FAT BODY PROTEIN GRANULES AND STORAGE PROTEINS IN THE SILKMOTH, *HYALOPHORA CECROPIA*

SUMIO TOJO, TEIICHI BETCHAKU, V. J. ZICCARDI†, and G. R. WYATT

From the Department of Biology, Yale University, New Haven, Connecticut 06520, the Laboratory of Applied Entomology, Faculty of Agriculture, University of Tokyo, Tokyo, Japan, and the Department of Biology, University of Pennsylvania, Philadelphia, Pennsylvania 19104. Dr. Tojo's present address is the Laboratory of Applied Entomology, Faculty of Agriculture, University of Tokyo, Bunkyo-ku, Tokyo, Japan. Dr. Betchaku's present address is Department of Pharmacology Yale University School of Medicine, New Haven, Connecticut 06510. Dr. Wyatt's present address, to which reprint requests should be directed, is the Department of Biology, Queen's University, Kingston, Ontario K7L 3N6, Canada.

ABSTRACT

Fat body cells of silkmoth pupae (*Hyalophora cecropia*) contain granules, showing a less dense outer zone and a denser, often crystalline, inner portion appear after cocoon spinning and increase until the larval-pupal ecdysis; more granules are formed in females than in males. Urate granules, appearing fibrous in internal structure, first form about the same time, but their accumulation is more gradual, and continues in the pupa. Both types have been isolated by centrifugation. Protein granules dissolve in buffers to yield proteins 1 and 2, with distinct electrophoretic and antigenic properties. These proteins have been isolated individually from pupal fat body extracts by using their different thermal stabilities in phosphate buffer containing $MgCl_2$ and $(NH_4)_2SO_4$, respectively, and purification was completed by gel chromatography. Protein 1 has a molecular weight of 480,000 and a subunit of 85,000 daltons, while protein 2 gives values of 530,000 and 89,000, respectively. Their amino acid compositions are similar but distinct. Proteins 1 and 2 accumulate in the hemolymph, beginning 3 days before spinning, reach maximal levels at spinning, and then decline in the hemolymph while granules are formed in the fat body, although the total hemolymph protein concentration does not decline at this time. It is concluded that the fat body of the late, feeding larva synthesizes two related "storage proteins" and secretes them in partially crystalline granules as protein reserves for metamorphosis.

KEY WORDS silkmoth · fat body · hemolymph · protein · granules · storage · metamorphosis · urate

The fat body of insects is a center for intermediary metabolism and the synthesis of hemolymph components which has been compared to the liver of vertebrates (24, 40, 52). It also serves for storage of nutrient reserves, a function which is most pronounced during the metamorphosis of holometabolous insects, when the fat body cells hypertrophy and become loaded with lipid, glycogen,

† Deceased.

and protein. Since the end of the last century (3), dense proteinaceous granules have repeatedly been described as accumulating in the fat body cells of various insects at the beginning of metamorphosis (40, 46). Since they disappear during the pupal-adult transformation, a role as a reserve for the growth of adult tissues has been generally accepted. Their origin, however, has been the subject of diverse speculation (50) but, until recently, little evidence.

In the skipper, *Calpodes ethlius*, Locke and Collins (28, 29) distinguished with the electron microscope several types of protein-containing granules, and presented evidence for their formation by a combination of processes including isolation of intracellular organelles within membranes derived from the Golgi complex, and pinocytotic uptake of protein from the hemolymph. Protein uptake was demonstrated with injected plant peroxidase as a tracer, and its natural occurrence was suggested by a marked decline in hemolymph protein level at the same time as the build-up of granules in the fat body. Thomasson and Mitchell (45) isolated granules from mature *Drosophila* larvae and showed that they contained radioactivity from previously injected ^{3}H-labeled hemolymph protein, but not from injected ^{14}C-leucine. In the blowfly, *Calliphora*, a protein called calliphorin, which makes up some 60% of the total soluble protein in the mature larva, is believed to have a storage role (36). Granules appear in the fat body at the same time as uptake of calliphorin from the hemolymph (32), and the formation of the granules from this protein has been inferred (46). The composition of isolated insect protein granules and its relationship to specific proteins of the hemolymph and fat body have not, however, heretofore been described.

We have studied the fat body of the silkmoth, *Hyalophora cecropia*, by light and electron microscopy, developed a procedure for isolating from it two classes of granules, characterized the major components of the protein granules, and followed changes in the distribution of these proteins, as well as some associated biochemical changes, during the larval-pupal transformation. Some of the results have been reported in preliminary form (52).

MATERIALS AND METHODS

Animals

Cecropia silkmoths, *H. cecropia*, were reared outdoors on wild cherry trees, and in the laboratory on an artificial diet (41). Mature larvae (1 day before spinning) were recognized by the empty midgut. The time from onset of spinning to the larval-pupal ecdysis was 8 days, at 25°C. Male and female larvae were distinguished by abdominal markings (22).

Tissue Preparation for Light and Electron Microscopy

For light microscopy, portions of fat body were fixed in 95% ethanol, dehydrated, and embedded in paraffin. Sections 5 μm thick were generally stained first by the argentaffin (methenamine silver nitrate) method for uric acid (18), and then with mercuric bromophenol blue for protein (33). Nucleic acids were detected by either methyl green-pyronin, Feulgen (21), or azure B reagent (15). The periodic acid-Schiff reaction (PAS) was used to demonstrate glycogen (21).

For electron microscopy, tissues were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4 for 4–5 h and post-fixed in 1% OsO_4 in 0.1 M cacodylate buffer for 1 h, all at 0–4°C. Dehydration and embedding in Epon 812 followed according to Luft (31). Some specimens were block-stained in 0.5% uranyl acetate in 0.05 M veronal acetate buffer at pH 5.0 for 1 h at 0–4°C before dehydration. Thin sections of 60–70 nm were cut with a diamond knife and stained with uranyl acetate and lead citrate. Observations were made with either a Philips 300 or an RCA EMU-4 electron microscope.

Chemical Analyses of Tissue Components

Fat body was rinsed with Ringer's solution (KCl 154 mM, NaCl 5.6 mM, $CaCl_2$ 2.3 mM, brought to pH 7.0 with about 0.1 mM $NaHCO_3$), and drained. 1 g portions were homogenized with 5 ml of water in a motor-driven, all-glass Potter-Elvehjem homogenizer, and duplicate 0.5-ml samples of the homogenate were dried to constant weight at 90°C, to give tissue dry weight.

Portions of the homogenate were fractionated for determination of uric acid, nucleic acids and protein (27). An equal volume of cold 1 N $HClO_4$ was added, and the precipitate collected by centrifugation was washed twice with 0.5 N $HClO_4$, the extracts being combined for the determination of soluble uric acid. The residue was washed twice with ethanol-ether (3:1) and twice with ether. To extract undissolved uric acid, the residue was suspended in 0.01 M Tris-HCl buffer, pH 7.0, and dialysed at 50°C against the same buffer, which was changed hourly until $A_{290\ nm}$ of the dialysate dropped below 0.01. The dialysates were combined for uric acid determination. Then the residue was extracted three times with 0.5 N $HClO_4$ at 70°C for 20 min, and the combined extracts were used for determination of DNA and RNA. From the final residue thus obtained, protein was extracted three times with 0.25 N NaOH.

Uric acid was determined by absorbance at 290 nm before and after treatment with uricase (1). The acid

extracts were first neutralized with KOH, and $KClO_4$ was removed by centrifugation. Combined values from the acid extracts and dialysates gave the total uric acid.

DNA was determined by the diphenylamine method (7), with calf thymus DNA as a standard. RNA was estimated from $A_{260\ nm}$ of the hot-acid extract, corrected for DNA (8, 27).

Protein in the alkali extract was determined by both the method of Lowry et al. (30) and the biuret reaction (13), with bovine serum albumin as a standard. In hemolymph samples, protein was measured by the biuret reaction directly after twofold dilution and centrifugation.

Isolation of Urate and Protein Granules

Fat body (2 g) from female pupae was homogenized with 20 ml of 0.3 M sodium-potassium phosphate buffer-0.01 M $MgCl_2$, pH 6.8, by 15 strokes in a motor-driven Teflon homogenizer (Dupont Instruments-Sorvall, Wilmington, Del.). The granules were centrifuged down at 600 *g* for 10 min, resuspended in buffer, and filtered through four layers of cheese cloth, which retained most of the nuclei. The filtrate was layered on 10 ml of 1.8 M sucrose in the same buffer and centrifuged at 600 *g* for 10 min, which caused urate granules to sediment to the bottom, while protein granules remained at the interface. The pellet was twice suspended in buffer and centrifuged down, yielding the urate granule fraction. The interface fraction was diluted with phosphate buffer, layered on a discontinuous gradient containing 7 ml each of 1.2 M, 1.5 M, and 1.8 M sucrose, and centrifuged at 22,500 *g* for 20 min (SW-25 rotor, Spinco L centrifuge Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The protein granules were retained partly at the interface between 1.2 and 1.5 M sucrose (fraction I), but mainly between 1.5 and 1.8 M sucrose (fraction II). Both were collected, sedimented, and washed. The isolated fractions were analyzed for protein, RNA, DNA, and uric acid by the methods described above.

Protein Extraction from Fat Body and Hemolymph

Rinsed, blotted fat body was homogenized with 10 vol of 0.05 M phosphate buffer, pH 6.8 (without $MgCl_2$), and centrifuged at 12,000 *g* for 20 min. The residue was reextracted in the same manner at least twice, to allow protein granules to dissolve, and the extracts were combined. Hemolymph was collected into a tube containing a known amount of buffer and a few crystals of phenylthiourea, then diluted to fivefold by adding buffer, and centrifuged at 12,000 *g* for 20 min.

Immunodiffusion Analysis

To prepare antisera, 0.5-ml portions of the soluble fraction from pupal fat body homogenized with 2 vol of phosphate buffer were emulsified with equal volumes of Freund's complete adjuvant and injected subcutaneously into rabbits. Two additional similar injections were given at weekly intervals, and the rabbits were bled 2 wk after the final injection. To render the antiserum specific for storage proteins, it was absorbed with hemolymph from early fifth instar larvae, which lack these proteins. Antisera were also prepared to the individual proteins by cutting the zones of proteins 1 and 2 from 12 acrylamide disc electrophoresis gels of fat body extracts, macerating them with 0.05 M phosphate buffer, and injecting them into rabbits without adjuvant.

Relative concentrations of the antigens were determined by the quantitative immunodiffusion technique of Oudin (44), with pooled hemolymph from male diapause pupae as a standard. When most of these analyses were carried out, the pure proteins as standards for absolute concentration were not available.

RESULTS

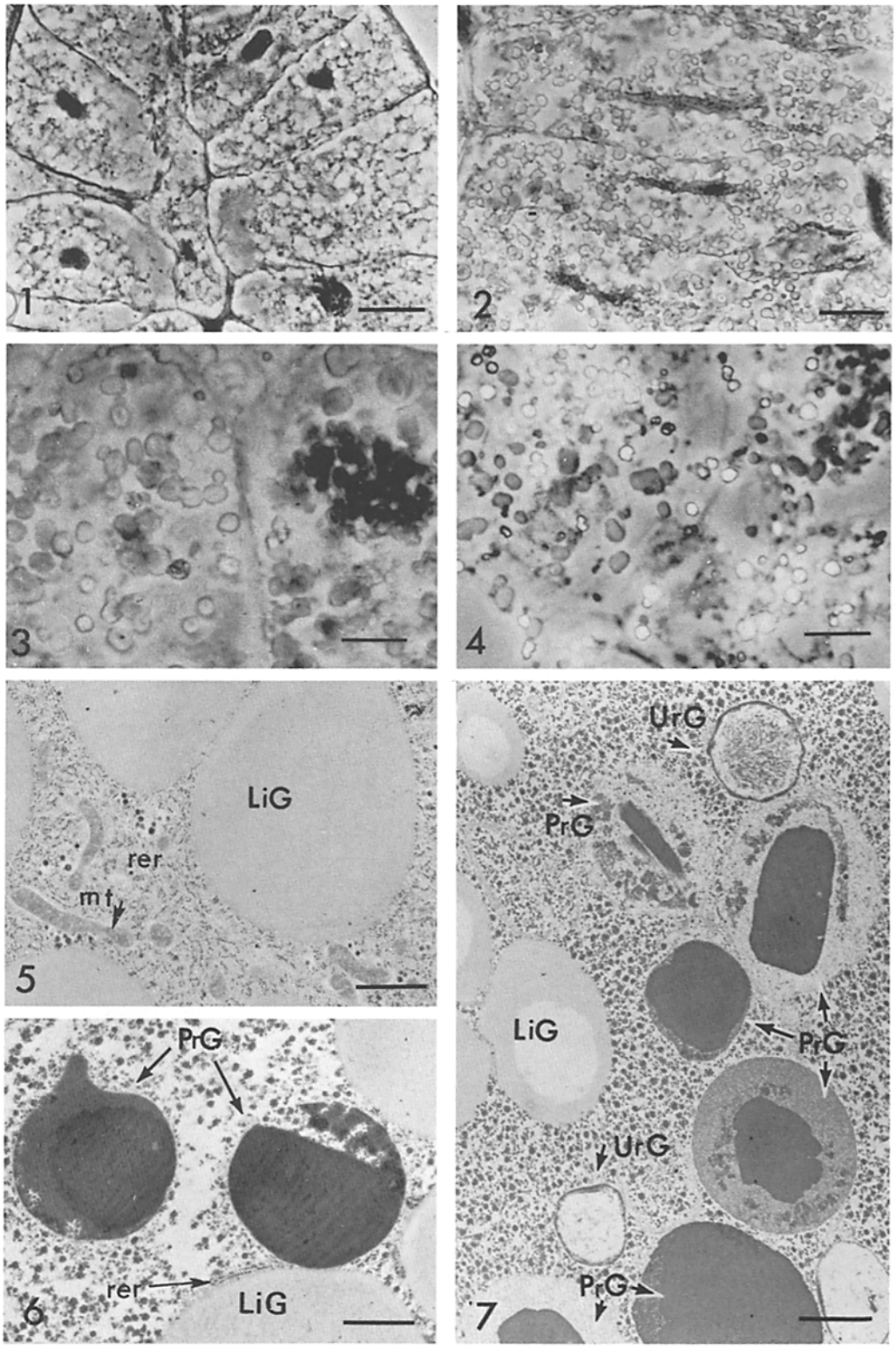
Light Microscopy

In sections or homogenates of fat body, two types of granule were observed: protein granules, which appeared orange by double staining with methenamine silver nitrate and mercuric bromphenol blue observed by phase microscopy, and urate granules, which stained bright yellow under these conditions (Fig. 4). With methenamine silver nitrate alone, the former were unstained, while the latter stained black, which is specific for uric acid. They could also be distinguished without staining by observation between crossed polaroid filters, which revealed strong optical activity in the urate granules but very little in the protein granules.

Sections of fat body from larvae up to the day of spinning (Fig. 1) showed roughly spherical nuclei and cytoplasm with many lipid droplets, but no dense granules. Beginning 2–3 days later, the nuclei became compressed and misshapen and the cytoplasm increasingly filled with storage granules (Fig. 2). The number and size of protein granules reached a maximum about the time of the larval-pupal ecdysis (Fig. 3). In diapause pupae (Fig. 4), the granules ranged from 1–8 μm, most commonly 1.5–3 μm, in diameter.

Urate granules could be observed in smeared fat body homogenate almost as early as the protein granules, although, being small at this stage, they were difficult to detect in sections. Thereafter, they gradually increased in size and number, becoming distinguishable among the protein granules in sections (Fig. 4).

By counting the granules in sections of pupal


1
2
3
4
LiG
rer
mt
5
PrG
rer
LiG
6
UrG
PrG
LiG
PrG
UrG
PrG
7

fat body, we found that the number of protein granules per unit area was almost twice as great in females as in males, whereas the urate granules were much more numerous in males. Some cells in male diapause pupae were packed almost exclusively with urate granules. The number of lipid droplets was about 50% greater in males.

Electron Microscopy

These developmental changes were further clarified at the fine-structural level. Sections of fat body from fully-grown feeding larvae (Fig. 5) showed cytoplasm with abundant mitochondria and ribosomes (apparently aligned on membranes), and scattered glycogen deposits, between the numerous large lipid droplets. By the day of spinning, ribosome-containing cytoplasm was largely restricted to zones around the lipid droplets, and glycogen had become generally distributed. Four days later (Fig. 6), cytoplasm was still more reduced, and dense protein granules were present, some of them still in the process of formation. Some of the marginal vesicles in the granules appeared to contain ribosomes. In pupae in diapause (Fig. 7), the fat body was packed with protein granules that showed considerable variation in form, along with urate granules, lipid droplets and glycogen, the total picture representing conversion of the tissue to a predominantly storage role.

The protein granules in pupal tissue were typically more or less oval in shape, with an electron-dense inner portion, often showing flat faces, and a less dense outer zone of variable thickness surrounded by a thin membrane (Figs. 7 and 8). In some granules, the dense inner part was clearly crystalline, with a regular lattice spaced at about 18 nm, and apparent addition of molecular layers at the surface (Fig. 8). The urate granules (Fig. 9) were also membrane-bounded, and showed a curiously fibrous internal structure.

Changes in the Composition of Fat Body and Hemolymph

Since the granules appeared sufficiently abundant to influence tissue gross composition, the total protein, RNA, DNA, and uric acid were determined in fat body from a series of developing stages. The results (Figs. 10–12), based on dry weight, must be influenced by changes in other major tissue components which were not measured. Lipid and glycogen accumulate rapidly in fat body just before the beginning of metamorphosis and then decrease somewhat between spinning and the larval-pupal ecdysis, and there is further gradual loss of fat-body glycogen (due to conversion to glycerol) in the cecropia pupa (2, 17). These changes may account for the apparent fall in nucleic acids and protein just

---

FIGURE 1 Light microscope section of fat body from a female cecropia silkmoth on the day of spinning. No cytoplasmic granules are evident. Methenamine silver nitrate-mercuric bromphenol blue; phase contrast. × 500. Bar, 20 μm.

FIGURE 2 As Fig. 1, 3 days after spinning. Abundant protein granules are visible. × 500. Bar, 20 μm.

FIGURE 3 As Fig. 1, 8 days after spinning (day of larval-pupal ecdysis). The cells are packed with protein granules. × 1,000. Bar, 10 μm.

FIGURE 4 As Fig. 1, 80 days after spinning (diapause pupa). Both protein granules (stained, often oval-shaped) and urate granules (bright, unstained, generally round) are visible. × 1,000. Bar, 10 μm.

FIGURE 5 Electron microscope section of fat body from a female cecropia fully-grown, feeding larva. Lipid globules (*LiG*) are abundant, rough endoplasmic reticulum (*rer*) is well developed, and mitochondria (*mt*) are numerous. There are no protein or urate granules. Fixation and staining as under Materials and Methods. × 11,000. Bar, 1 μm.

FIGURE 6 As Fig. 5, 4 days after spinning. Rough endoplasmic reticulum (*rer*) has almost completely disappeared, and can be seen only at the edge of one of the lipid globules. Protein granules (*PrG*) are forming, and the remainder of the cells is filled with glycogen. × 11,000. Bar, 1 μm.

FIGURE 7 As Fig. 5, 40-day old pupa. Protein granules are now numerous, showing varied internal structure. There are also some urate granules (*UrG*), which sometimes appear empty. Lipid globules (*LiG*) and glycogen persist. × 11,000. Bar, 1 μm.

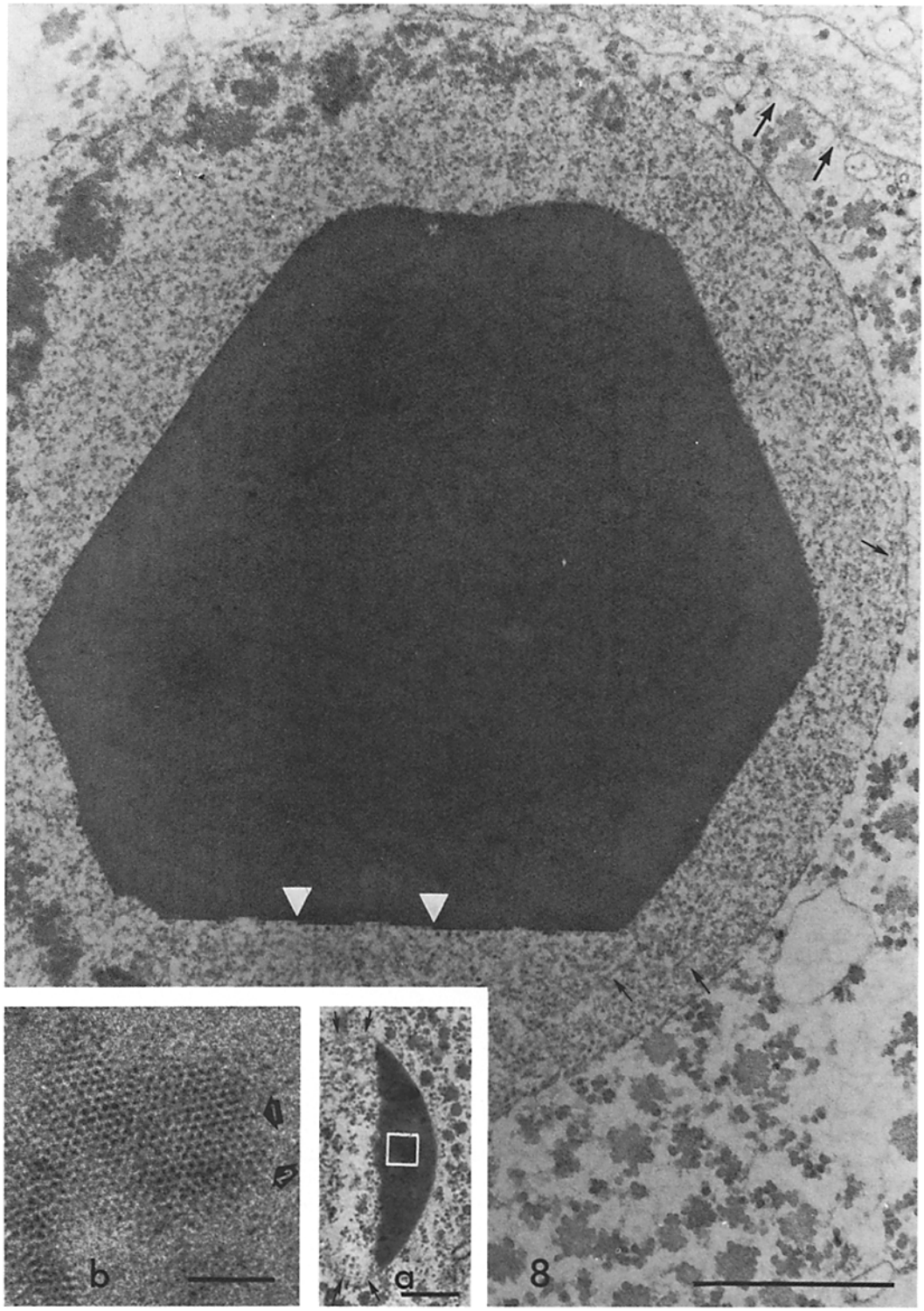


FIGURE 8 A protein granule in a section of fat body from a 40-day old pupa. The granule shows a surrounding membrane, an amorphous outer zone containing particles which may be storage protein (small arrows), and a dense core showing periodic structure and layers of crystallization at its surface (white triangles). The white-trimmed arrows point to the plasma membrane of the fat body cell, and glycogen granules are visible in the cytoplasm. × 60,000. Bar, 0.5 μm. (*a*) A growing protein granule, from an 8-day-old pupa. The arrows indicate the outer membrane of the granule extending beyond the region of dense protein deposition. The portion enlarged in *b* is outlined. × 13,000. Bar, 1 μm. (*b*) A portion of the growing granule from *a* enlarged to show crystalline structure. The repeat period in both directions 1 and 2 (arrows) is 18 nm. × 88,000. Bar, 0.1 μm.

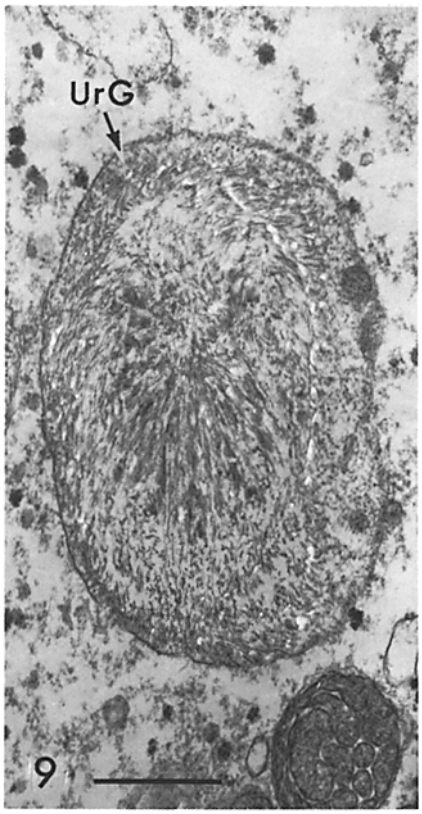


FIGURE 9 A urate granule (*UrG*) in a section of fat body from a 180-day old pupa. The interior of the granule appears fibrous. × 34,000. Bar, 0.5 μm.

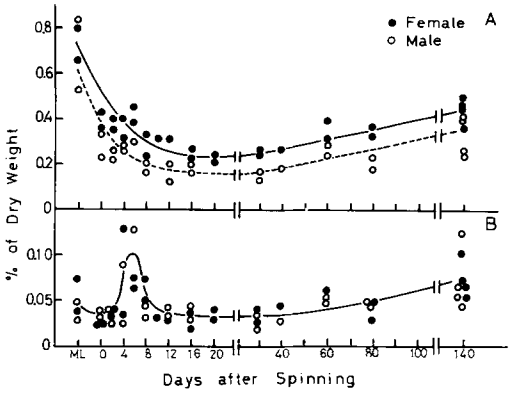


FIGURE 10 Content of nucleic acids in fat body, as a function of developmental stage. *ML*, mature larva. *A*, total RNA; *B*, DNA. In Fig. 10–13, points represent determinations on individual animals.

before spinning, and the gradual rise in nucleic acids during the pupal stage. The transient rise in fat body DNA after spinning (Fig. 10) may be due to uptake of degraded DNA from the degenerating silkgland, as already demonstrated in *Bombyx mori* (8).

The protein content of the fat body rose (much more in females than in males), during the week after spinning, indicating accumulation of protein, while RNA continued to decrease (Figs. 10 and 11). Uric acid increased greatly after spinning and continued to rise in the pupa, especially the male (Fig. 12). The results with respect to RNA, protein, and uric acid are therefore in accord with the microscope observations, and depict the transition of the fat body from a biosynthetic to a principally storage role.

The hemolymph (Fig. 13) showed a sharp rise in protein concentration, beginning in the mature, feeding larva, and continuing until about 4

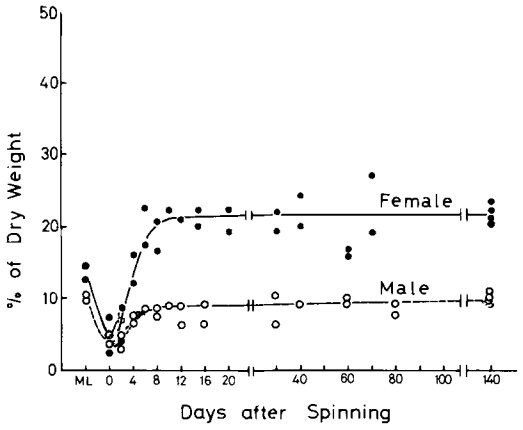


FIGURE 11 Protein content of fat body, as a function of developmental stage, determined by the biuret method.

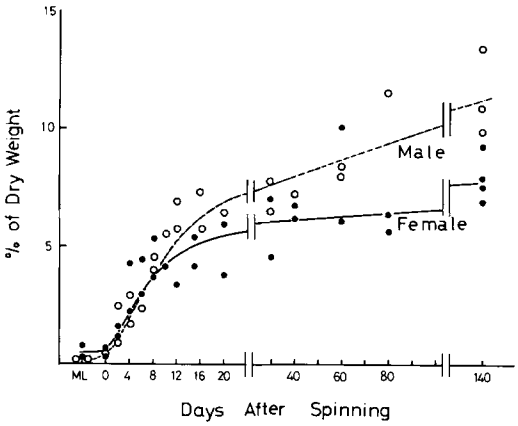


FIGURE 12 Uric acid content of fat body, as a function of developmental stage.

days after spinning, after which the level remained essentially constant. The protein concentrations averaged slightly higher in female than in male hemolymph.

Isolation of Urate and Protein Granules

After homogenization of fat body in most buffers, the protein granules tended to dissolve, leaving only membranes together with urate granules, which were more stable. Magnesium ions helped stabilization, and the best recoveries were obtained in sodium-potassium phosphate buffer, 0.3 M, pH 6.8, containing 10 mM $MgCl_2$,

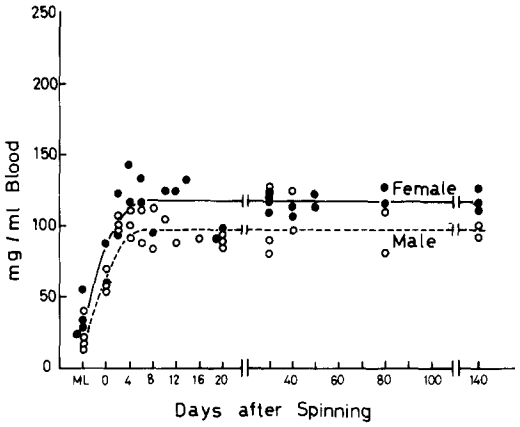


FIGURE 13 Protein content of hemolymph, determined by the biuret method, as a function of developmental stage.

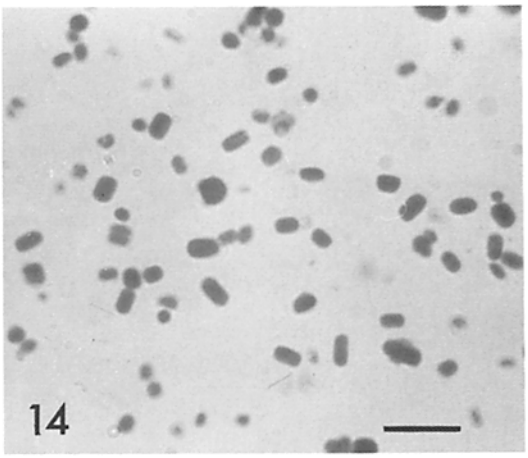
which was therefore used routinely. Nevertheless, repeated washing caused loss, and granules had to be prepared as rapidly as possible.

In addition to the urate granules, the isolation procedure (see Materials and Methods) yielded two fractions of protein granules. Protein granule fraction II was the cleaner, and under the microscope appeared virtually pure, as did the urate granule fraction (Figs. 14 and 15). The isolated protein granules stained orange with mercuric bromphenol blue, and the urate granules stained black with methenamine silver nitrate. Staining tests with PAS for glycogen, and Fuelgen, methyl green-pyronin and azure B for nucleic acids were negative. Under the electron microscope, protein granule fraction II showed intact granules with membranes, together with some ruptured granules (Fig. 16). The urate granule pellet was too hard for thin sectioning.

Chemical analyses showed, in the protein granule fractions, that protein made up about 98% of the substances assayed (Table I). The content of nucleic acids was very low. The urate granule fraction contained 75% uric acid and 24% protein. The homogeneity of the urate granule preparation under the microscope suggests that this protein is endogenous, and not due to contaminating protein granules.

Solubilization and Identification of Protein Components

Protein could be dissolved from the isolated



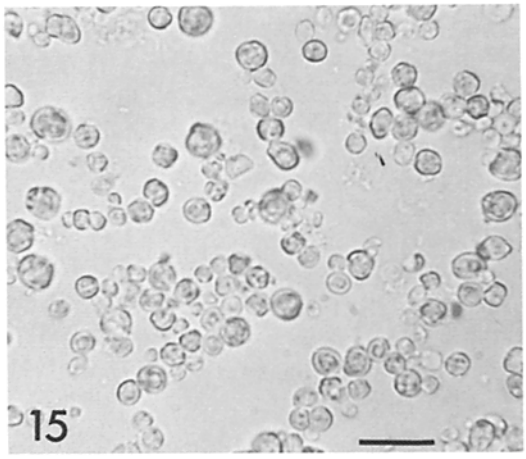


FIGURE 14 Isolated protein granules: fraction III prepared from the fat body of a female pupa. Stained with mercuric bromphenol blue; phase contrast. × 1,000. Bar, 10 μm.

FIGURE 15 Urate granules isolated from the fat body of a female pupa. Treated with mercuric bromphenol blue; the granules do not stain. Phase contrast. × 1,000. Bar, 10 μm.

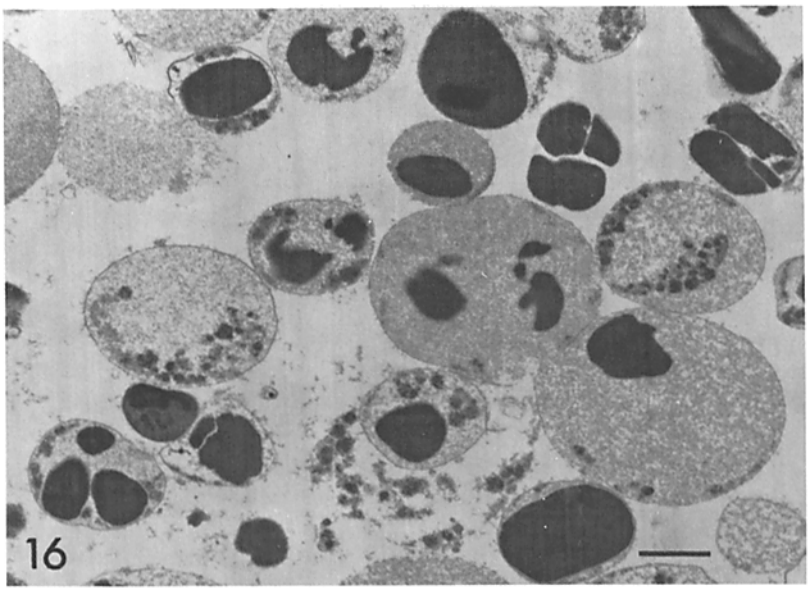


FIGURE 16 An electron micrograph of isolated protein granules, fraction II, prepared from the fat body of a 30-day-old female pupa, embedded and sectioned. Some granules seem to have swollen and some ruptured their outer membranes. × 9,400. Bar, 1 μm.

TABLE I

Chemical Composition of Urate Granule and Protein Granule Fractions Isolated from Fat Body of Female Pupae

	Percent of total substances determined			
Fraction	DNA	RNA	Uric acid	Protein (biuret)
Urate granule	0.28	0.58	75.1	24.1
Protein granule I	0.14	1.42	0.54	97.9
Protein granule II	0.01	0.58	0.54	98.9

granules by suspending them in 0.05 M phosphate buffer, pH 6.8. The urate granules dissolved only slowly, and protein was recovered from them by dialysis overnight at 4°C against phosphate buffer. The electrophoretic patterns of proteins from both types of granules are shown in Fig. 17, along with those from hemolymph and fat body extracts from different developmental stages. The protein granules yielded two major components, which migrate slowly and very close together, and are designated proteins 1 and 2. The urate granules yielded chiefly one broad, much faster-moving band.

In the hemolymph (Fig. 17*a*) of early fifth instar larvae, proteins 1 and 2 were not detectable; a band in this region had significantly different mobility. Proteins 1 and 2 first appeared in the hemolymph of late fifth instar larvae, and increased in concentration until the time of spinning. Protein 2 decreased markedly by five days after spinning, and both bands declined to minor status by the pupal stage. Electrophoretic bands 3 and 4 were additional major hemolymph proteins: band 3, which increased greatly in concentration from the late fifth instar, probably included the vitellogenin (38, 42). Other plasma proteins (44, 53) were poorly resolved or detected by the techniques used.

In fact body extracts (Fig. 17*b*), only traces of proteins 1 and 2 were detected before spinning, but by five days after spinning they became very prominent, and they remained so in the pupal stage. Proteins 3 and 4 appeared in the fat body in only relatively small amounts.

Purification and Characterization of Proteins 1 and 2

For purification of proteins 1 and 2, extracts of whole female fat body were used, so as to avoid the losses involved in isolation of granules. Pupal fat body was rinsed, drained, and homogenized in four times its weight of 0.1 M sodium phosphate buffer, pH 7.5, at 0°C. The homogenate was centrifuged at 10,000 *g* for 20 min, the residue was twice re-extracted, and the combined

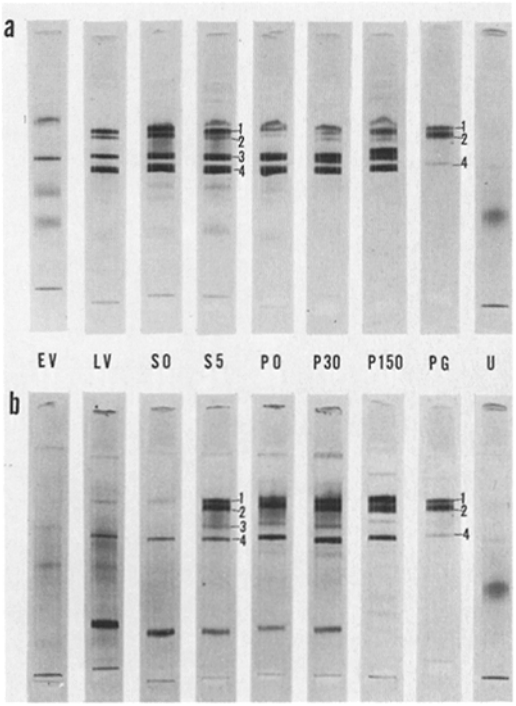


FIGURE 17 Acrylamide-gel electrophoretic patterns of proteins from female cecropia of different stages. *a*, Hemolymph; *b*, extract from fat body. *EV*, early fifth instar larva; *LV*, late fifth instar; *SO*, larva on the day of spinning; *S5*, 5 days after spinning; *PO*, freshly molted pupa; *P30*, 30-day pupa; and P150, 150-day pupa. Also shown are proteins solubilized from isolated protein granules (*PG*) and urate granules (*U*). Gels were prepared (12) with 4.5% acrylamide in 5 mM Tis-38 mM glycine buffer, pH 8.3, in glass tubes (6 × 60 mm), and were run at 2.5 mA/tube for 90 min; staining: 0.05% Coomassie blue in 12% trichloroacetic acid, destained in 7% acetic acid.

extracts were filtered through four layers of cheesecloth. Further extractions yielded less than an additional 2% of proteins 1 and 2.

After extended efforts to separate and purify proteins 1 and 2 by precipitation and chromatography, a simple method was developed based on their different thermal stability (Fig. 18). Addition of 0.05 M $MgCl_2$ to crude fat body extracts renders protein 2 labile while stabilizing protein 1, whereas 0.2 M $(NH_4)_2SO_4$ stabilizes protein 2. (The stability of the purified proteins is somewhat different, suggesting an influence of tissue components together with the added salts.) To 20 ml of extract (from 1.5 g fat body) was added 2 ml 1 M $MgCl_2$ (for the preparation of protein 1) or 8 ml 1 M $(NH_4)_2SO_4$ (for protein 2) and water to make 40 ml. The pH was adjusted to 7.5 with 0.01 M NaOH (protein 1 was unstable outside the range of pH 7.0–8.0). Then the solutions were apportioned in Pyrex tubes (Corning Glass Works, Coming, N. Y.) of 10 mm inside diameter and placed for 6 min in a water bath at 63°C for protein 1 or 72°C for protein 2. After precipitated protein was removed by centrifugation, the supernates showed single electrophoretic bands of protein 1 or 2, respectively (Fig. 19). To each solution, $(NH_4)_2SO_4$ was then added to make 60% saturation and the pH was adjusted to 7.5. Precipitated protein was collected and re-dissolved in 10 ml buffer (0.05 M Na phosphate, pH 7.5, for protein 1, and the same plus 0.05 M NaCl for protein 2); undissolved material was centrifuged off. The solutions were chromatographed on columns of Ultrogel ACA 22 previously equilibrated with the respective buffers (Fig. 20). Analyses showed that peaks *A*, *B*, and *C* contained predominantly glycogen, protein 1 or 2, and urate, respectively.

Recoveries from these procedures are shown in Table II. No increase in immunochemical purity is indicated by steps after the heat treatments, and the ammonium sulfate precipitation and Ultrogel chromatography served chiefly to concentrate the proteins and remove nonprotein contaminants. The estimates in the crude extracts indicate that proteins 1 and 2 together represented some 80% of the total protein in the soluble extract, or almost 60% of the total tissue

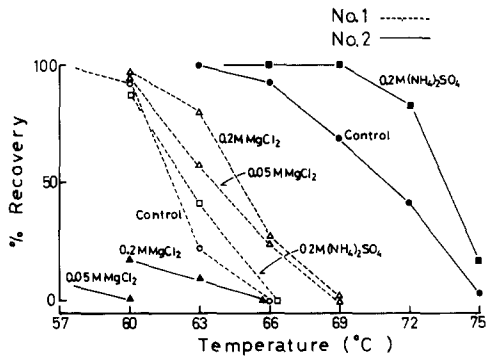


FIGURE 18 Heat-stability of storage proteins. Crude fat body extract was diluted so as to contain 1 mg/ml protein, 0.05 M sodium phosphate buffer, and $MgCl_2$ or $(NH_4)_2SO_4$ at the concentrations shown (control: no added salt). The mixtures were then heated at different temperatures for 6 min as described in the text, and precipitated protein was removed by centrifuging. Protein 1 and protein 2 remaining in solution were measured by quantitative immunodiffusion.

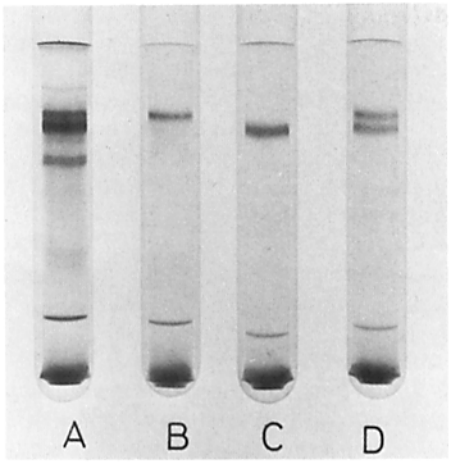


FIGURE 19 Polyacrylamide-gel electrophoresis of fat body extract and purified storage proteins. *A*, crude extract of female pupal fat body; *B*, protein 1 obtained by treatment of extract for 6 min at 63°C in 0.05 M Na phosphate buffer, pH 7.5, 0.05 M $MgCl_2$; *C*, protein 2 obtained by treatment of extract for 6 min at 72°C in 0.05 M Na phosphate buffer pH 7.5, 0.2 M $(NH_4)_2SO_4$; *D*, mixture of proteins 1 and 2. Acrylamide concentration, 5%.

protein (68.9 mg from 1.5 g of fat body).

Molecular weights of the native proteins were estimated, by electrophoresis on polyacrylamide gels of different concentrations, as 480,000 for protein 1, and 530,000 for protein 2 (Fig. 21). SDS-PAGE showed a single subunit component for each of the purified proteins, with mol wt 85,000 for protein 1, and 89,000 for protein 2 (Fig. 22).

The amino acid compositions of purified proteins 1 and 2 are shown in Table III.

Immunochemical Analysis of Proteins 1 and 2 in Hemolymph and Fat Body

Antiserum from rabbits immunized with fat body extract exhibited two zones of precipitation in the Oudin test when antiserum in agar was overlaid with pupal hemolymph, pupal fat body extract, or purified proteins 1 plus 2. This indicates that these two proteins are immunologically distinct. By a mutual dilution test (44), the two antigens in pupal fat body extract were shown to

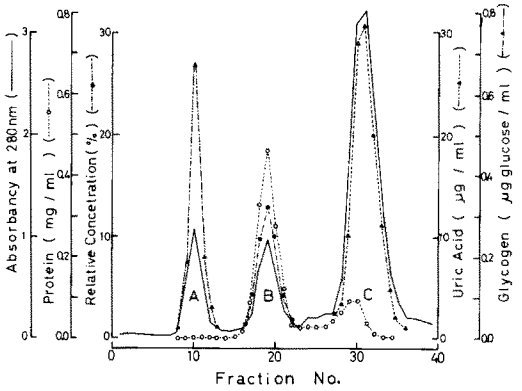


FIGURE 20 Column chromatography on Ultrogel of heat treated extract from fat body. Extract was prepared and heat-treated for protein 2 as described in the text and Table II, and the solution containing 8.1 mg protein was applied to a 2 × 20 cm column of Ultrogel ACA 22. Eluting buffer: 0.05 M NaCl-0.05 M Na phosphate, pH 7.5; flow rate, 12 ml/h; temperature, 10°C; fraction size, 4 ml. Total protein was determined by the method of Lowry et al. (30), protein 2 by quantitative immunodiffusion, uric acid by the method of Brown (6) and glycogen by the phenolsulfuric acid reaction (14). Protein recovered in peak *B* was 7.0 mg. The elution pattern from extract heat-treated for protein 1 was very similar.

TABLE II

Purification of Storage Proteins from Cecropia Fat Body

Step	Procedure for protein 1			Procedure for protein 2		
	Total protein	Protein 1*	Recovery	Total protein	Protein 2*	Recovery
	mg	*mg*	*%*	*mg*	*mg*	*%*
Crude	49.9	25.5	(100)	49.9	15.7	(100)
Heat treatment	8.8	10.6	42	15.7	12.6	80
$(NH_4)_2SO_4$ precipitation	4.9	6.7	26	8.1	9.3	59
Ultrogel chromatography	4.3	4.3	17	7.0	7.0	45

Fat body (3.0 g fresh wt) from female diapausing pupae was used. The crude extract was divided into two equal parts, which were processed separately for protein 1 and protein 2, as described in the text.

* Estimated by the immunodiffusion method of Oudin (see Materials and Methods), with male pupal hemolymph as a reference standard. Results were later converted to milligrams of each protein by assuming that the product from the final step was pure.

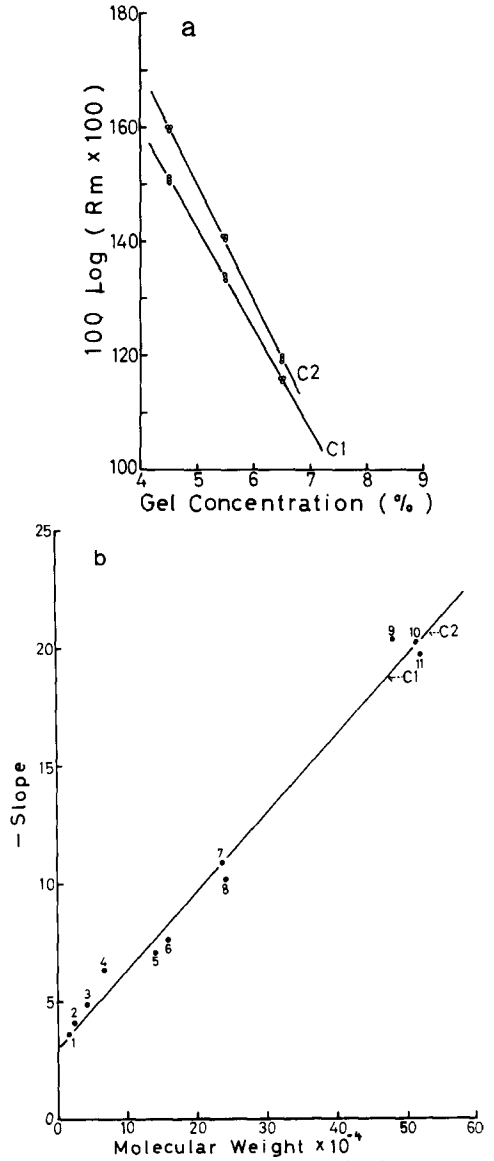


FIGURE 21 Molecular weight determination of native proteins 1 and 2. (*a*) Relative mobilities of proteins 1 and 2 in electrophoresis in acrylamide gels of different concentrations. (*b*) Relationship of molecular weights to the slopes of lines obtained as in (*a*) (19). Standard proteins: (1) horse heart myoglobin (17,800), (2) bovine pancreatic chymotrypsinogen (25,000), (3) hen ovalbumin (45,000), bovine serum albumin (67,000), rabbit muscle lactate dehydrogenase (140,000), rabbit muscle aldolase (158,000), rabbit muscle pyruvate kinase (237,000), bovine liver catalase (240,000), horse apoferritin (480,000), rice ribulose diphosphate carboxykinase (515,000), *E. coli* β-galactosidase (520,000). From the calculated regression line, the molecular weights of cecropia storage proteins 1 and 2 (*C1* and *C2*) are estimated as 480,000 and 530,000, respectively.

be common with those in the hemolymph. The slower and faster migrating precipitation zones corresponded to proteins 1 and 2, respectively.

Developmental changes in the concentrations of proteins 1 and 2 in hemolymph and fat body extracts were determined by quantitative immunodiffusion (Fig. 23). In the female, both proteins first appeared in the hemolymph 3 days before spinning, reached maximum concentrations about the time of spinning, and then declined. The fat body showed steady increase in content of both proteins from spinning to the early pupa, at the same time that they were decreasing in the hemolymph, until their concentrations were much greater in the fat body than in the hemolymph. These results support quantitatively the observations from gel electrophoresis. In the male, the hemolymph concentrations rose much less and then remained rather constant while there was a limited increase in the fat body.

DISCUSSION

In the fat body of pupating cecropia silkworms, we have recognized two types of dense cytoplasmic granules, and designated them, on the basis of their staining characteristics, their fine structure, and the analysis of isolated preparations, as urate granules and protein granules. Both are surrounded by membranes. The urate granules have a distinctive uneven, fibrous-appearing internal structure. The protein granules show two internal regions, an outer less dense zone and an inner electron-dense portion that is frequently crystalline.

Bodies showing fibrous structure in the electron microscope that may have been urate granules, although described as "myelin-like", have been observed in the fat body of other silkmoths (34, 48, 49). In other insects, urate granules do not seem to have been recorded, possibly because attention was usually focussed on the early stages of metamorphosis, when little urate would have accumulated. The urate in cecropia fat body exists largely as the potassium salt (26), and our demonstration of one major protein in the urate granules suggests the presence of a protein-potassium urate complex (20).

Partially crystalline protein bodies have been previously described in cecropia fat body (4). Dense cytoplasmic granules, variously named and showing some diversity of structure but including types probably essentially similar to the protein granules of cecropia, have been observed with the electron microscope in other silkmoths

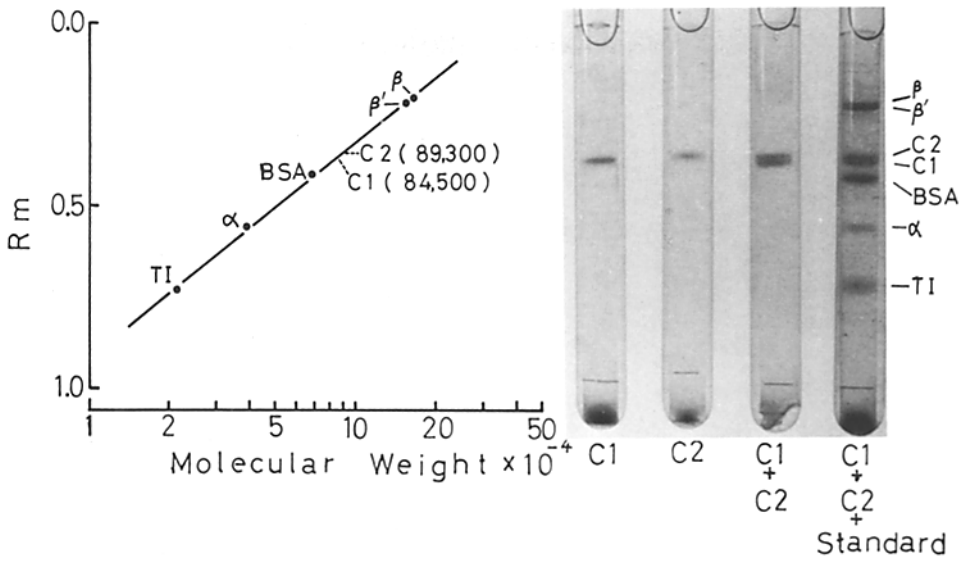


FIGURE 22 Molecular weight determination of subunits of proteins 1 and 2. Proteins were denatured by heating to 50°C from 120 min in 1% SDS-5% mercaptoethanol, and subjected to electrophoresis in 5% polyacrylamide gel containing 0.1% SDS. *TI*, soybean trypsin inhibitor (23,000); *α*, *β*, *β'*, *E. coli* RNA polymerase subunits (39,000, 165,000, 155,000); *BSA*, bovine serum albumin (67,000); *C1*, *C2*, cecropia storage proteins 1 and 2, respectively.

TABLE III
Amino Acid Composition of Cecropia Storage Proteins, Compared with Calliphorin

	Moles per cent of amino acids determined*		
	Cecropia protein 1	Cecropia protein 2	Calliphorin (reference 36)
Cysteine	trace	trace	0.5
Aspartate	13.3	13.4	11.7
Threonine	5.9	5.5	4.7
Serine	4.9	4.9	4.2
Glutamate	9.5	9.4	9.9
Proline	2.3	1.9	3.0
Glycine	5.4	3.9	5.3
Alanine	4.6	3.6	3.2
Valine	7.0	7.5	5.9
Methionine	7.0	4.9	4.5
Isoleucine	4.5	5.2	3.9
Leucine	8.6	10.6	5.8
Tyrosine	5.1	6.8	12.2
Phenylalanine	6.6	6.2	11.0
Lysine	8.5	10.0	8.1
Histidine	1.4	0.9	3.2
Arginine	5.6	5.1	3.2
Tryptophan	–	–	1.8

Proteins were hydrolyzed in 6 N HCl at 110°C for 24 h, and amino acids were determined with a JEOL JLC 6 AH analyzer (JEOL Analytical Instruments, Cranford, N. J.)

* Excluding cysteine and tryptophan, which were not determined for cecropia storage proteins.

(23, 34), the skipper, *C. ethlius* (28, 29), the queen bee (5), blowflies (39), and *Drosophila* (16). In *Calpodes* fat body, in addition to the relatively homogeneous protein granules, were found protein + RNA granules which contained fragments of rough endoplasmic reticulum and other organelles as well as occasional regions of protein crystallinity (28, 29). In cecropia, however, we did not find granules containing recognizable remnants of organelles, and the isolated granules contained little RNA.

Washed, isolated protein granules from cecropia pupal fat body dissolved readily in buffers to yield two major protein components with very close electrophoretic mobility. Isolation of protein granules from insect fat body has previously been reported only for *Drosophila* (45), but their morphology and composition were not described, and attempts to extract intact protein from them were unsuccessful. Proteins 1 and 2, the major components of cecropia protein granules, have native mol wts of 480,000 and 530,000, and subunits of 85,000 and 89,000, respectively. These values, suggesting hexameric structure, are very close to those for calliphorin and similar storage proteins of blowflies and *Drosophila* (25, 35, 47, 51). In amino acid composition, proteins 1 and 2 closely resemble each other and resemble calliphorin except that

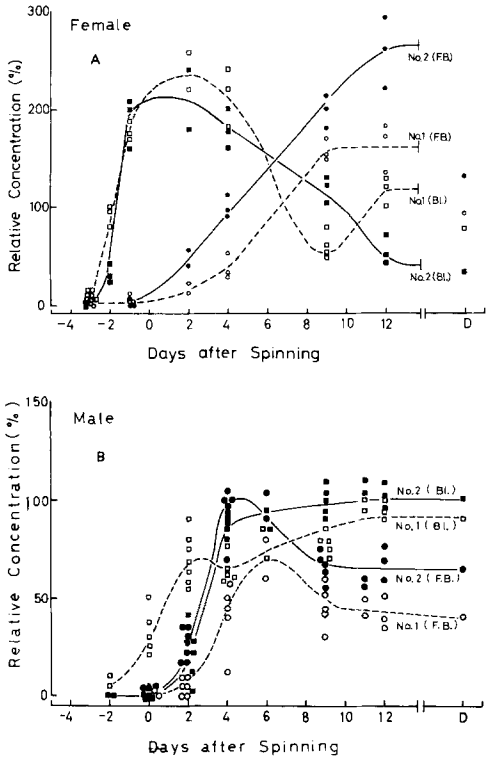


FIGURE 23 Changes in the content of proteins 1 and 2 in hemolymph and fat body of female (*A*) and male (*B*) cecropia during the larval-pupal transformation. Hemolymph and fat body extracts were prepared, and concentrations of antigens were determined by quantitative immunodiffusion, as described in Materials and Methods. Concentrations are expressed relative to a reference sample of pooled male pupal hemolymph. *FB*, fat body; *Bl*, hemolymph (blood), *D*, diapause. Points represent determinations on individual animals.

they lack the exceptionally high tyrosine and phenylalanine of the latter but contain more of other hydrophobic amino acids. A common evolutionary origin and function for these proteins is likely.

The concentrations of individual proteins in insect hemolymph, and their rates of synthesis in the fat body, change characteristically with stage of development (40, 44, 46, 53). The changes in hemolymph concentrations of proteins 1 and 2 of cecropia correspond to those previously described for antigen 6 by Telfer and Williams (44). Proteins 1 and 2 first appear in the larval hemolymph three days before spinning and reach maximal concentrations about the time of spinning. Synthesis and secretion of these proteins by cecropia fat body incubated in vitro with ^{3}H-leucine has been shown by Pan (37), who precipitated them from the medium with specific antiserum, and we have confirmed this (unpublished data). Production is active during several days before spinning, and falls off sharply by the day of spinning, to approach zero by the larval-pupal ecdysis. During the 8–10 days after spinning, the levels of the two proteins decline in the hemolymph and increase in the fat body at the same time as the appearance of visible intracellular protein granules. The observations indicate that these proteins are synthesized in massive amounts in the fat body during the final feeding period of the larva and secreted into the hemolymph for temporary storage, and then, during the first phase of metamorphosis, taken back into the fat body and deposited in semi-crystalline granules. Remarkable developmental regulation of fat body cell function is implied in the switch from an actively biosynthetic to a principally storage role. The capacity of the fat body to withdraw protein selectively from the hemolymph resembles the later activity of the ovary in taking up vitellogenins for deposition in yolk (43). Evidence indicating selective uptake of proteins from hemolymph into fat body has already been obtained for several other species on insects (9, 10, 11, 32).

The pattern just described is observed in the cecropia female. Males accumulate much less protein in the fat body, as previously reported for *Calpodes* (29), and the deposition of granules is not accompanied by a decrease in the levels of the specific proteins in the hemolymph. Possibly, the male fat body, not having to make vitellogenin, which the female fat body synthesizes from about the time of spinning (38), may continue to produce storage proteins for a longer time.

Although the fate of the granules and their constituent proteins has not been studied, it is probable that they provide reserves needed for synthesis in the developing adult tissues. Our observations, together with those of others on blowflies and other insects, indicate that the synthesis and deposition in the fat body of a class of storage proteins is a general major process in insect metamorphosis.

The authors are grateful to Dr. W. H. Telfer for initially proposing this investigation, to Dr. M. L. Pan for suggestions and assistance, to Dr. Y. Nagata for performing amino acid analysis, and to Dr. P. Couble for help with photography.

The work was supported by grants to W. H. Telfer from the National Science Foundation (GB-40154), and to G. R. Wyatt from the National Institutes of Health (HD-02176) and the Whitehall Foundation.

Received for publication 26 July 1977, and in revised form 15 May 1978.

REFERENCES

1. APPETT, A. W., and V. A. CVANCARA. 1969. An improved procedure for the determination of uric acid in biological homogenates. *Anal. Biochem.* **25:**588–590.
2. BADE, M. L., and G. R. WYATT. 1962. Metabolic conversions during pupation of the cecropia silkworm. I. Deposition and utilization of nutrient reserves. *Biochem. J.* **83:**470–482.
3. BERLESE, A. 1899. Osservazioni sur fenomeni che avvengono durante la ninfosi insetti metabolici. *Riv. Pathol. Veg.* **8:**1–163.
4. BHAKTHAN, N. M. G., and L. I. GILBERT. 1972. Studies on the cytophysiology of the fat body of the American silkmoth. *Z. Zellforsch. Mikrosk. Anat.* **124:**433–444.
5. BISHOP, G. H. 1958. Nuclear and cytoplasmic changes in fat body cells of the queen bee during metamorphosis. *J. Exp. Zool.* **137:**501–516.
6. BROWN, H. 1945. The determination of uric acid in human blood. *J. Biol. Chem.* **158:**601–608.
7. BURTON, K. 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem. J.* **62:**315–323.
8. CHINZEI, Y., and S. TOJO. 1972. Nucleic acid changes in the whole body and several organs of the silkworm, *Bombyx mori,* during metamorphosis. *J. Insect Physiol.* **18:**1683–1698.
9. CHIPPENDALE, G. M. 1971. Selective protein storage by the fat body of the angoumois grain moth, *Sitotroga cerealella. Insect Biochem.* **1:**122–124.
10. CHIPPENDALE, G. M., and B. A. KILBY. 1969. Relationship between the proteins of the haemolymph and fat body during development of *Pieris brassicae. J. Insect Physiol.* **15:**905–926.
11. COLLINS, J. V., and A. E. R. DOWNE. 1970. Selective accumulation of haemolymph proteins by the fat body of *Galleria mellonella. J. Insect Physiol.* **16:**1697–1708.
12. DAVIS, B. J. 1964. Disc electrophoresis-II. Method and application to human serum proteins. *Ann. N. Y. Acad. Sci.* **121:**406–427.
13. DITTEBRANDT, M. 1948. Application of the Weichselbaum biuret reagent to the determination of spinal fluid protein. *Am. J. Clin. Pathol.* **18:**439.
14. DUBOIS, M., K. A. GILLES, J. K. HAMILTON, P. A. RERERS, and F. SMITH. 1956. Colorimetric determination of sugars and related substances. *Anal. Chem.* **28:** 350–356.
15. FLAX, M. H., and M. H. HIMES. 1952. Microspectrophotometric analysis of metachromatic staining of nucleic acids. *Physiol. Zool.* **25:**297–311.
16. VON GAUDECKER, B. 1963. Uber den Formwechsel einiger Zellorganelle bei der Bildung der Reservestoffe im Fettkorper von Drosophila Larven. *Z. Zellforsch. Mikrosk. Anat.* **61:**56–95.
17. GILBERT, L. I. 1967. Lipid metabolism and function in insects. *Adv. Insect Physiol.* **4:**69–211.
18. GOMORI, G. 1952. Microscopic Histochemistry. University of Chicago Press, Chicago, Ill. 118–120.
19. HEDRICK, J. L., and A. J. SMITH. 1968. Size and charge isomer separation and estimation of molecular weights of proteins by disc electrophoresis. *Arch. Biochem. Biophys.* **126:**155–164.
20. HOPKINS, T. L., and P. A. LOFGREN. 1968. Adenine metabolism and urate storage in the cockroach, *Leucophaea maderae. J. Insect Physiol.* **14:**1803–1814.
21. HUMASON, G. L. 1962. Animal Tissue Techniques. W. H. Freeman and Company, San Francisco and London. 2nd Edition.
22. ISHEWATA, S. 1928. Sexual differences in the larvae of silkworms and some other moths. Proceedings of the Third Pan-Pacific Science Congress. **2:**2171.
23. ISHIZAKI, H. 1965. Electron microscopic study of changes in the subcellular organization during metamorphosis of the fat body cell of *Philosamia cynthia ricini* (Lepidoptera). *J. Insect Physiol.* **11:**843–855.
24. KILBY, B. A. 1963. The biochemistry of the insect fat body. *Adv. Insect Physiol.* **1:**111–174.
25. KINNEAR, J. F., AND J. A. THOMSON. 1975. Nature, origin and fate of major haemolymph proteins in *Calliphora. Insect Biochem.* **5:**531–552.
26. JUNGREIS, A. M., and S. TOJO. 1973. Potassium and uric acid content in tissues of the silkmoth *Hyalophora cecropia. Am. J. Physiol.* **224:**21–26.
27. LINZEN, B., and G. R. WYATT. 1964. The nucleic acid content of tissues of cecropia silkmoth pupae. Relations to body size and development. *Biochim. Biophys. Acta.* **87:**188–198.
28. LOCKE, M., and J. V. COLLINS. 1965. The structure and formation of protein granules in the fat body of an insect. *J. Cell Biol.* **26:**857–884.
29. LOCKE, M., and J. V. COLLINS. 1968. Protein uptake into multivesicular bodies and storage granules in the fat body of an insect. *J. Cell Biol.* **36:**453–483.
30. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:**265–275.
31. LUFT, J. H. 1961. Improvement of epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.* **9:**409–414.
32. MARTIN, M. D., J. F. KINNEAR, and J. A. THOMSON. 1971. Developmental changes in the late larva of *Calliphora stygia.* IV. Uptake of plasma protein

by the fat body. *Aust. J. Biol. Sci.* **24**:291-299.
33. MAZIA, D., P. A. BREWER, and M. ATFERT. 1953. Cytochemical staining and measurement of protein with mercuric bromphenol blue. *Biol. Bull (Woods Hole)*. **104**:57-67.
34. MORI, T., H. AKAI, and M. KOBAYASHI. 1970. Ultrastructural changes of the fat body in the silkworm during postembryonic development. *J. Seric. Sci. Jpn.* **39**:51-61.
35. MUNN, E. A., A. FEINSTEIN, and G. D. GREVILLE. 1971. The isolation and properties of the protein calliphorin. *Biochem. J.* **124**:367-374.
36. MUNN, E. A., and G. D. GREVILLE. 1969. The soluble proteins of developing *Calliphora erythrocephala*, particularly calliphorin, and similar proteins in other insects. *J. Insect Physiol.* **15**:1935-1950.
37. PAN, M. L. 1969. Synthesis of vitellogenic blood protein in the cecropia silkmoth. Ph. D. Thesis. University of Pennsylvania, Philadelphia, Pennsylvania. 83 pp.
38. PAN, M. L. 1971. The synthesis of vitellogenin in the cecropia silkworm. *J. Insect Physiol.* **17**:677-689.
39. PRICE, G. M. 1969. Protein synthesis and nucleic acid metabolism in the fat body of the larva of the blowfly, *Calliphora erythrocephala*. *J. Insect Physiol.* **15**:931-944.
40. PRICE, G. M. 1973. Protein and nucleic acid metabolism in insect fat body. *Biol. Rev. Camb. Philos. Soc.*, **48**:333-375.
41. RIDDIFORD, L. M. 1968. An artificial diet for cecropia and other saturniid silkworms. *Science (Wash. D. C.)*. **160**:1461-1462.
42. TELFER, W. H. 1954. Immunological studies of insect metamorphosis. II. The role of a sex-linked blood protein in egg formation by the cecropia silkworm. *J. Gen. Physiol.* **37**:539-558.
43. TELFER, W. H. 1965. The mechanism and control of yolk formation. *Annu. Rev. Entomol.* **10**:161-184.
44. TELFER, W. H. and C. M. WILLIAMS. 1953. Immunological studies of insect metamorphosis. I. Qualitative and quantitative description of the blood antigens of the cecropia silkworm. *J. Gen. Physiol.* **36**:389-413.
45. THOMASSON, W. A., and H. K. MITCHELL. 1972. Hormonal control of protein granule accumulation in fat body of *Drosophila melanogaster* larvae. *J. Insect Physiol.* **18**:1885-1899.
46. THOMSON, J. A. 1975. Major patterns of gene activity during development in holometabolous insects. *Adv. Insect Physiol.* **11**:321-398.
47. THOMSON, J. A., K. R. RADOK, D. C. SHAW, M. J. WHITTEN, G. G. FOSTER, and L. M. BIRT. 1976. Genetics of lucilin, a storage protein from the sheep blowfly, *Lucilia cuprina* (Calliphoridae). *Biochem. Genet.* **14**:145-160.
48. WAKU, Y., and K. SUMIMOTO. 1969. Light and electron microscopical study of the fat body cells in the metamorphosing silkworm. *Bull. Fac. Text. Fibers, Kyoto Univ. Ind. Arts Text. Fibers.* **5**:256-287.
49. WALKER, P. A. 1966. An electron microscope study of the fat body of the moth *Philosamia* during growth and metamorphosis. *J. Insect Physiol.* **12**:1009-1018.
50. WIGGLESWORTH, V. B. 1972. The Principles of Insect Physiology, 7th ed. Methuen & Co., Ltd., London, 442-447.
51. WOLFE, J., M. E. AKAM, and D. B. ROBERTS. 1977. Biochemical and immunological studies on larval serum protein. I. The major hemolymph protein of *Drosophila melanogaster* third-instar larvae. *Eur. J. Biochem.* **79**:47-53.
52. WYATT, G. R. 1975. Regulation of protein and carbohydrate metabolism in insect fat body. *Verh. Dtsch. Zool. Ges.* **1974**:209-226.
53. WYATT, G. R., and PAN, M. L. 1978. Insect plasma proteins. *Annu. Rev. Biochem.* **47**:779-817.