THE HEMOCYTE TYPES, DIFFERENTIAL AND TOTAL COUNT IN *PAPILIO DEMOLEUS* L. (LEPIDOPTERA: PAPILIONIDAE) DURING POST-EMBRYONIC DEVELOPMENT

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ABSTRACT: The present study was undertaken to recognize the types of hemocytes of *P. demoleus* by electron microscopy. Six types were identified: prohemocytes (PR), plasmatocytes (PL), granulocytes (GR), spherulocytes (SP), adipohemocytes (AD), oenocytoids (OE), with two additional types, vermicytes (VE) and podocytes (PO), having debatable identities. The PRs were mostly rounded small cells with characteristically large and filling nucleus showing an uneven surface due to the presence of vaguely concentric and elevated circles by SEM. The PLs were polymorphic but largely spindle–shaped. The GRs were rounded to ovoid with relatively small and eccentric nucleus. The SPs were also rounded to ovoid with cytoplasm appearing lobulated. The ADs were rounded to elongate with cytoplasm containing lipid droplets. The OEs were rounded, and mostly large. The PL and GR remained relatively high and SP, AD and OE low throughout the postembryonic development. The VEs, were greatly elongated and narrow cells with pointed ends. The POs were flat cells with three or more cytoplasmic arms projecting from a central body. These later cells appear for a limited period only in the early pupa. The THC steadily decreases during the developmental stages, attains its peak in the late 5th (prepupa) instar and steeply declines in the pupa.

KEY WORDS: Hemocytes, morphology, total hemocyte count, differential hemocyte count, *Papilio demoleus*

The free hemocytes in the hemolymph of insects, are responsible for the defense reactions against foreign agents that penetrate the hemocoel (Tepass et al., 1994; Falleiros & Gregorio, 1995; Inoue et al., 2001). They present variable morphology and functions (Gupta, 1985; Ratcliffe et al., 1985). The free hemocytes of lepidopterous insects have been studied in a wide range of species (Yeager, 1945; Munson, 1953; Shapiro, 1979; Boiteau & Perron, 1977; Akai and Sato, 1978, 1979; Arnold, 1982; Ashhurst, 1982; Beeman et al., 1983; Essawy et al., 1985; Saxena et al., 1988; Butt & Humber, 1989; Andrade et al., 2003). The aim of this study was to characterize the hemocytes in *Papilio demoleus* larvae, a major herbivore of lemon nursery, with the aid of scanning electron microscope as well as to quantify these cells through the total (THC) and differential (DHC) counts,once that the success of the immune response depends on the number and the types of hemocytes involved in these mechanisms(Russo et al., 2001).

MATERIALS AND METHODS

The insect culture was reared as previously described by Sendi et al. (1993). For blood smear slide preparation, a small drop of heat – fixed hemolymph was obtained by clipping of the proleg present on the 7th abdominal segment of the larva or piercing the cuticle of the pupa. The drop was then drawn into a thin film by the edge of another slide and the film air – dried before staining. For staining, the stock solution of Giemsa stain prepared by the method of Yeager (1945) was diluted 10 times with distilled water. The air dried smear was stained with the diluted stain for 20 minutes and subsequently differentiated in dilute lithium carbonate solution for red staining structures and then in Hcl acidified distilled water for blue staining structures. The slide was rinsed in distilled water and mounted in DPX. For differential hemocyte count (DHC), the first instar larvae through pupae were chosen for the studies. And to determine the DHC, cell categories were counted in 200 cells chosen from random areas of the stained blood smear by a laboratory blood cell counter.

The data obtained from the total and differential counts were analyzed through ANOVA and Tukey's test ($P<0.05$).

For the total hemocytes counts(THC), the hemolymph was drawn into a Thoma white blood cell pippete up to the 0.5 mark and diluted up to the 11 mark with Tauber-Yeager fluid (Tauber and Yeager, 1935). The pipette was shaken for several minutes and the first three drops were discarded. A double line with improved Neubaur ruling hemocytometer was filled with diluted hemolymph and the hemocytes were counted in their four corners and one control (1 mm^2) sqares. The number of circulating hemocytes per cubic millimeter (mm^3) was calculated using the following formula of Jones (1962):

$$\frac{\text{Hemocytes in five 1 mm}^2 \times \text{Dilution} \times \text{Depth factor of chamber}}{\text{No. of squares counted}}$$

Where dilution = 20 times (except in 2nd and 3rd instar larvae where it was 100 times), depth factor of the chamber = 10 (constant) and No. of squares counted = 5.

For scanning electron microscopy (SEM) , the thin blood smear prepared on a cover – slip by mixing a drop of hemolymph and anticoagulant, was firstly fixed with 2.5% glutaraldelyde in phosphate buffer (PH 7.2) for 1h at 4 degree centigrade, then with 1% osmium tetraoxide for 2 h at the same temperature, and dehydrated in acetone grades. It was then immersed in amyl acetate, critical point dried, gold – coated in a sputter coater and later was observed under the electron microscope (Jeol 100 cx – II, Japan) at 40 KV with 20^o tilt. The hemocyte types were classified based on pioneering work of Jones (1962) which is still the most acceptable one.

RESULTS

Based on Jones' classification (Jones, 1962) six types of hemocytes were identified in *Papilio demoleus*. They are the prohemocytes (PRs), plasmatocytes (PLs), granulocytes (GRs), spherulocytes (SP_s), oenocytoids (OEs), adipohemocytes (ADs). Two more types, the vermicytes (VEs) and podocytes (POs) have also been observed.

The PR (Fig . 1) appears mostly rounded but some are oval or spindle – shaped, and small in size (3.8 – 7.0 µm), with characterically large and filling nucleus that reduce the cytoplasm to a narrow peripheral band. Their percentage is relatively high (40%) in the 1st instar larvae and low in the remaining instars. The surface of these cells appear somewhat uneven due to its being raised into vaguely concentric circles. Fig. 2 represents a cell type which due to its small size (cf. fig.1) could be regarded as PR but due to its surface structures (cytoplasmic projections) comes close to the GR (cf. Figs. 11, 12) and, therefore, it can be regarded as a cell intermediate between PR and GR.

The Pls (Figs. 3-10) are pleomorphic cells and are accordingly rounded fusiforms or spindle shaped, triramus or tetraramus They are large in size (7.5 – 15.3 µm) with a relatively smaller nucleus. In the I and II instars, there is no variation in their shapes but in the 3rd instar onwards, they tend to become three or four- armed. Their population ranges between 15.3 (pupa) and 49 (IV instars) percent. We could detect 6 variants of these cells based on their shape, size and surface specialization. They are: (a) the typical spindle – shaped cells with pseudopodia (Fig. 3) that sometimes contact with other cells (Fig. 4), (b) cells with a gaping surface – opening (Fig. 5), (c) cone – shaped cells with depressed bottom bearing pores and projection (Fig. 6), (d) cells with numerous surface projections of varying shapes and sizes (Figs. 7-8), (e) flat thin cells resembling PLs in amoeboid movement (Fig.9), and sickle – shaped cells with smooth margins (Fig. 10).

The GRs (Fig. 11-12) are usually rounded to ovoid in shape and 5.3-8.2 µm in size. The population of these cells fluctuates in different instars, being the lowest (10.5%) in the pupa, highest (37.8%) in 5th instar and variable in other instar larvae. The GRs along with PLs constitute the major hemocyte population during larval development. The cell surface appears covered with rather flat projections which are smaller and fewer in some (Fig. 11) and larger and many in some others (Fig. 12).

The SPs are rounded to ovoid in shape. They are larger (6.4 – 9.3 µm) than the GRs, and have a centrally located nucleus. Their population remains low in the early (I-IV) instars, attains a peak in the V instar and declines there after. The cells appear lobulated due to the marginal bulges possibly caused by accumulation of spherules. The size and number of the lobes seem to vary (Figs. 13-14). The surface may appear rough due to the presence of cytoplasmic projections (Fig. 13) or relatively smooth due to the lack of them (Fig. 14).

The Ads are somewhat rounded cells whose size ranges from 5.5-10.6 µm with a centrally locatead nucleus. These cells appear ovoid to elongate and are recognizable by their surface bulging caused by the lipid globules (Fig.15). Except for the areas bearing lipid inclusions, the rest of the surface is smooth, devoid of any surface structures.

The OEs are rounded, small to large (12 – 28 µm) cells. Compared to cell size, the nucleus is small and is eccentrically located, the population of these cells is generally low with their peak (8.4%) in the pupa. They appear spherical with a rough surface (Fig. 16) and occasionally kidney – shaped with smooth surface (Fig . 17).

The VEs (Figs. 18-19) appear only in the early stage of the pupa and disappear from its late stage. They are greatly elongated and narrow cell with pointed ends. Their length varies from 18-35µm and the nucleus is a small compact, deeply staining and centrally located. These cells show the presence of several pores on their surface but with no pseudopodial projections.

The Pos are flat cells with three or more cytoplasmic arms projecting out from a central body (Figs. 20-22). Their nucleus is centrally located, compact and fairly large in size. Their cells also appear in the early pupal stage along with the VES. They show numerous pores on their central body in some cases, the arms may be greatly shortened (Fig. 22). Such a cell may be representing a different phase in its activity.

The THC could be determined only in 2nd instar onwards, since the 1st instar failed to yield an adequate amount of hemolymph. As depicted in Table 1 the THC starts at a low value in the 2nd instar larva, increases gradually in the 3rd instar and early 5th instar larva, takes a dip in the late 4th instar, fluctuates in the early part of the 5th instar larva and shoots up in its later part, declines steeply in the pupa stage. During the moulting process, the THC starts at a low level in the early 4th (Table 2) and attains a peak prior to ecdysis. Declining slightly immediately after the moult, it increases again to attain a peak prior to pupa moult and falls thereafter.

The study of DHC showed the highest PRs population in the 1st instar larvae and the lowest in the pupae, the PLs and GRs remain relatively high and SPs, Ads and OEs low throughout the postembryonic development. The VEs and Pos appear for a limited period in the early pupa only to disappear later before the imaginal moult (Table 3).

DISCUSSION

The hemocytes of *P. demoleus* may be arranged in 6 classes on the basis of distinctive morphological and cytological features revealed by light (LM), phase (PCM) and scanning electron microscopy (SEM). They are the PRs, PLs, GRs, SPs, Ads, OEs, and two additional subtypes, the VEs, and POs.

The PRs have been reported in all insect orders studied except for Thysanura and Odonata (Gupta, 1985a). They have been described as oval or rounded cells with high nuclear to cytoplasm ratio and are

considered as the stem cells (Arnold, 1952; Srivastava & Richards, 1965; Lai–fook, 1973 and Beeman et al., 1983) which give rise to some of the hemocyte types, such as the SPs and ADs (Yeager, 1946; Jones 1959), or the PLs, GRs and SPs, (Arnold, 1970). In *P. demoleus*, the PR, remains low in number throughout the larval development except in the Ist instar where their percentage is high. As their population declines in the later instars and the percentage of PLs and GRs increases, it may be taken to indicate the conversion of the former (PRs) into the latters (PLs and GRs). By SEM, it was observed that cells resemble PRs in size but due to the presence of surface projections, they are similar to GRs entitling them to be called PR – GR intermediates (cf. Fig.6), as it has been reported in some other insects (Arnold & Sohi, 1974; Arnold & Hinks, 1976; Pelc, 1986). In *P. demoleus* also frequent mitotic figures are encountered in these cells indicating their stem – cell – nature.

The PLs have been reported in all insect orders (Gupta, 1985a). Some authors had difficulty in differentiating PRs from PLs. The PRs have been considered as young PLs and therefore, the latter has been regarded as stem cells that gave rise to the other cell types (Taylor, 1935; Gupta and Sutherland, 1966). But this view is not held by the others (Arnold, 1979). In *P. demoleus*, the PRs and PLs possess all the typical features of their own and their separate identities are confirmed by all the techniques adopted in this study. Being pleomorphic, PLs are known to present themselves in different forms. In *P. demoleus*, we came across rounded, spindle – shaped (with or without pseudopodia), tri- and tetraramus PLs and their six variants. The type with pseudopodia shows few surface pores, while the one without pseudopodia lacks them. Essawy *et al.* (1985) in their SEM study noticed a large number of surface pores in the PLs of *Heliothis armigera*, whereas Saxena et al. (1988) reported only fewer of them in the cells of *Spodoptera litura* . The latter condition is in agreement with that of in *P. demoleus*. As shown by TEM picture of Essawy et al. (1985), these pores in *H. armigera* are only superficial depressions without any apparent significance. The present results are also in agreement with that of Andrade et al. (2003) in *Anticarsia gemmetalis*.

The GRs are considered as plesiomorphic hemocytes, and are the only hemocyte type that has been reported from all major arthropod groups and Onychophora (Gupta, 1985a). Brehelin and Zachary (1986), on the basis of ultrastructural characteristics, divided them into four subtypes: GR1, GR2, GR3 cells into 2 subtypes on the basis of surface ultrastrcture: one with many and the other with fewer surface projections (pseudopodia) which possibly represent active and inactive phases of the cell, respectively. A phagocytic role has been assigned to the GRs by several authors (Crossley, 1964; Arnold, 1970; Akai and Sato, 1978, 1979; Neuwirth, 1974). Such a role for them is indicated in *P. domoleus*, also, where they are seen to establish contacts with injected India ink particles and subsequently engulf them (Figs. 26, 28). Gupta (1985a) has mentioned 3 steps in the process of encapsulation: establishment of

contact with the foreign body, release of granules or granule extrusions, and attraction of other phagocytic cells towards the foreign body. We have also succeeded in capturing the first two steps by PCM (Figs. 26, 27). It is reported by some investigators (Gupta and Sutherland, 1966; Arnold and Salkad, 1967) that the GRs are capable of giving rise to SPs, ADs and COs. In *P. demoleus*, the relative percentage of GRs decreases in the late V instar onwards and that of ADs correspondingly increases an interconversion of the former into the latter cannot be ruled out. Saxena et al. (1988) have also arrived at the same conclusion on the basis of differential counts of these cells in *S. litura* larvae.

The SPs – Hollande (1909) was the first to have named them spherule cells and the term has stayed up-to-date Although their relative population is generally low (Arnold, 1974), they are easily identified by their conspicuous spherules filling in the cytoplasm. In the present study, the SEM gave the best confirmatory results by revealing clearly the typical morphology (marginal spherule bulges) of these cells. While some authors regard SPs as the derivations of GRs (Gupta & Sutherland, 1966; Arnold & Salkeld, 1967). Millara (1947) considered them only as a phase in the life of these cells. In *P. demoleus,* there is no reason to believe in the interconversion of GRs into SPs, since the Population of both these cell types tend to simultaneously increases during the early part of the larval development and decreases in V instar onwards. Gupta and Sutherland (1966) suggested that the SPs are capable of transformation into AD and COs, but in the present insect, the transformation of SPs into ADs is not indicated, because, while the population of SPs increases from I through V instar larvae, that of the ADs fluctuate. Even though in the late V instar onwards, the SPs slightly decline and the ADs greatly increase, the population change is not proportionate to claim the conversion of SPs into ADs.

The ADs – Ashhurst & Richard (1964), Jones (1967) and Shapiro (1968) have reported the occurrence of ADs in *Galleria mellonella*, while Neuwirth (1973) failed to detect them in the same insect in her ultrastrctural study. Andrade et al. (2003) also did not report ADs from *A. gemmatalis*. The consideration of ADs as a hemocyte category has often been questioned, and it has been regarded as a functional stage of the GRs (Raina & Bell, 1974). In *P. demoleus,* we can undoubtedly regard them as cell category on the basis of features revealed by PCM (cytoplasm filled with sharply defined lipid droplets, Jones, 1959) and SEM (Characteristic globular surface–projection, Saxena & Tikku, 1990). Several authors (Ludwig & Wugmeister, 1953; Clark and Chadbourne, 1960; Gupta and Sutherland, 1966) have found that under the conditions of reduced metabolism such as chilling, starvation and diapause, ADs increase in number. Since in *P. demoleus,* the ADs increase in number in the non-feeding pupal stage, the above view could hold good in this insect, too. Gupta and Sutherland (1966) have shown the transformation of GRs into ADs . The decline in the percentage population of GRs and

corresponding increase in that of the ADs in the prepupa and pupal stage of this insect tends to support the above findings.

The OES- In different species of Noctuidae OE, are reported to be round to ovoid, rod shaped, elongated, fusiform or even triramus (Arnold, 1983). In cockroach *Gromphadorhina portentosa*, they have been found to be crescent – shaped (Gupta, 1985b). In *Anticarsia gemmatalis*, they have been reported to be the largest celluar types observed, being round with small eccentric nucleus, homogenous cytoplasm and plasmatic membrane without projections (Andrade et al., 2003). In *P. demoleus*, they are round to ovoid or sometimes even kidney – shaped, only. Since OEs, being unstable *in vitro*, undergo rapid transformation into hyaline cells, they have been often mistaken for COs in the literature (Lea and Gilbert, 1966). In almost all insects studied so far, these have been described as large-sized and non-dividing cells with small eccentric nucleus. In *P. demoleus*, the morphology of these cells and their non-dividing nature is similar to what has been described by the other workers However, Saxena et al. (1989) have described the presence of a large and centrally- located nucleus in them in *Spodoptera litura*. In *P. demoleus*, the nucleus is small and eccentric. Pelc (1986) in *Mamestra brassicae* found these cells to be dividing mitotically. In *P. demoleus*, mitotic figures have not been observed.

The VEs- The term VE was first employed by Yeager (1945) for describing extremely elongated hemocytes. Many workers (Gupta & Sutherland, 1966; Lea & Gillbert, 1966; Andrade et al., 2003) consider these cells as variant of the PLs. Arnold (1974) stated that these cells mainly appear prior to pupation. Saxena et al. (1988) in their study of *S. litura* by SEM, considered VEs and POs as separate hemocyte types. In *P. demoleus*, these cells appear only during the early pupal stage and never before and after it. Due to their elongated spindle-shape, they seem to be derived by the elongation of the PLs. Our data on their relative percentage (unpublished) tend to support this view. However, the presence of numerous pores on their surface which are only a few in the PLs, gives VEs an additional feature.

The POs- The term PO was also first employed by Yeager (1945). According to Arnold (1974), these cells have been identified correctly only in *Prodenia* (Yeager, 1945, Jones, 1959) and possibly in *Sarcophaga bullata* (Jones, 1956). Gupta (1969) found them in the nymphs of *Periplaneta americana*. Authors generally do not accept them as a separate hemocyte category. Instead, they regard them as a variant of the PLs (Riziki, 1953, 1962; Gupta & Sutherland, 1966; Devauchelle, 1971). In *P. demoleus* the POs with a flat central disc and several cytoplasmic arms compare well with the POs described by Saxena et al. (1988). Since the VEs and POs appear only in the early pupal stage, it is likely that they also perform some functions relevant to the early pupal development (i.e. metamorphosis).

The pattern of the THC changes during postembryonic development is largely similar to what has been reported in the literature on

holometabola-i.e., it increases during the larval stages, attains its peak by end of 5th instar (prepupa) and declines in the pupa.The reason that seems more plausible could be an elevated rate of mitosis that characterizes all other tissues during this period of active growth and it may also increase the number of hemocytes as observed. Besides the hemocytes are known to be involved in intermediary metabolism such as protein synthesis, transport of nutreints, phenol metabolism, growth stimulation, etc. (Wigglesworth, 1959; Crossley, 1979). Due to active growth during the larval stages, intermediary metabolism process should be higher and thertefore needs the services of a large number of hemocytes. Some authors demonstrated that ecdysone enhances the rate of mitosis in hemocytes (Hoffman, 1970; Hinks and Arnold, 1977; Prasada Rao et al., 1984). Since the ecdysone titre is high towards the latter part of each instar (Hinks and Arnold, 1977) and especially when larva is becoming prepared to undergo larval –pupal moult, the sudden rise in the hemocyte count observed in the prepupal stage of *P.demoleus* could be the effect of this hormone. The steep decline in hemocyte count in the pupa of the present insect (from 12326 / mm^3 in late prepupa to 6688 / mm^3 in 0 day pupa) is in agreement with most of the other reports. Andrade et al. (2003) reported decreasing tendency in the number of hemocytes in *A. gemmastalis* during larval period which was also observed in the present study.

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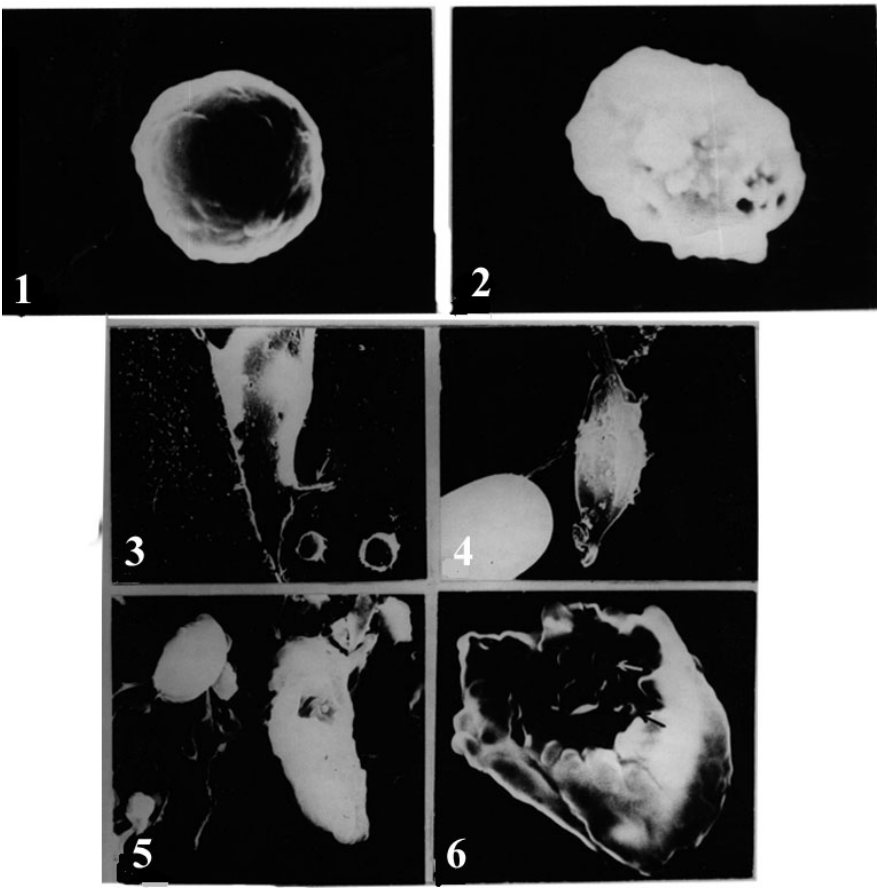

Plate I. Fig. 1. PR with surface raised into somewhat concentric circles. SEM, X 30000. Fig.2. PR-GR intermediate, (size of PR and surface structures like GR). SEM, X 45000. Fig. 3. PL-fusiform with pseudopodia (arrow) and PRs. SEM, X 9000. Fig. 4. PL-fusiform establishing a cytoplasmic contact with an OE. SEM, X 9000. Fig. 5. PL with a gaping mouth on its surface and with a GRs and two PRs. SEM, X 22500. Fig.6. PL-cone-shaped with a depressed bottom bearing pores (arrows). SEM, X 22500.

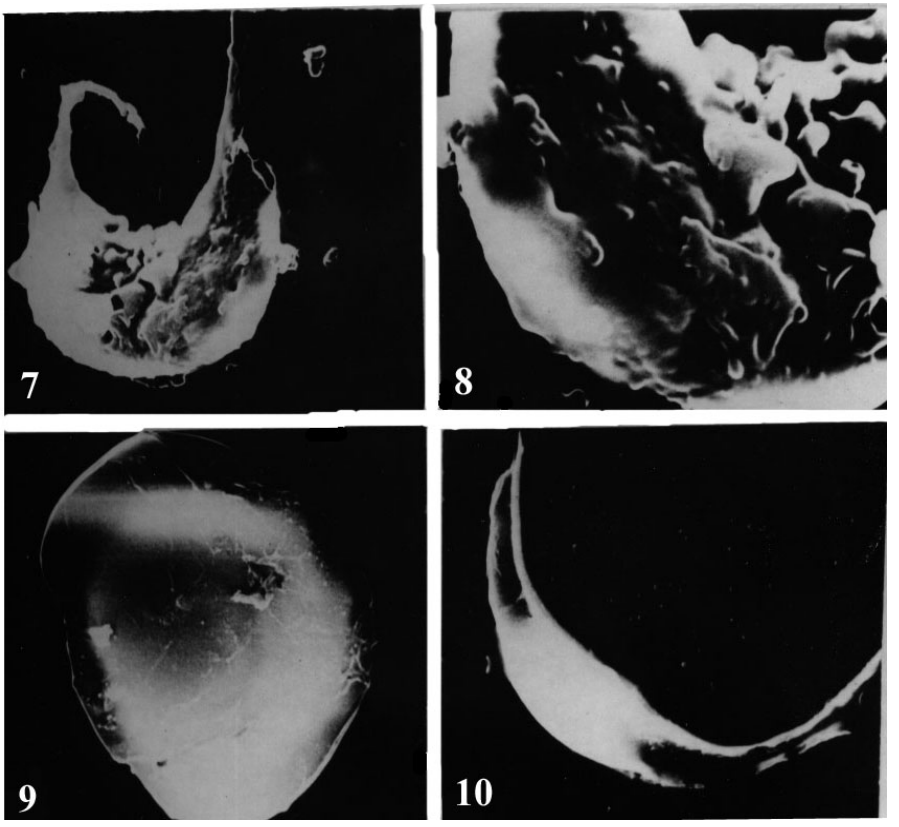

Plate II. Fig. 7. PL with numerous pseudopodial projections of variable shapes and sizes. SEM, X 9000. Fig. 8. A portion of the same magnified. SEM, X 22500. Fig. 9. PL inamoeboid movement (thin cytoplasm, irregular boundary). SEM, X 9000. Fig10. PL-sickle-shaped and smooth-margined. SEM, X 15000.

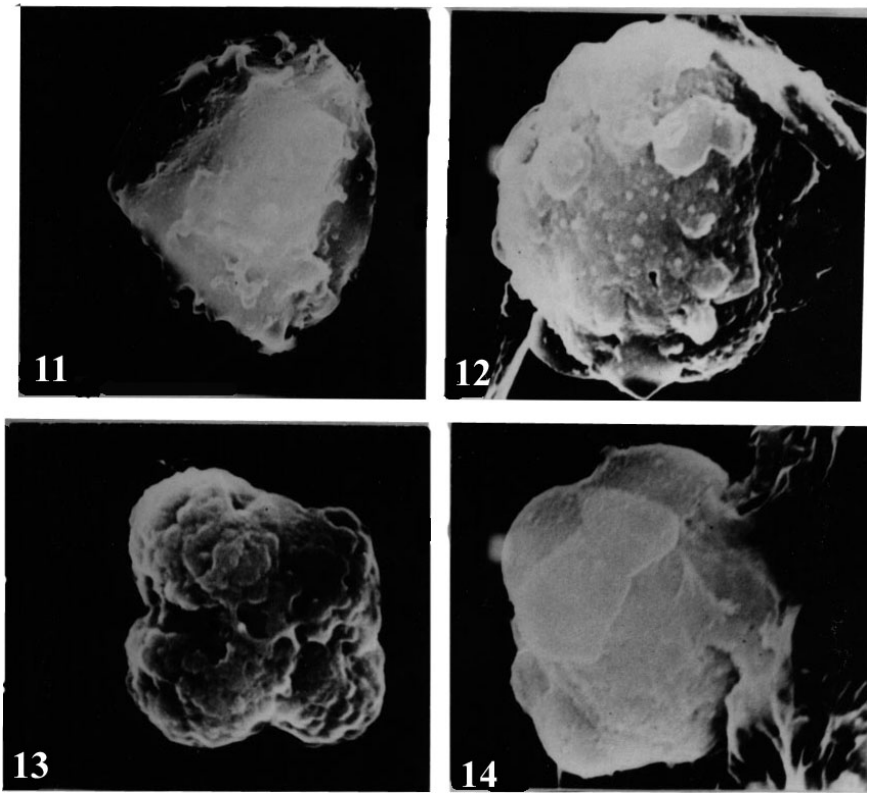

Plate III. Fig. 11. GR with fewer and smaller surface projections. SEM, X 15000. Fig. 12. GR with numerous, large and small surface projections. SEM, X 22500. Fig. 13. SP showing marginal spherule-bulges and rough surface projections. SEM, X 22500. Fig. 14. SP with marginal spherule-bulges but smooth surface due to lack of surface projections. SEM, X 22500.

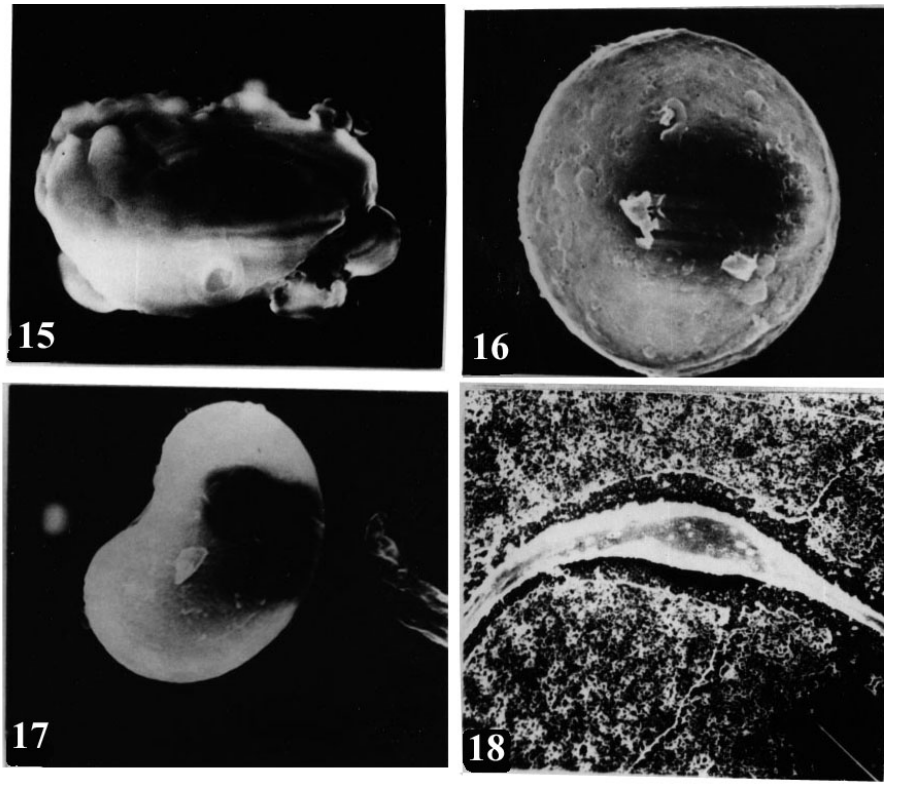

Plate IV. Fig. 15 AD with lipid globules projecting through the surface. SEM, X9000. Fig. 16. OE-spherical and rough surfaced. SEM, X 15000. Fig. 17. OE-kidney-shaped and smooth surfaces. SEM, X 15000. Fig. 18. VE showing surface pores and lack of surface projections. SEM, X 4500.

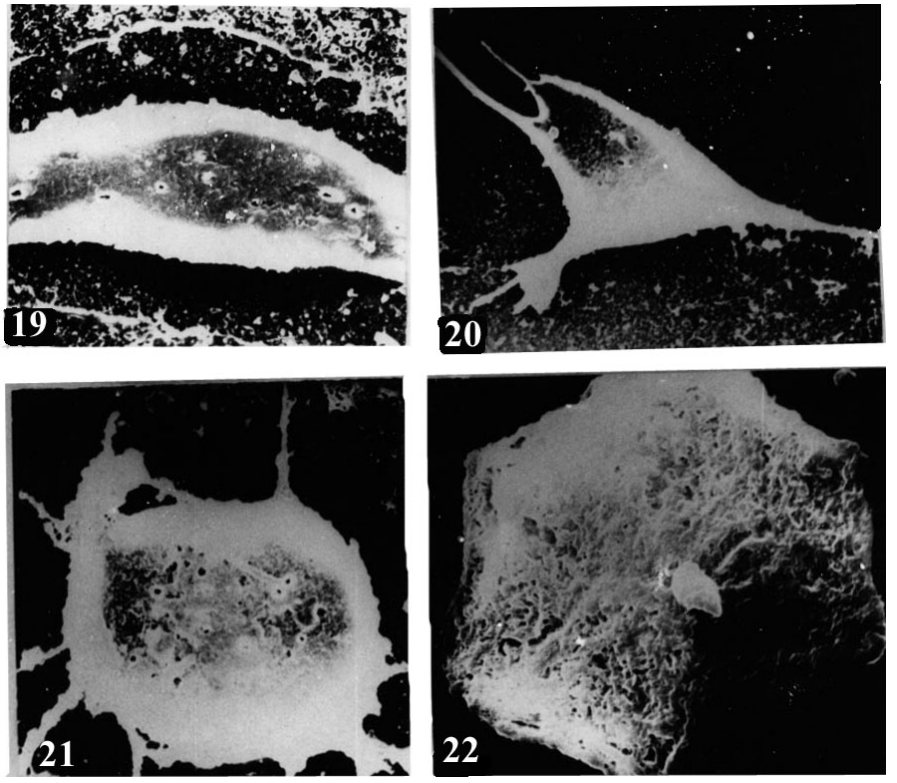

Plate V. Fig. 19. A portion of Fig. 18, magnified. SEM, X 9000. Figs. 20-22. Pos–possibly in different phases of activity, showing surface pores and variable number and sizes of arms which are greatly shortened in some cells (Fig. 22). SEM, X 4500, 9000, 4500.

Table 1.THC changes during the postembryonic development.

Instars	Insect No.	THC/mm^3±SE
II	10	2008.0±65.4
III	10	3872.0±102.0
IV	10	9244.0±84.8
V1	10	6505.4±661.7
V2	10	9440.0±822.7
V3	10	8016.0±539.8
V4	10	9557.0±516.5
V5(mid-prepupa)	10	14100.5±391.6
Pupa1	10	4840.0±492.7
Pupa2	10	3269.3±89.7
Pupa3	10	1603.0±472.8
Pupa4	10	648.0±42.3

Figures subscripted to instars indicate age.

Table 2.THC changes in relation to ecdysis.

Instars	Insect No.	THC/mm^3±SE
IV1	10	3582.6±630.0
IV4	10	7705.0±840.8
V1	10	6505.4±661.7
V4	10	9557.0±5166
V5(late prepupa)	10	12326.6±757.0
Pupa0	10	6688.0±497.7
Pupa4	10	648.0±42.3

Figures subscripted to instars indicate age.

Table 3. DHC changes during the postembryonic development.

Instars	Insect No.	percentage hemocyte types ± SE							
		PR	PL	GR	SP	AD	OE	VE	PO
I-mid	10	40.0±5.0	29.0±4.3	18.3±3.0	8.4±1.5	4.3±1.7	0.0	-	-
II-mid	10	15.0±3.6	38.6±3.2	25.0±2.3	10.0±1.0	7.0±0.6	4.4±1.1	-	-
III-mid	10	5.7±0.4	42.0±5.0	30.8±4.3	12.3±2.6	6.0±0.7	3.2±2.7	-	-
IV-mid	10	5.6±0.8	49.03.4	27.6±0.8	12.5±3.0	2.8±0.4	2.51.5	-	-
V-mid	10	3.6±1.0	33.5±3.9	37.8±3.1	15.3±3.6	3.3±1.1	6.3±0.5	-	-
V-late(prepupa)	10	2.5±0.5	40.5±4.1	22.5±3.8	13.8±3.3	13.90.3	6.8±1.3	-	-
Pupa1	10	2.0±0.8	5.3±3.6	10.5±2.5	12.2±3.0	18.2±2.8	8.4±1.7	26.0±5.0	7.4±3.6