PROTEIN UPTAKE INTO MULTIVESICULAR BODIES AND STORAGE GRANULES IN THE FAT BODY OF AN INSECT

M. LOCKE and JANET V. COLLINS

From the Developmental Biology Center, Case Western Reserve University, Cleveland, Ohio 44106

ABSTRACT

The fat body in *Calpodes ethlius* (Lepidoptera, Hesperiidae) takes up protein from the blood throughout the larval stage before pupation. Depending upon the phase of development, the protein appears in multivesicular bodies, in large storage granules, and in structures of intermediate form. There are three phases in the 8 days of the last larval stage; the first devoted to growth (molting to 66 hr), the second to synthesis for storage or export (M + 66 to M + 156 hr), and the third to preparation for pupation (M + 156 to pupation at M + 192 hr). From M + O to M + 156 and from M + 180 to M + 188 hr, protein is taken up into multivesicular bodies. Larger MVB's form a continuous series with the protein granules formed from M + 162 to M + 180 hr. Blood proteins increase in concentration and amount from M + 66 to M + 156 hr at the same time as the fat body cells have a high rate of incorporation of amino acids and a structure appropriate for protein synthesis. During granule formation, both amino acid incorporation and blood protein concentration decrease. Since foreign proteins injected into the blood appear in the granules, they are probably made mainly from sequestered blood. Protein uptake involves two stages: concentration between the cells, and ingestion in pinocytotic vesicles. The vesicles fuse to become MVB's or storage granules, depending upon their rates of growth and the addition of lytic enzymes. Since MVB's do not accumulate in the fat body and since many of them contain acid phosphatase and appear empty, they are presumed to be concerned in protein turnover.

INTRODUCTION

Insect fat body is functionally similar to mammalian liver. It contains a single cell type which synthesizes, stores, and mobilizes glycogen, lipid, and protein. In *Calpodes ethlius*, the fat body cells engage in these activities sequentially as larval development progresses. These cells, therefore, provide excellent material for studying the morphological changes involved in the transition from one type of activity to another.

The formation of protein granules is one of several changes taking place in the fat body of *Calpodes ethlius* in the larval stage before pupation. The granules are of several sorts which differ in their content and method of formation. The main features of their structure and some aspects of their formation have already been described (26). In that report, the origin of the granules was ascribed to four processes: (1) the packaging of protein by the Golgi complex; (2) the isolation of cell components by paired membranes for immediate or delayed lysis; (3) growth of granules by fusion at various stages of their development; and (4) growth of granules by the addition of microvesicles at the granule surface. The above

description does not explain the formation of the large protein storage granules arising in the fat body at pupation. The problem has been (1) to determine the origin of the protein in the granules, and (2) to define the mechanisms of granule formation. Tracing the fate of injected plant peroxidase has revealed that the fat body sequesters blood proteins for hydrolysis in multivesicular bodies during the intermolt and for storage at pupation (7, 27), indicating that a fifth process operates in granule formation, in addition to those previously described. This paper completes the description of the origin of protein granules in the fat body.

MATERIALS AND METHODS

The Experimental Animal

Calpodes ethlius Stoll (Lepidoptera, Hesperiidae) has several advantages for studies in developmental cytology. It is easily reared, is disease free, and has a relatively short life cycle. It has five larval instars. The 5th stadium is the longest (192 ± 13 hr at 22°C). The pupal stadium lasts about 10 days. The changes in the larva which mark its progress from one stage to the next occur at precise times in the stadium, thus making it easy to perform experiments on large numbers of animals of the same physiological age. In addition, the ultrastructure of several tissues is now known in detail, and provides a useful basis for comparison and correlation of the changes observed in the fat body.

This study has concentrated on events in the 5th larval stadium, including the 4th to 5th and 5th to pupal molts. The larvae were reared in a greenhouse; towards the end of the 4th or 5th stadium they were transferred to a 22°C incubator in which they were observed for the timing of various external changes marking their physiological stage. The most useful changes for this study occur during the last 30 hr of the 5th stadium.

Tracer Proteins

We used several foreign proteins as tracer molecules to observe the sequestration process. The aim was to use molecules which could be localized by means of histochemical techniques for light or electron microscopy. Among the molecules employed (plant lipase and acid phosphatase, ferritin, rabbit serum globulin, and fluorescent anti-rabbit serum globulin, algal protein-^{14}C, horseradish peroxidase), the peroxidase proved to be the most useful for both light and electron microscopy and was used routinely. It is readily visualized as the brown product of the reaction with benzidine or diaminobenzidine. In addition to the direct observation of the brown color, the site of peroxidase localization could be detected by fluorescence microscopy, since the brown product quenched the natural yellow fluorescence of the fat body protein granules.

A standard procedure was developed for using the horseradish peroxidase as an in vivo protein tracer. Larvae of known ages were injected with a solution of peroxidase (Sigma Type II; Worthington HPOD) in insect Ringer's (NaCl, 11.0 g/l; KCl, 1.4 g/l; $CaCl_2$, 1.1 g/l), and were allowed 4 hr for incorporation. In the earlier experiments, the usual dose was 0.1 ml of a solution containing 1 mg/0.1 ml; this dose was later reduced as the technique for visualizing the enzyme was refined. The methods used for demonstrating the sites of peroxidase activity are modifications of those developed for the light microscope by Straus (49, 50) and for the electron microscope by Graham and Karnovsky (19). After many trials, we found the following procedure to be satisfactory. Fixed fat body was first equilibrated for 10 min on an Eberbach oscillatory shaker in an incubation medium of 0.03% benzidine hydrochloride or diaminobenzidine in 0.05 M veronal-acetate buffer at pH 7.0. Hydrogen peroxide was then added to make a final strength of 0.03% and the mixture shaken for 6 min more. The penetration of the reagent into the tissues was greatly facilitated by the vigorous agitation of the oscillatory shaker. For light microscopy, the tissues were first reacted with the benzidine mixture and then embedded in ester wax for routine sectioning. For electron microscopy, they were postfixed in osmium tetroxide and processed as usual. The sites of peroxidase activity could be visualized because of the increased electron opacity deriving from the reaction between the osmium and the oxidized diaminobenzidine. For survey work and controls, thick sections were photographed unstained. For better resolution, thin sections were lightly stained in either lead citrate or uranyl acetate.

The Determination of Blood Protein Concentration

The protein concentration of the hemolymph was measured by the Biuret reaction, according to the method of Dittebrandt (12). Blood samples were collected from larvae of known ages by pricking a proleg, or from pupae by cutting the tip of the proboscis. Duplicate samples of 20–50μl were taken with either a 50-μl micropipette or a 100-μl serological pipette calibrated to deliver a specified volume.

The samples were immediately diluted to a final volume of 2.5 ml (including the Biuret reagent). They were incubated at 37°C for 30 min and read on a Beckman spectrophotometer at 550 mμ. Bovine serum albumin was used for all standard solutions.

Tissue Preparation for Light and Electron Microscopy

The larvae were fixed by standard procedures for light and electron microscopy (26). They were fixed in glutaraldehyde (5% with 0.05 M cacodylate buffer, pH 7.4) at 0–4°C for 2–18 hr and stored at 0–4°C in 10% sucrose with 0.05 M cacodylate buffer at pH 7.4. For the localization of peroxidase, it is essential that sucrose not be added to the glutaraldehyde. For electron microscopy, the tissues were postfixed in 1% osmium tetroxide buffered at pH 7.4 and containing 4% sucrose, dehydrated in alcohol or acetone, and embedded in Araldite. Thin sections were stained routinely for 10 min with saturated uranyl acetate in 70% ethanol and absolute methanol (1:1) (24) and then for 5 min with lead citrate, pH 12.0 (38). During staining, the grids were handled in a device which allows easy manipulation and consistently gives no contamination (26). Light microscope observations were made either on thick sections cut from Araldite blocks or on 4–8-μ ester wax sections.

The electron micrographs were taken on an RCA EMU 3 F microscope operated at 100 kv with a 25-μ objective aperture and a high magnification specimen holder, or at 50 kv with a 35-μ objective aperture and a low magnification holder. Micrographs were taken in a through-focus series, at magnifications of 4,000–22,000.

Histochemistry

Acid phosphatase activity was observed with both the light and electron microscopes on tissue prepared by the methods of Miller and Palade (31). The clearest contrast between reaction product and resolvable cell components was obtained after staining in lead citrate only.

The contents of the granules in the fat body cells were characterized with several histochemical techniques and protein stains including bromphenol blue, Millon's reagent, the Morel-Sisley diazotization with 1-amino-8-naphthol-4-sulfonic acid (S-acid), and the dinitro-fluoro-benzene (DNFB) method followed by diazotization and treatment with 1-amino-8-napthol-3:6-disulfonic acid (H-acid).

Radioautography

Tritiated tyrosine (Schwarz BioResearch, Orangeburg, N.Y.) with a specific activity of 0.5 c/mmole was injected into larvae of various ages in the 5th stadium, in doses of 20 μc/g of larval weight. Larvae were fixed after 2 hr of incorporation and prepared for light microscopy. Tissue sections were coated with Kodak nuclear emulsion NTB 3 and were exposed at −10°C for 14 days.

RESULTS

Preliminary Observations on the Fat Body of 5th Instar Larvae

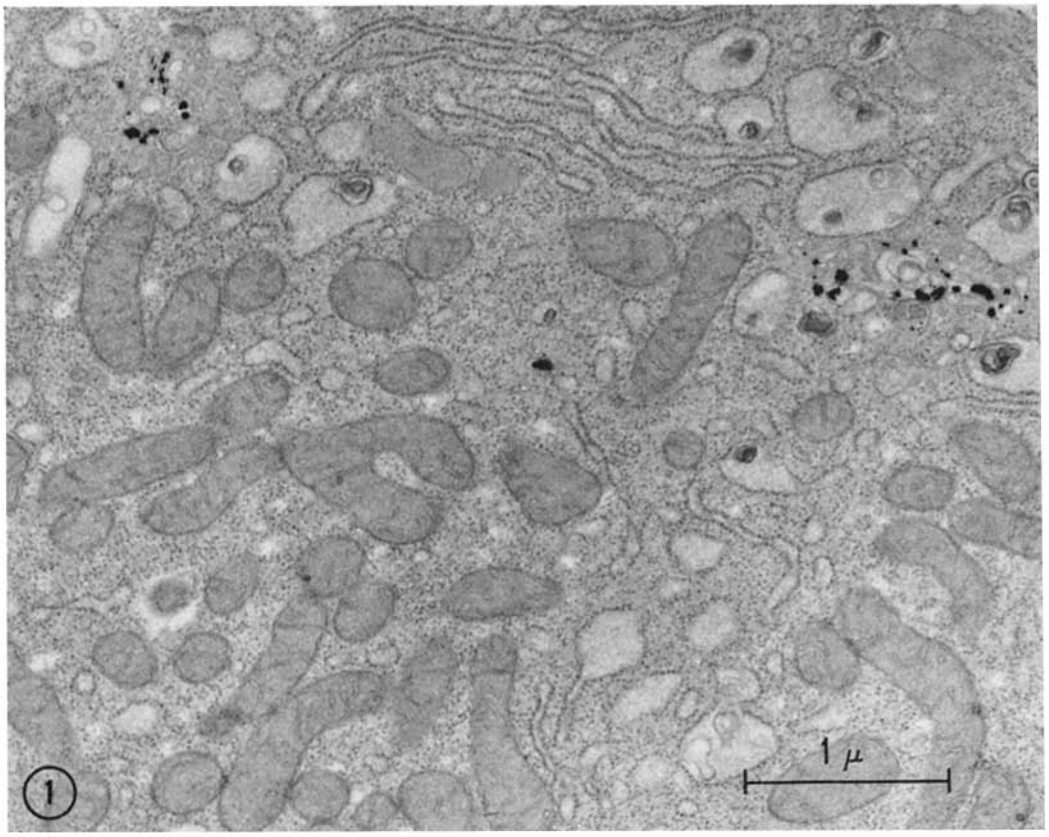
The fat body of the larva is composed of sheets of cells, two layers thick, forming thin ribbons attached in a few places to the integument but otherwise floating freely in the hemolymph. The entire outer surface of the tissue is covered by a basement membrane which is not a barrier to macromolecules. At pupation, the cells lose their adhesions to one another, and the ribbons become clumps. The cells are then more nearly spherical and are kept in place only by the basement membrane.

The 5th stadium may be divided into three phases. The first phase occupies the period from ecdysis at the 4th to 5th molt up to the critical period for the operation of the brain hormone on the prothoracic glands 66 hr later. Radioautographic studies show that this is a phase of nuclear replication and RNA synthesis.[1] Figs. 1 and 2 show the appearance of the fat body cytoplasm at the beginning of this period. The cells are small and contain many mitochondria. The saccules of the Golgi complexes contain acid phosphatase but there are no large secretory vacuoles. By 66 hr after molting (M + 66 hr), the fat body cells have small droplets and no glycogen. They are intensely basophilic with many free ribosomes but little endoplasmic reticulum (RER), although the Golgi complexes are beginning to have vacuoles of secretory material (Fig. 3).

During the second phase, from M + 66 to M + 156 hr, there is a variety of syntheses: free ribosomes decrease in number as the rough endoplasmic reticulum (RER) appears. Dense material accumulates in the cisternae, and the Golgi complex contains much larger vacuoles of secretion (Fig. 4). Small lipid droplets become larger, occupying 40% of the cell volume, and extensive glycogen deposits are laid down (Fig. 5). The basophilia is restricted to the cytoplasm between these reserves.

The third phase is initiated at the time of the critical period for the operation of the prothoracic glands upon the tissues to induce molting (M + 156 hr).The larva is then at its maximal size. Shortly afterwards the gut empties, in preparation for pupation. Protein granules appear suddenly

[1] Locke, M. 1967. Unpublished observations.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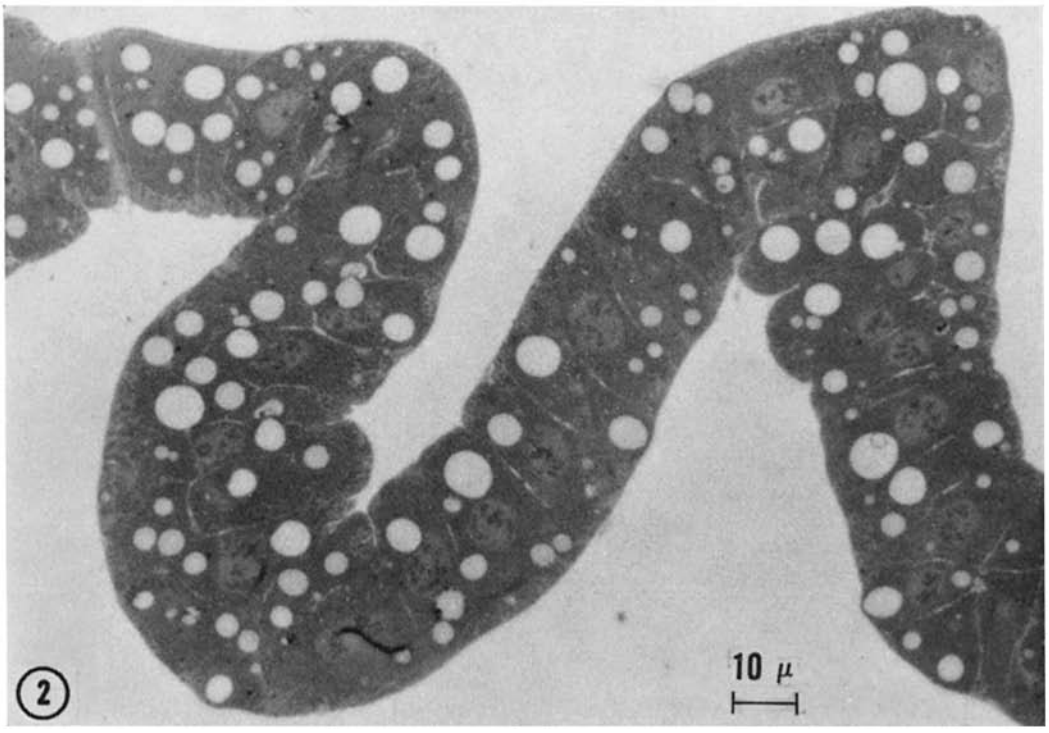
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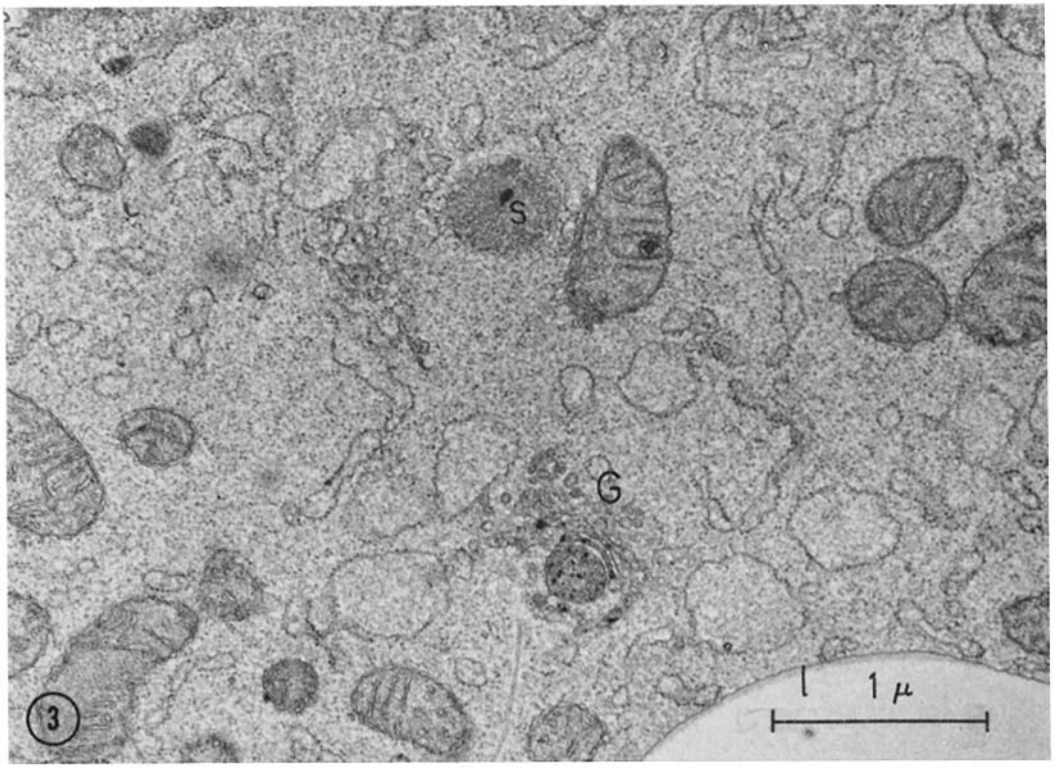

FIGURE 3 The fat body at the end of the first phase (M + 68 hr). By this time, the cells have undergone a period of RNA synthesis. The population of free ribosomes has increased markedly, but there is still little rough ER. Small lipid droplets are present, and the Golgi complex has begun to produce secretory vacuoles, some of which contain acid phosphatase. Acid phosphatase; stained with lead citrate and uranyl acetate. *G*, Golgi complex; *l*, lipid; *s*, Golgi secretion. 50 kv. × 28,000.

in the fat body at a time when the gut is half empty (M + 162 hr). They continue to form over the next 18–20 hr, after which they occupy 15–25% of the cell volume. There is a marked sex difference in the amount of protein stored by the fat body (Fig. 6). In the female, the granules occupy about 25% of the cell, but in the male only about 15%. Several histochemical stains were used to characterize the granules. The intensity of staining showed that the granules contain protein with a high proportion of aromatic amino aids. The granules also have a strong natural yellow fluorescence when excited by ultraviolet light. In some older granules the center becomes crystalline, like the albuminoid crystalloids described by Hollande (20).

Just before and coincident with the formation of the first protein granules, there is a phase of

FIGURE 1 The fat body at the beginning of the first phase of the stadium 2 hr after the 4th–5th molt. The cells contain numerous mitochondria and free ribosomes, but little rough ER and no lipid droplets. The Golgi vesicles and saccules contain some secretory products such as acid phosphatase, but there are no large secretory vacuoles. Acid phosphatase; stained with lead citrate and uranyl acetate. 50 kv. × 26,000.

FIGURE 2 The fat body at the beginning of the first phase of the stadium (M + 23 hr). The lipid droplets are small, and the cytoplasm is dense and basophilic. Araldite section stained with methylene blue. × 700.

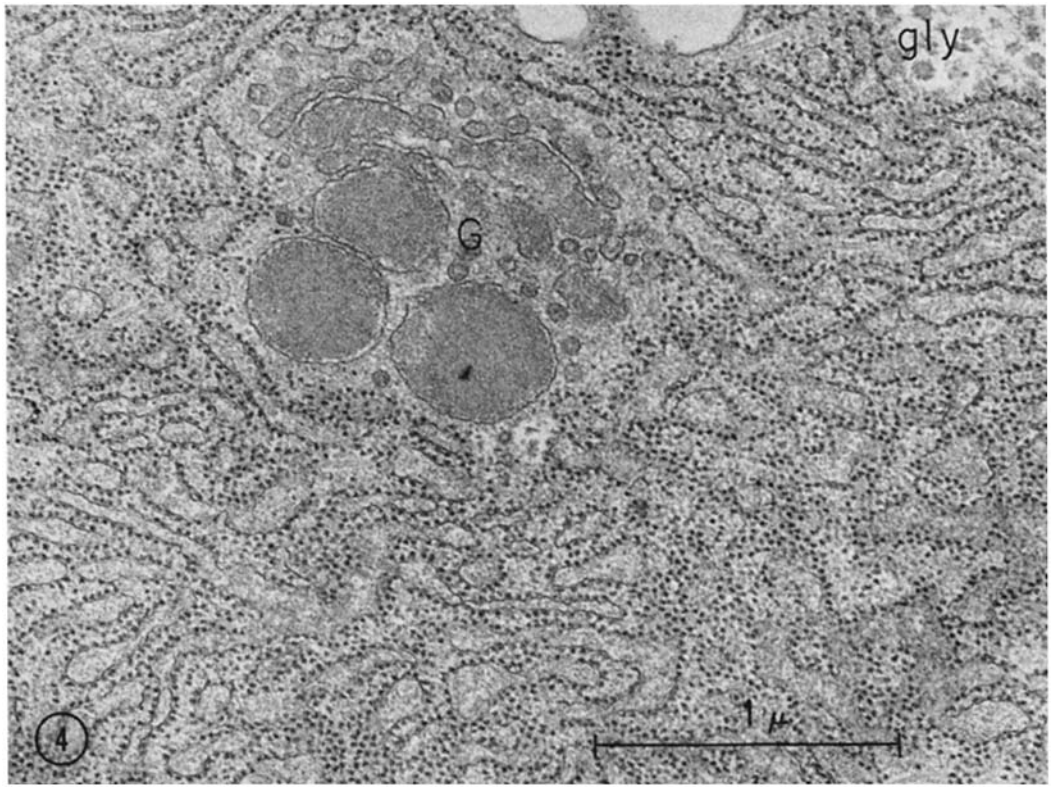

FIGURE 4 The fat body during the phase of synthesis and secretion (M + 66 to M + 156 hr). The cells are engaged in the synthesis of lipid, glycogen, and protein, and the rough ER is extensive. Large secretory droplets arise from the Golgi complex. Stained with lead citrate and uranyl acetate. *G*, Golgi complex; *gly*, glycogen. 100 kv. × 40,000.

autolysis: the mitochondria are isolated by paired membranes in bodies which fuse to become large autophagic vacuoles (25, 26). Immediately after protein granule formation ceases (at about M + 182 hr), there is another phase of autolysis: the ER is isolated and concentrated into granules containing RNA and protein (26). Thus, the main phase of protein granule formation is flanked in time by bursts of autolytic activity.

These observations established the basic structure and developmental history of the fat body cells and the timing and extent of protein granule formation summarized in Fig. 25. This paper is concerned primarily with the origin and mechanism of formation of the protein granules.

Protein Synthesis and Granule Formation

The rate of accumulation of granules when they suddenly appear indicates intense activity on the part of the fat body. If the fat body protein is synthesized in situ from amino acids from the blood, then there should be a very high rate of amino acid incorporation. A series of radioautographic experiments was performed so that we could determine whether the protein granules contained newly synthesized proteins.

Tyrosine-^{3}H (20 μc/g) was injected into the hemocoel of a batch of 70 larvae of various ages in the 5th stadium. Tyrosine was chosen primarily because the histochemical preparations showed that the proteins of the granules are rich in aromatic amino acids. An experiment with leucine-^{3}H gave qualitatively similar results but was not quantified. The larvae were allowed to incorporate the precursor for 2–16 hr, and were then prepared for radioautography. Fig. 7 shows the results after a 2-hr incorporation period for one series of various ages selected to span the stadium. Early

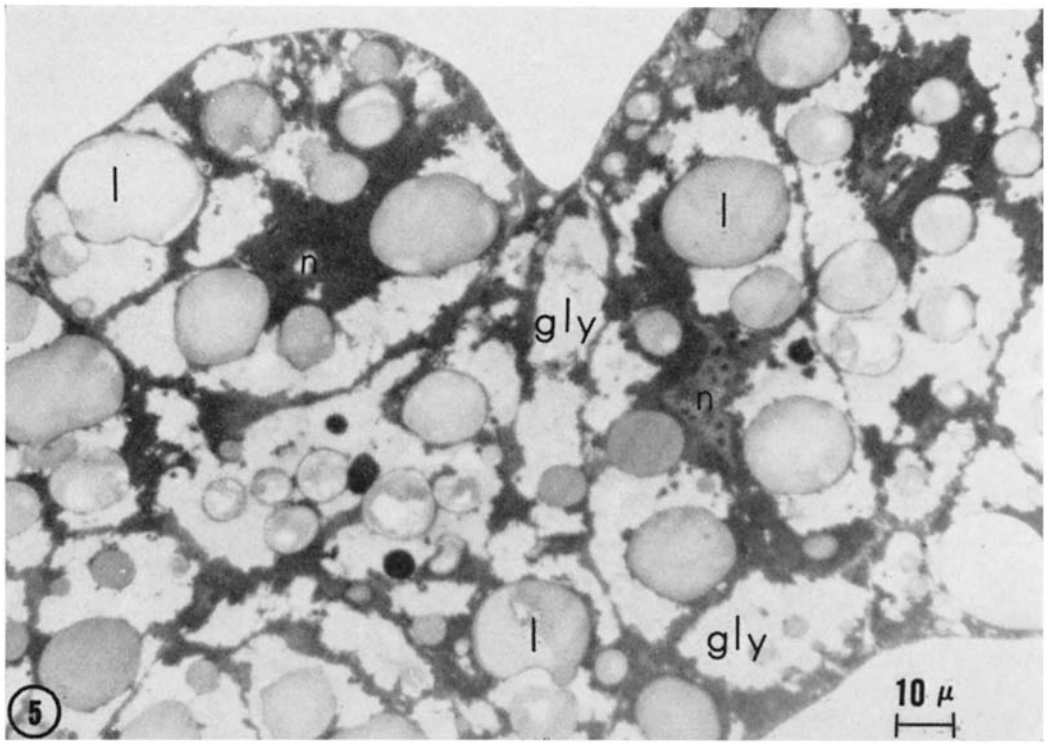

FIGURE 5 The fat body during the phase of synthesis and secretion (M + 66 to M + 156 hr). The ER is restricted to strands around the nucleus and lipid droplets. Araldite section stained with methylene blue. The lipid droplets and areas containing glycogen are unstained. *n*, nucleus; *l*, lipid; *gly*, glycogen. × 700.

in the stadium, the incorporation is low. The incorporation is highest in the central phase between the critical periods and falls again at the time of granule formation. The results agree with the electron microscope observations which show that the RER is not extensive until after the head critical period. From M + 66 to M + 156 hr, the Golgi complexes appear most active with large vacuoles. Just before protein granule formation, the Golgi vacuoles are again small or absent.

The important point with respect to the granules is that the grain count on the radioautographs is low during their formation relative to other times, and that there are few grains over the granules themselves. Longer periods of incorporation did not result in any more labeled protein. The grain count over the granules is also very low relative to that in the epidermis during cuticle formation. Cuticle deposition is an example of protein secretion comparable in magnitude to the accumulation of granules in the fat body. The grains over newly secreted cuticle in these preparations are too dense to be counted.

The low incorporation of amino acids into the fat body at the time of granule formation contrasts with the higher incorporation during the preceding 90 hr. The results clearly show that protein synthesis in the fat body does not match the appearance of the granules. We may conclude that little protein is synthesized during granule formation or that the protein synthesized turns over very rapidly. The granules, therefore, contain little protein which has been newly synthesized in situ.

The Relationship Between the Blood Proteins and Granule Formation

Granule formation involves a massive increase in the amount of protein stored in the fat body. If the blood is the source of this protein, we might expect its protein content to show an equally

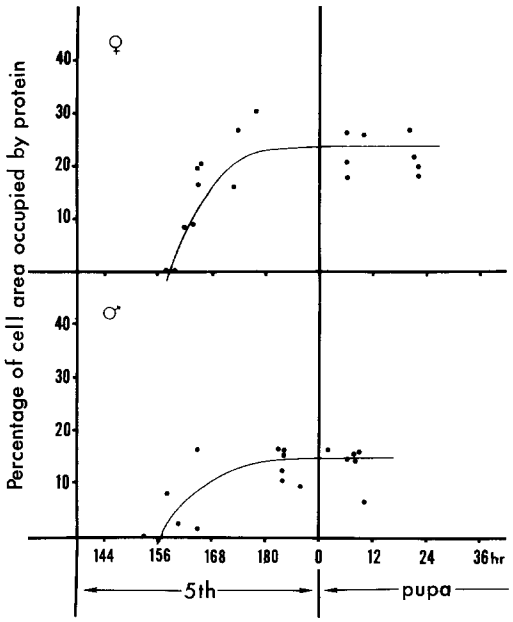

FIGURE 6 The proportion of the cell occupied by protein granules at pupation. Each point represents data from 10 counts on sections of one animal. The values were calculated from the number of dots which lay over protein granules, using a randomly dotted ocular grid with 50 dots (8). The proportion of the cell area containing granules begins to increase 30 hr before pupation. The fat body of the female contains more protein in granules than that of the male.

large reduction due to granule formation: there might be a rise in blood protein correlated with the observed synthesis of protein by the fat body and then a fall in blood protein as the protein is removed from circulation.

The blood protein concentration was measured in a series of larvae of all ages in the 5th stadium; measurements were also made during the last 24 hr of the 4th stadium and the first 24 hr of the pupal stadium. The mean values for each larva are plotted against age in Fig. 8.

The blood protein concentration is constant for the first 66 hr. Following the critical period for the action of the brain hormone on the prothoracic glands, there is a steady increase in blood protein concentration. The peak concentration occurs just before the fat body begins to sequester protein to make the granules, at about the time of the critical period for the initiation of molting at M + 156 hr. After the critical period, the blood protein concentration declines rapidly to about the same value as at M + 0 to M + 66 hr.

The blood volume was also measured in a series of larvae throughout the stadium. The volume increased with feeding and gain in body weight, but remained constant in proportion at 30% of the body volume. Thus, the increase in blood protein concentration during the middle third of the stadium and the decrease during protein granule formation reflect changes in the amount of blood protein.

The main increase in blood protein occurs when the larva is feeding and there is general growth of the tissues. The formation of protein storage granules, on the other hand, occurs only at the end of this phase and is not proportional to the protein level of the blood. The sharp decline in the blood protein level correlates exactly with the increase in density of protein granules in the fat body.

There is a marked sexual difference in the amount of protein circulating in the blood, the female having more blood protein than the male. Just before granule formation, the average blood protein concentration in females is 120 mg/ml, while in males the corresponding value is 80 mg/ml (Fig. 8). The rate of increase in blood protein concentration is also greater in females. A comparable sex difference was noted in the proportion of protein stored in granules. This evidence, coupled with the measured decline in the blood protein concentration, suggests strongly that the blood makes a significant contribution to the protein in the granules.

The Sequestration of Protein to Form Granules

From ecdysis to M + 156 hr, the fat body has no stored protein visible by light microscopy. There is then an abrupt increase to 15–25% of the cell volume at a time when the blood loses protein and the fat body is not engaged in massive synthesis. The hypothesis that the fat body takes up and stores protein from the blood was therefore explored.

A single dose of peroxidase was injected into larvae for incorporation periods of 1 min–24 hr at various times to span the 5th stadium. Except during the last 4 hr before pupation, when the cells are more or less spherical with large spaces between them, the peroxidase was always concentrated in channels and intercellular spaces. From M + 162 to M + 180 hr, the peroxidase was readily identified in variable concentrations

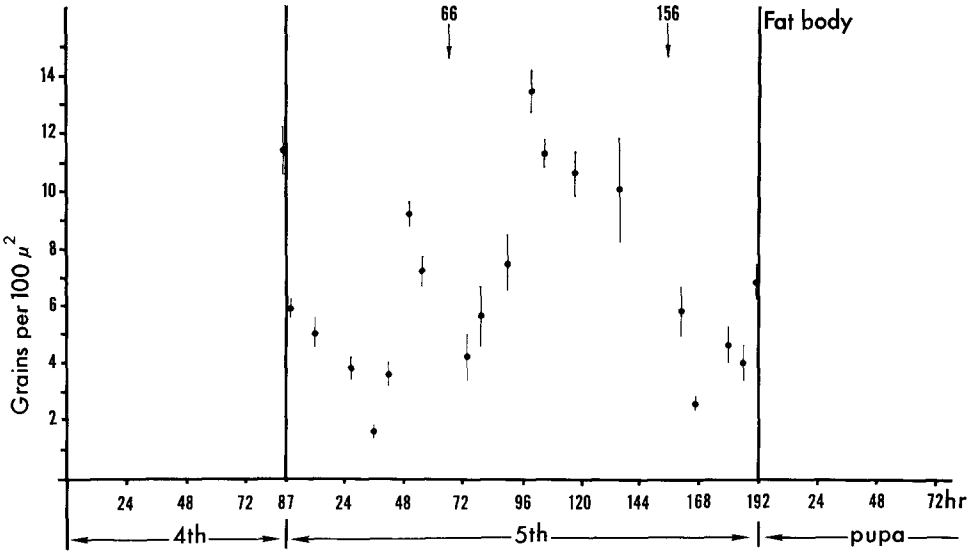

FIGURE 7 The relative amounts of tyrosine-^{3}H incorporated into the fat body of 5th instar larvae of different ages throughout the stadium, after a 2-hr incorporation period. The arrows at 66 and 156 hr mark the critical periods for the action of the brain and prothoracic glands, respectively. The points on the graph are the means from two separate sets of grain counts on a series of radioautographs selected, to span the stadium, from the group of 70 larvae injected. These data are similar to those obtained from counts on the entire series. The two high values at 55–60 hr are probably related to the change in synthetic activity taking place at 66 hr. The incorporation is low during the third phase when protein granules accumulate.

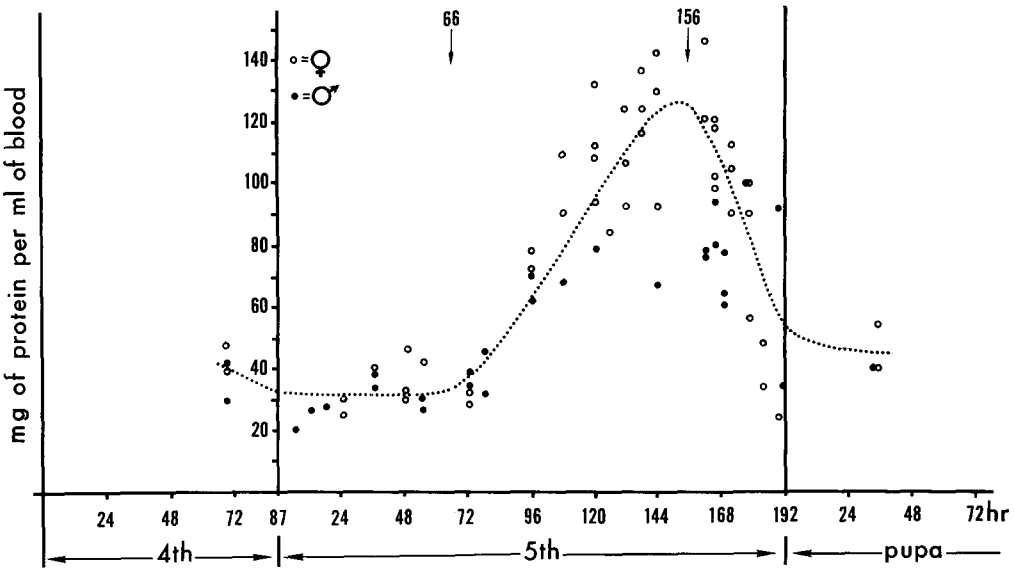

FIGURE 8 The blood protein concentration during the 5th stadium. Each point represents data from a single animal. The curves were fitted to the points from regression lines calculated for the intervals: M + 0 to M + 78 hr, M + 72 to M + 168 hr, and M + 168 to 192 hr. The concentration is constant for about 70 hr after ecdysis, increases steadily from 70 to 156 hr, and then declines after 168 hr.

in many of the protein granules (Fig. 9). The rate of peroxidase accumulation into granules was estimated from the area of granules containing peroxidase seen in sections (Fig. 10). The highest rate of accumulation of peroxidase into protein granules coincided with the most active period of granule formation (Fig. 6), and with the time at which blood protein was depleted (Fig. 8).

Protein Uptake by the Fat Body Before and After Granule Formation

Although the concentration of blood protein is high during much of the stadium, the large granules only appear in the fat body shortly before pupation. Superficially, it appeard that protein sequestration only occurred then. It was, therefore, a considerable surprise to find, on the contrary, that protein uptake takes place throughout the stadium and is not correlated solely with the formation of large storage granules.

Between M + 0 hr and the formation of large granules, the peroxidase could be identified in very small structures, ½—5 μ in diameter, often around the nucleus (Fig. 11). The last granules formed just before pupation are again "tiny" and appear around the nucleus as granules do during the intermolt (Fig. 12). All uptake of peroxidase stops 4 hr before ecdysis.

The time taken for peroxidase to be concentrated and to appear in the "tiny" granules was estimated on a series of mid instar larvae (aged M + 80 to 100 hr) after various incorporation periods. After 1 min, peroxidase could be detected in "corners" between the cells, and 4 min was long enough for it to concentrate in other areas between the cells. During the next 2 hr these areas enlarged and the reaction became more distinct, appearing also in the projections into the cell which were later identified as pinocytosis channels. By 3 hr after injection, there was a distinct aggregation of "tiny" granules around the nucleus. These granules enlarged slightly over the next hour and showed little change for the next 24 hr.

Protein Uptake into MVB's and Granules

The peroxidase observed by light microscopy between the fat body cells, in the "tiny" granules and in the large storage granules, was taken to indicate the presence of sequestered protein. However, it is difficult to resolve the presence of natural blood protein in these positions by light microscopy with conventional stains, and the possibility remains that artefacts might be induced by the administration of the peroxidase itself. As the ultrastructure associated with the naturally sequestered protein became clearer, it was compared with the structure seen in similar larvae which had sequestered peroxidase. No qualitative difference in ultrastructure was detected between larvae with and larvae without peroxidase. We may, therefore, discard the notion that the structures seen after the injection of peroxidase are merely artefacts induced by a foreign protein. Although we cannot exclude the possibility that foreign proteins stimulate processes involved in their own uptake, we may assume that the route of entry of peroxidase into the cells is similar to the route of entry of natural proteins.

During most of the intermolt (M + 0 to M + 156 hr), the cells are joined by few desmosomes and are separated from each other by a system of spaces and channels containing dense material. This material is presumed to be protein because of its similarity to the protein contained in the granules. It is more dense than fixed blood which is occasionally encountered between other tissues. Microtubules frequently occur in the cytoplasm around the spaces filled with concentrated blood, as though they are maintaining cortical rigidity (Fig. 13). The face of the fat body cell which abuts on the hemocoel also has a ramifying network of channels opening in many places below the basement membrane. These channels do not usually contain dense deposits and they may be different from the channels on the other faces. Intracellular channels lead from the spaces between cells, and their contents enter the cytoplasm as small protein-filled vesicles which coalesce to form large vesicles and granules (Fig. 14). The tips of the intracellular channels often have a structure similar to that of the coated vesicles of Roth and Porter (41, 42), and some coated vesicles about 1,000 A in diameter may also occur within the cells. Coated vesicles have often been implicated in the transport of protein into cells (16) and they may have this function in the fat body. However, they are not so frequent as vesicles without coats, and both sorts may have a role in the transport of blood proteins to make the granules.

The electron microscope localization of peroxidase showed that the dense material between the cells contained peroxidase in the first stages of its sequestration. Fig. 15 shows an unstained section from a late intermolt larva 4 hr after the

injection of peroxidase into the hemocoel. The peroxidase activity is concentrated in the intercellular spaces and in intracellular channels which bud off into small vesicles.

Fig. 16 shows the formation of protein granules by the fusion of small vesicles and the accretion of vesicles to granules. These granules, about 0.5–1.0 μ in diameter, are the "tiny" granules seen by light microscopy. The dense amorphous contents of sequestered protein are bounded by a single unit membrane. There are few from M + 0 to M + 66 hr; thereafter, they become progressively larger and more frequent and often contain microvesicles (Fig. 17). Those granules with the highest density of microvesicles have the characteristic appearance of the multivesicular bodies described by Sotelo and Porter (47). The granules which become multivesicular bodies continue to contain peroxidase in their matrices (Fig. 18). Several authors have noted that sequestered foreign proteins appear in the matrix of multivesicular bodies (15, 17, 40). However, in the fat body the peroxidase seems to be present in at least some of the microvesicles (Fig. 19). Many of the feeder microvesicles carrying protein from the cell surface must pop through the MVB limiting membrane and survive intact for awhile. In support of this interpretation, some microvesicles within MVB's appear to have the structure of coated vesicles. This interpretation also makes it easier for us to understand how some MVB's can appear almost filled with microvesicles and yet still be concerned with protein sequestration. There is a wide variation in the size of the granules and the number of microvesicles they contain. Late in the intermolt, there are all intermediates between dense granules and membrane-bounded vacuoles empty except for a few microvesicles. The dense granules may occur anywhere in the strands of ER between lipid and glycogen deposits or around the nucleus. The multivesicular bodies typically lie around the nucleus, especially the more empty ones which are interpreted to be in later stages after the lysis of their contents.

The loss of protein from the matrix of the intermolt granules after they have incorporated microvesicles suggested that at least some of the microvesicles might be primary lysosomes carrying hydrolytic enzymes. The distribution of acid phosphatase confirmed this suggestion. Light microscopy showed that acid phosphatase was localized in granules of about the same size and distribution as those granules shown to incorporate peroxidase from the blood. (At some stages there are also autophagic vacuoles and residual bodies containing acid phosphatase. These will not be described). Electron microscopy showed that acid phosphatase was localized in the multivesicular bodies, with lead deposits in both the matrix and some microvesicles (Fig. 20). In addition, there were sites of acid phosphatase activity in some Golgi saccules and vacuoles and a few vesicles (Figs. 1, 3). The Golgi vesicles containing the enzyme are similar in size and appearance to the microvesicles within the multivesicular bodies. Just as some of the microvesicles in MVB's which had sequestered peroxidase did not contain the foreign protein, so many of the microvesicles in the acid phosphatase–reacted material did not contain acid phosphatase. We may conclude that the microvesicles may have a mixed origin. The localization of acid phosphatase provides circumstantial evidence that the lytic enzymes of the MVB are transported from the Golgi complex in microvesicles, some of which may survive intact within the MVB.

From about M + 156 to M + 162 hr, there is a phase of transition from the production of small multivesicular bodies to large storage granules. The gap between these activities is bridged by the formation of large multivesicular bodies intermediate in size between the intermolt granules and the storage granules (Figs. 21, 22). These large multivesicular bodies are so numerous that they spread into the reserves of glycogen. Like their intermolt counterparts most of them lose their contents, but while doing so, they often develop a characteristic fibrous or bristly texture before becoming empty-looking vacuoles (Fig. 23). A few of these granules remain dense and have a few or no microvesicles (Fig. 24). Presumably, these are the ones which are larger by M + 162 hr and continue to accrete vesicles and grow into storage granules.

The formation of the protein storage granules from M + 162 to M + 180 hr is a major event in the life history of the fat body. The cells become more globular and adhere less tightly to one another so that the intercellular spaces become even larger than in the intermolt and may contain more concentrated protein. The events leading to the formation of the storage granules are similar to those occurring in the formation of the intermolt granules but the channel systems and vesicles

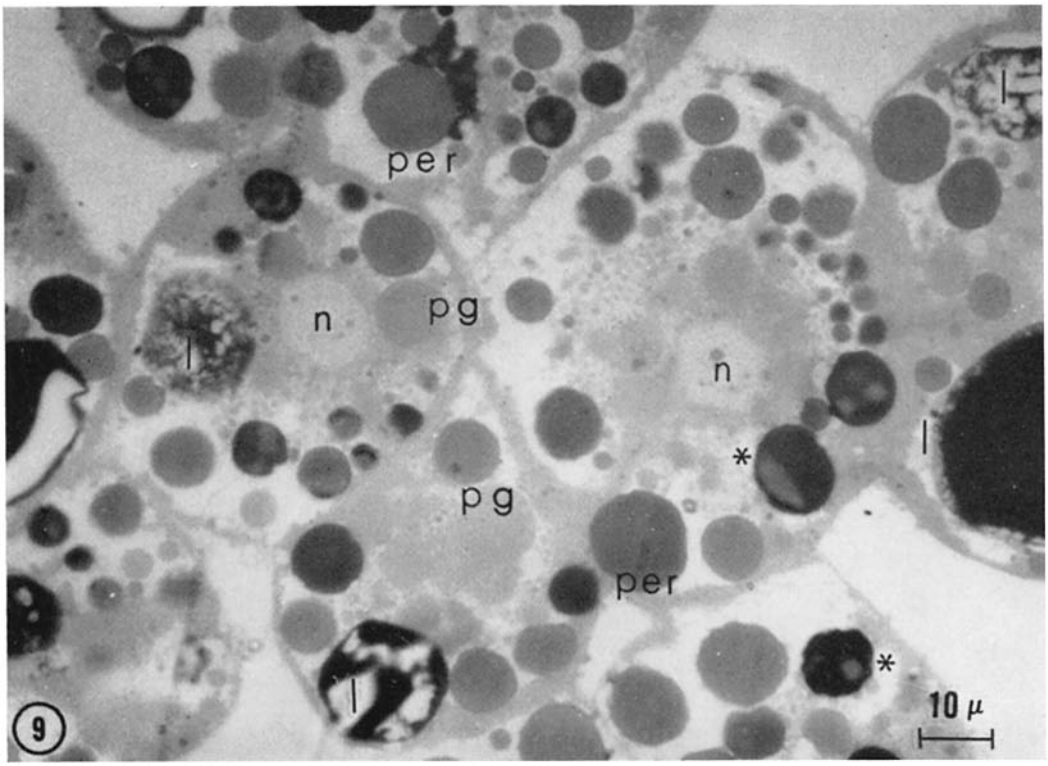

FIGURE 9 The sequestration of peroxidase into protein granules at pupation. The larva was injected with peroxidase at M + 168 hr and fixed at M + 178 hr. The granules containing peroxidase are more dense, and some have crystalline deposits (*). Diaminobenzidine reaction; otherwise unstained. *n*, nucleus; *pg*, protein granule without peroxidase; *per*, peroxidase; *l*, lipid. Araldite section. × 900.

are even more obvious, resembling typical, actively pinocytosing surfaces as in insect oocytes (41, 47). At this time, the main differences between the intermolt granules and the storage granules are those of size and speed of growth. Also, the storage granules remain densely packed within a single membrane although they may have a few peripheral microvesicles. Later, the core of the storage granules often becomes crystalline.

The studies on the incorporation of peroxidase show that the formation of multivesicular bodies and protein storage granules is basically similar, involving the following steps: (1) The concentration of blood proteins between the cells, (2) the transport of concentrated protein into the cell in vesicles; and (3) the fusion of the vesicles to make granules. Thereafter, the granules either become storage granules by continuing these processes, or multivesicular bodies by the addition and survival of microvesicles. The switch from the intermolt to the molt involves either an increase in the rate of protein accumulation or a decrease in lytic vesicle production, or both.

DISCUSSION

The Relation to Previous Work

The precise timing of the changes occurring in the fat body of *Calpodes* at metamorphosis has made it relatively easy for us to observe the events leading to the formation of different sorts of granules, with a clarification of the complexity described in other insects by various authors. We regard this description as an essential preliminary to further studies. The main events in the life history of the fat body in the 5th stadium are outlined in Fig. 25. According to their origin, the granules may be classified as heterophagic or autophagic.

The granules formed from sequestered blood

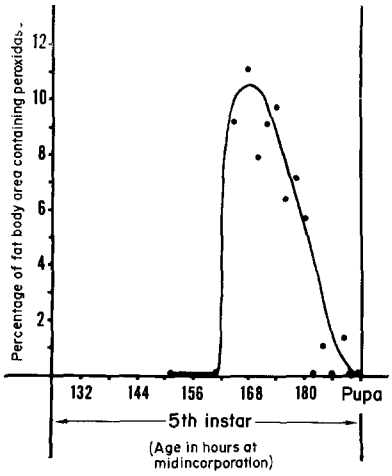

FIGURE 10 The rate of peroxidase accumulation in granules at metamorphosis. Each point is the mean of measurements on five cells from one animal after 4 hr of incorporation. The percentage of the cell area containing peroxidase was calculated from the average area occupied by peroxidase granules and the cross-sectional area. The peroxidase is sequestered in granules from M + 162 to M + 182 hr.

proteins are heterophagic vacuoles. In *Calpodes* the multivesicular bodies and the granules of stored protein form a continuous series, differing only in size and in the timing of their formation and lysis (Fig. 26). They account for the bulk of the protein stored in the fat body in *Calpodes* and probably in other insects as well. Thus, the "albuminoid granules," described by earlier authors as characteristic of metamorphosing insects, are probably composed of sequestered blood proteins as well as isolated cell organelles. Although Berlese (2) suggested that the granules in *Calliphora* were formed from albuminoid substances absorbed from the blood, Hollande (20) found no evidence that the fat body in *Nymphalis io* and *Aglais urticae* could take up nucleic acids and peptones injected into the blood, and therefore concluded that the contents of the granules were being made in situ. More recently, it has been suggested that the fat body in *Calliphora* larvae synthesizes and exports proteins to the blood, and also takes up blood proteins (37), the two processes occurring in equilibrium (36). We have used the peroxidase technique to confirm the uptake of blood proteins by the fat body in

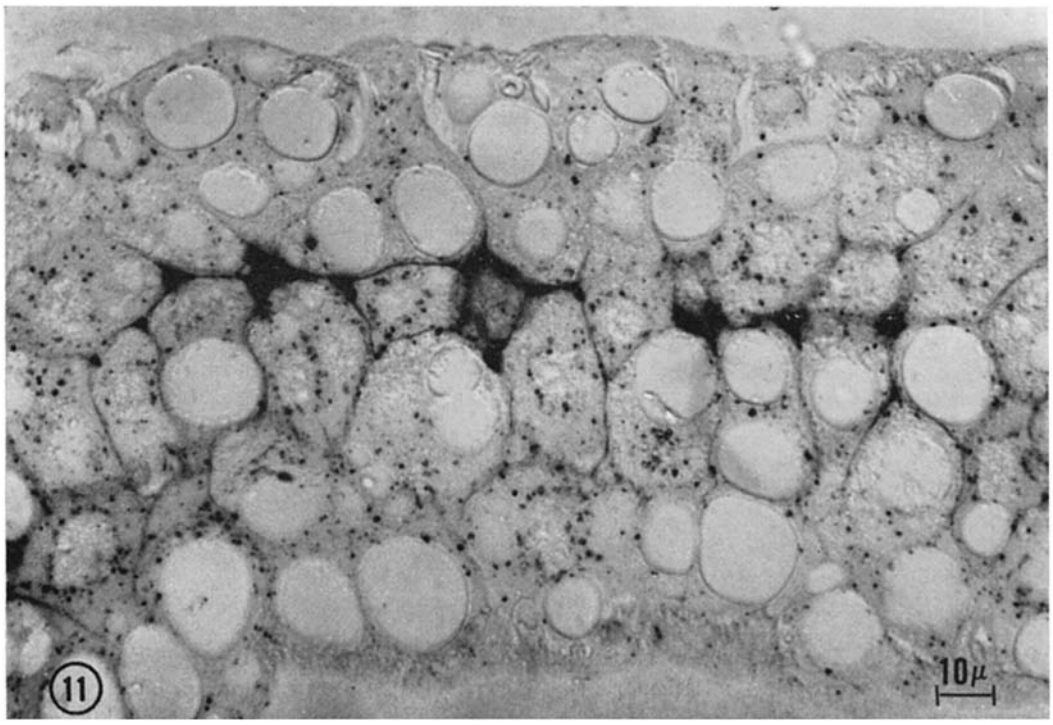

FIGURE 11 The uptake of peroxidase by the intermolt fat body after allowing incorporation from M + 70 to M + 78 hr. The enzyme is concentrated in tiny granules and in the intercellular spaces. Diaminobenzidine reaction; otherwise unstained. 4-μ ester wax section. × 700.

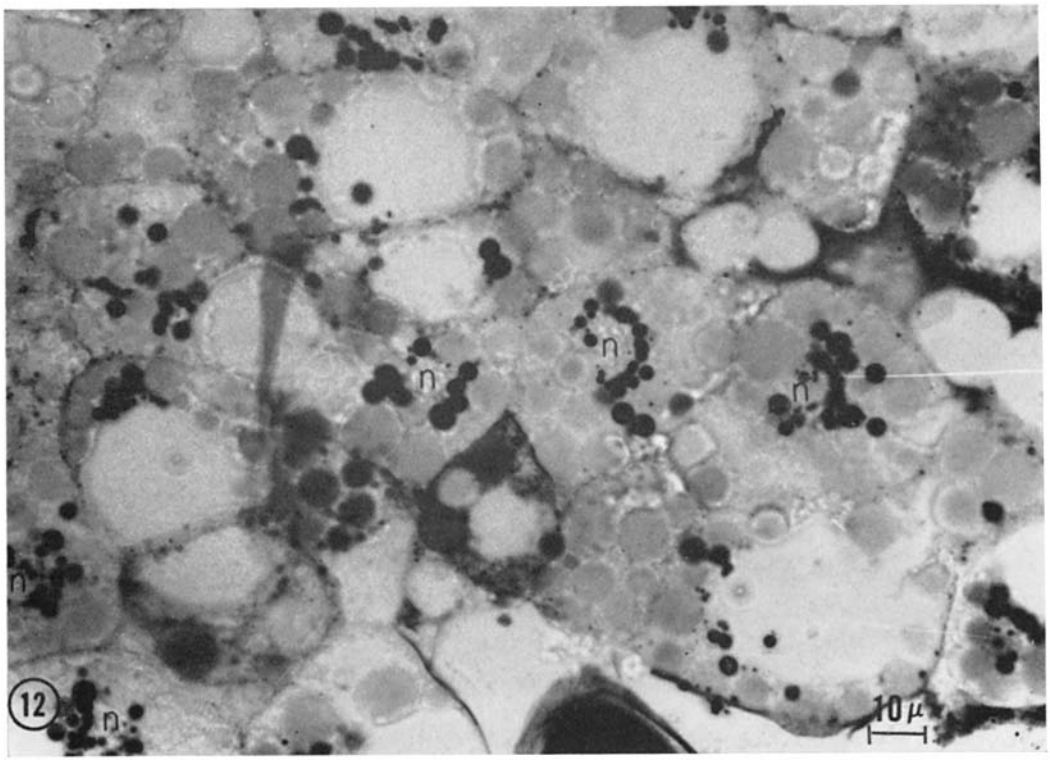

FIGURE 12 The sequestration of peroxidase into perinuclear granules from M + 184 to M + 192 hr. This incorporation time has spanned the transition from the formation of large protein storage granules to tiny perinuclear granules. Diaminobenzidine reaction; otherwise unstained. 4-μ ester wax section. *n*, nucleus. × 700.

Calliphora. If the results in *Calpodes* and *Calliphora* are taken to have general relevance in insects, we may conclude that Berlese was correct in his hypothesis that the contents of some granules arise from the blood.

The general mechanism for all autophagy in *Calpodes* involves the isolation of organelles by paired membranes (26). The isolation of mitochondria for autolysis produces transient granules which are soon lost (26). Several authors have described fat body granules which stain with Janus Green (4, 10) and fuchsin (22), indicating that they contain mitochondria, and such granules have been seen in electron micrographs of *Drosophila* fat body (18). The formation of albuminoid granules from degenerating mitochondria has also been described in the fat body of metamorphosing *Philosamia* (51). The ER isolation bodies of *Calpodes* store protein and RNA in the pupa. They are probably equivalent to the basophilic granules described in the literature (2, 3, 5, 20 32, 35, 44, 52) and to the granules containing isolated ER in *Drosophila* (18). In addition to the basophilic granules, some authors have described granules which are first basophilic and later become acidophilic (20, 44, 52). At the time of mitochondrial autolysis, similar granules were seen in *Calpodes* to arise by the isolation and lysis of rough ER, resulting in storage granules without RNA (26).

These observations on the sequential formation of various granule types in *Calpodes* clarify the confusion arising from the descriptions of granules in other insects. The formation of protein granules during metamorphosis is the result of three processes: (1) the sequestration of blood proteins; (2) the isolation and autolysis of cell organelles; and (3) the isolation, incomplete autolysis, and temporary storage of the rough endoplasmic reticulum.

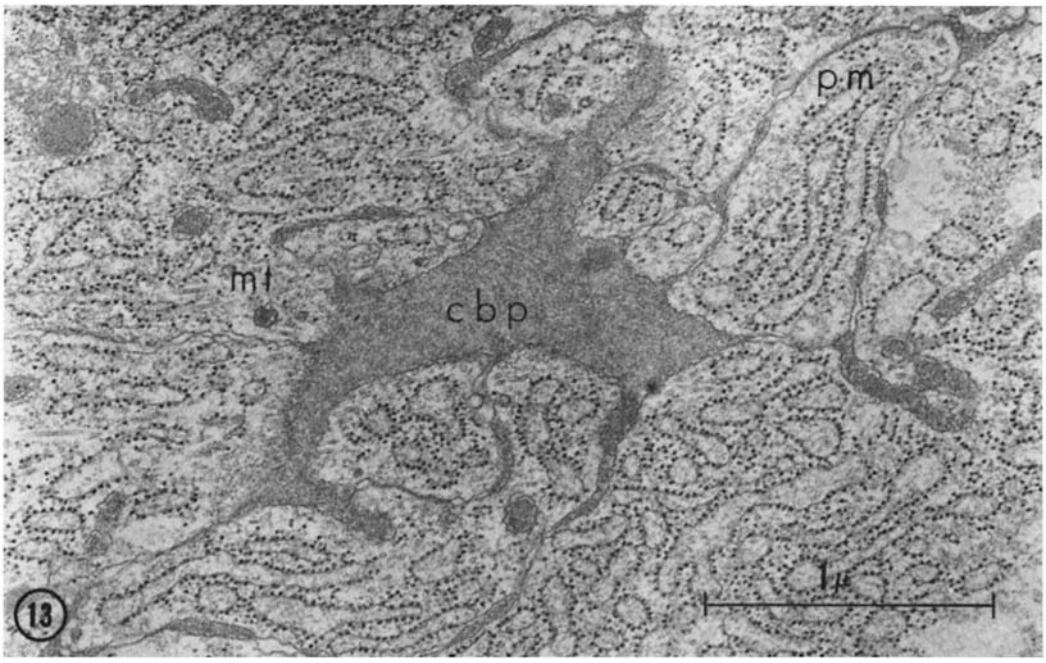

FIGURE 13 The concentration of blood protein between the cells (M + 145 hr). There are many protein-filled folds and tubular extensions of the plasma membrane into the cells, some of which appear to break off to form small protein-filled vesicles. Concentrations of microtubules within the cells border the intercellular spaces. Stained with lead citrate and uranyl acetate. *pm*, plasma membrane; *mt*, microtubules; *cbp*, concentrated blood protein. 100 kv. × 39,000.

Mechanisms of Protein Uptake

Protein uptake in the fat body of *Calpodes* differs from that of previous reports of pinocytosis in being a two-stage process; blood protein is first concentrated in intercellular spaces and channels before being ingested in vesicles. The mechanism involved may differ according to the stage being considered.

In the first stage, protein is concentrated in masses which may be a micron or more from a cell surface. The uptake is nonspecific, allowing the sequestration of proteins as foreign as plant peroxidase and algal protein. The simplest hypothesis would suppose that whole blood is concentrated between the cells by the loss of water following the active transport of ions and/or metabolites. The fat body is one of many tissues which take up peroxidase in *Calpodes* (28). The concentration of protein may, to some extent, be inevitable in cells which are actively taking up precursors for the synthesis of proteins, lipids, and glycogen. Concentration could also be effected by the movement of ions as in *Carausius* which maintains a potential gradient of 15–20 mv across the fat body (29). Miller et al. (30) have suggested that an APTase may be involved in the uptake of peroxidase by the convoluted tubule loop of Henle in rat kidney slices, since the process is Ouabain sensitive. The concentrating stage in *Calpodes* might be the result of a similar process, but, so far, we have been unable to demonstrate an ATPase with the convincing localization reported by Berridge and Gupta.[2]

The specificity of the second stage of uptake may be influenced by the size of the pinocytotic vesicles. If specificity is due to an immunological type of reaction between protein and the membrane surface and if there is no mechanism for concentrating material in the center of the vesicle, then all vesicles would be more or less specific. If the vesicles contain contents in addition to that

[2] M. J. Berridge and B. L. Gupta. 1968. Fine structural localization of adenosine triphosphatase in the rectum of *Calliphora*. *J. Cell Sci.* In press.

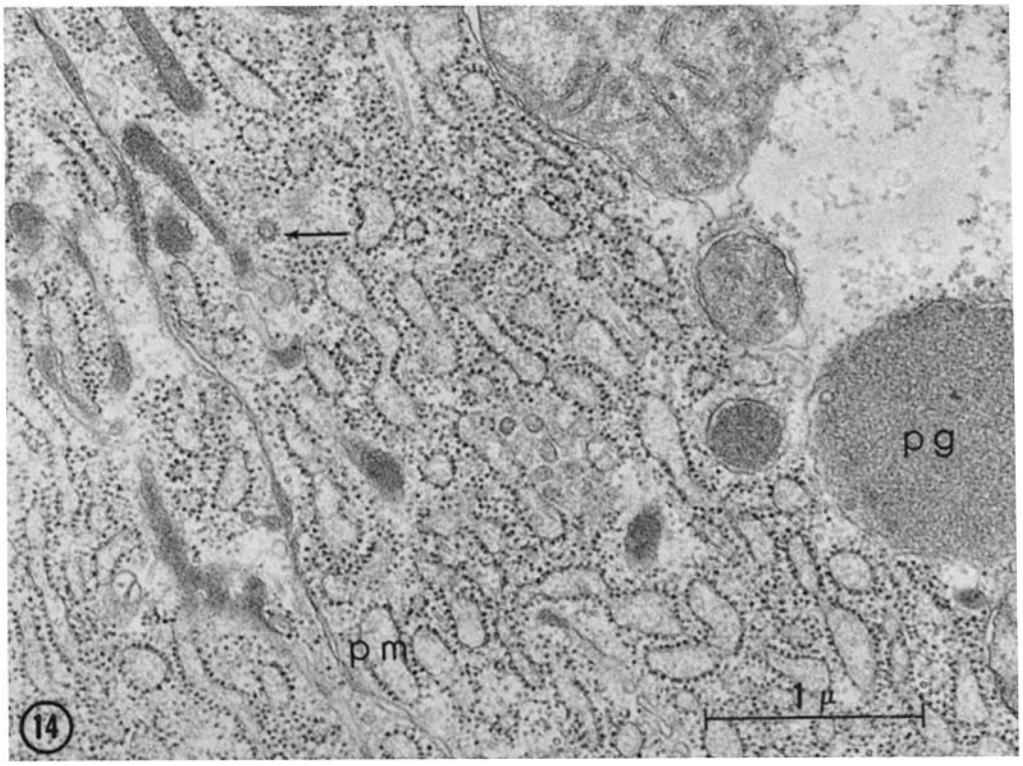

FIGURE 14 The formation of small protein granules. Protein-filled vacuoles bud off from the intracellular channels and fuse to form larger vacuoles which become small protein granules (M + 145 hr). Stained with lead citrate and uranyl acetate. *pg*, protein granules; *pm*, plasma membrane; ↑, coated vesicle. 100 kv. × 34,000.

adsorbed, then the smaller vesicles would be more specific. For example, it can be seen from Fig. 27 that a microvesicle with an external diameter of of about 1,000 A, which has a 75-A-thick layer of a specific protein adsorbed to its inner surface, could carry specific: nonspecific contents in the ratio of 2:3. The lack of specificity shown by *Calpodes* may be due to the large size of the vesicles (about 1800 A diameter) and the nonspecific concentrating mechanism which would be expected to fill the centers of the vesicles.

Multivesicular Bodies and Protein Turnover

During the intermolt, and again shortly before pupation, the fat body takes up blood protein into granules which become multivesicular bodies. The granules and the multivesicular bodies contain lytic enzymes and do not accumulate during the intermolt, so that we may assume that the empty-looking MVB's are truly evidence for the hydrolysis of the exogenous protein sequestered in the granules.

During the intermolt, the fat body synthesizes proteins in several insects (36, 37, 45, 46). The rise in blood protein during the latter part of the intermolt when the fat body takes up amino acids and when it has active-appearing ER and Golgi complexes, suggests that in *Calpodes* also the fat body synthesizes protein for export to the blood. Thus, the fat body both synthesizes and breaks down proteins. Although we do not yet know the relative magnitudes of the two processes, the products of breakdown presumably reappear as the products of synthesis, and we may regard the MVB's as organelles for the turnover of blood proteins. Further studies are needed to determine the specificity of uptake in relation to the proteins synthesized.

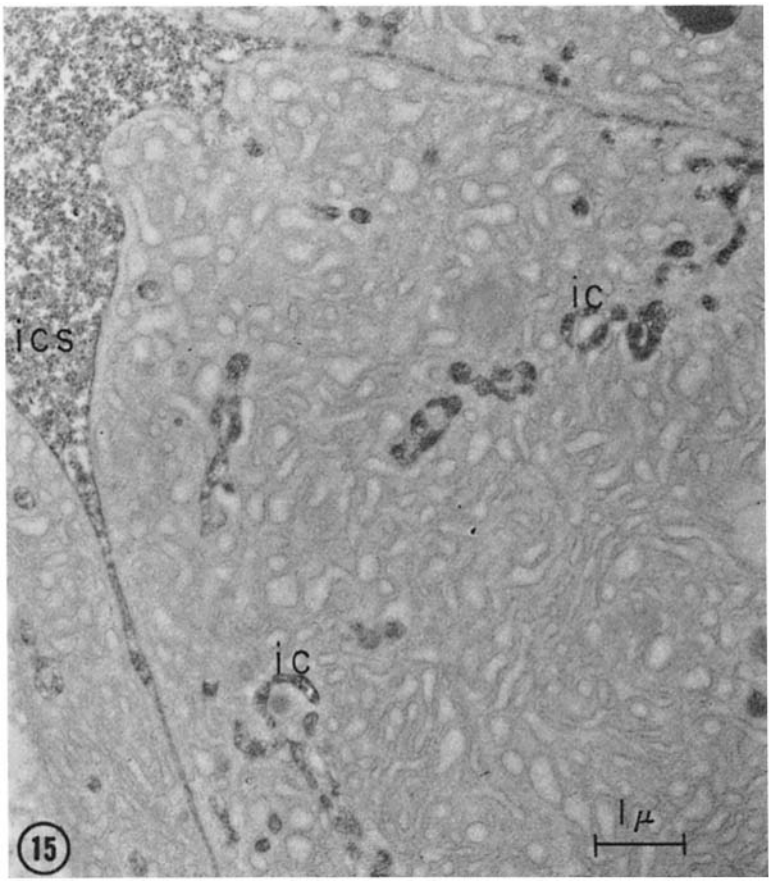

FIGURE 15 The uptake of peroxidase before granule formation. Unstained section from a late intermolt larva fixed 4 hr after the injection of peroxidase. Diaminobenzidine reaction. The peroxidase is concentrated in the intercellular spaces and in intracellular channels. *ics*, intercellular spaces; *ic*, intracellular channels. 50 kv. × 12,000.

The Membrane Turnover Involved in Protein Uptake

One of the consequences of pinocytosis is the loss of plasma membrane from the cell surface. Membrane must also be removed from the granules, since their surface area is much less than the combined surface area of the constitutent small vesicles. During the formation of granules, membrane must be removed either from the granule envelope if fusion occurs, or from within the granule if the microvesicles pop through the limiting membrane intact. It is of interest to estimate the magnitude of membrane turnover to test the plausibility of the concept of membrane flow in vesicle formation proposed by Bennett (1). "Turnover" as used here refers to the possible movement of membranes from the surface of MVB's or granules and back again. Nothing is implied about possible degrees of degradation and synthesis of membrane in the proposed cycle.

At the time of their maximum size, the cells have a cross-section of about $3 \times 10^3\ \mu^2$, a volume of about $1.3 \times 10^5\ \mu^3$, and a surface area of about $1.2 \times 10^4\ \mu^2$. In the female, each cell would contain $3.3 \times 10^4\ \mu^3$ of protein in granules. About 10^7 pinocytotic vesicles, 1,800 A in diameter, would be needed per cell to transport this protein. The vesicles would have a combined surface area

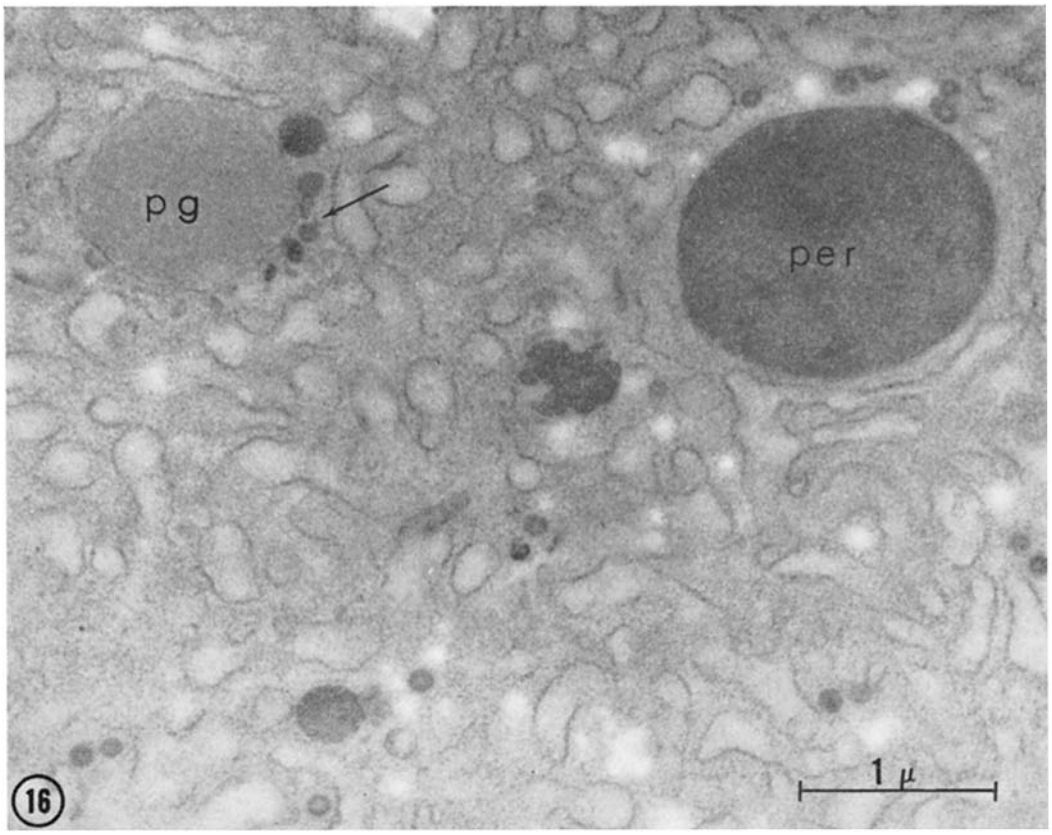

FIGURE 16 The incorporation of peroxidase into protein granules. Unstained thick section from a larva injected with peroxidase at M + 162 hr and fixed after 4 hr. Diaminobenzidine reaction. The peroxidase is present in vesicles and granules. The arrow points to vesicles lying close to the membrane of a granule as if on the point of fusing with it. *per*, granule containing peroxidase; *pg*, granule without peroxidase. 50 kv. × 26,000.

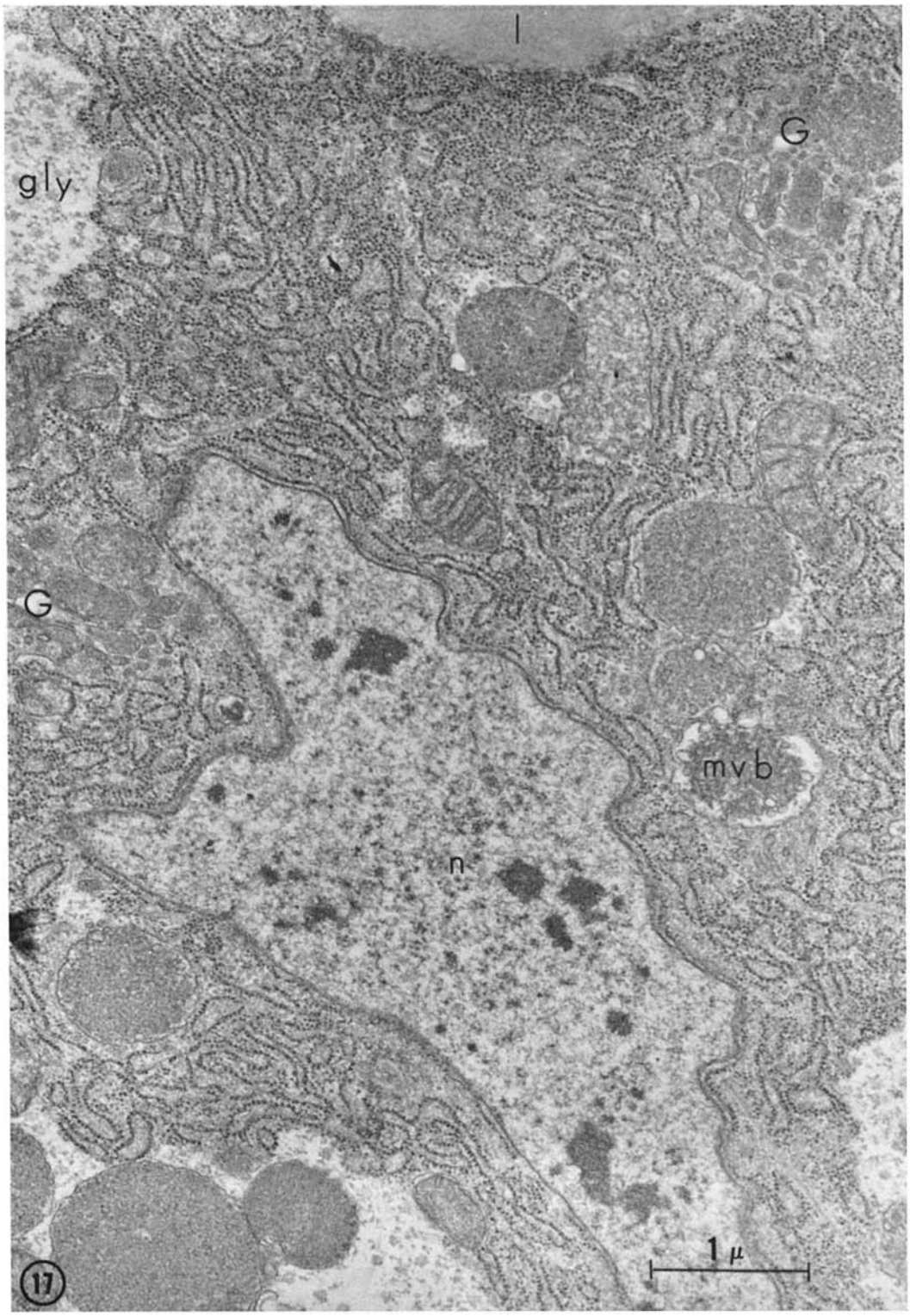

FIGURE 17 Multivesicular bodies at the end of the intermolt (M + 156 hr). A series can be distinguished around the nucleus, from granules containing little but protein to others with many small vesicles. These small granules and multivesicular bodies are the "tiny" perinuclear granules seen after peroxidase incorporation. Stained with lead citrate and uranyl acetate. *n*, nucleus; *mvb*, multivesicular body; *gly*, glycogen; *G*, Golgi complex; *l*, lipid. 100 kv. × 24,000.

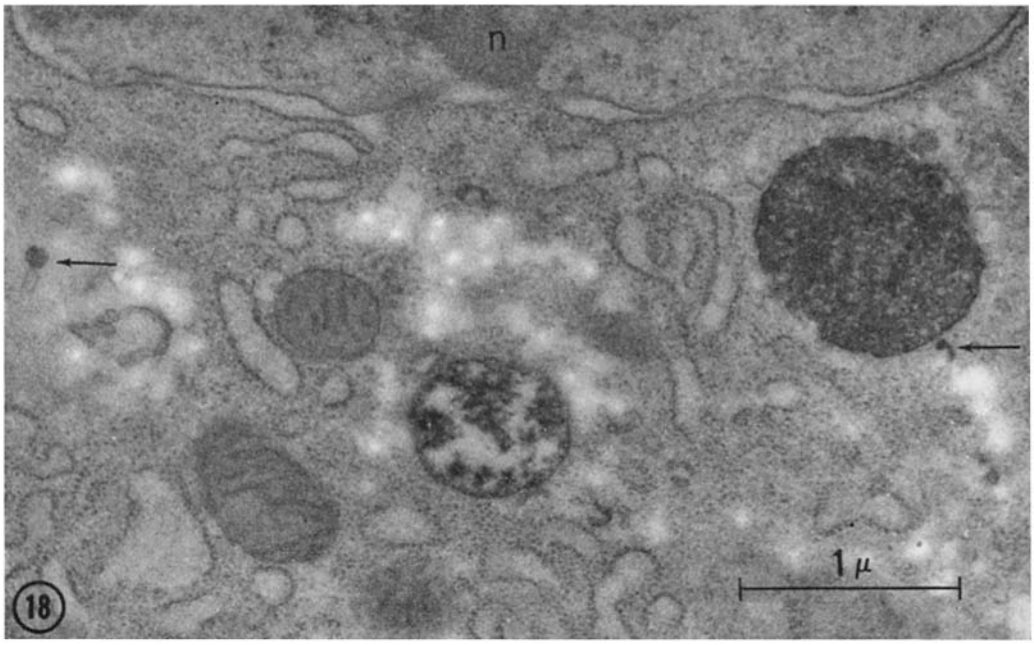

FIGURE 18 The incorporation of peroxidase into MVB's. Unstained section from a larva injected with peroxidase at M + 180 hr and fixed at M + 190 hr. By this time, the formation of large protein granules has ceased and the peroxidase is localized in multivesicular bodies only. Diaminobenzidine reaction. *n*, nucleus; ↑, microvesicles containing peroxidase. 50 kv. × 28,000.

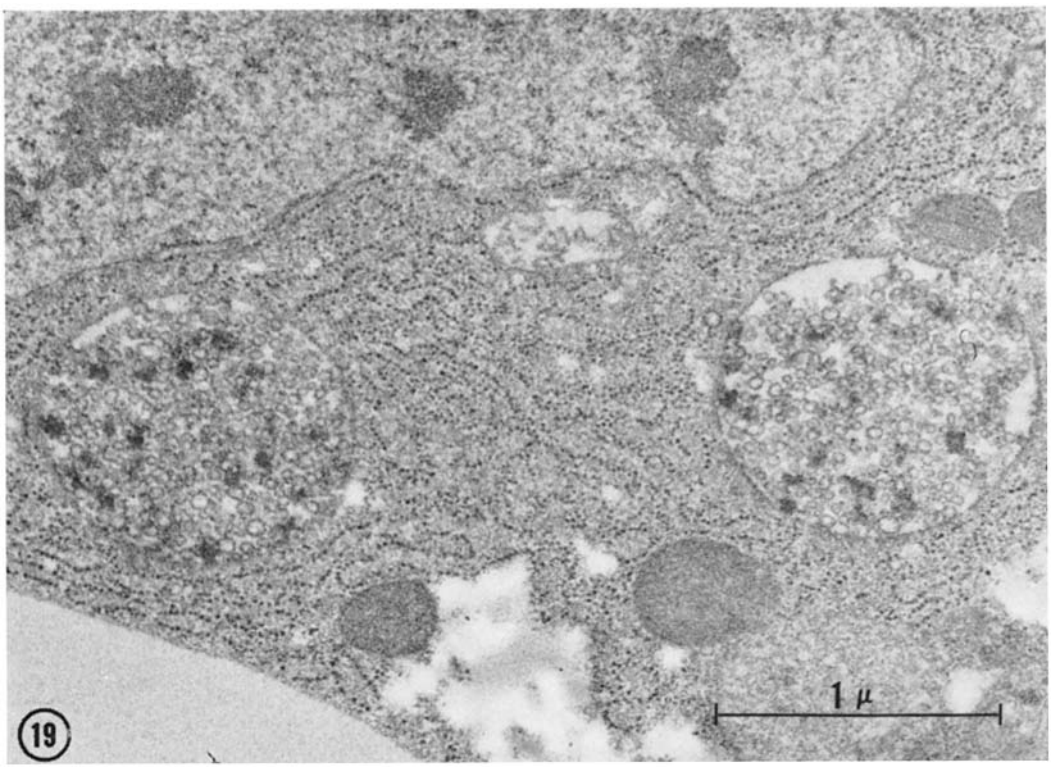

FIGURE 19 Peroxidase in microvesicles. Multivesicular bodies from a larva injected with peroxidase at M + 123 hr and fixed 3 hr later. The benzidine reaction product shows discrete localization, and is probably associated with some microvesicles. Section stained with uranyl acetate. 50 kv. × 37,000.

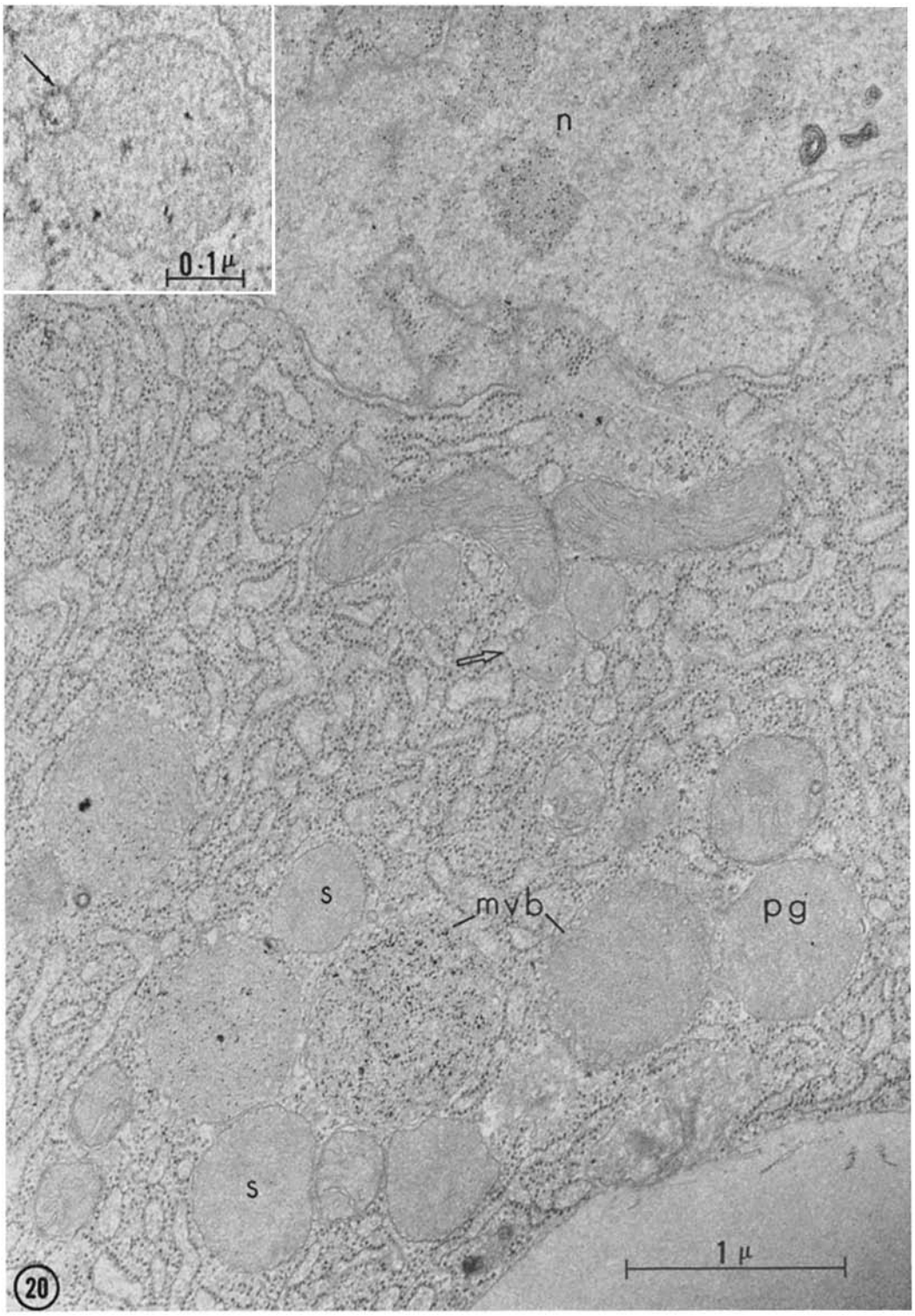

FIGURE 20 Acid phosphatase in small protein granules and MVB's in the intermolt (M + 120 hr). The intensity of the acid phosphatase is not obviously correlated with the frequency of microvesicles. The Golgi secretion in vacuoles, recognized by its fingerprint-like pattern, does not usually contain acid phosphatase. *Inset:* a small vesicle containing acid phosphatase is being joined by a microvesicle which may also be carrying acid phosphatase. Stained with lead citrate only. *n*, nucleus; *pg*, small intermolt granule; *mvb*, multivesicular body; *s*, Golgi secretion; ↑, vesicle enlarged in inset. 50 kv. × 33,000. Inset, × 115,000.

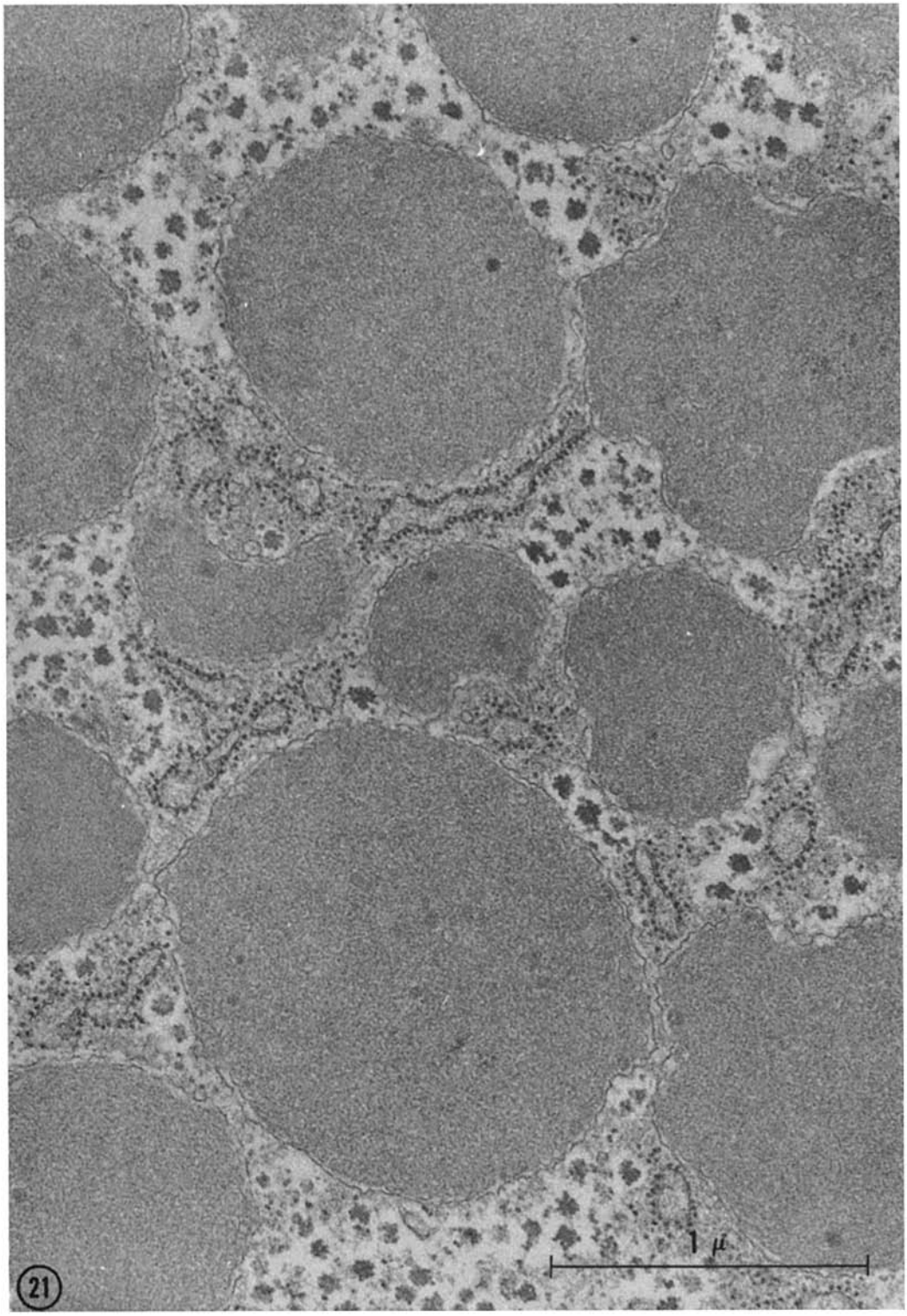

FIGURE 21 Granules of sequestered protein at M + 158 hr. The switch from the phase of intermolt multivesicular body production to the formation of protein storage granules is accompanied by the formation of large multivesicular bodies from granules of this sort. Stained with lead citrate and uranyl acetate. 100 kv. × 48,000.

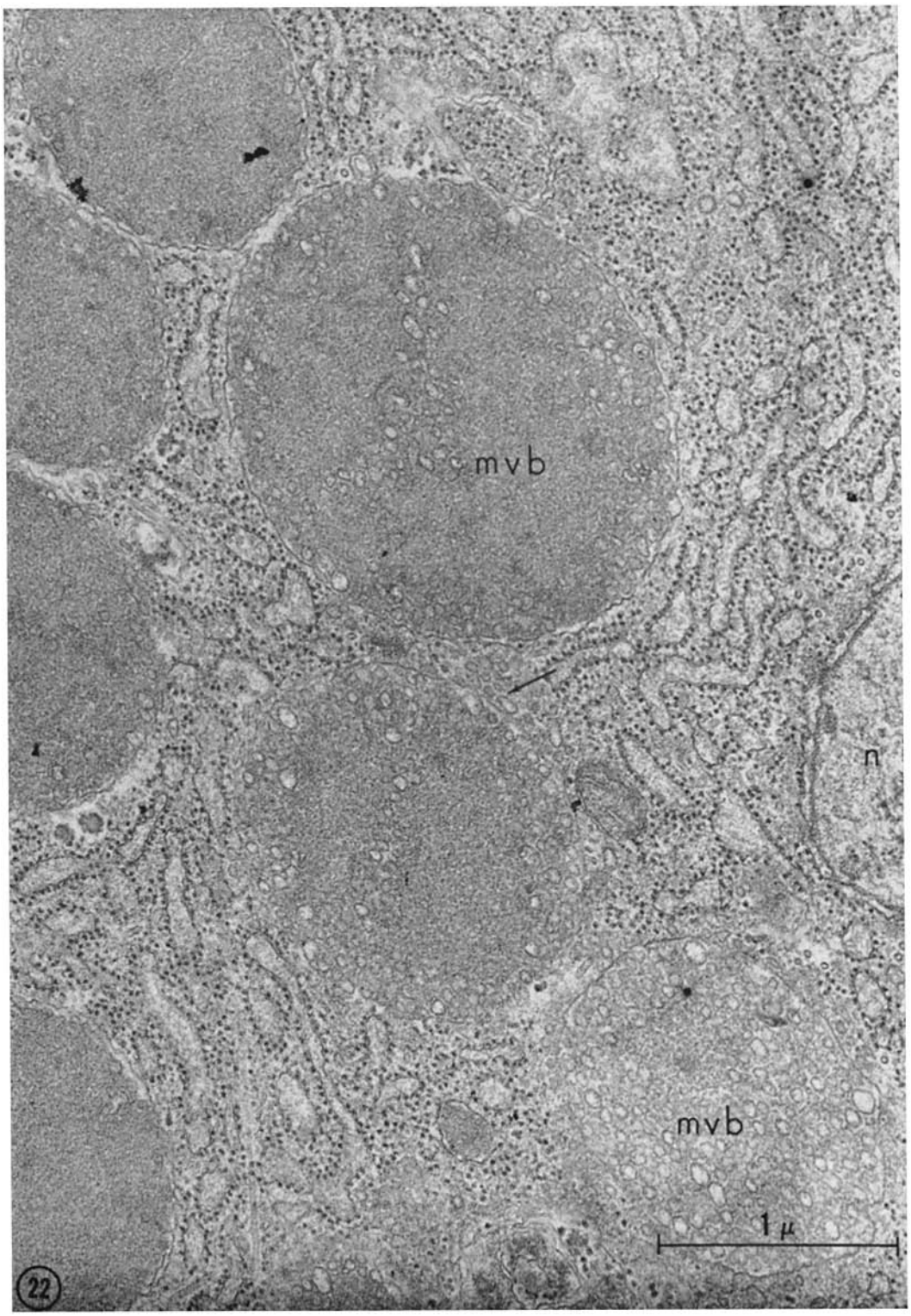

FIGURE 22 Multivesicular bodies in a larva at M + 158 hr. Granules of sequestered protein incorporate microvesicles and become multivesicular bodies. Stained with lead citrate and uranyl acetate. *mvb*, multivesicular body; ↑, microvesicles in the cytoplasm; *n*, nucleus. 100 kv. × 37,000.

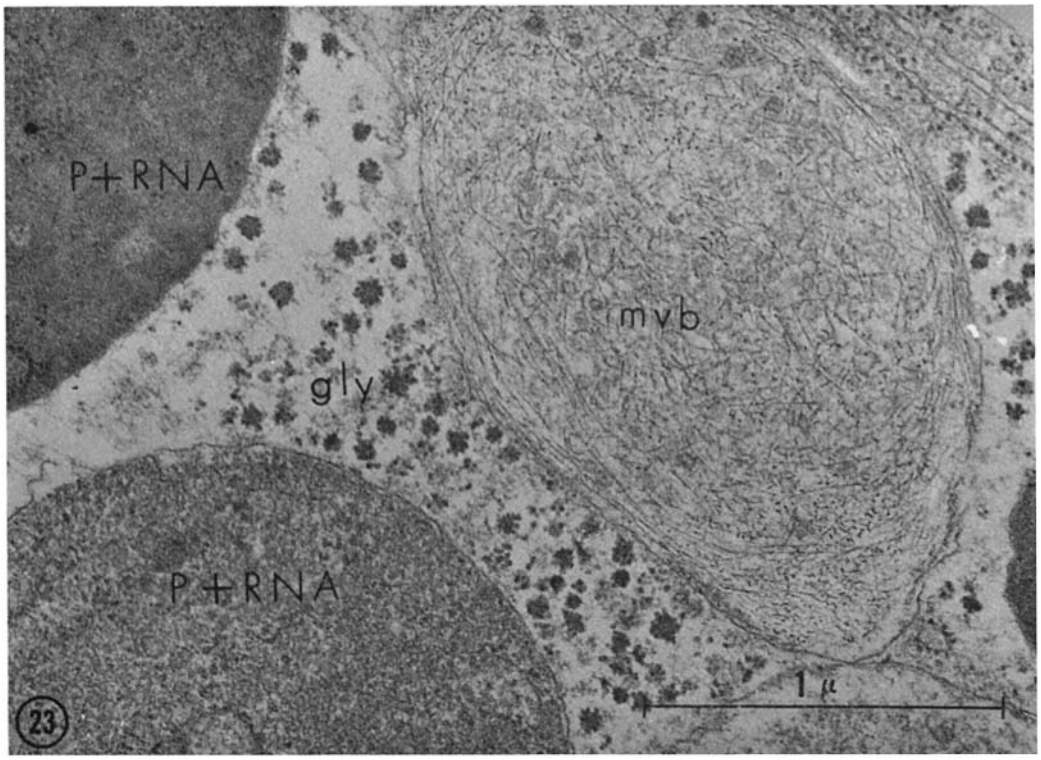

FIGURE 23 The large fibrous MVB's occurring at metamorphosis have a characteristic fibrous texture during lysis. The P + RNA granules arise by the isolation and partial hydrolysis of regions of rough ER. At this stage, they contain concentrated protein and some ribosomes. Stained with lead citrate and uranyl acetate. *mvb*, multivesicular body; *gly*, glycogen. 100 kv .× 47,000.

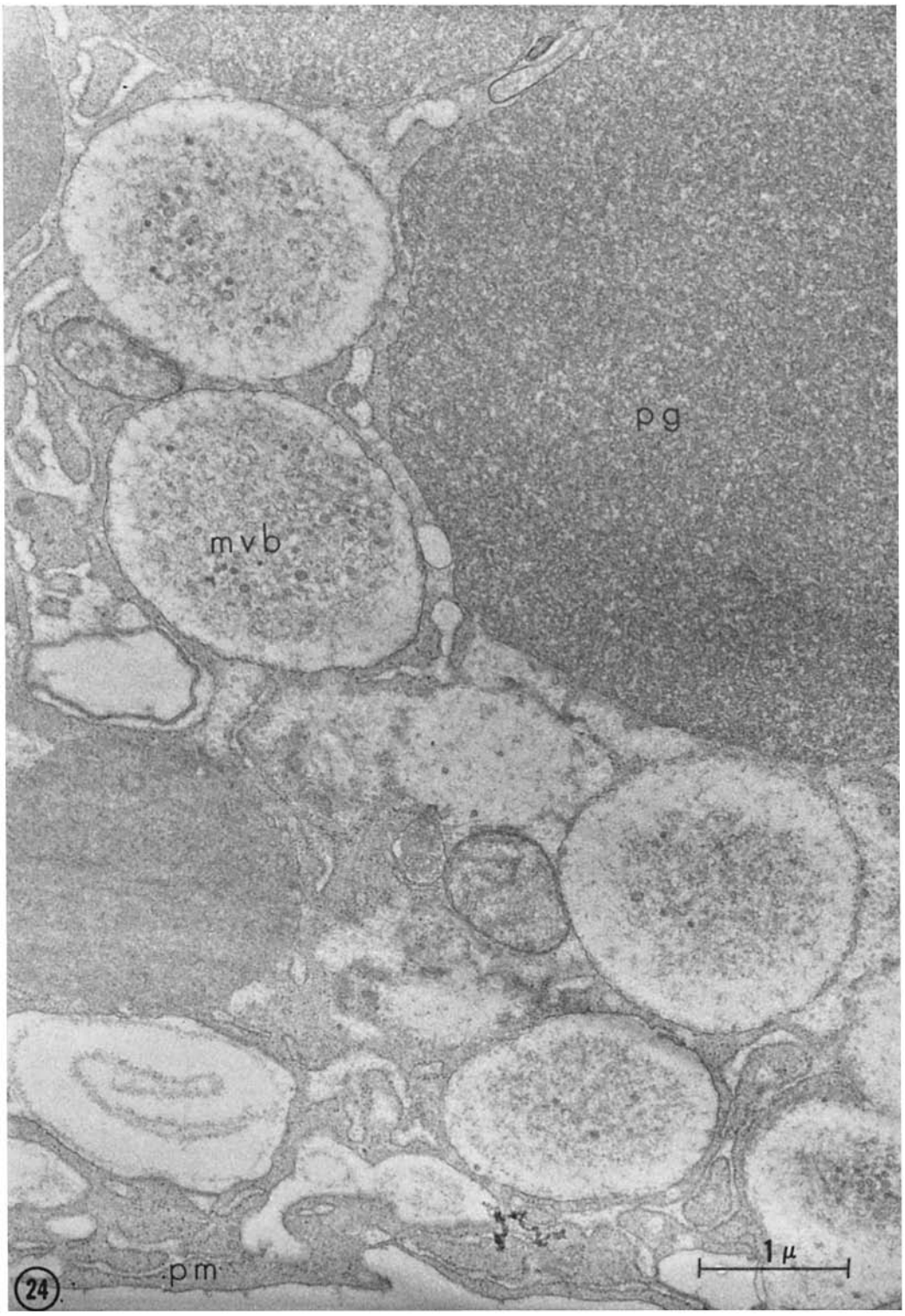

FIGURE 24 Late MVB's and protein storage granules from an early pupa. The dense contents of the MVB's are lost before pupation, leaving "empty" residual bodies. Stained with lead citrate and uranyl acetate. *pg*, protein granule; *mvb*, multivesicular body; *pm*, plasma membrane. 100 kv. × 23,000.

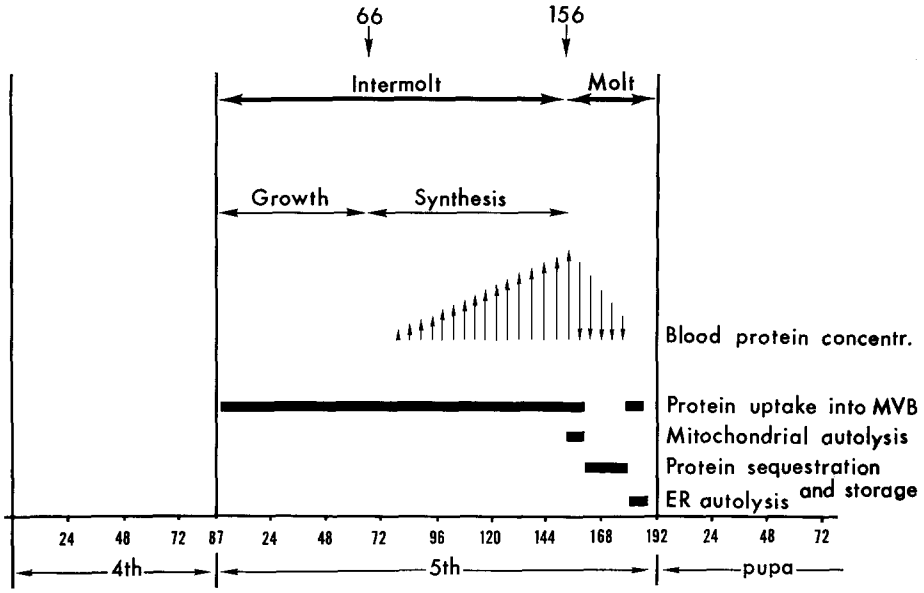

FIGURE 25 The principal events in the life history of the fat body during the 5th stadium. The arrows at 66 and 156 hr mark the critical periods for the action of the head and prothoracic glands. The first phase from molting to 66 hr is one of growth. Between 66 and 156 hr, there is a phase of synthesis and secretion during which the blood protein concentration increases to a peak at 156 hr. Blood protein is taken up by the fat body to form multivesicular bodies throughout most of the stadium except during the formation of storage granules. Isolation and autolysis of the mitochondria occur early in the third phase (M + 156 to M + 192 hr). From M + 156 to M + 162 hr, there is a transitional phase of giant multivesicular body formation after which protein storage granules are formed from M + 162 to M + 180 hr. During granule formation, the blood protein concentration decreases. Following the sequestration of blood proteins into storage granules, there is another phase of MVB formation, during which time the ER is isolated and undergoes partial autolysis. (Figs. 2, 5, and 9 show the characteristic appearance of the fat body during the three main phases.)

of about 10^6 μ^2, or 100 times the surface area of the cell, so that an area equivalent to the surface membrane might be needed every 10 min to form vesicles. Since the combined surface area of the protein granules is only about 2×10^4 μ^2 per cell, almost all the vesicle membrane must be returned to the cellular pool.

After determining the rate of pinocytosis in *Chaos chaos*, Christiansen and Marshall (6) calculated that the half life of the surface membrane could be a little over an hour. A comparable figure for mammalian cells (21) is about 4 hr. If membrane does constantly form vesicles at the rate at which protein is taken up in *Calpodes*, then the rate of membrane turnover seems improbably high. Although there may be some membrane loss in the formation of microvesicles sequestered within MVB's, it is perhaps more plausible to think of the protein granules as being filled from clouds of vesicles which repeatedly exchange their contents with one another by fusing and separating during random movement. As long as a granule does not refill a vesicle which temporarily fuses with it, protein could move through the cloud of vesicles without any one vesicle shuttling for more than a fraction of the route.

During their formation, protein granules do not contain microvesicles in proportion to their increasing volume. The MVB's, on the other hand, may appear almost filled with microvesicles, most of which do not contain acid phosphatase. Many microvesicles seem empty or may contain peroxidase, suggesting that they are part of the pinocytosis system rather than the Golgi complex. Many microvesicles appear broken in the MVB's with little contents, so that the MVB's may be concerned in the breakdown of both the membrane

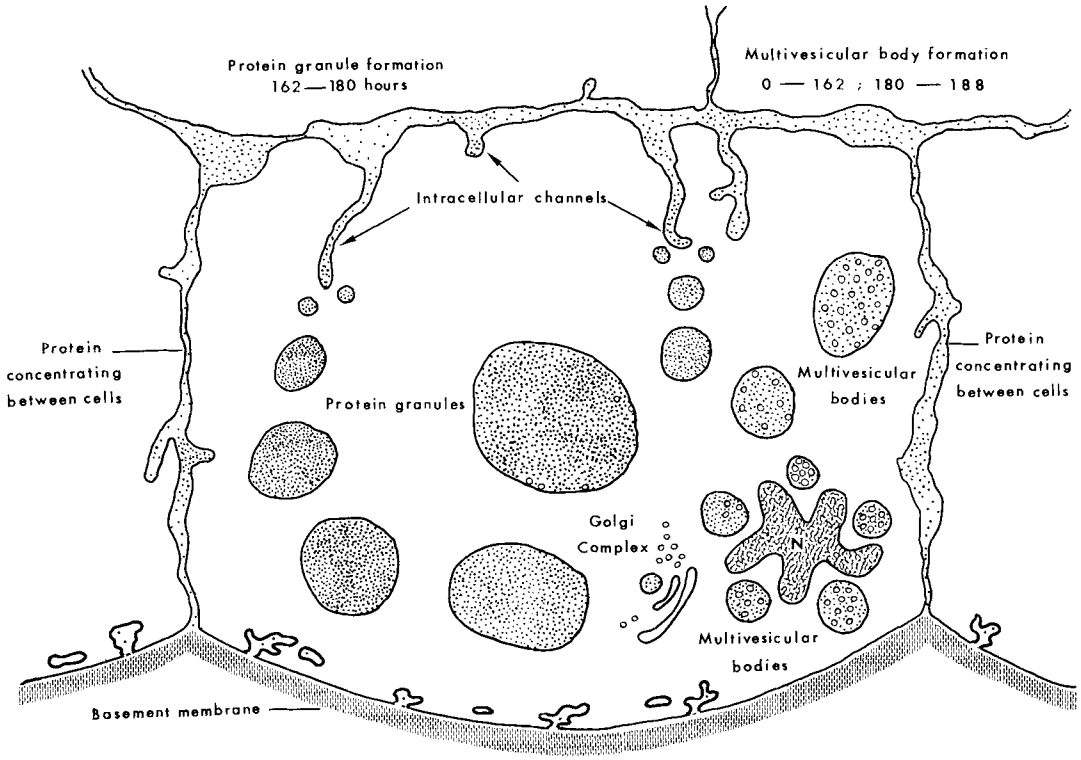

FIGURE 26 The sequestration of blood protein to form granules and multivesicular bodies. Blood protein is concentrated in the spaces between cells and in channels and tubular extensions within the cells. Vesicles and vacuoles pinch off from the intracellular channels and fuse to form small granules which grow by fusion with other granules and by accretion of vesicles from the cell surface. The granules incorporate microvesicles from the cytoplasm to become MVB's during most of the stadium (M + 0 to M + 162 hr; M + 180 to M + 188 hr). Protein storage granules are formed in the same way as MVB's, but do not incorporate microvesicles to become multivesicular (M + 162 to M + 180 hr).

and contents of microvesicles. It is not generally realized how large the proportion of this membrane may be. Fig. 27 shows the theoretical proportions of membrane and contents for the range of microvesicle sizes typically met with in cells, assuming membrane thicknesses of 75 and 100 A. It can be seen that the volume of a typical microvesicle measuring about 0.1 μ in diameter is 40–50% membrane, and that a very small vesicle of 300 A in diameter, the limiting size according to Robertson (39), is 85–95% membrane. Thus, if microvesicles are used up in conveying their contents from the cell surface to a MVB or protein granule, they would be transporting more membrane than contents. We should keep an open mind on whether microvesicles and MVB's are primarily autophagic or heterophagic in function, or both. Some MVB's might be organelles engaged primarily in membrane turnover for changing the surface properties of a cell, and others might be more important in digesting external proteins.

Functions of Multivesicular Bodies

The term multivesicular body was first used to describe a granule occurring in rat ova which is bounded by a single membrane and contains a variable number of microvesicles and a nucleoid (47). Numerous descriptions of these bodies in a variety of cell types have appeard since then, but it is clear that many of the structures described are not identical. Those present in eggs are probably similar to the MVB's originally described, but an equally common type occurs in cells which sequester exogenous protein (13–15, 17, 28, 40

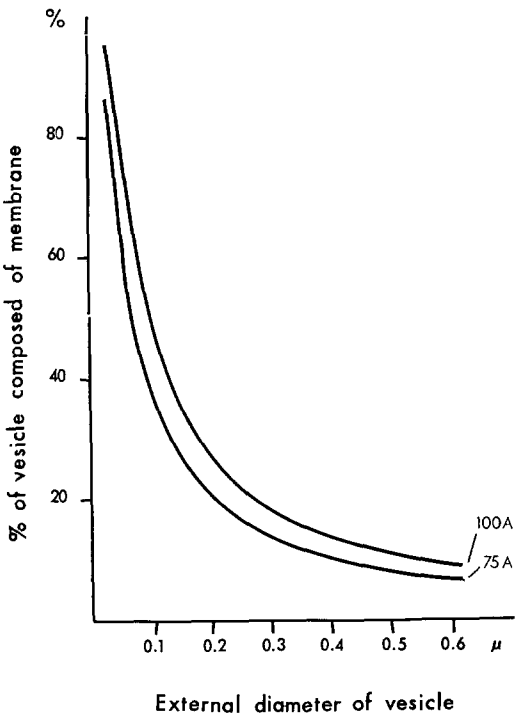

FIGURE 27 The theoretical proportions of membrane and contents for small vesicles of various sizes. Curves are calculated for membrane thicknesses of 100 and 75 A. The curves also show how specific the composition of vesicles of various sizes could be if the vesicles carried nonspecific protein in the center and had specific protein adsorbed to the membrane in a layer 75 or 100 A thick. The ordinate represents the per cent of vesicle composed of membrane.

43). In such cells, the protein is present in the matrix of the granule; and in one example, it is present in the microvesicles (23). In *Calpodes*, peroxidase occurs in both the matrix and the microvesicles of the MVB's which, therefore, belong to the class which sequesters foreign protein. The presence of acid phosphatase confirms the view that the MVB's in *Calpodes* are heterolysosomes (11) containing lytic enzymes as well as sequestered protein (13, 14, 33, 41). However, it has been claimed that the MVB's of eggs are not lysosomes even though they contain acid phosphatase (9). In the secretory neurons of the goldfish brain, the MVB's were thought to be stages in the formation of neurosecretory granules and unrelated to MVB's containing nucleoids (34). In insect epidermis, MVB's may have yet another function since they contain a natural peroxidase and appear to be concerned in the formation of hard cuticle.[1]

These considerations lead us to conclude that the term "multivesicular body" is a purely descriptive one which should not be used to imply function or even a similar origin.

We are grateful to Drs. M. J. Berridge and P. A. Lawrence for stimulating discussions and to Dr. J. L. Oschman who criticized the manuscript. We also thank Miss Bonnie J. Sedlak and Mr. J. T. McMahon for technical assistance. The work was generously supported by a grant from the United States Public Health Service, GM 09960, and a fellowship from the Government of Jamaica (JVC).

Received for publication 26 July 1967, and in revised form 11 October 1967.

BIBLIOGRAPHY

1. BENNETT, H. S. 1956. The concepts of membrane flow and membrane vesiculation as mechanisms for active transport and ion pumping. *J. Biophys. Biochem. Cytol.* **2:** (4, Suppl.)99.
2. BERLESE, A. 1901. Osserverzion sur fenomeni che avvengono durante la ninfosi degli Insetti metabolici. *Riv. Patol. Veget. Firuza.* Anno. 11. (Not seen in the original.)
3. BISHOP, G. H. 1922. Cytological changes accompanying growth and histolysis of the fat body of *Apis mellifica*. *J. Morphol.* **36**:567.
4. BISHOP, G. H. 1958. Nuclear and cytoplasmic changes in fat body cells of queen bee during metamorphosis. *J. Exptl. Zool.* **137**:501.
5. BUTTERWORTH, F. M., D. BODENSTEIN, and R. C. KING. 1965. Adipose tissue of *Drosophila melanogaster*. I. An experimental study of larval fat body. *J. Exptl. Zool.* **158**:141.
6. CHRISTIANSEN, R. G., AND J. M. MARSHALL. 1956. A study of phagocytosis in the Amoeba *Chaos chaos*. *J. Cell Biol.* **25**:443.
7. COLLINS, J. V., and M. LOCKE. 1966. The sequestration of protein by fat body cells in an insect. *J. Cell Biol.*, **31**:42. (Abstract).
8. CURTIS, A. S. G. 1960. Area and volume measurements by random sampling methods. *Med. Biol. Illustr.* **10**:261.
9. DALCQ, A. M. 1963. The relation to lysosomes of the *in vivo* metachromatic granules. Lysosomes. Ciba Foundation Symposium. A. V. S. de Reuck and M. P. Cameron, editors. Little, Brown and Company, Boston, Massachusetts.
10. DEBOISSEZON, P. 1930. Contribution a l'étude de la biologie et de l'histo-physiologie de *Culex pipiens* L. *Arch. Zool. Exptl. Gen.* **70**:281.

11. DE DUVE, C., and R. WATTIAUX. 1966. Functions of lysosomes. *Ann. Rev. Physiol.* **28**:435.
12. DITTEBRANDT, M. 1948. Application of the Weichselbaum biuret reagent to the determination of spinal fluid protein. *Am. J. Clin. Pathol.* **18**:439.
13. EASTON, J. M., B. GOLDBERG, and H. GREEN. 1962. Demonstration of surface antigens and pinocytosis in mammalian cells with ferritin-antibody conjugates. *J. Cell Biol.* **12**:437.
14. FARQUHAR, M. G., and G. E. PALADE. 1960. Segregation of ferritin in glomerular protein absorption droplets. *J. Biophys. Biochem. Cytol.* **7**:297.
15. FARQUHAR, M. G., and G. E. PALADE. 1962. Functional evidence for the existence of a third cell type in the renal glomerulus. *J. Cell Biol.* **13**:55.
16. FAWCETT, D. W. 1965. Surface specialization of absorbing cells. *J. Histochem. Cytochem.* **13**:75.
17. FRIEND, D. S. 1966. Peroxidase absorption in the vas deferens of the rat. *J. Cell Biol.* **31**:37A. (Abstr.)
18. GAUDECKER, B. 1963. Über den formwechsel einiger Zellorganelle bei der bildung der reservestoffe im fettkorper von *Drosophila* larven. *Z. Zellforsch.* **61**:56.
19. GRAHAM, R. C., JR., and M. J. KARNOVSKY. 1966. The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.* **14**:291.
20. HOLLANDE, A. C. 1914. Formations endogenes des cristalloides albuminoides et des urates des cellules adipeuses des Chenilles des *Vanessa* io et *Vanessa urticae*. *Arch. Zool. Exptl. Gen.* **53**:559.
21. HOLTZER, H., and S. HOLTZER. 1960. The *in vitro* uptake of fluorescein-labelled plasma proteins. I. Mature cells. *Compt. Rend. Trav. Lab. Carlsberg.* **31**:373.
22. HOSSELET, C. 1931. Contribution a l'étude du chondriome chez les insectes (Culicides et Phryganides). *Arch. Zool. Exptl. Gen.* **72**:1.
23. LAUFER, H., and M. GOLDSMITH. 1965. Ultrastructural evidence for a protein transport system in *Chironomus* salivary glands and its implication for chromosomal puffing. *J. Cell Biol.* **27**:57A (Abstr.)
24. LOCKE, M. 1966 *a*. The structure and formation of the cuticulin layer in the epicuticle of an insect, *Calpodes ethlius* (Lepidoptera, Hesperidae). *J. Morphol.* **118**:461.
25. LOCKE, M. 1966 *b*. Isolation membranes in insect cells at metamorphosis. *J. Cell Biol.* **31**:264. (Abstr.).
26. 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.
27. LOCKE, M., and J. V. COLLINS. 1966. The sequestration of protein by the fat body of an insect. *Nature.* **210**:552.
28. LOCKE, M., and J. V. COLLINS. 1967. Protein uptake in multivesicular bodies in the molt/intermolt cycle of an insect. *Science.* **155**:467.
29. MADDRELL, S. H. P., and J. E. TREHERNE. 1966. A neural fat body sheath in a phytophagous insect (*Carausius morosus*). *Nature.* **211**:215.
30. MILLER, A. I., JR., D. M. HALE, and K. D. ALEXANDER. 1965. Histochemical studies on the uptake of horseradish peroxidase by rat kidney slices. *J. Cell Biol.* **27**:305.
31. MILLER, F., and G. E. PALADE. 1964. Lytic activities in renal protein adsorption droplets. An electron microscopical cytochemical study. *J. Cell Biol.* **23**:519.
32. NAIR, K. S. S., and J. C. GEORGE. 1964. A histological and histochemical study of the larval fat body of *Anthrenus vorax* Waterhouse (Dermestidae, Coleoptera). *J. Insect Physiol.* **10**:509.
33. NOVIKOFF, A. B., and SHIN, W-Y. 1964. The ER in the Golgi zone and its relations to microbodies, Golgi apparatus and autophagic vacuoles in rat liver. *J. Microscop.* **3**:187.
34. PALAY, S. 1960. The fine structure of secretory neurones in the preoptic nucleus of the goldfish, *Carassius auratus*. *Anat. Record.* **138**:417.
35. PEREZ, C. 1910. Recherches histologiques sur la Metamorphose des muscides *Calliphora erythrocephala* Ny. *Arch. Zool. Exptl. Gen.* **44**:1.
36. PRICE, G. M. 1966. The *in vitro* incorporation of ^{14}C (U) valine into fat body protein of the larva of the blowfly. *Calliphora erythrocephala*. *J. Insect Physiol.* **12**:731.
37. PRICE, G. M., and T. BOSMAN. 1966. The electrophoretic separation of proteins isolated from the larva of the blowfly, *Calliphora erythrocephala*. *J. Insect Physiol.* **12**:741.
38. REYNOLDS, E. S. 1963. The use of lead citrate of high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* **17**:208.
39. ROBERTSON, J. D. 1964. Cellular Membranes in Development. M. Locke, editor. Academic Press Inc., New York.
40. ROSENBLUTH, J., and S. L. WISSIG. 1964. The distribution of exogenous ferritin in toad spinal ganglia and the mechanism of its uptake by neurons. *J. Cell Biol.* **23**:307.
41. ROTH, T. F., and K. R. PORTER. 1962. Specialized sites on the cell surface for protein uptake. Electron Microscopy. Fifth International Congress on Electron Microscopy Held in Philadelphia, Pa., August 29–September 5, 1962.

Sydney S. Breese, Jr., editor. Academic Press, Inc., N. Y. Vol. II, LL4.

42. ROTH, T. F., and K. R. PORTER. 1964. Yolk protein uptake in the oocyte of the mosquito *Aedes aegypti* L. *J. Cell Biol.* **20**:313.
43. RYSER, H., J. B. CAULFIELD, and J. C. AUB. 1962. Studies on protein uptake by isolated tumor cells. I. Electron microscopic evidence of ferritin uptake by Ehrlich ascites tumor cells. *J. Cell Biol.* **14**:255.
44. SCHMEIDER, R. G. 1928. Observations on the fat body in Hymenoptera. *J. Morphol.* **45**:121.
45. SHIGEMATSU, H. 1958. Synthesis of blood protein by the fat body in the silkworm, *Bombyx mori* L. *Nature.* **182**:880.
46. SHIGEMATSU, H. 1960. Protein metabolism in the fat body of the silkworm, *Bombyx mori. Bull Sericult. Expt. Sta. (Tokyo).* **16**:141.
47. SOTELO, J. R., and K. R. PORTER. 1959. An electron microscope study of the rat ovum. *J. Biophys. Biochem. Cytol.* **5**:327.
48. STAY, B. 1965. Protein uptake in the oocytes of the cecropia moth. *J. Cell Biol.* **26**:49.
49. STRAUS, W. 1964 *a.* Factors affecting the cytochemical reaction of peroxidase with benzidine and the stability of the blue reaction product. *J. Histochem. Cytochem.* **12**:462.
50. STRAUS, W. 1964 *b.* Factors affecting the state of injected horseradish peroxidase in animal tissues and procedure for the study of phagosomes and phagolysosomes. *J. Histochem. Cytochem.* **12**:470.
51. 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.
52. WIGGLESWORTH, V. B. 1942. The storage of protein, fat glycogen and uric acid in the fat body and other tissues of mosquito larva. *J. Exptl. Biol.* **19**:56.