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Electrofocusing and electrophoresis of proteins present in the fat body of Diatraea grandiosella (Lepidoptera, Pyralidae)

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Analytical isoelectric focusing and electrophoresis with sodium dodecyl sulphate were used to examine the proteins present in the fat body of the southwestern corn borer, *Diatraea grandiosella*. In extracts from mature non-diapausing larvae about 60 protein bands were resolved with isoelectric focusing, and about 45 bands with dodecyl sulphate electrophoresis. The isoelectric points of most fat body proteins fell between pH 4.6 and 8.1. Molecular weights of major polypeptides ranged from about 30 000 to about 80 000 daltons. Several changes occurred in the protein patterns during metamorphosis. Selective release of some proteins was demonstrated *in vitro*. Larval diapause was associated with high titres of fat body and haemolymph proteins which appear to function in lipid transport.

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1. Introduction

The fat body of insects is a metabolic centre similar in many ways to the hepatopancreas of molluscs and crustaceans, and combining several of the functions of the liver and the adipose tissue of vertebrates (Kilby 1963). The fat body is engaged in the synthesis and storage of lipids, proteins and carbohydrates (Price 1973, Wyatt 1975, Downer 1978, Keeley 1978). It is also a source of insect plasma proteins (Wyatt & Pan 1978). Although many studies have concerned insect fat body proteins, more information is needed on the properties of individual insect proteins. The present study employed three methods, analytical isoelectric focusing, electrophoresis with sodium dodecyl sulphate, and non-dissociating disc electrophoresis, to examine the proteins present in the fat body of the southwestern corn borer, *Diatraea grandiosella* (Lep., Pyralidae). These techniques can be used to obtain information on fundamental properties of proteins, i.e. their isoelectric points and subunit molecular weights (Laemmli 1970, Righetti & Drysdale 1974, Anderson & Anderson 1978).

2. Materials and methods

A. Test insects and tissues

Larvae of *D. grandiosella* were reared at 30° with a daily light-dark rhythm of 16 h light, 8 h dark (LD 16:8) (non-diapausing) or at 23° with LD 12:12 (diapausing), on an artificial diet (Chippendale 1975). Non-diapausing larvae reached maturity around 14 days and attained 50 % pupation by 17 days, whereas pre-diapausing larvae reached maturity between 35 and 40 days and entered diapause.

Perivisceral fat bodies were dissected out, and carefully rinsed and homogenized in cold saline solution (50 mM KH_2PO_4, 150 mM NaCl, pH adjusted to 7.2 with KOH). The homogenate was centrifuged at 21 000 *g* for 30 min at 4°. Total protein was determined from the supernatants with bovine serum albumin or bovine gamma globulin as standard (Bradford 1976).

In incubation studies *in vitro*, perivisceral fat bodies were removed from surface-sterilized 40-day-old mature pre-diapausing larvae under cold macromolecule-free Grace's medium (Grand Island Biological Co.). The right sheet was homogenized in the medium (500 µl), and the left sheet was incubated in the medium (500 µl) for 1 to 20 h at 23° with occasional stirring. After incubation the left sheet was rinsed and homogenized in 500 µl of Grace's medium. The homogenates and the medium were centrifuged at 21 000 *g* for 30 min at 4° and the supernatants were used for disc electrophoresis.

Treatments with C_{17}-juvenile hormone were performed topically on 19-day-old non-diapausing larvae kept at 25°, and LD 16:8. Larvae were treated with 3 µg juvenile hormone in 3 µl acetone/day and kept during treatments at 25° and LD 12:12. Under these conditions control larvae pupated without the intervention of diapause, whereas hormone-treated larvae transformed into a pigment-free morph characteristic of larval diapause.

B. Isoelectric focusing and electrophoresis

Gels for isoelectric focusing (100 × 5.5 mm) were prepared to contain 7.2 % (w/v) acrylamide monomer, with 2.4 % of this as BIS acrylamide, 5 % (w/v) glycerol, and 2 % (w/v) ampholyte (Bio-Lyte 3/10, Bio Rad Laboratories). Riboflavin 5'-phosphate (8.4×10^{-6}M) was used for photopolymerization of the acrylamide.

Samples (30 to 200 µg protein) were applied as a 30 % solution of sucrose to the anodal top end of the gels, and were overlayered with 100 µl of 20 % sucrose and with 100 µl of 10 % sucrose. The tubes were then filled with 0.4 % H_2SO_4. The anode vessel contained precooled 0.4 % H_2SO_4 and the cathode vessel contained precooled 0.4 % 2-aminoethanol. A constant current of 0.5 mA/tube was applied for 8 to 9 h, in which time the proteins were focused. The pH gradient was determined by slicing the gels (Bio Rad model 190 slicer) into 5-mm sections which were then incubated in 1 ml sterile distilled water for 24 h at room temperature before the pH was measured. Parallel gels were stained for proteins in an aqueous solution of Coomassie blue (0.1 %) containing methanol, sulphosalicylic acid and trichloroacetic acid (Vesterberg 1971), and destained in ethanol: water: acetic acid (25:65:8; v/v/v).

Disc electrophoresis in 5 % and 7 % acrylamide in the separation gel and 2.5 % acrylamide in the stacking gel (non-dissociating conditions) was carried out at 4° in 125 × 5.5 mm I.D. tubes, with Tris-glycine (pH 8.3) as the bath buffer (Davis 1964, Smith 1976). Samples (50 to 200 µg protein) were applied in a 40 % solution of sucrose. Each run was terminated after about 4 h at 2 mA/tube. Bromophenol blue was used as the marker dye and Coomassie blue (0.25 % in water containing 12.5 % trichloroacetic acid) was used for staining.

Electrophoresis with sodium dodecyl sulphate using a discontinuous buffer system was carried out in 10 % acrylamide gels. The stacking gel (0.125 M Tris-HCl, pH 6.8) and the separation gel (0.375 M Tris-HCl, pH 8.8) were prepared according to Payne (1976). The electrode buffer contained 25 mM Tris and 192 mM glycine (pH 8.3). Samples (5 to 50 µg protein in 25 µl) were adjusted to contain 62.5 mM Tris-HCl (pH 6.8), 2 % sodium dodecyl sulphate (Bio Rad), 5 % 2-mercaptoethanol, and 10 % sucrose, and heated in a boiling water bath for 5 min. Bromophenol blue was used as the marker dye. Standards for molecular weight determination were lysozyme (14 300 daltons), soybean trypsin inhibitor (21 000), carbonic anhydrase (30 000), ovalbumin (43 000), bovine serum albumin (68 000) and phosphorylase B (94 000), obtained from Bio Rad Laboratories. A constant current of 2.5 mA/tube was applied for 3 to 4 h at room remperature. The gels were stained in water: methanol: trichloroacetic acid (5:5:1; v/v/w), containing 0.05 % Coomassie blue, and destained in water: methanol: trichloroacetic acid (38:2:3; v/v/w) (Payne 1976). Electropherograms were obtained by scanning (610 nm) with a Photovolt densitometer.

3. Results

This study examined the value of different electrophoretic techniques for resolving the proteins present in the fat body of *D. grandiosella* during metamorphosis. Comparison of isoelectric focusing and disc electrophoresis showed that, after separation of fat body proteins of mature non-diapausing larvae by isoelectric focusing in the pH gradient range 3 to 10, about 60 bands could be detected visually in the gels. On disc electrophoresis in 10 % acrylamide gels with sodium dodecyl sulphate the number of bands resolved was about 45, and on non-dissociating disc electrophoresis in 7 % acrylamide gels about 30. The results show that isoelectric focusing is well suited for separating insect proteins and complements other electrophoretic techniques in the study of protein metabolism.

Although electrophoretic mobility alone is not a sufficient criterion for distinguishing between proteins, the mobility values of the proteins are included in this study to facilitate discussion. It can be argued that proteins from different tissues are not necessarily identical, despite showing the same mobility.

Isoelectric focusing of fat body proteins

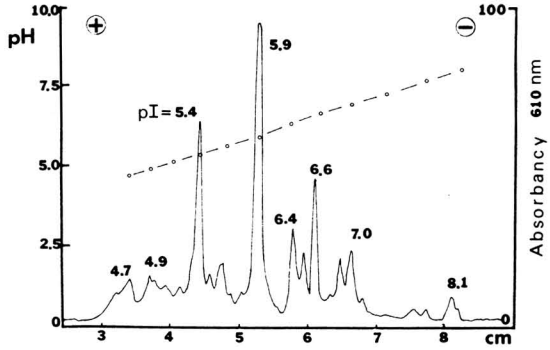

Fig. 1. Densitometric scan of a gel after isoelectric focusing of fat body proteins (115 µg) of a 40-day-old newly diapaused female larva in the pH gradient range 3 to 10. In this and subsequent scans (except in Fig. 5) proteins from a portion of a single fat body are shown. Coomassie blue staining. Isoelectric points (pI) shown for major proteins. Protein concentrations are presented in arbitrary absorbancy units.

showed that the isoelectric points (pI) of most proteins fell between 4.6 and 8.1 (Fig. 1). Corresponding values for most haemolymph proteins were found to be 4.3 and 6.6. The major protein band present in the eggs has a pI of about 5.8 (Fig. 2A). Several protein bands with isoelectric points from 4.7 to 5.4 can be distinguished in the fat body throughout metamorphosis (Fig. 2B-F). Of these, a protein with an isoelectric point at pH 5.4 was prominent in mature larvae and in pupae, whereas another protein with an isoelectric point at pH 4.5 was prominent in eggs. The data show that fat body proteins of 2-day-old post-emergence adult males differ distinctly from those of 2-day-old postemergence females (Fig. 2E, F).

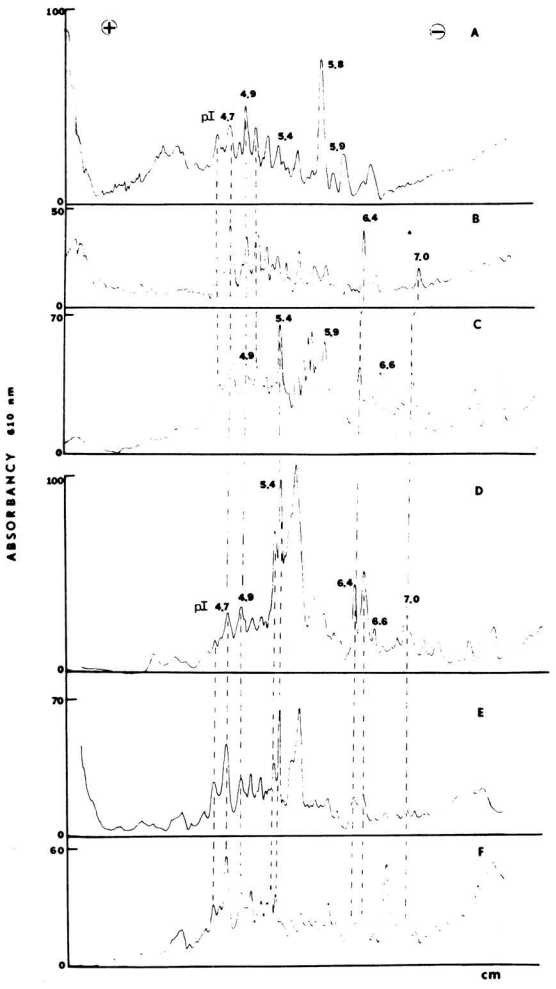

Fig. 2. Densitometric scans of gels after isoelectric focusing of egg and fat body proteins (50—100 μg) in the pH gradient range 3 to 10. A: eggs, B: 9-day-old 4th instar larvae, C: 18-day-old mature 6th instar female larvae, D: ca. 12-h-old male pupae, E: 1-day-old male adults. F:1-day old female adults.

Comparison of protein patterns of the different developmental stages using non-dissociating electrophoresis indicated that several changes take place during metamorphosis (Fig. 3). Four major protein bands were found to be prominent in the eggs, with relative mobilities, Rm, of 0.05, 0.08, 0.40 and 0.43 (Fig. 3A). The rate of protein synthesis increases from the fourth instar, and this increase may be associated with the appearance of some proteins in the fat body towards the end of the larval stage (Fig. 3C). Pupation is associated with changes in the proteins of the fat body, as shown by an examination of the proteins present in this tissue a few hours after pupation, i.e. before the onset of tissue histolysis (Fig. 3D). The data further indicate that proteins present in the fat body of adult males differ from those present in the fat body of adult females (Fig. 3E, F).

Separation of the proteins of the fat body from mature larvae, pupae and adults under dissociating conditions indicates that the molecular weights of major individual peptides range from about 30 000 to about 80 000 daltons (bands 9 — 26, Fig. 4A). Some very-high-molecular-weight peptides present in the fat body and the haemolymph do not penetrate the separation gel containing 10 % acrylamide. Thus, under these dissociating conditions, a prominent high-molecular-weight haemolymph peptide band migrates only a short distance into the stacking gel containing 5 % acrylamide. The major peptides observed in the fat body of mature non-diapausing and pre-diapausing larvae were found to have the following approximate molecular weights: 26 000 (peptide no. 6), 30 000 (9), 35 000 (12), 50 000 (18), 58 000 (21), and 70 000 (26).

Many of the proteins of the fat body were shown to be discharged from this tissue upon *in vitro* incubation in macromolecule-free Grace's medium (Fig. 5). The right perivisceral fat body sheet was used as a control and was homogenized after removal (Fig. 5A), and the left sheet was incubated for 20 h in Grace's medium. After incubation the left sheet (Fig. 5B) and the medium (Fig. 5C) were analysed separately for proteins. The results show that such release of proteins is clearly selective, the major protein

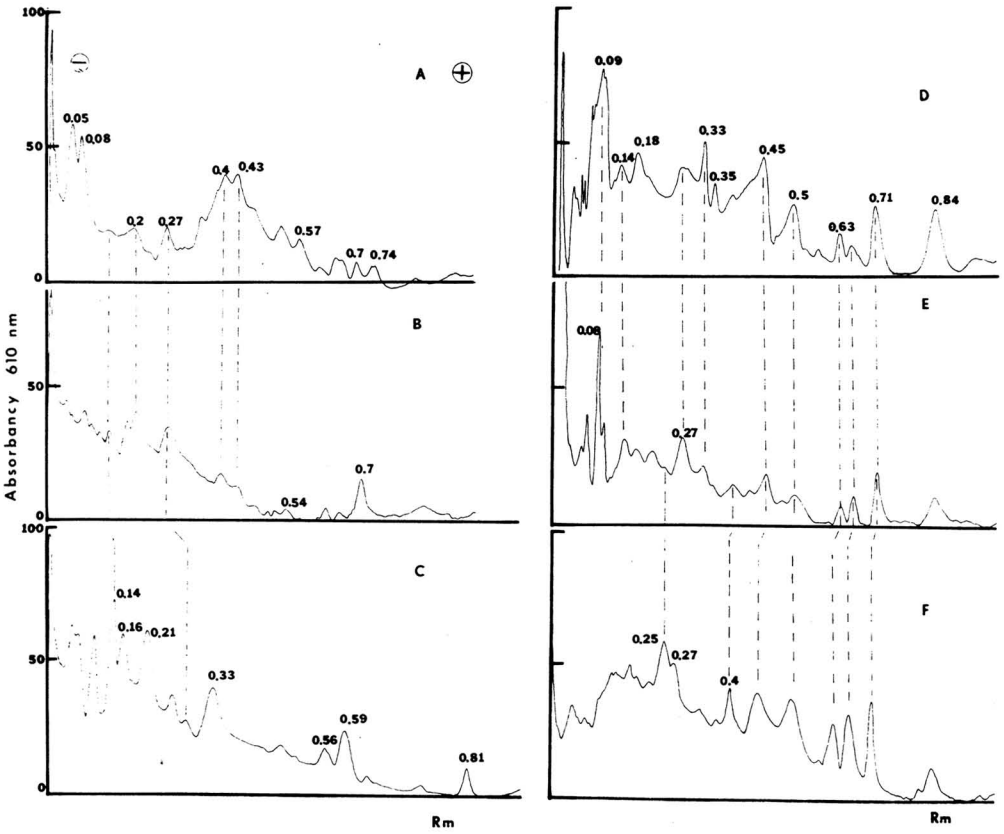

Fig. 3. Densitometric scans of gels after non-dissociating electrophoresis of egg and fat body proteins. A: eggs, B: 9-day-old 4 th instar larvae, C: 14-day-old 5th instar female larvae, D: ca. 12-h-old male pupae, E: 1-day-old male adults, F: 1-day-old female adults. Rm = relative mobility.

released into the medium being the band at Rm 0.14 (corresponding to peptide 26). The mobility of this protein corresponds to that of a major lipid carrier protein present in the larval haemolymph, suggesting that the fat body is the source of this haemolymph lipoprotein. Another fat body protein released, although more slowly than the protein at Rm 0.14, is the one at Rm 0.56 (corresponding to peptide 12).

The possible functions of some key fat body proteins may include transport of lipid molecules after the proteins are released into the haemolymph. Thus, the critical overwintering period of these larvae seems to have a profound effect on the protein composition of the larval fat body. Non-dissociating electrophoresis of fat body proteins in 5 % acrylamide gels showed that, as compared with 18-day-old non-diapausing larvae, 36-day-old pre-diapausing and 155-day-old diapausing larvae contain much higher titres of several high-molecular-weight proteins (Fig. 6A-C). The haemolymph of 155-day-old diapausing larvae also contained high titres of proteins with approximate relative mobilities of 0.07, 0.14, 0.20, and 0.31 (Fig. 6D). These four haemolymph proteins proved to be Sudan-black-positive, suggesting that they play a role in lipid transport. For comparison, the approximate mobilities of these haemolymph proteins in 7 % acrylamide are 0.02, 0.05, 0.08 and 0.14. Additional tests showed that juvenile hormone may influence the titre of some of these lipoproteins. Thus, non-diapausing larvae treated with C_{17} juvenile hormone had increased titres of at least two haemolymph lipoproteins.

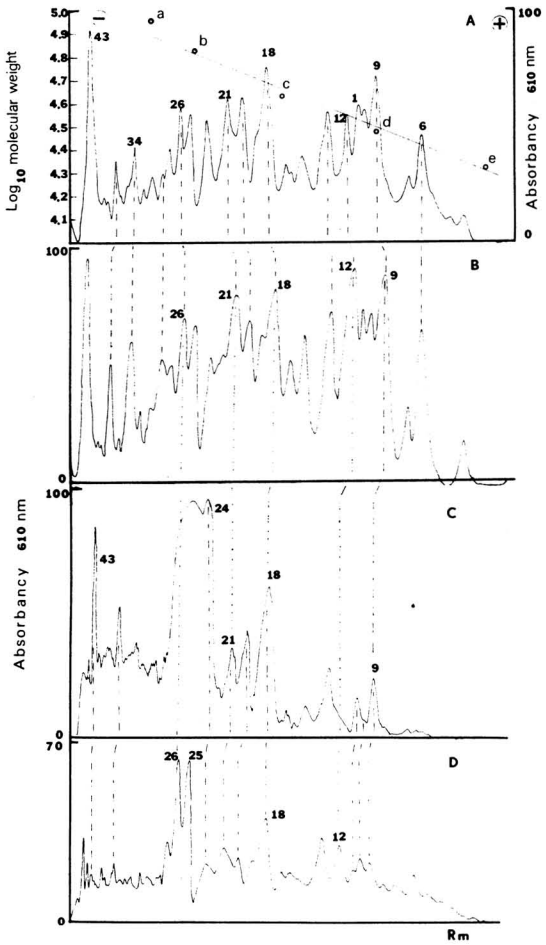

Fig. 4. Densitometric scans of gels after electrophoresis of fat body proteins (50—60 μg) with sodium dodecyl sulphate in 10 % acrylamide gels. A: 18-day-old mature 6th instar female larvae, B: 40-day-old newly diapaused female larvae, C: ca. 12-h-old male pupae, D: 1-day-old male adults. Standards for molecular weight determination are: (a) phosphorylase B, (b) bovine serum albumin, (c) ovalbumin, (d) carbonic anhydrase, and (e) soybean trypsin inhibitor. Log_{10} molecular weight scale on left.

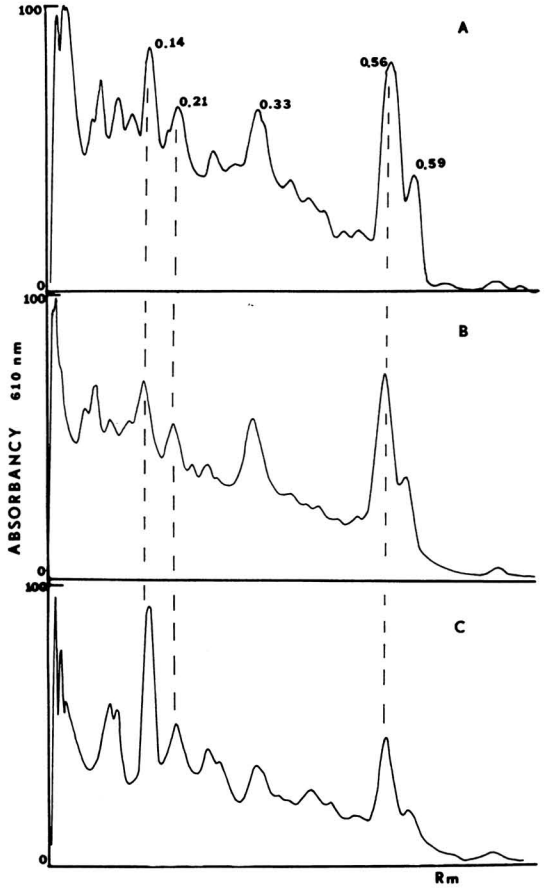

Fig. 5. Densitometric scans of gels after non-dissociating electrophoresis of proteins in 7 % acrylamide showing *in vitro* release of proteins from the pooled perivisceral fat bodies of 3 newly diapaused (42-day-old) female larvae. A: right fat body sheets analysed before incubation (control) (0.1 portion, ca. 150 μg protein), B: left fat body sheets after a 20 h incubation in macromolecule-free Grace's medium (0.1 portion), C: medium after the 20 h incubation of left sheets (0.2 portion), C: medium after the 20 h incubation of left sheets (0.2 portion of released proteins).

4. Discussion

This study compared three methods for resolving the proteins present in the fat body of the southwestern corn borer at different stages of development. Isoelectric focusing has been found to be a convenient means of separating insect proteins. However, probably owing to differences in the proportions of the different proteins and to the ionic composition, haemolymph proteins do not focus as well or as quickly as do those of the fat body. Under the conditions used, fat body proteins were found to be focused within 7 or 8 h with sample sizes up to 300 μg/gel using ampholytes in the pH gradient ranges 3 to 10 and 5 to 7. Less uniformity was sometimes observed in the basic region of the pH gradient, there being some variation in the relative distances between the protein bands.

The results suggest that in *D. grandiosella*

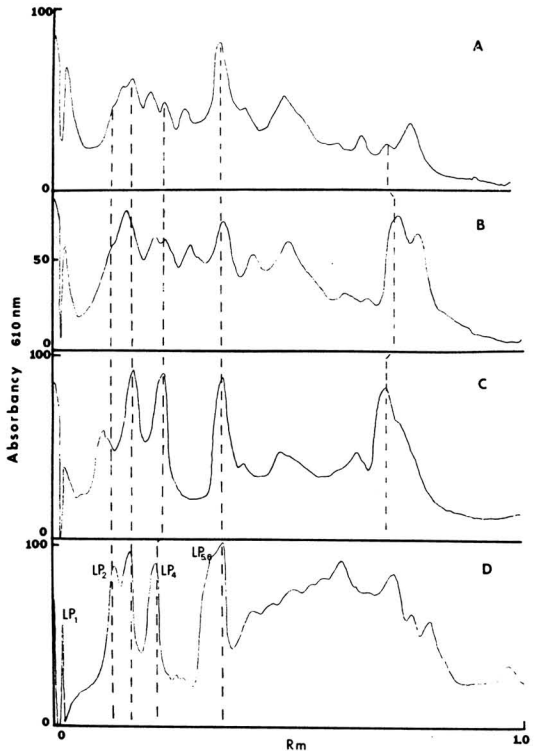

Fig. 6. Densitometric scans of gels after non-dissociating electrophoresis of proteins in 5 % acrylamide showing the presence of slowly migrating lipoproteins (LP). A: fat body (0.1 portion) of 18-day-old mature non-diapausing larvae, B: fat body (0.1 portion) of 37-day-old pre-diapausing larvae, C: fat body (0.1 portion) of 155-day-old diapausing larvae, D: haemolymph (2 μl) of 155-day-old diapausing larvae.

several changes occur in the fat body protein pattern during metamorphosis. Temporal changes in the proteins of the larval fat body of this species have been studied previously (Brown & Chippendale 1978, Brown et al., 1977). The major protein accumulated in the fat body of pre-diapausing larvae (peptide 12) has a native molecular weight of about 35 000 and an isoelectric point at pH 5.9 (Turunen & Chippendale 1979). Peptide 26 has a molecular weight of about 70 000 and seems to be a component of a major lipid carrier protein present in larval and pupal haemolymph. The pI of this haemolymph protein is approximately 5.5—5.6. Both these proteins are discharged from the fat body of diapausing larvae during *in vitro* incubation. These data agree with those from several other species of insects which have been suggested to synthesize the majority of their interstitial proteins in the fat body (Pan & Wallace 1974, Wyatt 1975, Agosin 1978, Chen 1978).

The present data also implied the fat body origin of several major lipid carrier proteins of the haemolymph. Lipids are known to be the chief source of energy for diapausing larvae of *D. grandiosella* (Turunen & Chippendale 1976). Thus lipoproteins must play a critical role in successful overwintering. The data also indicated that treatment of non-diapausing larvae with C_{17} juvenile hormone increased the haemolymph titres of some lipoproteins. It is possible that in *D. grandiosella* juvenile hormone functions *in vivo* by influencing the synthesis of lipid carrier proteins to ensure continued transport of lipids during diapause.

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References

Agosin, M. 1978: Functional role of proteins. — In: Rockstein, M. (ed.), Biochemistry of insects: 94—144. Acad. Press, New York.

Anderson, N. L. & Anderson, N. G. 1978: Analytical techniques for cell fractions. XXII. Two-dimensional analysis of serum and tissue proteins: Multiple gradient-slab gel electrophoresis. — Anal. Biochem. 85:341—354.

Bradford, M. M. 1976: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. — Anal. Biochem. 72:248—254.

Brown, J. J. & Chippendale, G. M. 1978: Juvenile hormone and a protein associated with the larval diapause of the southwestern corn borer, Diatraea grandiosella. — Insect Biochem. 8:359—367.

Brown, J. J., Chippendale, G. M. & Turunen, S. 1977: Larval esterases of the southwestern corn borer, Diatraea grandiosella: temporal changes and specificity. — J. Insect Physiol. 23: 1255—1260.

Chen, P. S. 1978: Protein synthesis in relation to cellular activation and deactivation. — In: Rockstein, M. (ed.), Biochemistry of insects: 145—203. Acad. Press, New York.

Chippendale, G. M. 1975: Ascorbic acid: an essential nutrient for a plant-feeding insect, Diatraea

grandiosella. — J. Nutr. 105: 499—507.
Davis, B. J. 1964: Disc electrophoresis — II. Methods and application to human serum proteins. — Ann. N. Y. Acad. Sci. 121:404—427.
Downer, R. G. H. 1978: Functional role of lipids in insects. — In: Rockstein, M. (ed.), Biochemistry of insects; 57—92. Acad. Press, New York.
Keeley, L. L. 1978: Endocrine regulation of fat body development and function. — Ann. Rev. Entomol. 23:329—352.
Kilby, B. A. 1963: The biochemistry of the insect fat body. — Adv. Insect Physiol. 1:112—174.
Laemmli, U. K. 1970: Cleavage of structural proteins during the assembly of the head of bacteriophage T 4. — Nature (Lond.) 227:680—685.
Pan, M. L. & Wallace, R. A. 1974: Cecropia vitellogenin: isolation and characterization. — Amer. Zool. 14:1239—1242.
Payne, J. W. 1976: Electrophoresis of proteins on sodium dodecyl sulphate polyacrylamide gels. — In: Smith, I. (ed.), Chromatographic and electrophoretic techniques. II. Zone electrophoresis: 321—346. Year Book Medical Publishers, Chicago.
Price, G. M. 1973: Protein and nucleic acid metabolism in insect fat body. — Biol. Rev. 48:333—375.
Richetti, P. G. & Drysdale, J. W. 1974: Isoelectric focusing in gels. — J. Chromatogr. 98:271—321.
Smith, I. 1976: Acrylamide gel disc electrophoresis; Section I: Techniques of disc electrophoresis. — In: Smith, I. (ed.), Chromatographic and electrophoretic techniques. II. Zone electrophoresis: 210—237. Year Book Medical Publishers, Chicago.
Turunen, S. & Chippendale, G. M. 1976: Use of fat body and midgut lipids by diapausing larvae of the southwestern corn borer, Diatraea grandiosella. — Ann. Entomol Soc. Amer. 69:551—555.
Turunen, S. & Chippendale, G. M. 1979: Possible function of juvenile hormone-dependent protein in larval insect diapause. — Nature (Lond.) 280: 836—838.
Vesterberg, O. 1971: Staining of protein zones after isoelectric focusing in polyacrylamide gels. — Biochim. Biophys. Acta 243:345—348.
Wyatt, G. R. 1975: Regulation of protein and carbohydrate metabolism in insect fat body. — Verh. Deutsche Zool. Ges. 1974:209—226.
Wyatt, G. R. & Pan, M. L. 1978: Insect plasma proteins. — Ann. Rev. Biochem. 47:779—817.

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