tr>
<tr>
<td></td>
<td>5 M HCl (after 22 hours)</td>
<td>375 nm ($\varepsilon_{0.14}=0.236$)</td>
<td>478 nm ($\varepsilon_{0.14}=0.343$)</td>
<td>0.68</td>
</tr>
</tbody>
</table>

$^{(a)}$: Either [$\varepsilon_{0.14}$ at $\lambda_{\text{Max}}$ 1]/[$\varepsilon_{0.14}$ at $\lambda_{\text{Max}}$ 2] or [(A at $\lambda_{\text{Max}}$ 1)/(A at $\lambda_{\text{Max}}$ 2)].

$^{(b)}$: Data from Linzen, 1974.
(Fig. 22 and Table 2). Both the positions and the relative intensities of the absorbance maxima of chromophore in water and in 5 M HCl were in good agreement with values reported for ommatin D (Table 2).

Butenandt et al. (1960) reported that over the course of 26 hours, at room temperature, in 5 M HCl, the sulfate ester bond of ommatin D is cleaved to yield sulfate and xanthommatin; this reaction can be followed spectrophotometrically by monitoring the disappearance of ommatin D and the appearance of xanthommatin (Table 2). For chromophore released from YCP, spectrophotometric changes indicative of such a reaction were observed (Fig. 23). Again, the positions and the relative intensities of the absorbance maxima were in agreement with values published for ommatin D (Table 2). Taken together, these results suggested that ommatin D was the chromophore of YCP.

The chromophore of YCP was sensitive to treatment with arylsulfatase. During the incubation, the orange-colored chromophore became yellow. Spectrophotometrically, this change manifested itself as a shift in the position of the absorbance maximum with a concomitant increase in intensity (Fig. 24). Presumably, the presence of both xanthommatin and ommatin D in the incubation mixture caused the appearance of the absorbance maximum at 462 nm. The position and the intensity of this absorbance maximum are intermediate between those of xanthommatin and ommatin D (Table 2). The absorbance
Fig. 22:

Absorbance spectra of chromophore at neutrality and in acid

Methanol-chloroform extraction and gel filtration chromatography were used to release the chromophore from purified YCP. The absorbance spectrum of chromophore in H₂O (solid line) showed maxima at 370 nm (A₃₇₀ nm = 0.088) and at 490 nm (A₄₉₀ nm = 0.190). In 5 M HCl (dotted line), the chromophore displayed maxima at 305 nm (A₃₀₅ nm = 0.388), at 438 nm (A₄₃₈ nm = 0.192) and at 683 nm (A₆₈₃ nm = 0.053).
**Fig. 23:**

Absorbance spectra of chromophore incubated in acid

In 5 M HCl, the chromophore initially showed absorbance maxima at 305 nm ($A_{305\text{ nm}} = 0.388$) and at 438 nm ($A_{438\text{ nm}} = 0.192$) (solid line). After 3.5 hours at room temperature, the absorbance spectrum had changed (dash-dot line). After 22 hours at room temperature, the sample showed absorbance maxima at 375 nm ($A_{375\text{ nm}} = 0.236$) and at 478 nm ($A_{478\text{ nm}} = 0.343$) (dotted line).
Fig. 24:

Absorbance spectra of chromophore treated with arylsulfatase

Chromophore in 67 mM phosphate, pH 7, was treated with arylsulfatase. At the start of the incubation, the chromophore showed absorbance maxima at 367 nm ($A_{367\ nm} = 0.075$) and at 488 nm ($A_{488\ nm} = 0.170$) (solid line). After 72 hours at room temperature, the maxima had shifted to 360 nm ($A_{360\ nm} = 0.207$) and 462 nm ($A_{462\ nm} = 0.235$) (dotted line); the color of the incubation mixture had shifted from orange to yellow. Chromophore lacking enzyme and chromophore incubated with β-glucosidase showed no such changes.
maximum at 360 nm observed in the incubation mixture indicated that some ommatin D was still present since xanthommatin displays no absorbance maximum in that region (Table 2). Apparently, the partial conversion of ommatin D to xanthommatin could be effected enzymatically.

Rhodommatin, the O-β-glucoside of xanthommatin, displays certain characteristics that are similar to those of ommatin D. Rhodommatin has absorbance maxima at 306 nm and 437 nm in 5 N HCl, and at 377 nm and 497 nm in phosphate buffer, pH 7.4 (Linzen, 1974). For rhodommatin, incubation in 1 N HCl is sufficient to hydrolyze the glucosidic bond, causing the release of glucose and xanthommatin (Butenandt et al., 1963). For chromophore from YCP, the rate of appearance of xanthommatin during the incubation in HCl was closer to that reported for ommatin D than for rhodommatin. Furthermore, rhodommatin is sensitive to treatment with β-glucosidase (Butenandt, et al., 1963). Incubating chromophore from YCP with β-glucosidase did not cause changes in the absorbance spectra. Therefore, ommatin D appeared to be the only ommochromes released from YCP.
CHAPTER 5

BIOLOGY OF THE YELLOW-COLORED PROTEIN
MATERIALS AND METHODS:

ANTI-YCP ANTISERUM

Antiserum directed against YCP was prepared in a New Zealand white rabbit. Pre-immune serum was obtained by bleeding the rabbit through the main ear vein prior to the injection in the hind limb of 340 μg YCP emulsified in Freund's complete adjuvant. Booster injections of 340 μg YCP in Freund's incomplete adjuvant were given three and five weeks after the initial inoculation. Blood was collected two weeks after the second booster injection. The blood was allowed to clot overnight at 4°C and the antiserum was aliquoted and stored at -80°C.

IN VITRO INCUBATION OF FAT BODY

The procedure of Prasad et al. (1986) was followed. Fat bodies were dissected from four feeding stage fifth instar larvae and were incubated in vitro in the presence of 56 μCi of [35S]methionine. After treatment of the medium with pre-immune serum, YCP secreted into the medium was immunoprecipitated with anti-YCP, subjected to SDS-PAGE, and visualized by autoradiography.

IMMUNOBLOTTING

Immunoblotting was used to determine if the YCP polypeptide was present in M. sexta throughout the life cycle, to detect the YCP polypeptide in chromatographic fractions and to examine the hemolymph of other insects for the presence of immunologically cross-reacting proteins.
Samples

Samples were hemolymph from *M. sexta* at each larval instar. For fifth instar larvae, hemolymph was obtained both from an animal in the feeding stage and from an animal in the wandering stage. Pupae and adults were sexed before bleeding. Finally, a buffer-soluble extract of dissected and homogenized oocytes was prepared.

Hemolymph samples from the species listed in Table 4 were examined for the presence of proteins cross-reacting with the anti-YCP antiserum. Hemolymph from *Hyalaphora cecropia* was a gift from Dr. William Telfer. *Bombyx mori* hemolymph was a gift from Ann Peterson. Dr. Michael Kanost kindly supplied the other hemolymph samples listed in Table 4.

Electrophoresis and immunodetection

SDS-PAGE was performed under reducing conditions (Laemmli, 1970) in gradient gels, as previously described (Chapter 2). Prestained protein molecular weight markers (Amersham, Arlington Heights, IL 60005) were used. The markers were: lysozyme (14.4 kDa), soybean trypsin inhibitor (21.5 kDa), carbonic anhydrase (29 kDa), ovalbumin (45 kDa), bovine serum albumin (66.2 kDa), phosphorylase B (92.5 kDa), and myosin (200 kDa). After electrophoresis, proteins were electroblotted from the gel onto nitrocellulose (Towbin et al., 1979).

Immunodetection was performed using the anti-YCP antiserum and a kit containing biotinylated goat anti-rabbit
IgG and avidin-linked peroxidase (Vector Laboratories, Burlingame, CA 94010).

CONCENTRATION DETERMINATIONS

The Oudin immunodiffusion procedure as presented by Telfer et al. (1983) was used to determine the concentration of YCP polypeptide in various samples.

Hemolymph Samples

Hemolymph samples were collected from individual animals whose stage of development had been determined. The stage of development of larvae was determined by the weight and developmental events such as molts, head-cap slippage, exposure of the dorsal vessel, onset of wandering, and pupation. Larval hemolymph was collected on each day of the fourth and fifth instars. At pupation, the animals were separated by sex. Pupae of each sex were bled on the first day after pupation and every third day thereafter until emergence of the adults. Also, hemolymph was collected from newly emerged adults. A sample was also prepared from oocytes. Older adult females (i.e. two to four days after emergence) were dissected to remove the oocytes. The largest of these were homogenized in an Eppendorf tube and centrifuged to pellet insolubles. The resulting supernatant was treated the same way as hemolymph.

Larvae were bled through incisions in the prolegs or in the dorsal vessel. For pupae, the proboscis was removed and hemolymph was obtained by low speed centrifugation of the
animal. Adults were decapitated prior to low speed centrifugation.

Equal volumes of collected hemolymph and of 2 mM phenylthiourea (PTU) were mixed in Eppendorf tubes maintained on ice. The samples were placed under nitrogen and were centrifuged for five minutes in a benchtop centrifuge to remove hemocytes and insolubles. In no instance was darkening or clotting of the hemolymph observed. After centrifugation, each hemolymph supernatant was removed from the underlying pellet and stored at -20°C until needed.

**YCP standards**

The protein concentration of a stock solution of purified YCP in PBS was determined using the bicinchoninic acid (BCA) protein assay (Pierce Chemical Co., Rockford, IL) according to the method of Smith et al. (1985); bovine serum albumin served as the protein standard. Ten successive serial dilutions of the YCP stock solution were made. The concentrations of the first seven standards fell within the detection range of the BCA protein assay and their protein concentrations were determined: 8.08 mg/ml, 4.04 mg/ml, 2.02 mg/ml, 1.07 mg/ml, 0.52 mg/ml, 0.24 mg/ml, and 0.12 mg/ml. Four other YCP standards were too dilute to be assayed for protein; their concentrations were estimated at 60 µg/ml, 30 µg/ml, 15 µg/ml and 8 µg/ml.

**Oudin Test**

Following the procedure of Telfer et al. (1983), glass
tubes (60 mm x 3 mm i.d.) were sealed at one end and precoated by filling and emptying them with a solution of 0.1% (w/v) agarose. The tubes were left to air dry for two days. An agarose stock solution of 1.5% (w/v) agarose in PBS was prepared. Working at 45°C, each glass tube was filled with 300 μl of a solution containing by volume: 70% PBS, 20% agarose stock (at 100°C), and 10% anti-YCP rabbit antiserum. The tubes were stored at 4°C in a humidified environment until needed.

The experiment was initiated by overlaying an antiserum agar tube with 100 μl of sample. The tubes were sealed with Parafilm to prevent evaporation and kept at room temperature. All samples including the standards, were assayed in duplicate. To avoid subsequent bias in the measurements, the hemolymph samples were assayed out of sequence and duplicates did not succeed one another.

The distance between the meniscus of the antiserum agar and the leading edge of the milky precipitation zone (h, in mm) was measured for each sample. These measurements were made 18, 44.5, 66 and 169 hours after the start of the experiment.

Data analysis

Data were analyzed on an Apple Macintosh IIcx computer using Cricket Graph software. Each measurement (h) was converted to K, where K=h/√t and t was the elapsed time in hours. For the YCP standards, the K values of duplicates were
averaged to yield $K_{av}$. A plot of $K_{av}$ against $\log[YCP]$ ([YCP] in mg/ml) was made and the best-fitting curve having a second-order polynomial equation was identified. A coefficient of simple determination ($R^2$) gave an estimate of the correlation between actual data and values predicted from the polynomial equation; $R^2 = 1$ indicated a perfect fit.

$K_{av}$ values obtained for the hemolymph-derived samples were converted to $\log[YCP]$ using the constants from the second-order polynomial equation. $\log[YCP]$ was converted to [YCP] which was multiplied by two (to account for dilution with 2 mM PTU) to obtain the concentration of YCP polypeptide in the original hemolymph sample. The [YCP] values which were obtained for a given sample at the different time points, were averaged and the standard deviation was recorded.

ASSOCIATION OF CHROMOPHORE WITH PROTEIN

Calibrated gel filtration column

To investigate the association of the chromophore with the YCP polypeptide, the calibrated Sephadex G-75 Superfine gel filtration column described in Chapter 3 was used.

Sample preparation

Hemolymph was obtained from feeding stage fifth instar larvae, fifth instar wandering stage larvae, adult males and adult females. In all, three different hemolymph samples were obtained from fifth instar wandering stage larvae. The first was obtained from larvae that were in the second or third day of wandering. The second sample was collected from larvae in
the first day of wandering and the third sample was from larvae in the third day of wandering. The adults were bled by the "flushing-out" method of Chino et al. (1987); larvae were bled through incisions in the prolegs or in the dorsal vessel. Hemolymph was treated with reduced glutathione and diisopropyl fluorophosphate and subjected to KBr density-gradient ultracentrifugation, as described in Chapter 2. After ultracentrifugation, the lipid-free proteins in the subphase were recovered and dialyzed against PBS. The subphase proteins precipitating between 60% and 95% ammonium sulfate saturation were obtained; they were dialyzed against PBS, concentrated by ultrafiltration and applied to the calibrated Sephadex G-75 Superfine gel filtration column. Fractions (2.3 ml each) were monitored for absorbance at 405 nm and were assayed for protein using the BCA protein assay. Relevant fractions were further examined by SDS-PAGE and immunoblotting.

Chromophore to protein ratio

For hemolymph obtained from larvae in the first day of the wandering stage and for hemolymph obtained from larvae in the third day of wandering, YCP-containing fractions collected from the gel filtration column (fractions 23 and 24) were characterized in greater detail. For each of these fractions, protein content was measured using the BCA assay, the absorbance at 405 nm was recorded, and the YCP polypeptide concentration was determined using the Oudin immunodiffusion
procedure. For YCP derived from the hemolymph of adult females, fractions 23, 24 and 25 from the gel filtration column were pooled and concentrated before the measurements were made.
RESULTS AND DISCUSSION:

YCP is a protein from the hemolymph of *M. sexta* which derives its yellow color from a chromophore which appears to be ommatin D. As such, YCP has two components: the chromophore and the 31 kDa polypeptide chain. Antibodies raised against YCP react with the polypeptide chain whereas the chromophore is responsible for the absorbance at 405 nm.

SITE OF SYNTHESIS

The antiserum directed against YCP served to identify the fat body as a site of synthesis for the polypeptide component of YCP. Fat bodies from feeding stage fifth instar larvae were incubated *in vitro* in the presence of \[^{35}\text{S}]\)-methionine. YCP polypeptide was immunoprecipitated from the incubation medium and visualized by autoradiography after electrophoresis (Fig. 25). A cross-reacting protein of approximately 41 kDa was also detected. In Fig. 25, this 41 kDa protein produces a stronger signal than YCP. Either the 41 kDa protein is synthesized in greater amounts than YCP or it contains more methionine residues (and hence more \(^{35}\text{S}\)) than does YCP. It is not known whether the cross-reacting protein is immunologically-related to YCP or whether the YCP immunogen was contaminated with trace amounts of the 41 kDa protein. Sometimes, the 41 kDa protein was also observed in immunoblots of hemolymph proteins. However, it always gave a much weaker signal than YCP. Furthermore, if the anti-YCP antiserum was diluted at least 1:500, the 41 kDa protein was not detectable.
Fat bodies were dissected from feeding stage fifth instar larvae and were incubated in vitro in the presence of \(^{[35}S\)methionine. The YCP polypeptide was precipitated from the incubation medium with anti-YCP antiserum. Following electrophoresis, it was visualized by autoradiography (arrows). Shown here are autoradiographs obtained after exposures of 48 hours (left) and 72 hours (right). The samples are: Lane 1, incubation mixture; Lane 2, \(^{[14}C\)-labelled molecular weight marker proteins; Lane 3, immunoprecipitated material.
It was not surprising to find the YCP polypeptide synthesized by the fat body since the majority of proteins found in the hemolymph of *M. sexta* are synthesized by that tissue (for review, see Kanost et al., 1990). A notable exception is insecticyanin which is synthesized by the epidermis (Riddiford, 1982).

Recently, additional evidence was obtained which also suggested that the YCP polypeptide is synthesized by the fat body. Screening a cDNA library derived from larval fat body with anti-YCP antiserum led to the isolation of a clone which appeared to encode the N-terminus of YCP (Ho, C.M., Yepiz-Plascensia, G.M., Martel, R.R., and Law, J.H., preliminary results). The deduced amino acid sequence of the clone was:

```
Ser-Lys-Asp-Cys-Val-Ser-Val-Asn-Gly-Lys-Asn-
Tyr-Gly-Lys-Glu-Val-Leu-Lys-Asp-Asn-Ile-
His-Gln-Ala-Tyr-Gln-Leu-Ser-Phe-Asp-Pro-
```

This sequence is in good agreement with the N-terminal sequence of YCP determined by automated Edman degradation (Chapter 3), with the exception of residues 4 (Cys vs Ser) and 31 (Pro vs. Glu). Sequencing the YCP polypeptide indicated that either Ser or Thr was present at the sixth residue; in the cDNA clone, Ser was found at that position.

**OCCURRENCE AND TITERS OF YCP POLYPEPTIDE IN HEMOLYMPH**

An immunoblot with anti-YCP antiserum indicated that the YCP polypeptide was present in the hemolymph throughout the life cycle and in the oocyte (Fig. 26).
Proteins from an oocyte extract and from hemolymph samples were subjected to SDS-PAGE and examined for the presence of YCP polypeptide by immunoblotting using anti-YCP antiserum (diluted 1:750). The following samples are shown. Lanes 1, 6 and 14: prestained molecular weight marker proteins, the molecular masses (in kDa) are shown. Soluble oocyte extract (Lane 2). Hemolymph from larvae: first instar (Lane 3), second instar (Lane 4), third instar (Lane 5), and fourth instar (Lane 7); fifth instar: feeding stage (Lane 8) and wandering stage (Lane 9). Hemolymph from pupae: from females (Lane 10) and males (Lane 11). Hemolymph from adults: females (Lane 12) and males (Lane 13).
Having detected the YCP polypeptide in each sample examined, the titer of the polypeptide in the hemolymph was determined, from the start of the fourth larval instar until emergence of the adult. Titers were measured using the Oudin immunodiffusion procedure (Telfer et al., 1983). For purified YCP standards, the best-fitting second-order polynomial equation expressing log[YCP] as a function of Kav had the correlation coefficient R²=0.987 for data collected at 18 hours, R²=0.997 at 44.5 hours and at 169 hours, and R²=0.996 at 66 hours. In all, results from the Oudin test were highly reproducible both for replicates at a single time point and for individual samples over time.

The titer of YCP polypeptide in the hemolymph is shown in Fig. 27. During the fourth larval instar, YCP polypeptide concentration rose from 0.010 ± 0.002 mg/ml on the first day to 0.021 ± 0.003 mg/ml after head-cap slippage. During the molt to the fifth instar, the concentration fell to 0.009 ± 0.002 mg/ml after which it rose rapidly to 0.41 ± 0.06 mg/ml on the second day of the wandering stage. The highest levels of YCP polypeptide were found on the first day of pupation: 0.47 ± 0.10 mg/ml in females and 0.44 ± 0.05 mg/ml in males. Subsequently, the titer decreased steadily over the course of metamorphosis to 0.16 ± 0.01 mg/ml in males and 0.12 ± 0.01 mg/ml in females, just prior to eclosion. That females contained 25% less YCP polypeptide than did males was in all likelihood due to the incorporation of YCP polypeptide into
Fig. 27:

Titers of YCP polypeptide in the hemolymph.

The concentration of YCP polypeptide in hemolymph was determined in fourth and fifth instar larvae (●), in pupae and adults: female (○) and male (■). Concentration determinations were made using anti-YCP antiserum in the Oudin immunodiffusion procedure.
developing oocytes since the polypeptide was detected in dissected oocytes by immunoblotting. Furthermore, YCP polypeptide concentration in soluble oocyte extract was 0.010 ± 0.003 mg/ml. On emergence of the adult moths, there was a slight increase in the hemolymph concentration of YCP polypeptide. Rather than being due to new synthesis of YCP polypeptide, this increase probably resulted from a reduction in the hemolymph volume.

The amount of YCP polypeptide per animal increased considerably during the fifth larval instar. From the beginning of the fifth instar until the beginning of wandering, YCP polypeptide concentration rose from 0.009 ± 0.002 mg/ml to 0.25 ± 0.04 mg/ml, a 45-fold increase. In addition, *M. sexta* larvae grow substantially during that period. Over the course of the fifth instar, the fraction of fresh body weight that is the hemolymph, was reported by Beckage and Riddiford (1982). Using those values and the weights of the experimental animals, YCP polypeptide content increased about 100-fold from approximately 0.008 mg/larva at the beginning of the fifth instar to approximately 0.8 mg/larva at the onset of wandering. Overall, the rise and fall of YCP polypeptide titers in hemolymph resembled the changes in hemolymph concentration of both arylphorin and total protein (Kramer et al., 1980).

ASSOCIATION OF CHROMOPHORE WITH YCP POLYPEPTIDE

The subphase obtained from the density gradient
ultracentrifugation of hemolymph from wandering stage fifth instar larvae was distinctly green in color. In contrast, subphase obtained from the ultracentrifugation of hemolymph from feeding fifth instar larvae was blue. Only YCP appeared to be responsible for this change in color yet the hemolymph of feeding stage fifth instar larvae contained enough YCP polypeptide to impart at least a greenish tinge on the subphase provided however, that chromophore was associated with the polypeptide. To determine whether chromophore was indeed associated with the YCP polypeptide at various stages of the life cycle, hemolymph was collected from i) fifth instar feeding stage larvae, ii) fifth instar wandering stage larvae, iii) male moths, and iv) female moths. After ultracentrifugation and ammonium sulfate fractionation, the four hemolymph-derived samples were applied separately to the calibrated Sephadex G-75 Superfine gel filtration column. Figure 28 shows the results of protein assays and measurements of absorbance at 405 nm, made on successive fractions collected from the column. The volumes at which molecular weight standards and purified YCP eluted, are shown in Fig. 28-A. For all four hemolymph-derived samples, the absorbance at 405 nm that reached a maximum at 44 ml was due to the blue-colored biliprotein, insecticyanin (Cherbas, 1973). In the samples derived from fifth instar wandering larvae (Fig. 28-C) and female moths (Fig. 28-E), yellow color and absorbance at 405 nm were detected, reaching a maximum at 56 ml. This was
Fig. 28:

Association of chromophore with YCP polypeptide at different life stages

The calibration of a gel filtration chromatography column is shown, including the position at which purified YCP eluted (A). Elution profiles show protein concentration (●) and absorbance at 405 nm (○) of hemolymph-derived samples subjected to gel filtration chromatography. The samples were derived from the hemolymph fifth instar larvae, both feeding stage (B) and wandering stage (C), and from adult males (D) and females (E). The hemolymph samples were enriched for YCP by ultracentrifugation and ammonium sulfate fractionation before gel filtration chromatography. YCP polypeptide was detected by immunoblotting in the fractions marked with arrowheads (▼).
YCP. Protein-associated chromophore was not detected in the samples derived from the hemolymph of feeding fifth instar larvae (Fig. 28-B) or of male moths (Fig. 28-D).

Protein-containing fractions collected from the gel filtration column were further examined by SDS-PAGE and by immunoblotting using the anti-YCP antiserum. The results of these examinations are presented in: Fig. 29 for feeding stage fifth instar larvae, Fig. 30 for wandering stage fifth instar larvae, Fig. 31 for male moths, Fig. 32 for female moths. For all four hemolymph-derived samples, the YCP polypeptide was visible in Coomassie-stained gels and detectable with the anti-YCP antiserum in the fractions marked with arrowheads in Fig. 28. Thus, while YCP polypeptide was present in all hemolymph samples examined, only in wandering stage larvae and female moths did it have detectable amounts of chromophore associated with it. The presence in hemolymph from feeding stage fifth instar larvae and from adult males, of the YCP polypeptide lacking detectable levels of chromophore suggested that apoYCP (i.e. the polypeptide without the chromophore) was present in those animals.

To characterize further the association of chromophore with protein during the wandering stage, YCP was partially purified from the hemolymph of: i) fifth instar larvae in the first day of the wandering stage, ii) fifth instar larvae in the third day of the wandering stage, iii) adult females. The hemolymph samples were subjected to ultracentrifugation,
A sample derived from the hemolymph of feeding stage fifth instar larvae was enriched for YCP by ultracentrifugation and ammonium sulfate fractionation. The sample was subjected to gel filtration chromatography and fractions collected from the column were analyzed. The top panel shows the protein concentration in mg/ml (grey bars) and the absorbance at 405 nm (black bars) of successive fractions. This panel presents data that were also used in Fig. 28-B. The fractions were examined by SDS-PAGE (central panel) and by immunoblotting with anti-YCP antiserum (bottom panel). In the SDS-PAGE gel, the bands corresponding to the YCP polypeptide are marked with arrows and the fraction numbers are shown. Molecular weight marker proteins are in the first and last lanes; their molecular masses (in kDa) are indicated. For bands in the immunoblot, the corresponding fraction number is marked.
Fig. 30:

YCP in hemolymph from wandering stage fifth instar larvae: chromatographic, electrophoretic and immunological analysis

A sample derived from the hemolymph of wandering stage fifth instar larvae was enriched for YCP by ultracentrifugation and ammonium sulfate fractionation. The sample was subjected to gel filtration chromatography and fractions collected from the column were analyzed. The top panel shows the protein concentration in mg/ml (grey bars) and the absorbance at 405 nm (black bars) of successive fractions. This panel presents data that were also used in Fig. 28-C. The fractions were examined by SDS-PAGE (central panel) and by immunoblotting with anti-YCP antiserum (bottom panel). In the SDS-PAGE gel, the bands corresponding to the YCP polypeptide are marked with arrows and the fraction numbers are shown. Molecular weight marker proteins are in the first and last lanes; their molecular masses (in kDa) are indicated. For bands in the immunoblot, the corresponding fraction number is marked.
Fig. 31:

**YCP in hemolymph from male moths:**
chromatographic, electrophoretic
and immunological analysis

A sample derived from the hemolymph of male moths was enriched for YCP by ultracentrifugation and ammonium sulfate fractionation. The sample was subjected to gel filtration chromatography and fractions collected from the column were analyzed. The top panel shows the protein concentration in mg/ml (grey bars) and the absorbance at 405 nm (black bars) of successive fractions. This panel presents data that were also used in Fig. 28-D. The fractions were examined by SDS-PAGE (central panel) and by immunoblotting with anti-YCP antiserum (bottom panel). In the SDS-PAGE gel, the bands corresponding to the YCP polypeptide are marked with arrows and the fraction numbers are shown. Molecular weight marker proteins are in the first and last lanes; their molecular masses (in kDa) are indicated. For bands in the immunoblot, the corresponding fraction number is marked.
YCP in hemolymph from female moths: chromatographic, electrophoretic and immunological analysis

A sample derived from the hemolymph of female moths was enriched for YCP by ultracentrifugation and ammonium sulfate fractionation. The sample was subjected to gel filtration chromatography and fractions collected from the column were analyzed. The top panel shows the protein concentration in mg/ml (grey bars) and the absorbance at 405 nm (black bars) of successive fractions. This panel presents data that were also used in Fig. 28-£. The fractions were examined by SDS-PAGE (central panel) and by immunoblotting with anti-YCP antiserum (bottom panel). In the SDS-PAGE gel, the bands corresponding to the YCP polypeptide are marked with arrows and the fraction numbers are shown. Molecular weight marker proteins are in the first and last lanes; their molecular masses (in kDa) are indicated. For bands in the immunoblot, the corresponding fraction number is marked.
ammonium sulfate fractionation and gel filtration chromatography. For samples derived from the hemolymph of wandering stage larvae, the elution profiles of the gel filtration column are shown in Fig. 33. In Fig. 33 as in Fig. 28, the absorbance at 405 nm recorded around 44 ml was due to insecticyanin. For both samples, the absorbance at 405 nm reaching a maximum at 55 ml (i.e. fraction 24) was due to YCP. This was confirmed by obtaining the absorbance spectrum of fraction 24 derived from each sample (Fig. 34). Relative to insecticyanin, sample from larvae in the first day of wandering (Fig. 33-A) contained substantially less YCP-associated chromophore than did sample from larvae in the third day of wandering (Fig. 33-B). For YCP partially purified from the hemolymph of adult females, the absorbance spectrum is also shown in Fig. 34. YCP-containing fractions collected from the gel filtration column and marked with arrowheads in Fig. 33 were examined in more detail as was YCP derived from female moths. The samples were assayed for total protein, YCP polypeptide and chromophore, using respectively, the BCA protein assay, the Oudin immunodiffusion procedure and absorbance at 405 nm (Table 3).

The ratio of absorbance at 405 nm to YCP polypeptide concentration (in mg/ml) rose from 0.08 on the first day of wandering to 0.23 on the third day of wandering, to 0.36 in adult females. In other words, the amount of chromophore per milligram of YCP polypeptide tripled between the first day and
Fig. 33:

Association of chromophore with YCP polypeptide in wandering stage fifth instar larvae

Hemolymph samples were examined from fifth instar larvae in the first day (A) and in the third day (B) of the wandering stage. Elution profiles show protein concentration (●) and absorbance at 405 nm (○) of hemolymph-derived samples subjected to gel filtration chromatography. The hemolymph samples were enriched for YCP by ultracentrifugation and ammonium sulfate fractionation before gel filtration chromatography. Fractions marked with arrowheads (i.e. fractions 23 and 24) were assayed for protein, for YCP polypeptide and chromophore. The absorbance spectrum of the fraction marked with an asterisk was also obtained.
Fig. 34:

Absorbance spectra of partially-purified YCP from hemolymph obtained at different stages

Samples were enriched for YCP by ultracentrifugation, ammonium sulfate fractionation and gel filtration chromatography. Spectra were obtained for YCP-containing fractions collected from the calibrated gel filtration column. The sources of hemolymph from which YCP was partially purified were: fifth instar larvae in the first day of wandering (A), in the third day of wandering (B), and adult females (C). The spectra indicated that each sample contained authentic YCP.
Table 3:

YCP polypeptide and chromophore content in selected samples

<table>
<thead>
<tr>
<th>Hemolymph Source</th>
<th>Fraction from Sephadex G-75</th>
<th>Protein Conc. (mg/ml)</th>
<th>$A_{405 \text{ nm}}$</th>
<th>YCP Polypeptide Conc. (mg/ml)</th>
<th>Ratio: $A_{405 \text{ nm}}$ to YCP Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fifth Instar Wandering Day 1</td>
<td>23</td>
<td>2.30</td>
<td>0.076</td>
<td>0.95 ± 0.11</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>2.25</td>
<td>0.088</td>
<td>1.06 ± 0.15</td>
<td>0.08</td>
</tr>
<tr>
<td>Fifth Instar Wandering Day 3</td>
<td>23</td>
<td>0.71</td>
<td>0.096</td>
<td>0.41 ± 0.03</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.84</td>
<td>0.149</td>
<td>0.64 ± 0.05</td>
<td>0.23</td>
</tr>
<tr>
<td>Adult Females</td>
<td>23, 24 and 25</td>
<td>1.89</td>
<td>0.158</td>
<td>0.44 ± 0.02</td>
<td>0.36</td>
</tr>
</tbody>
</table>
the third day of the wandering stage. This increase was accompanied by a rise in titer of YCP polypeptide in the hemolymph from $0.25 \pm 0.04$ mg/ml on the first day to $0.41 \pm 0.04$ mg/ml on the third day (Fig. 27). Therefore, the hemolymph concentration of protein-bound chromophore increased nearly five-fold during the wandering stage. In the hemolymph of adult females, there was 1.6 times more chromophore per unit of YCP polypeptide than there was in larvae in the third day of wandering (Table 3); however, YCP polypeptide titers were substantially lower in adult females (Fig. 27).

The change of apoYCP to YCP at the onset of wandering correlates with the start of ommochrome synthesis in the epidermis of *M. sexta* (Hori and Riddiford, 1982). During the feeding stage of the fifth instar, larvae accumulate kynurenine and 3-hydroxykynurenine in the epidermis; at wandering and under hormonal regulation, these compounds are converted to ommochromes (Hori and Riddiford, 1982). In view of these observations, it is possible that apoYCP rather than YCP is present in the hemolymph of feeding stage fifth instar larvae because the chromophore has not yet been synthesized.

To account for observing YCP in the hemolymph of female moths and apoYCP in the hemolymph of male moths, both sex-dependent chromophore release into the hemolymph and sex-dependent chromophore uptake from the hemolymph were considered as explanations. Increased ommochrome synthesis in females could account for the presence of YCP. Being
tryptophan metabolites, ommochromes would have to arise either from a sex-specific excess of tryptophan in females or from sex-dependent regulation of the tryptophan to ommochrome pathway. Excess tryptophan could arise in females from increased levels of protein synthesis occurring to support egg maturation. This would include the synthesis of microvitellogenin and especially, the synthesis of vitellogenin. Since *M. sexta* does not feed after the onset of wandering, all proteins synthesized in pupae and in adults incorporate amino acids that were ingested and stored by the larvae. Adults synthesize proteins using amino acids recovered from the degradation of larval proteins (Kramer et al., 1980). If the amount of tryptophan released from the degradation of larval proteins exceeds the amount required for incorporation into adult proteins, then the excess tryptophan can be catabolized to ommochromes thereby providing apoYCP with chromophore.

In *M. sexta*, vitellogenin is synthesized by the female fat body, secreted into the hemolymph and incorporated into nascent oocytes in which it is converted to the yolk protein, vitellin. This protein comprises two types of polypeptide chains: apovitellogenin I (177 kDa) and apovitellogenin II (45 kDa) (Osir et al., 1986b). In the hemolymph of female moths, vitellogenin is an abundant protein: its apoproteins are clearly visible in lanes 2 to 5 of the SDS-PAGE gel in Fig. 32. However, vitellogenin is very poor in tryptophan
residues: the holoprotein has a tryptophan content of approximately 0.3 mol% (Osir et al., 1986b). Most of the amino acids in vitellogenin are probably derived from arylphorin, since the latter is the major hemolymph protein when feeding ceases. The tryptophan content of arylphorin is approximately 1.1 mol% (Willott et al., 1989). Thus, synthesizing vitellogenin with amino acids from arylphorin would produce a tryptophan surplus which could lead to increased ommochrome levels in females.

An analogous situation occurs in the silkmoth, B. mori. Linzen (1974) reports that in the two days immediately preceding and following the onset of cocoon spinning, the concentration of free tryptophan and of its metabolites greatly increases, presumably because silk, which is poor in tryptophan, must be synthesized from proteins of average composition.

Alternatively, the tryptophan to ommochrome pathway may be regulated in a sex-dependent fashion. Linzen (1974), summarizing the results of studies on the rb mutant of B. mori, indicates that beginning on the fifth day after pupation, levels of tryptophan metabolites diverge in males and females. Males accumulate kynurenine whereas females accumulate 3-hydroxykynurenine. Just prior to eclosion, there is twice as much kynurenine in males as there is in females. These differences are due to the activity of kynurenine-3-hydroxylase (which converts kynurenine to 3-hydroxykynurenine)
in the ovaries. If a similar difference in the activity of kynurenine-3-hydroxylase occurs in *M. sexta* and if ommochrome synthesis proceeds unimpeded from 3-hydroxykynurenine, then this can account for the presence of YCP in females and of apoYCP in males.

Finally, ommochrome from YCP may be sequestered in male moths but not in females. Dihydroxanthommatin (i.e. the reduced form of xanthommatin) has been identified as a pigment of testes in other lepidoptera: *Pieris brassicae* (Kayser, 1979), *Ephistia kühniella* (Linzen, 1974) and *Papilio xuthus* (Umebachi and Uchida, 1970). For *M. sexta*, the testes were white in fifth instar larvae, cream-colored in pupae, and yellow in adults (unpublished observations). Whether the yellow color observed in adult testes was due to ommochromes remains to be investigated.

**IMMUNOLOGICAL CROSS-REACTIVITY**

Hemolymph samples from the insects listed in Table 4 were examined by immunoblotting for proteins cross-reacting with the anti-YCP antiserum. In the non-lepidopteran samples, no immunologically-related proteins were observed. However, in each of the four lepidopteran species examined, a cross-reacting protein was detected (Fig. 35). Furthermore, these cross-reacting proteins had nearly the same relative mobility in SDS-PAGE gels as the YCP polypeptide from *M. sexta*. It is probable that these proteins are analogous to YCP although their capacity to bind ommochrome remains to be examined.
### Table 4:

Insects examined for hemolymph proteins cross-reacting with anti-YCP antiserum

<table>
<thead>
<tr>
<th>Lepidoptera</th>
<th>Manduca sexta</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Celerio sp.</td>
</tr>
<tr>
<td></td>
<td>Bombyx mori</td>
</tr>
<tr>
<td></td>
<td>Heliothis zea</td>
</tr>
<tr>
<td></td>
<td>Hyalophora cecropia</td>
</tr>
<tr>
<td>Coleoptera</td>
<td>Cotinus mutabilis</td>
</tr>
<tr>
<td></td>
<td>Derobrachus geminatus</td>
</tr>
<tr>
<td></td>
<td>Tenebrio molitor</td>
</tr>
<tr>
<td>Diptera</td>
<td>Calliphora sp.</td>
</tr>
<tr>
<td>Hemiptera</td>
<td>Thasus acutangulus</td>
</tr>
<tr>
<td>Homoptera</td>
<td>Cicada sp.</td>
</tr>
<tr>
<td>Hymenoptera</td>
<td>Apis mellifera</td>
</tr>
<tr>
<td>Odonata</td>
<td>Dragonfly</td>
</tr>
<tr>
<td>Orthoptera</td>
<td>Locusta migratoria</td>
</tr>
<tr>
<td></td>
<td>Schistocerca gregaria</td>
</tr>
<tr>
<td></td>
<td>Nauphoeta cinerea</td>
</tr>
<tr>
<td></td>
<td>Periplaneta americana</td>
</tr>
</tbody>
</table>
Fig. 35:

Immunoblot with anti-YCP antiserum of hemolymph from various lepidopteran species

Hemolymph samples were subjected to SDS-PAGE and immunoblotting with anti-YCP antiserum. Hemolymph was obtained from *M. sexta* (Lane 1), *H. cecropia* (Lane 3), *Celerio* sp. (Lane 4), *H. zea* (Lane 6), and *B. mori* (Lane 7). Prestained molecular weight marker proteins are visible (Lanes 2 and 5) and their molecular masses (in kDa) are indicated.
THE FUNCTION OF YCP

The biochemical analysis of YCP has led to the following hypothesis regarding its function. The 31 kDa polypeptide component of YCP appears to be involved in the transport of ommochrome through the hemolymph. Present in hemolymph throughout the life cycle, apoYCP is synthesized by the larval fat body and is secreted into the hemolymph, especially during the fifth instar. During the wandering stage, the polypeptide binds increasing amounts of chromophore, apparently ommatin D, thus forming YCP. The ommatin D is thought to arise from the solubilization of dihydroxanthommatin which had previously accumulated in the tissues (e.g. the dorsal epidermis). Since association with protein is not required to render the chromophore soluble, the protein may serve to guide the chromophore from one tissue to another. Because apoYCP was observed in adult males, there also appears to be subsequent transfer of chromophore from its protein-bound form in the hemolymph to other tissues. These other tissues may include the testes or the gut, possibly by way of the Malpighian tubules. This transfer of chromophore may also be accompanied by a chemical transformation. For instance, ommatin D may be converted back to dihydroxanthommatin through the loss of a sulfate moiety.

Proteins immunologically related to YCP appear to be common in lepidoptera. Whether they have the same function as YCP remains to be determined.
FUTURE STUDIES

The primary structure of the YCP polypeptide will soon be deduced from the nucleotide sequence of a cDNA clone that encodes YCP. Sequence homologies and analogies between YCP and other proteins have the potential to elucidate the function of YCP. The cDNA can also serve as a tool to investigate the distribution and regulation of expression of the YCP gene in vivo.

To confirm the identification of the chromophore, physical techniques such as nuclear magnetic resonance, mass spectroscopy and infrared spectroscopy will have to be used. Preliminary studies using each of these three techniques were performed. None provided data that were inconsistent with the chromophore being ommatin D, yet the data were not conclusive either. The labile nature of the chromophore, the lack of readily available reference compounds and perhaps the ability the chromophore to chelate metals are significant problems. The chemical synthesis of ommatin D has been reported (Butenandt et al., 1960) but proved to be difficult to reproduce. Nevertheless, the chromophore will have to be unambiguously identified.

Identification of the chromophore should provide insights as to how radioisotopes can be incorporated into this molecule, be it in vivo or in vitro. Radiolabelled chromophore in reconstituted YCP can be used to determine the fate of the chromophore in vivo.
Since the interaction between the YCP polypeptide and the chromophore appears to be noncovalent, the binding constant can be derived and inhibitors of binding can be developed. The effects of such inhibitors on M. sexta would in all likelihood provide further insight into the physiological function of this protein.

Finally, it has been shown that proteins immunologically related to YCP are common in lepidoptera. It will be important to determine whether the results obtained in the study of M. sexta can be extended to other organisms.
REFERENCES


Hori and Riddiford (1981) Isolation of ommochromes and 3-hydroxykynurenine from the tobacco hornworm, Manduca sexta. Insect Biochem. 11, 507-513


