CYCLIC FLUCTUATIONS IN ARYLPHORIN, THE PRINCIPAL SERUM STORAGE PROTEIN OF *LYMANTRIA DISPAR*, INDICATE MULTIPLE ROLES IN DEVELOPMENT

Sharon T. Karpells[1], David E. Leonard[2] and Joseph G. Kunkel[1,*]

[1]Department of Zoology and [2]Department of Entomology, University of Massachusetts,
Amherst, MA 01003, U.S.A.

Abstract—Arylphorin (Ap) was isolated and partially characterized from larval serum of the gypsy moth, *Lymantria dispar* (Lepidoptera: Lymantriidae). Non-denaturing and SDS–polyacrylamide gel electrophoresis suggest a 440,000 Da hexamer composed of nonidentical subunits of M_r 73,000 and 80,000. Tyrosine and phenylalanine comprise 15.6% of the total protein amino acid. *L. dispar* Ap is immunologically related to *Manduca sexta* Ap. Hemolymph Ap concentrations throughout the larval stadia were determined using quantitative immunoelectrophoresis. Cyclic fluctuations in hemolymph concentrations are correlated with each molting cycle. A general increase in Ap concentration during each intermolt is followed by a sharp decline between apolysis and ecdysis. The last days of each instar were found to be the best time to sample protein titer with minimum variance. Hemolymph space, estimated by measuring the dilution of an injected foreign protein, is a constant 28% (v/w) of body weight. Total serum Ap per animal was calculated. A divergent allometric relationship between Ap accumulation and weight gain throughout the larval period results in the ultimate domination of the serum protein profile by Ap in the ultimate larval instar of the female in particular. An additional instar of Ap accumulation in the female gypsy moth is suggested to compensate for the lack of a predominantly female-specific storage protein in this species.

Key Word Index: arylphorin, *Lymantria dispar*, storage protein, hemolymph volume

INTRODUCTION

Insect hemolymph at the time of metamorphosis is dominated by serum storage proteins. Identified in both the Hemimetabola and Holometabola, these proteins are characterized by a hexameric structure and a subunit molecular weight of approx. 75–85,000 Da. They accumulate in late larval or pupal hemolymph as well as in fat body extracts (Locke and Collins, 1968; Tojo *et al.*, 1978, 1980). One class of these proteins has an unusually high content of aromatic amino acids (Munn and Greville, 1969; Kunkel and Pan, 1976; Wyatt and Pan, 1978; Duhamel and Kunkel, 1983; Riddiford and Law, 1983; Telfer *et al.*, 1983; Levenbook, 1985). The generic name arylphorin (Ap) has been proposed for such proteins (Telfer *et al.*, 1983). The protein calliphorin, the principal serum storage protein of the blowfly, *Calliphora erythrocephala*, serves as the prototype for this class (Munn and Greville, 1969). An additional class of storage protein has been identified in lepidopterans. This class has an average content of aryl amino acids, is more prevalent in females and in one case has been called larval serum protein (LSP) (Ryan *et al.*, 1985). Dipterans (Munn *et al.*, 1971; Kefaliakou-Bourdoulou *et al.*, 1981) and dictyopterans (Kunkel and Pan, 1976; Duhamel and Kunkel, 1983) have two immunologically distinct types of

high aryl serum storage protein. It is clear that other serum storage proteins exist whose classes have yet to be well defined (Telfer and Massey, 1987).

Many lepidopteran Aps have been described (Tojo *et al.*, 1980; Kramer *et al.*, 1980; Telfer *et al.*, 1983; Haunerland and Bowers, 1986; Ryan *et al.*, 1986; Palli and Locke, 1987c) and although Ap tends to be the most abundant protein in larval hemolymph, the scope of its functions is not yet known. It has been demonstrated that, like most other serum proteins, Aps are synthesized by the larval fat body and released in to the hemolymph (Levenbook, 1985; Venkatesh and Chippendale, 1986). However, other tissues have also been implicated in Ap synthesis (Palli and Locke, 1987a,b). There is evidence that Aps are degraded in the developing adult either in the fat body or their tissue of use to supply necessary amino acids for remodeling of tissues and sclerotization of cuticle (Scheller *et al.*, 1980; Riddiford and Law, 1983; Levenbook and Bauer, 1984; Levenbook, 1985; Konig and Scheller, 1986). It also has been reported that the Ap of *Heliothis zea* binds certain insecticides suggesting a carrier and transport function (Haunerland and Bowers, 1986). It is clear that more data are needed to link specific serum storage proteins to physiologial functions.

We report herein the purification and partial characterization of Ap from the gypsy moth, *Lymantria dispar*. We also present detailed profiles of the Ap concentration and total Ap mass relative to animal

---

*To whom all correspondence should be addressed.

growth and sex through larval development. These profiles further challenge the conventional perception of Ap as functioning exclusively as a metamorphic reserve and suggest independent roles in larval molting cycle and the female reproductive cycle.

METHODS AND MATERIALS

Insect cultures

The New Jersey strain of gypsy moth was obtained as egg masses from the USDA Animal and Plant Health Inspection Service Methods Development Laboratory, Otis, Mass. Larvae were reared from hatching at 24°C, 16-h photophase on the artificial diet of Bell et al. (1981). For developmental studies, individual larvae were reared in 60 × 15 mm Petri dishes until fifth instar and thereafter in 100 × 15 mm Petri dishes. Animals were fed ad libitum with food replaced at weekly intervals. Larvae were checked daily for staging. The first day of head capsule forward was designated as apolysis. Prepupal stage was subdivided into: (1) early—after wandering and when silking was first noted, (2) mid—when the sparse silk cocoon was constructed and larvae began to contract and (3) late—when prepupae were fully contracted. The sex of larvae was determined by dissection.

Collection of hemolymph

For purification of arylphorin, early sixth instar non-vitellogenic female larvae were anesthetized for 10–15 s with carbon dioxide. Hemolymph was collected by a Pasteur pipette from an incision made in a proleg. A few crystals of phenylthiourea (PTU) were immediately added to the pooled hemolymph to prevent melanization. Following centrifugation to remove hemocytes (10 min, 8730 g, 4°C, Beckman Microfuge B), the hemolymph was dialyzed for 24 h at 4°C against PBS (0.15 M NaCl, 0.10 M Na₂HPO₄, pH 7.2).

For Ap titer measurement, hemolymph was sampled from individual, staged larvae with microcapillary tubes (Drummond, Micro Cap), diluted 1:40 (v/v) in PBS and stored at 4°C with a crystal of PTU. Individual samples were stored for no longer than 24 h before quantitative immuno-electrophoresis was performed.

Glycerol gradient centrifugation

Twenty five μl aliquots of 20% hemolymph in PBS were layered onto the tops of linear 5–25% glycerol gradients prepared in PBS. Gradients were spun for 19 h at 34,000 rpm (196,000 g) in a Beckman L-62 Ultracentrifuge using a SW 41 Ti rotor. Gradients were eluted from top to bottom with 50% glycerol and 0.002 M potassium hydrogen pthalate to signal the bottom of the gradient. The eluent was monitored at 280 nm. Fractions were collected using a Buchler Fractomette Alpha 20. Serum Protein I of the German cockroach, Blattella germanica, was used as a 16S sedimentation standard (Kunkel and Pan, 1976).

Polyacrylamide gel electrophoresis (PAGE)

Samples denatured by heating (95°C, 5 min) in 2% sodium dodecyl sulfate (SDS) reducing buffer (Laemmli, 1970) were subjected to SDS–PAGE in 7.5% acrylamide slabs with an acrylamide/bis-acrylamide ratio of 37.5 and a stacking gel of 4% in the discontinuous buffer system of Laemmli (1970). Native gradient pore PAGE (N–PAGE) was performed in 3.5–15% acrylamide gradient slabs at 4°C for 6000 Vh using the buffer system of Ornstein and Davis (1964).

Densitometry

To determine the stoichiometric relationship between the two Ap subunits, serial dilutions of purified Ap made in PBS, pH 7.2, were subjected to SDS–PAGE as described above. Following electrophoresis, the gel was stained for

12 h with 0.1% Coomassie Blue R-250/40% methanol/10% acetic acid and destained with 40% methanol/10% acetic acid. The density of the stained bands was measured using the Megavision system running on a Micro Vax computer. The scan data were ported to an AT style microcomputer and plotted using Microsoft Works.

Molecular weight estimation

Native molecular weights were estimated by N–PAGE using the Pharmacia high-molecular weight electrophoresis calibration kit and a plot of molecular weight vs relative migration. Subunit molecular weight was determined using SDS–PAGE and a plot of log molecular weight vs relative migration of known standards (BioRad SDS–PAGE high molecular weight standards).

Amino acid analysis

Eight hundred μg of Ap was lyophilized and hydrolyzed in 6N HCl at 120°C in vacuo for 24 h. Analysis was performed as previously described (Duhamel and Kunkel, 1983). Tryptophan content was determined spectrophotometrically according to Spande and Witkop (1967).

Production of antiserum

An antiserum against purified Ap was obtained by immunizing a male white New Zealand rabbit using a standard protocol (Kunkel, 1988). The initial immunization used 250 μg of purified Ap emulsified with Freund's complete adjuvant. The rabbit was boosted at 1 month with 250 μg of protein in Freund's incomplete adjuvant. One month later a series of five daily intravenous injections of 50 μg Ap in PBS was administered. Blood was collected 1 week after the last injection, immunoglobulins were precipitated with ammonium sulfate and the specificity of the resulting antiserum was characterized using immunoelectrophoresis (Kunkel, 1988), quantitative immunoelectrophoresis (Laurell, 1966) and Western blotting (Burnette, 1981).

Immunoblotting

Immunoblotting experiments were performed using both the antiserum developed in this laboratory againt L. dispar Ap and antisera against Manduca sexta Ap (Kramer et al., 1980) and M. sexta larval serum protein (LSP), a predominantly female-specific protein (Ryan et al., 1985). Purified proteins or whole hemolymph samples were subjected to SDS–PAGE and transferred electrophoretically to nitrocellulose according to Burnette (1981). Unbound sites on the nitrocellulose were blocked with gelatin. The nitrocellulose membrane strips were then incubated with specific antiserum, washed to remove unbound antibody, incubated with goat anti-rabbit horseradish peroxidase (BioRad) and washed again. The membranes were developed with HRP Color Development Reagent containing 4-chloro-1-naphthol (BioRad).

Affinity chromatography

To further test the homogeneity of the 16S fraction obtained by glycerol gradient centrifugation, lectin affinity chromatography was carried out according to Ryan et al. (1986). Hemolymph from wandering females was pooled and centrifuged to remove hemocytes as described above. Three ml of the supernatnat was brought to a total volume of 20 ml with PBS and made 44% (w/w) KBr. Six ml of this solution was transferred to each of three 12 ml Beckman Polyallomer centrifuge tubes and overlayed with 5.5 ml 0.9% NaCl. Following centrifugation at 34,000 rpm (196,000 g) for 32 h. Fifty minutes at 4°C in a SW 41 Ti rotor, lipophorin was separated from the other serum proteins by flotation at the interface. The remaining serum proteins were collected and dialyzed extensively against PBS. Twenty five μl aliquots of the dialyzed serum proteins were layered onto the tops of linear glycerol gradients and treated as described above. Fractions corresponding

to 16S were pooled and concentrated using Centricon microconcentrators (Amicon).

The 16S fraction was chromatographed on a 1.0×8.0 cm concanavalin A-Sepharose 4B (Sigma) column equilibrated with 10 mM Tris–HCl, pH 7.4, 0.5 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.02% NaN_3. Subsequent to washing the column with 3 column volumes of starting buffer while continuously monitoring the eluent at 280 nm, adsorbed material was eluted with starting buffer containing 0.5 M α-methyl-D-mannoside. Although no proteins eluted with starting buffer, a major protein fraction was eluted from the column with starting buffer plus α-methyl-D-mannoside.

Quantitation of arylphorin

Ap was measured using quantitative immunoelectrophoresis (QIEP) (Laurell, 1966; Kunkel, 1988). Purified Ap was used for calibration of Ap weight vs QIEP rocket height. A solution of purified Ap was divided into two aliquots. One aliquot was delipidated, dried down to constant weight and weighed in a Mettler microbalance. The second aliquot was analyzed for u.v. absorbance, protein content (Bradford, 1976) using bovine serum albumin (BSA) as standard and also used to generate a QIEP standard curve for Ap.

Hemolymph volumes

Hemolymph volume was estimated by protein dilution. Animals were injected with 1 μl of *Periplaneta americana* adult hemolymph containing Serum Protein I. Following an incubation period of 15 min, 1–5 μl of hemolymph were collected from a proleg and diluted 1:10 in Tris–citrate buffer, pH 8.6. The dilution of the known volume of hemolymph injected was determined using QIEP and anti-*P. americana* Serum Protein I (Duhamel and Kunkel, 1987).

Weight and size measurements

Size and weight of animals were used as covariates in studying Ap titers and hemolymph volumes. Weight for stages I and II animals sampled was measured in a Cahn 21 Automatic Electrobalance sensitive to 1 μg which is <0.2% of the weight of the smallest animals measured. Animals older than stage II were measured in a Mettler Balance sensitive to 0.1 mg which is 1.0% of the weight of the smallest animals measured. Head capsule width was measured with a filar ocular micrometer (American Optical) mounted on a fixed objective dissecting microscope (American Optical) and calibrated with a stage micrometer rule.

Statistical analysis

The data were analyzed using the general linear model and factorial design (Rao, 1968). Initially, fully parametized models, including interaction between the main effects, were fit to the data. Subsequently the significance of the interaction and main effects were tested for exclusion from a final model. An attempt was made to maintain, where possible, an equal sample size of five animals at each age and sex combination.

RESULTS

Isolation and identification

Arylphorin was isolated from sixth-stage, day 3 female larval hemolymph according to its sedimentation rate in 5–25% linear glycerol gradients. The glycerol gradient optical density profile, Fig. 1, reveals a simple pattern of two protein peaks, a minor 5S lipophorin peak and a major peak with a sedimentation coefficient of 16S. The 16S peak was pooled and its purity evaluated by PAGE and SDS–PAGE.
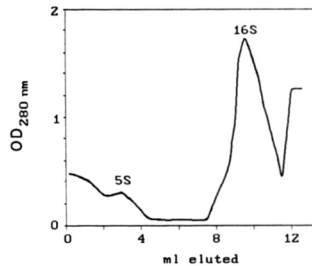

Fig. 1. Representative elution profile of density gradient ultracentrifugation. 25 μl of hemolymph diluted 1:5 in PBS were layered onto the tops of 11.5 ml linear 5–25% glycerol gradients. Gradients were spun for 19 h at 34,000 rpm (196,000 *g*) in a Beckman L-62 ultracentrifuge using an SW 41 Ti rotor. Gradients were eluted from top to bottom with 50% glycerol and monitored at A280. A minor lipophorin peak occurs at the top of the gradient with a sedimentation coefficient of 5S whereas the major arylphorin peak has a sedimentation coefficient of 16S.

The extinction coefficient of a purified sample of Ap was determined by first measuring the $O.D._{280}$ of an aliquot and then delipidating, ethanol:ether extracting and weighing the protein. The $E_{280}^{1\%}$ was calculated to be 10.9 O.D.

Native gradient pore PAGE demonstrated that the 16S protein peak contained one major protein band of molecular weight ~440,000. It also showed that this protein is the major band in the hemolymph of the last larval instar (Fig. 2). SDS–PAGE revealed that this protein consisted of subunits which appear as a doublet of bands with apparent molecular weights of 73,000 and 80,000 [Fig. 3(A)]. Densitometry of serial dilutions of Ap denatured on SDS gels demonstrated that the subunits occur in a ratio of 4:1 (73,000:80,000 Da subunit) (Fig. 4).

A determination of the amino acid content was performed on two independently purified Ap samples each hydrolyzed for 24 h. The *L. dispar* Ap composition correlated well with those of several other lepidopteran Aps including the unusually high content—15.6 mol%—of aromatic amino acids, tyrosine and phenylalanine (Table 1).

SDS–PAGE of the protein eluted from the concanavalin A Sepharose column when α-methyl-D-mannoside was added to the starting buffer demonstrated that this protein corresponded to arylphorin. Binding of arylphorin to concanavalin A Sepharose indicates the presence of covalently linked oligosaccharide residues, most likely of the high mannose type.

Immunological identification of L. dispar Ap

Immunoblotting experiments were performed on the Ap protein and female larval hemolymph of two stages using antisera against *Manduca sexta* Ap and LSP. The immunoblot demonstrated a strong homology between the Aps of the two species [Fig. 3(B)]. The *M. sexta* antiserum reacts with both subunits of *L. dispar* Ap although it is not visible in this photograph. No cross-reacting LSP was found in gypsy moth samples although a strong cross-reacting
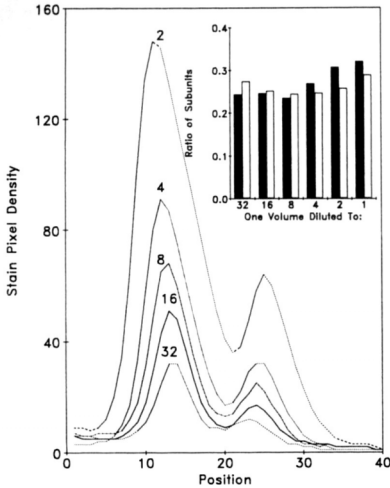
Fig. 4. Stoichiometric relationship of arylphorin subunits. Densitometry was performed on a 7% SDS–PAGE stained with Coomassie Blue R-250 in which serial dilutions of arylphorin had been run. Density is plotted vs position as measured from the beginning of a band (the bottom of the gel serving as origin). The tall peak corresponds to the 73,000 Da subunit while the short peak corresponds to the 80,000 Da subunit. Two independent dilution series were scanned and the inset graph shows that the ratios of the peak areas for the two series (solid vs open bars) maintain a subunit ratio of 1:4 over a broad range of sample dilution.

LSP was found in hemolymph of several other lepidopteran species (not shown). According to the general chemical characteristics of the gypsy moth Ap and the results of the immunoblotting experiments, the protein was identified as bona fide Ap.

The antiserum raised against *L. dispar* Ap contained strong antibodies for only one native serum protein of whole hemolymph of the last larval instar.

Table 1. Amino acid analysis of *L. dispar* Ap compared to the Observed Average Protein (OAA) composition (King and Jukes, 1969)

Amino acid	mol%*	OAA mol%
Asx	10.46	10.8
Thr	4.53	6.5
Ser	6.44	8.5
Glx	9.99	10.0
Pro	5.11	5.2
Gly	9.42	7.8
Ala	5.72	7.8
Val	6.42	7.1
Met	1.42	1.9
Ile	4.31	4.0
Leu	7.31	8.0
Tyr	8.26	3.5
Phe	7.37	4.2
His	2.66	3.0
Lys	7.19	7.5
Arg	2.90	4.4
Try†	0.49	1.2
	100.00	100.0

*Mean of two 24 h hydrolysates.
†Spectrophotometrically determined.

The specificity of the resulting antiserum was confirmed by immunoelectrophoresis (Fig. 5). Immunoblots (Fig. 6) of fresh preparations of Ap as well as whole hemolymph from wandering and pupal females demonstrated that the antiserum recognized the major subunit of Ap.

Prediction of hemolymph volume

Hemolymph volume was needed to turn concentrations into absolute amounts of Ap per animal. In a series of fourth through sixth instar larvae, hemolymph volume was 28.0% ($\pm 1.2\%$ SE; $r^2 = 0.986$; $n = 19$) of total body weight. This result allows us to use body weight to estimate hemolymph volume. This method applies for the majority of the animal's larval life; however during the gut-empty stage at the end of the ultimate larval instar there is a decrease in weight which most probably corresponds to a decrease in hemolymph volume (Jones, 1977).

Development profiles for Ap

The results of QIEP show a general increase in Ap concentration in the hemolymph throughout larval development. For the third through ultimate larval instars, the protein concentration increases markedly during the intermolt period, reaches a peak at apolysis and rapidly declines before ecdysis [Fig. 7(A)].
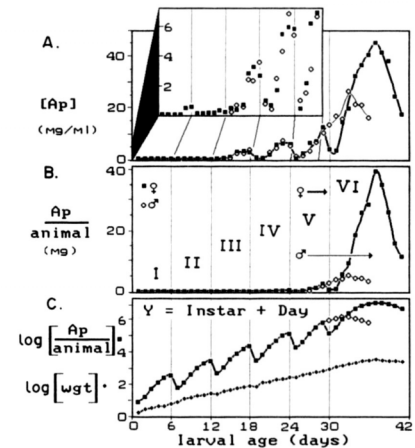

Fig. 7. Daily profile of arylphorin in serum of *L. dispar* throughout larval development. The means for five animals are plotted. Roman numerals in panel B identify larval instars. (A) Average Ap concentration plotted against age. An insert expands the low concentration titers of instar I through day 4 of instar V. (B) Serum Ap/animal plotted against age. Total Ap in each animal's serum was calculated by multiplying its Ap titer by its estimated blood volume. Averages for male and female larvae coincide until the mid-fifth instar of the male, when the male values (thin line connecting ○s) become distinct. (C) Both log (Ap/animal) and log (weight) are predicted by a linear combination or just two fixed effects, an instar effect and an age effect within the instar (Y = instar + age within instar). The points plotted represent the expected values of Ap/animal and weight. No significant sex effect or interactions between sex, instar or age were detected ($P > 10\%$).
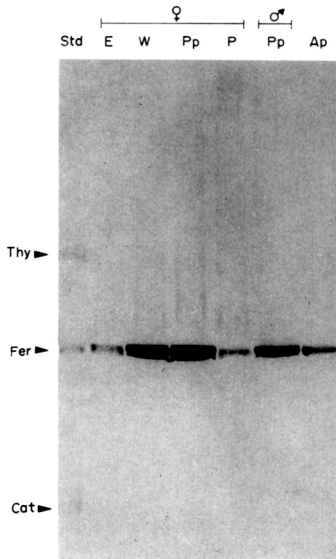
Fig. 2. Molecular weight estimation of arylphorin by native gradient pore polyacrylamide gel electrophoresis (PAGE). Native gradient pore PAGE was performed in 3.5–15% acrylamide slabs at 4 C for 6000 Vh using the buffer system of Laemmli (1970). Lane 1: molecular weight standards including thyroglobulin (Thy), ferritin (Fer), and catalase (Cat); Lane 2: 640 nl hemolymph from early sixth instar (E) *L. dispar* females; Lane 3: 640 nl hemolymph from wandering (W) *L. dispar* females; Lane 4: 640 nl hemolymph from early prepupal (Pp) *L. dispar* females; Lane 5: 640 nl hemolymph from pupal (P) *L. dispar* females; Lane 6: 640 nl hemolymph from prepupal (Pp) *L. dispar* males; Lane 7: 12.5 μg *L. dispar* arylphorin (Ap). While the lanes containing whole hemolymph samples demonstrate more than one protein band, only one band can be seen in the lane containing Ap. Based upon relative migration to that of standard proteins, the molecular weight of arylphorin was estimated to be 440,000 Da.
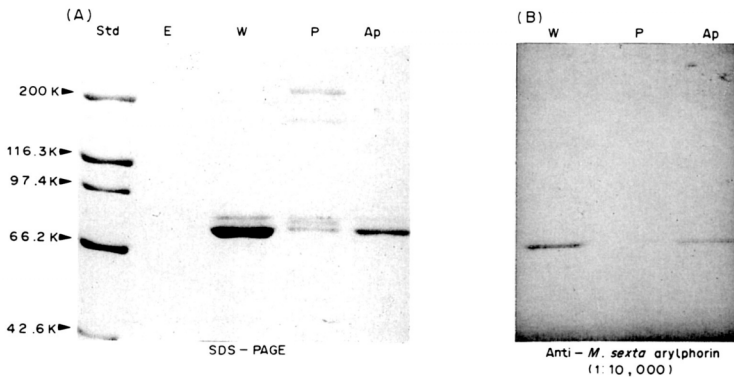

Fig. 3. (A) Molecular weight of polypeptide subunits of arylphorin by SDS–PAGE. SDS–PAGE was performed in 7.5% acrylamide mini slabs with a stacking gel of 3.5% in the discontinuous buffer system of Laemmli (1970). Lane 1: molecular weight standards; Lane 2: 63 nl *L. dispar* female early sixth instar hemolymph (E); Lane 3: 63 nl *L. dispar* female wandering hemolymph (W); Lane 4: 63 nl *L. dispar* female pupal hemolymph (P); Lane 5: 3.5 μg *L. dispar* arylphorin (Ap). While samples of whole hemolymph contain multiple protein bands, *L. dispar* Ap is composed of nonidentical subunits of M_r 73,000 and 80,000 Da based upon relative migration to that of standard proteins. (B) Immunoblot of *L. dispar* hemolymph samples and arylphorin probed with *Manduca sexta* anti-arylphorin. One half of the gel was transferred electrophoretically to nitrocellulose and the membrane was probed with an antiserum against *M. sexta* arylphorin. The *M. sexta* antiserum recognizes those polypeptides in *L. dispar* hemolymph corresponding to arylphorin demonstrating a strong homology between the proteins of the two species.
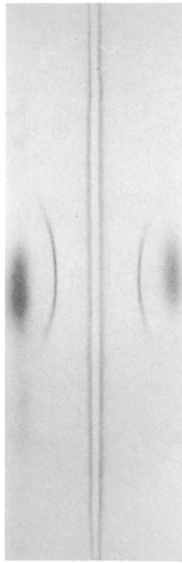
Fig. 5. Immunoelectrophoresis of *L. dispar* hemolymph proteins and purified arylphorin. Samples were electrophoresed from top to bottom in 1% agarose for 4 h at 5 mA and 4°C. A trough was cut between the two sample lanes and the antiserum against *L. dispar* arylphorin was added until the trough was filled. Diffusion of the samples was stopped in 48 h by rinsing in PBS. Lane 1: 3500 nl *L. dispar* hemolymph from wandering female larvae; Lane 2: 7.0 µg *L. dispar* arylphorin. Although there appear to be four major hemolymph proteins, a precipitin line was formed between the arylphorin antiserum and only one hemolymph protein which corresponds with the arylphorin sample.
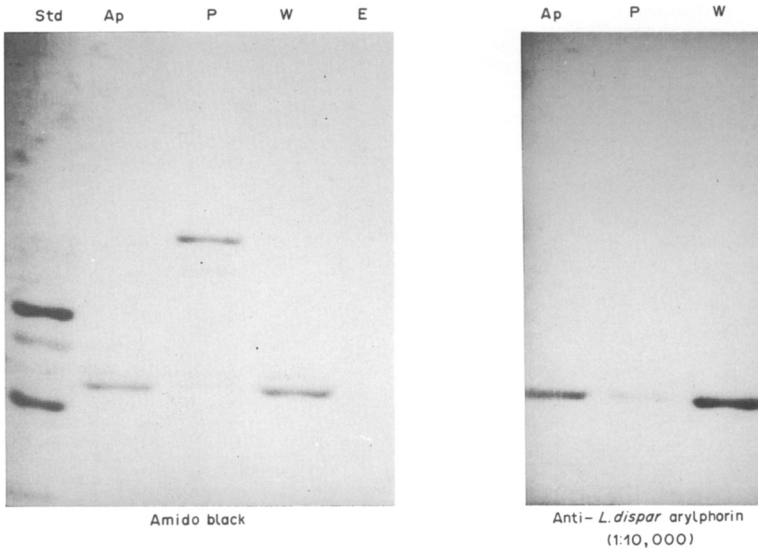

Amido black

Anti– *L.dispar* arylphorin
(1:10,000)

Fig. 6. Immunoblot of whole hemolymph samples of *L. dispar* and arylphorin probed with *L. dispar* anti-arylphorin. Samples were subjected to SDS–PAGE in 7.5% acrylamide mini slabs and transferred electrophoretically to nitrocellulose. One half of the membrane was stained with amido black while the other half was probed with antiserum developed against *L. dispar* arylphorin. Lane designations are as they appear in Fig. 3. The blot demonstrates that only the arylphorin subunits are recognized by the arylphorin antiserum.

Males achieve a maximum concentration of 26.2 mg/ml in 5 larval instars and females reach a maximum of 44.8 mg/ml in 6 larval instars.

Plotting the absolute amounts of serum Ap during development [Fig. 7(B)] emphasizes accumulation in the last larval instar for contribution to metamorphosis. Males accumulate 5.5 mg of Ap/animal and females a maximum of 39.3 mg. When expressed this way, the most relevant instar for Ap accumulation for metamorphosis is the last larval instar. It also should be noted that the decrease of serum Ap/animal just prior to pupation may actually be more drastic than calculated due to the reduction in hemolymph volume associated with pupation.

We distinguish the increase of Ap from the concommitant increase in overall weight that occurs during this exponential larval growth. The log transform of the Ap per animal and animal weight were computed in order to eliminate exponential effects and both sets of data were submitted to analysis of variance. That analysis revealed that both Ap per animal and weight could be predicted by knowledge of 2 factors, the instar and the age within the instar. Figure 7(C) illustrates the predicted values on a log scale which emphasizes the similarity of the rhythmic cycling that Ap undergoes in each molting cycle. No significant statistical ($P > 0.1$) or visual improvement in the fit of an equation to the actual data was obtained by including sex as a factor or interactions among any of the main factors (instar, age, sex). On the log scale the two quantities, Ap/animal and weight, are seen to diverge allometrically over the course of 6 instars. While weight increases 3.6-fold (= 0.56 log units) per instar the Ap titer increases at twice that rate (7.2-fold = 0.86 log units). This regular but different exponential increase throughout larval development results in dramatic differences when interpreted on the normal concentration scales of Fig. 7(A), (B).

The optimum time for serum sampling

Standard errors were not included in Fig. 7 in the interest of clarity, however, the variation of Ap within each sampling period was informative. Figure 8 illustrates the coefficient of variation in Ap titer plotted against the day within each instar for males and females. Even though no significant difference for the sexes was detected in the averages displayed in Fig. 7, the CVs were plotted separately to illustrate their reproducibility. CV is high at the beginning of each instar when Ap titer is low. CVs reach a low point by about day 6 of each instar and remain low for days 7–12 of the last larval instars. This is of substantial significance since the termination of feeding on day 8 corresponds to the peak of Ap titer as well as a particularly stable time to sample a population of animals.

DISCUSSION

A single *L. dispar* 16S serum storage protein was found and purified from the hemolymph of female larvae by density gradient centrifugation. It was identified according to its size, sedimentation coefficient, subunit structure, amino acid composition and immunological properties as an Ap. The size of
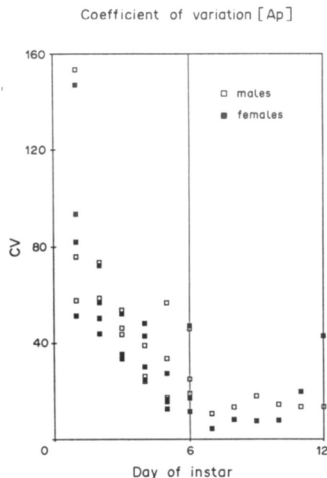

Fig. 8. The coefficient of variation for arylphorin titers. The coefficient of variation for arylphorin titers of Fig. 7(A) is plotted vs day within each instar in instars III to V and VI for male (□) and females (■) respectively. Only metamorphic animals have measurements from days 7 to 12.

L. dispar Ap, M_r 440,000, is consistent with the generality that serum proteins in insects have Stoke's radii > 50 Å. This allows storage accumulation in the hemolymph of such macromolecules without the routine filtered turnover seen for smaller molecules (Duhamel and Kunkel, 1987). *L. dispar* Ap is a heterohexamer composed of subunits with apparent molecular weights of 73,000 (major subunit) and 80,000 (minor subunit). In other lepidopteran species where Ap is composed of two types of subunits, the subunits occur in apparently equal amounts (Kramer *et al.*, 1980; Tojo *et al.*, 1980). However, some dipteran Aps have been shown to be composed of unequal subunit ratios (Marinotti and deBianchi, 1986; Levenbook and Bauer, 1980).

Insect serum often reaches high total protein concentrations (6–10%) (Wyatt and Pan, 1978). These peak levels are often transitory in the dynamics of insect development. We have illustrated the dynamics of Ap accumulation in the gypsy moth growing under ideal laboratory conditions of continuous food availability and regulated light, humidity and temperature. It is clear that the peak of Ap titer, corresponding to the final day of feeding, lasts for a very short time and is greatest in the female. We suggest that the peak of Ap titer corresponds to an integrated measure of the nutritional experience that the larva has undergone. The curves in Fig. 6 are presented as a baseline for our further studies of the nutritional reserves of the gypsy moth. Several aspects of gypsy moth biology lend themselves to this quantitative approach (Leonard, 1970; Leonard and Kunkel, 1989).

In addition to the species of Lepidoptera cited previously in which Aps have been identified, Aps

have been described in Diptera (Munn et al., 1971; Thompson et al., 1976; Kefalialou-Bourdopoulou et al., 1981; Mintzas and Reboutsicas, 1984; Marinotti and deBianchi, 1986), Hymenoptera (Ryan et al., 1984), Orthoptera (Phillips and Loughton, unpublished; deKort and Koopmanschap, 1987), and Dictyoptera (Kunkel and Lawler, 1974; Kunkel and Pan, 1976; Duhamel and Kunkel, 1983).

A second class of hexameric storage protein has been designated as Serum Protein 1 in Bombyx mori (Tojo et al., 1980), Proteins 1 and 2 in Hyalophora cecropia (Tojo et al., 1978) and LSP in Manduca sexta (Ryan et al., 1985). This hexameric class is average in phenolic residues and high in methionine. In two instances in which it was titered in males and females it was found to be of higher concentration in females, was only present in hemolymph late in the ultimate larval instar and was preferentially stored in the fat body during metamorphosis. This suggests a possible sexual dimorphic role in supplying the greater nutritional needs of the female during its metamorphosis and reproductive cycle (Ogawa and Tojo, 1981; Ryan et al., 1985).

However, no major serum storage protein with the properties of LSP was detected in L. dispar with the searching techniques applied: glycerol gradient centrifugation, native gel electrophoresis, affinity chromatography on concanavalin A-Sepharose and direct and indirect immunology. The absence of this protein in a species which does not feed in the adult stage adds weight to other forms of nutrient storage. This may be a major reason why Ap is found in 8-fold greater quantity in female gypsy moth last instar larvae than in males: Ap may provide one extra protein reserve necessary for the female gypsy moth to produce a substantial egg mass with its protective coverings including setae and chorion.

Arylphorin peak concentration increases from stage to stage throughout larval development. Within each stage, however, the Ap concentration follows a pattern, increasing markedly to a peak at apolysis and decreasing rapidly to baseline close to ecdysis. Some regulation of this accumulation may exist since in each larval instar Ap titer starts out with a high CV but declines to low CV by the last day of the instar. This cycling of arylphorin in correlation with molting is of considerable interest in itself. Telfer et al. (1983) described a similar occurrence of cyclic change in arylphorin concentration in H. cecropia. In H. cecropia, arylphorin is present in the hemolymph during the feeding stage of the last three larval instars, yet falls to undetectable levels during each larval–larval molt. Further fluctuations in storage protein concentrations have been shown in the Hemimetabola. In the cockroach, Blattella germanica, concentrations of larval-specific protein were found to rise to similar levels during each intermolt, peaking at apolysis prior to a decline through ecdysis (Kunkel and Lawler, 1974). A greater accumulation was noted whenever the stadium was lengthened by metamorphosis or forced regeneration (Kunkel, 1975). Duhamel and Kunkel (1978, 1987) demonstrated in a second cockroach species, Blatta orientalis, that all four major larval serum proteins (two arylphorins, a lipophorin and an unidentified fourth

serum protein) exhibit a similar pattern of molting-cycle related changes. The larval-specific protein, an Ap, however, shows the greatest fluctuation. It was suggested that this protein provides the reserves for cuticle production and tissue growth during the molting phase when the cockroach stops eating.

As demonstrated by Scheller and co-workers (1980) in Calliphora vicina, the Ap, calliphorin, is utilized during metamorphosis in the formation of puparial cuticle. In L. dispar, Ap is most likely involved in the production of both larval and pupal cuticles since the clearance of Ap from the hemolymph in each molting cycle corresponds to that time when feeding has ceased yet pre-ecdysial cuticle is still being synthesized.

The only other lepidopterans for which partial larval development profiles of arylphorin concentration are available are M. sexta and H. cecropia. In M. sexta arylphorin concentrations of approx. 37 mg/ml are reached in the ultimate instar (Kramer et al., 1980). During the last larval instar of H. cecropia males, arylphorin concentrations of 30–40 mg/ml had been attained by pupal ecdysis (Telfer et al., 1983). In L. dispar males, arylphorin concentrations reach 26.2 mg/ml in the final instar while females achieve arylphorin concentrations of 44.8 mg/ml in their ultimate instar. L. dispar females achieve concentrations 1.7-fold greater than L. dispar males and 12% greater than both M. sexta and H. cecropia at analogous times of development. It is worth noting, however, that the concentration of the L. dispar Ap we report is based on the determination of an absolute weight of the purified protein standard and not by the often used Bradford (1976) method which shows a wide variability in its sensitivity to various proteins (Pierce and Suelter, 1977). In fact, it is to be expected that our methods would be the more accurate but be systematically greater than the values obtained using the colorimetric methods of other workers in the field. Using the Bradford method and BSA as a standard the peak of Ap titer in L. dispar females would have been reported as 32.4 mg/ml, 27% lower than we report. This suggests that the M. sexta and H. cecropia peak titers should be inflated by about 30%. We argue for more careful assays for insect proteins when purified standard is available. This is particularly important for the insect storage proteins since theories about their storage function may depend on their absolute and not merely relative amounts.

In another novel approach we used an injected foreign protein, P. americana Serum Protein 1, to estimate hemolymph volume instead of inulin. Since Serum Protein 1 is a macromolecule similar in size to Ap, only the passive space penetrated by native Ap was measured.

Sexual dimorphism in the amount of serum Ap accumulated is achieved in an interesting and perhaps unique way by the gypsy moth. Sexual dimorphism in the number of larval instars results in an apparent sexual dimorphism in the Ap levels reached in the respective ultimate larval instars [Fig. 7(A) and (B)]. The male pupates after the fifth larval instar while the female pupates after the sixth. The ultimate larval instar in each sex, during which serum Ap reaches maximum concentrations, is extended to include a

longer feeding period, a wandering phase and a prepupal period.

Plotting the log Ap per animal vs age [Fig. 6(C)] provides insights into the apparent sexual dimorphism in Ap accumulation. There is an obvious rhythmic component to the Ap accumulation curve. The rhythmic component was found to be independent of a linear additive component correlated with increasing instar number. Subtracting the rhythmic component from the Ap accumulation curve leaves a monotonic exponential increase with an instar specific component which is twice the rate of weight gain per instar of the gypsy moth larva. There is an approximate 2-fold allometric departure of Ap and weight per instar. Since male and female are essentially identical in Ap accumulation and weight gain during the first five instars, the greater accumulation of Ap in females can be most simply interpreted as the purely additive effects of an extra 3.6-fold weight gain of the female's sixth instar plus the 2-fold allometric increase in Ap synthesis. This relationship indicates that the higher titer and absolute amount of Ap in prepupal females is a result of sexual dimorphism in the number of larval instars (V in males, but VI in females) rather than a sex specific dimorphism in amplification of Ap synthesized. This demonstrates the functional importance of the additional instar in the female.

Since the additional larval instar leads to a higher Ap titer in the female, a supplemental storage protein such as a female specific-type may be unnecessary in female *L. dispar*. It is possible that in this species the drive for greater storage potential (for egg production) in the female has lead to a selection for females with an additional instar, such that the normal allometric increase in Ap is maintained, resulting in the amplification of total Ap. It would be interesting to examine whether the tendency to use Ap as a storage reserve for reproductive females is seen in other insects which have non-feeding adults. In two such species, *H. cecropia* and *B. mori*, a second protein, which is predominantly female specific, appears to have served as an alternate solution to similar reproductive pressures.

In Diptera the appearance of Ap is treated as a quantal phenomenon associated with the third larval instar (Levenbook and Bauer, 1980). We have demonstrated that there is no metamorphosis related change in this gypsy moth storage protein profile. Its course in the last larval instars is a simple extrapolation of a modest 2-fold allometric departure first observed in the early instars. This gradual change in the Ap gene expression level contrasts sharply with traditional views of the dramatic metamorphosis of holometabolic species.

REFERENCES

Bell R. A., Owens C. D., Shapira M. and Tardiff J. R. (1981) Development of mass-rearing technology. In *The Gypsy Moth: Research Toward Integrated Pest Management* (Edited by Doane C. C. and McManus M. L.). *USDA For. Serv. Tech. Bull.* **1584**, 608.

Bradford M. M. (1976) A rapid and sensitive method for the quantitization of microgram quantities of protein utilizing the principle of protein-dye binding. *Analyt. Biochem.* **7**, 248–254.

Burnette W. N. (1981) "Western blotting": electrophoretic transfer of proteins from SDS–PAGE to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Analyt. Biochem.* **112**, 195–203.

Duhamel R. C. and Kunkel J. G. (1978) A molting rhythm for serum proteins of the cockroach, *Blatta orientalis*. *Comp. Biochem. Physiol.* **60B**, 33–337.

Duhamel R. C. and Kunkel J. G. (1983) Cockroach larval-specific protein: a tyrosine-rich serum protein. *J. biol. Chem.* **258**, 14461–14465.

Duhamel R. C. and Kunkel J. G. (1987) Moulting-cycle regulation of haemolymph protein clearance in cockroaches: possible size-dependent mechanism. *J. Insect Physiol.* **33**, 155–158.

Haunerland N. H. and Bowers W. S. (1986) Binding of insecticide to lipophorin and arylphorin, two proteins of *Heliothis zea*. *Archs Insect Biochem. Physiol.* **3**, 87–96.

Jones J. C. (1977) *The Circulatory System of Insects*. Thomas, Springfield, Ill.

Kefaliakou-Bourdopoulou M., Christodoulou C. and Marmaras V. J. (1981) Storage proteins in the olive fruit fly, *Dacus oleae*: characterization and immunological studies. *Insect Biochem.* **11**, 707–711.

King T. H. and Jukes J. L. (1969) Non-darwinian evolution. *Science* **164**, 788–798.

Konig M. and Scheller K. (1986) Influence of 20-OH-ecdysone on the incorporation of arylphorin in larval trachea of *Calliphora vicina*. *Zool. Jahrb.* **90**, 65–76.

deKort C. A. D. and Koopmanschap A. B. (1987) Isolation and characterization of a larval hemolymph protein in *Locusta migratoria*. *Archs Insect Biochem. Physiol.* **6**, 191–203.

Kramer S. J., Mundall E. C. and Law J. H. (1980) Purification and properties of manducin, an amino acid storage protein of the hemolymph of larval and pupal *Manduca sexta*. *Insect Biochem.* **10**, 279–288c.

Kunkel J. G. (1975) Larval-specific serum protein in the order Dictyoptera—II. Antagonistic effects of ecdysone and regeneration on LSP concentration in the hemolymph of the Oriental cockroach, *Blatta orientalis*. *Comp. Biochem. Physiol.* **51B**, 177–188.

Kunkel J. G. (1988) Analytical immunological techniques. In *Immunological Techniques: Arthropods* (Edited by Gilbert L. I. and Miller T. A.), pp. 1–41. Springer, Berlin.

Kunkel J. G. and Lawler D. M. (1974) Larval-specific serum protein in the order Dictyoptera-immunologic characterization in larval *Blattella germanica* and cross-reaction throughout the order. *Comp. Biochem. Physiol.* **47**, 697–710.

Kunkel J. G. and Pan M. L. (1976) Selectivity of yolk protein uptake: comparison of vitellogenins of two insects. *J. Insect Physiol.* **22**, 809–818.

Laemmli U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Laurell C.-B. (1966) Quantitative estimation of protein by electrophoresis in agarose gels containing antibodies. *Analyt. Biochem.* **15**, 45–52.

Leonard D. E. (1970) Effect of starvation on behaviour, number of larval instars, and developmental rate of *Porthetria dispar*. *J. Insect Physiol.* **16**, 25–31.

Leonard D. E. and Kunkel J. G. (1989) Nutritional ecology: Lymantria dispar as a model system for study of serum storage proteins. In *Proc. Symp. Comp. New and Old World Tussock Moths* (New Haven, 1988) (Edited by Wallner W. E.). USDA Forest Service Experiment Station Tech. Rpt NE-123, pp. 367–380.

Levenbook L. (1985) Insect storage proteins. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology* (Edited by Kerkut G. A. and Gilbert G. I.), Vol. 10, chapter 9, pp. 307–346.

Levenbook L. and Bauer A. C. (1980) Calliphorin and soluble protein of hemolymph and tissues during larval growth and adult development of *Calliphora vicina*. *Insect Biochem.* **10**, 693–701.

Levenbook L. and Bauer A. C. (1984) The fate of the larval storage protein calliphorin during adult development of *Calliphora vicina*. *Insect Biochem.* **14**, 77–86.

Locke M. and Collins J. (1968) Protein uptake into multivesicular bodies and storage granules in the fat body of an insect. *J. cell. Biol.* **36**, 453–483.

Marinotti O. and de Bianchi A. G. (1986) Structural properties of *Musca domestica* storage protein. *Insect Biochem.* **16**, 709–716.

Mintzas A. C. and Reboutsicas D. (1984) Isolation, characterization and immunological properties of ceratitin-4, a major hemolymph protein of mediterranean fruit fly *Ceratitis capitata*. *Insect Biochem.* **14**, 285–291.

Munn E. A. and Greville G. D. (1969) The soluble proteins of developing *Calliphora erythrocephala*, particularly calliphorin and similar proteins in other insects. *J. Insect Physiol.* **15**, 1935–1950.

Munn E. A., Feinstein A. and Greville G. D. (1971) The isolation and properties of the protein calliphorin. *Biochem. J.* **124**, 367–374.

Ogawa K. and Tojo S. (1981) Quantitative changes of storage protein and vitellogenin during pupal–adult development in the silkworm, *Bombyx mori* (Lepidoptera: Bombycidae). *Appl. Ent. Zool.* **16**, 288–296.

Ornstein L. and Davis B. J. (1964) Disc electrophoresis I and II. *Ann. N.Y. Acad. Sci.* **121**, 321–349, 404–427.

Palli S. R. and Locke M. (1987a) The synthesis of hemolymph proteins by the larval midgut of an insect *Calpodes ethlius* (Lepidoptera: Hesperiidae). *Insect Biochem.* **17**, 561–572.

Palli S. R. and Locke M. (1987b) The synthesis of hemolymph proteins by the larval epidermis of an insect *Calpodes ethlius* (Lepidoptera: Hesperiidae). *Insect Biochem.* **17**, 711–722.

Palli S. R. and Locke M. (1987c) Purification and characterization of three major hemolymph proteins of an insect, *Calpodes ethlius* (Lepidoptera: Hesperiidae). *Archs Insect Biochem. Physiol.* **5**, 233–244.

Pierce J. and Seulter C. (1977) An evaluation of the Coomassie Brilliant Blue G-250 dye binding method for quantitative protein determination. *Analyt. Biochem.* **81**, 478–480.

Rao C. R. (1968) *Linear Statistical Inference and Its Applications.* Wiley, New York.

Riddiford L. and Law J. H. (1983) Larval serum proteins of Lepidoptera. In *The Larval Serum Proteins of Insects* (Edited by Scheller K.), pp. 75–85.

Ryan R. O., Schimidt J. O. and Law J. H. (1984) Arylphorin from the hemolymph of the larval honeybee, *Apis mellifera*. *Insect Biochem.* **14**, 515–520.

Ryan R. O., Keim P. S., Wells M. A. and Law J. H. (1985) Purification and properties of a predominantly female-specific protein from the hemolymph of the larva of the tobacco hornworm, *Manduca sexta*. *J. biol. Chem.* **260**, 782–787.

Ryan R. O., Wang X., Willott E. and Law J. H. (1986) Major hemolymph proteins from larvae of the black swallowtail butterfly, *Papilio polyxenes*. *Archs Insect Biochem. Physiol.* **3**, 539–550.

Scheller K., Zimmerman H-P. and Sekeris C. E. (1980) Calliphorin, a protein involved in cuticle formation of the blowfly, *Calliphora vicina*. *Z. Natur.* **35C**, 387–389.

Spande T. F. and Witkop B. (1967) Determination of tryptophan content of proteins with *n*-bromosuccinimide. *Meth. Enzym.* **VI**, 498–506.

Telfer W. H. and Massey H. C. (1987) A storage hexamer from *Hyalophora* that binds riboflavin and resembles the apoprotein of hemocyanin. In *Molecular Entomology* (Edited by Law J. H.), pp. 305–314. Liss, New York.

Telfer W. H., Keim P. S. and Law J. H. (1983) Arylphorin, a new protein from *Hyalophora cecropia*: comparisons with calliphorin and manducin. *Insect Biochem.* **13**, 601–613.

Thompson J. A., Radok K. R., Shaw D. C., Whitten M. J., Foster G. G. and Birt M. L. (1976) Genetic of lucilin, a storage protein from the sheep blowfly, *Lucilia cuprina* Calliphoridae. *Biochem. Genetics* **14**, 145–160.

Tojo S., Nagata M. and Kobayashi M. (1980) Storage proteins in the silkworm, *Bombyx mori*. *Insect Biochem.* **12**, 289–303.

Tojo S., Betchaku T., Ziccardi V. J. and Wyatt G. R. (1978) Fat body protein granules and storage proteins in the silkmoth, *Hyalophora cecropia*. *J. cell. Biol.* **78**, 823–838.

Venenkatesh K. and Chippendale G. M. (1986) Synthesis and release of proteins from cultured larval fat body of the southwestern cornborer, *Diatrea grandiosella*. *Insect Biochem.* **16**, 917–927.

Wyatt G. R. and Pan M. L. (1978) Insect plasma proteins. *A. Rev. Biochem.* **47**, 779–817.