An Electron Microscopical Study of the Ultrastructure and Classification Hemocyte of the Grasshopper, Melanoplus Differentials (Thomas) (Orthoptera: Acrididae)

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AN ELECTRON MICROSCOPICAL STUDY OF THE
ULTRASTRUCTURE AND CLASSIFICATION
HEMOCYTE OF THE GRASSHOPPER, MELANOPLUS
DIFFERENTIALIS (THOMAS) (ORTHOPTERA: ACRIDIDAE).

by
Andrejs Liepins

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Submitted to the
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Of The
Degree of Master of Arts

Western Michigan University
Kalamazoo, Michigan
April, 1972
The hemocytes of the fifth instar nymphs of the grasshopper *Melanoplus differentialis* (Thomas) (Orthoptera: Acrididae) have been the subject of this study. The hemocytes of this insect, based on the fine structures of its cells, have been found to fall into four main categories or groups: Nucleocytes, Plasmatocytes, Granulocytes and Coagulocytes. The bases for their classification are differences in the ultrastructure of the cytoplasmic organelles as well as cytoplasmic inclusions as revealed by electron microscopy.
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and ideas, for my better understanding of the American
culture, outside the academic environment.
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GENERAL INTRODUCTION

The hemocoele and the hemolymph or circulating body fluids of insects have been the subject of many investigations. Of particular interest have been the cellular inclusions of hemolymph, called the hemocytes, otherwise referred to as blood cells.

Since the time of Swammerdam (1737), the insects have been known to possess hemocytes. However, even today, there does not exist a single workable classification of these cells, applicable to any group of insects. Lack of consistency or uniformity in classification may be due, at least in part, to the variety of fixation and cytochemical techniques employed by different workers in the classification of these cells.

What is urgently needed is perhaps an understanding of the interrelationship between the morphologically different cells with respect to their origin and function. With respect to the origin of hemocytes, Hoffmann has shown that in Locusta migratoria, at the area of the dorsal diaphragm, there is an agglomeration of reticular and blood cells that present the characteristics of a rudimentary hemocytopoietic tissue.

Functionally, the hemocytes of Calliphora erythrocephala have been shown to participate in the breakdown of larval muscles (Crossley, 1965). There is no evidence
that they are involved in respiration, which is accomplished by means of a tracheal system. However, the phagocytic role of hemocytes has been supported by their abundance in the hemolymph before pupation and by the presence of slender processes or pseudopodia which appear to engulf droplets of hemolymph, histolizing larval muscles and other tissues not destined to survive into the adult stage (Crossley, 1965). They have also been shown to participate in defense mechanisms against invading foreign bodies and bacteria by their capacity to form a capsule which surrounds the invading elements (Salt, 1967).

Other functions attributed to hemocytes are those of blood coagulation, connective tissue formation, metabolism, and the manufacture of hemolymph proteins (Wigglesworth, 1965).

In view of the recently discovered functions of the hemocytes, there seems to be a need to provide morphological evidence for a more workable classification of hemocytes as well as their possible role in the different functions mentioned above.

Although there are hemocytes that are transitional in appearance between the clear cut groups of a classification system, a system of this sort, even though it may be arbitrary, would be useful to identify the cells morphologically if their functions are to be discovered (Jones, 1962).
In the present study, the ultrastructure and classification of the hemocytes of the larvae of *Melanoplus differentialis* has been worked out with the aid of electron microscopy. Particular attention has been given to cell shape, nuclear-cytoplasmic ratios, fine structure of the cytoplasmic organelles, cytoplasmic inclusion, as well as the possible functional role of the hemocytes in relation to their different morphological appearance.

Hemocytes, under the electron microscope, vary considerably in their histological appearance. These cells possess a rich variety of cytoplasmic structures that differ in degree or extent among cell types found in the fifth larval stage of the insect under study. The results of this electron microscope study have been correlated with those obtained by light microscopists in order to retain the prevalent terminology used in the classification of insect hemocytes.
LITERATURE REVIEW

General Considerations

Insect hemocytes have been extensively investigated (Hrdy, 1959; Rizki, 1962; Crossley, 1964), but a coherent picture of the types as well as the function of these cells has not yet emerged.

Since the first review published in 1937 by Roseboom (Avery, 1963), extensive morphological and cytochemical studies using light and phase contrast microscopy have been carried out. Wigglesworth (1959) and Jones (1962) have given a detailed review on this topic, including extensive lists of references. Little could be added to their reviews, until recently when electron microscope studies have shed new light. Different workers have employed different characteristics of classification in the characterization of hemocytes into different groups or categories.

The main criteria of identification used to classify hemocytes were summarized by Avery (1963). They are:
1. Size and shape of the cell, 2. staining properties,
3. cytoplasmic inclusions, 4. nuclear-cytoplasmic ratio, and 5. function.

Yeager (1945), working on the larvae of Prodermia eridania (Cramer) was able to recognize ten classes of hemocytes with 32 types. The classes were:
a. Proleucocytoids, b. small chromophil cells derived from (a), c. Oenocytoids, d. Plasmatocytes with finely vacuolated cytoplams, e. podocytes, f. vermiform cells, g. cytocytes with rounded eosinophil inclusions, h. spheroidocytes with colourless vacuoles, i. eruptive unstable cells, and j. degenerative cells.

Using phase contrast microscopy, histochemistry and electron microscopy on Calliphora larvae, Crossley (1964) classified the hemocytes into seven types that he labeled: Z, A, B, C, D, E, and F. Rizki (1957, 1962) described only two main types of hemocytes in the larvae of Drosophila melanogaster: the plasmatocytes and the crystal cells. Toward the end of larval stage, a modified plasmatocyte appears which he calls podocyte. In addition, another cell type which appears at the time of pupation was referred to as lamellocyte.

According to Rizki (1962), intermediate forms to the above mentioned cell types do exist. This led him to put forth the hypothesis that the podocytes and lamellocytes are variants of the plasmatocyte type blood cell.

In cricket, Acheta domesticus L., four main types have been distinguished (Hrdy, 1959): 1. Nucleocytes, 2. plasmatocytes, 3. rhegmatocytes, and 4. granulocytes. Naked nuclei and other degenerative forms were also described.
The different hemocyte classifications mentioned above are a small sample of the taxonomic diversity encountered in the literature. More complete lists of classification and terminology may be found in the reviews of Jones (1962) and Wigglesworth (1959).

Lack of standard techniques, the pleomorphicity of hemocytes and their capacity to give different staining reactions may have contributed to the diverse classifications mentioned. The problem is further complicated by mutants within species which result in further variations of hemocyte behavior with respect to their function in defense mechanisms (Rizki, 1962).

Finally, a lack of consistent terminology as well as the diversity of organisms studied has further complicated the task of any comprehensive comparative review of insect hemocytes. To illustrate this point, Avery (1963) compiled a list of names employed to describe a single hemocyte which is found in the majority of insects studied. This characteristic hemocyte is small; has a relatively large and round nucleus, surrounded by a small amount of basophilic cytoplasm; and contains little or no vacuolation. Some of the names attributed to it are: Amoebocyte (Cuenot, 1896); Leucoblast (Arvy, et al. Bounhiol, 1952; Lhoste, 1957); Lymphosite (Cameron, 1934); Nucleocyte (Hrdy, 1959); Plasmatocyte (Rizki, 1953 and 1957); Prohemocyte (Arnold, 1952; Jones, 1950, 1956, 1959)
and 1962) etc. Although this list is by no means complete, it is sufficient to illustrate the diversity in names ascribed to a particular type of cell.

As indicated previously, fixation and staining techniques employed in light microscopy studies of hemocytes may be the reason for the different names ascribed to what may well be the same cell. To resolve this problem of classification of hemocytes, a study of the ultrastructure of these cells appears to be necessary.

Review of Electron Microscopical Studies

From the foregoing account, it is clear that most of the studies on the morphology of insect hemocytes were conducted by light or phase contrast microscopy. They have revealed little information on the fine structure of hemocytes, except for terms of such general descriptive nature as granules, spheroids, or vacuoles to describe structures found in the cytoplasm of these cells. From these studies, little can be said about the possible function of the cytoplasmic inclusions as well as that of the cells in which they are found.

Electron microscopical studies have revealed some information about the ultrastructure of hemocytes with possible correlation of structure and function. Hoffmann (1966), studying the ultrastructure of larval and adult Locusta migratoria granular hemocytes, was able to
distinguish two types of cells based on the fine structure of the cytoplasmic granules. Type one was characterized as having numerous, small, electron-dense, non-structured granules; and the second type as having larger but fewer globular cytoplasmic inclusions which present a tubular internal structure, and an extensive swollen or dilated endoplasmic reticulum. He suggested that these two types of cells are highly specialized and that one might be derived from the other, each having a different physiological role. The possible function of these cells was not indicated by the author.

In a later and more extensive study, Hoffmann et al. (1968b) further examined the hemocytes of adult *Locusta migratoria*, and distinguished three fundamental categories or groups of cells: the plasmatocytes, the granulocytes, and the coagulocytes. The plasmatocytes were shown to contain dense bodies suggesting a "reabsorptive" nature or function for these cells. The granulocytes were characterized by the presence in their cytoplasm of numerous dense bodies of irregular dimensions and little developed endoplasmic reticulum forming short strands in the neighborhood of mitochondria. These cells were found to be generally elongated with invaginations along the cell membrane. The granulocytes, according to the author, are capable of changing into a modified cell type called the oenocytoid, which is characterized by an accumulation of
tubular material within the perinuclear space as well as the endoplasmic reticulum. The third main type, the coagulocytes, are characterized by their richness in free ribosomes; extensive endoplasmic reticulum with a tendency to be expanded or dilated; well developed Golgi apparatus and fewer electron dense bodies than in the granulocytes.

Referring to his previous work, mentioned above, the author divides the granular hemocytes into two categories: the granulocytes as such, and the coagulocytes. He considered the oenocytoids as representing a particular evolutionary form of granulocytes. This transformation would be brought about by a change in the synthesizing activities of the granulocyte's endoplasmic reticulum, which would be responsible for the production and storage of the tubular material in the perinuclear and endoplasmic reticular spaces. The author suggests that this phenomena is similar to storage of glycoproteins (Russel bodies) in the plasmatocytes of mammals. This "process of storage" is accompanied by a decrease in the number of electron dense bodies.

Shortly after the studies of Hoffmann (1968), Cassier and Fain-Maurel (1968) studied the accumulation of microtubules in oenocytoids of the migratory grasshopper, Locusta migratoria migratoroides (R. and F.). He established by means of cytochemical techniques that the material accumulated in the perinuclear and endoplasmic
reticular spaces were sulfur-containing proteins and not muco-polysaccharides as had been previously suggested. It could not be pinpointed, however, whether these proteins were extra-tubular, tubular, or intra-tubular.

With respect to the origin of hemocytes, Jones (1962) pointed out that experimental proof for the presence of hemocytopoietic organs in insects, while convincing in many cases, was still lacking. He suggested that in many instances, hemocytes can become trapped during fixation, and these artificial accumulations had probably been mistaken for hemocytopoietic tissue.

In the Orthopterans, *Gryllus bimaculatus* and *Locusta migratoria*, Hoffmann et al. (1968a and 1968c) have shown that the "phagocytic organs" described by Cuenot (1896) present the structural organization of a hemocytopoietic organ. In *Gryllus*, it is a compact structure with a system of fibers comparable to that of vertebrate's hematopoietic organs, whereas in *Locusta* this tissue is organized in a diffuse manner.

When bleeding experiments were carried out in *L. migratoria* by Hoffmann (1969), the total hemocyte count was found to increase immediately after the experimental bleeding. Differential hemocyte counts showed that this increase is due to the sudden release of poorly differentiated blood cells, the cytological characteristics of which are identical to those of the reticular cells of
the hemocytopoietic organs described previously. From differential hemocyte counts, the author was able to show the coagulocytes were the first differentiated cell type to appear in the hemolymph and the granulocytes were the last. This observation, for which the author gives no explanation, is in direct opposition to Jones' (1962) hypothesis of granulocytes giving rise to coagulocytes.

Histological observations on the hemocytopoietic organ after bleeding, showed a considerable increase in the number of small groups of like cells (hemocytes) in the process of differentiation, connected to each other by means of desmosomes and surrounded by a compact tissue (Hoffmann, 1968c).

Other possible sources of hemocytes is their division by mitosis. Avery (1963) studied the postembryonic development of the grasshopper *Melanoplus differentialis* in which he found that during the early stadia of development, the number of potentially dividing cells is of sufficient quantity to replace not only hemocytes lost through degeneration and death but also to add to the total number of hemocytes. As the insect progressed through various stages of development, the number of dividing cells was found to be reduced so that they are no longer able to substantially add to the total number of cells, but are still able to replace the cells being lost by degeneration. Avery (1963) assumes an absence of hemocytopoietic organs
in the nymphs of Melanoplus differentialis. And he sug­
gests that changes in hemolymph volume and hemocyte
adherence to tissues as a possible explanation for the
changing hemocyte over-all picture in the post-embryonic
development.

Hoffmann (1970), however, gives strong evidence,
based on electro-microscopic studies, that the "phagocy­
tic tissues" of the dorsal diaphragm in the Orthopterans
Gryllus bimaculatus and Locusta migratoria are hemocyto­
poietic in nature. In these two species, the hemocytes
are shown to develop from a large number of reticular
cells of mesodermic origin. Thus, undoubtedly, the
changing pattern of hemocytes during embryonic and post­
embryonic development in these two forms are due to the
presence of the hemocytopoietic tissues.
MATERIALS AND METHODS

Grasshoppers were obtained from the Argonne National Laboratory. In the present study, the last (fifth) instar was used as a source of hemolymph.

The posterior tip of the fifth stadium was cut off, and by a gentle squeeze, the hemolymph was directly poured into a centrifuge tube containing chilled paraformaldehyde glutaraldehyde. Fixation was done for one hour, essentially following the technique of Karnovsky (1965). The fixed material was centrifuged at 2,000RPMs for five minutes yielding a pellet and the supernatant. The supernatant was centrifuged once again to insure that all the residual cells were now in a pellet.

The pellet was washed in 0.1M phosphate buffer and post-fixed in 1 per cent osmium tetroxide pH 6.8, for one hour. The pellet was then cut into small pieces and dehydration was done through a series of alcohols, ten minutes each in 50 per cent, 70 per cent, 90 per cent absolute alcohol. It was washed once again in absolute alcohol for another ten minutes.

After dehydration, the material was washed in propylene oxide and transferred to one:one mixture of epoxy: propylene oxide contained in glass vials. After about one-two hours, more epoxy (half as much as the total volume of the mixture in the vials) was added to bring
its level to 75 per cent of the mixture in the vial. This was left overnight. Then it was embedded in epoxy contained in gelatin capsules. The blocks were hardened at 60° degrees centigrade for 12 hours. Gelatin capsules were dissolved away in hot tap water. The numbering of the blocks containing the experimental material was purely arbitrary.

Sections were cut with glass knives in an LKB microtome III, and mounted on carbon coated copper grids. Sections were stained with uranyl acetate for one hour (Watson, 1958) and lead citrate (Venable and Loggeshall, 1965), and examined under an R.C.A.-E.M.U.-3, or Siemens Elmiskop IA, electron microscope.

Block 347 contains a pellet of hemolymph.
Block 348 contains a pellet of supernatant of the material centrifuged for Block 347.
Block 349 contains a hemolymph pellet treated with glutaraldehyde and phosphate buffer (Na Na).
Block 350 contains the hemolymph pellet treated with glutaraldehyde and phosphate buffer (Na K).
RESULTS

General Considerations

Under the electron microscope, the hemocytes vary with respect to their size and shape. The nucleus is well defined in all the cells observed in this study, and its shape appears to follow the general outline of the particular cell. The chromatin within the nucleus is compact and closely associated with the nuclear membrane, with periodical interruptions where the less dense nuclear matrix is seen to come in contact with the nuclear membrane.

The cytoplasm in the less differentiated cells, presents an extensive network of rough endoplasmic reticulum. The membranes of this endoplasmic reticulum are seen to follow the general outline of the nucleus and are less abundant toward the periphery of the cell. Free ribosomes are abundant in the cytoplasm as well as attached to the membranes of the endoplasmic reticulum and the outer nuclear membranes (Plates I and II).

Electron dense granules are abundant in certain cells where they occupy the greater portion of the cytoplasm. Less electron dense vacuoles of various sizes, which appear to be of pinocytic origin, with granular or flocculent contents, are observed toward the cell periphery.
and interspersed with the electron dense granules.

Mitochondria vary in their size and shape and are found interspersed among the electron dense granules throughout the cytoplasm. Occasionally, microtubules are observed within the cytoplasm arranged longitudinally along the longer axis of the cell. The Golgi apparatus is less ubiquitous than the other cytoplasmic inclusions mentioned above.

Hemocyte Classification

The electron micrographs presented in this work show that the hemocytes of the fifth instar of the grasshopper Melanoplus differentialis (Thomas) can be divided into four main types based on their ultrastructural characteristics. Following the prevailing literature terminology, these groups are: nucleocytes, plasmatocytes, granulocytes and coagulocytes.

The nucleocytes (Jones', 1962, prohemocytes) are generally large, ovoid or slightly elongated cells with a prominent large nucleus that occupies the greater portion of the cell cytoplasm (Plates I and II). The cell membrane is generally smooth, but in some cases, presents finger-like projections or pseudopodia. The nuclear membrane follows the general outline of the cell membrane. Within the nucleus, the chromatin material forms dense compact masses, closely associated with the nuclear
membrane. The cytoplasm of these cells is rich in free as well as membrane-bound ribosomes. The rough endoplasmic reticulum is located in the perinuclear area closely following the outline of the nuclear membrane. Mitochondria are numerous throughout the cytoplasm and vary in their size and shape. Microtubules are seen in the outermost portion of the cytoplasm running in the direction of the longer axis of the cell. Membrane-bound electron dense and electron light bodies in the cytoplasm are of variable size and relatively small in number. Vacuoles with a flocculent content of presumable pinocytotic origin, are observed in the outer portion of the cytoplasm in the vicinity of the electron denser bodies.

The plasmatocytes are elongated cells with irregular surface that frequently present digitations or small pseudopodia. The nucleus occupies a smaller portion of the cytoplasm than it does in nucleocytes and presents occasional invaginations that are followed by ribosome studded membranes of the endoplasmic reticulum.

Chromatin is characteristically compact and closely associated with the nuclear membrane. As for nucleocytes, the cytoplasm is rich in free and membrane bound ribosomes. Mitochondria are numerous and of various sizes and shapes. Microtubules running in the longitudinal direction of the cell and are seen generally in the vicinity of mitochondria. Electron dense and electron light
bodies as well as vacuoles are of variable size and shape and generally found toward the periphery of the cell cytoplasm. The Golgi apparatus when seen, is well defined and presents numerous small vesicles in its vicinity (Plates III and IV).

The granulocytes are the most abundant group of cells observed. They are characterized by the numerous electron dense bodies found throughout the cytoplasm (Plate V). The nucleus occupies a smaller portion of the cytoplasm than in the two previous cell types described, increasing the area of the cytoplasm which is largely occupied by different size electron dense bodies. Vacuoles and electron light bodies are fewer in number than the darker electron dense bodies. The vacuoles containing a flocculent material are generally found toward the periphery of the cell. Free ribosomes and mitochondria are seen dispersed among the electron dense bodies. The rough endoplasmic reticulum remain well defined and follows the contours of the nucleus. Only short cisternae of the endoplasmic reticulum are seen interspersed with mitochondria among the ubiquitous dense bodies. An unusual horseshoe shape double membrane is observed in the cytoplasm of some of these cells and appear to be surrounding clumps of free ribosomes (Plate VI, Figures a and b).
The fourth group distinguished are the coagulocytes (Hrdy, 1959, rhegmatocytes). The cytoplasm of these cells is rich in free ribosomes and are characterized by an extensive but expanded endoplasmic reticulum. These expanded membranes of the endoplasmic reticulum are seen to be continuous with the outer nuclear membrane (Plate VI and VII). The outer nuclear membrane as well as the membranes of the expanded endoplasmic reticulum are studded with ribosomes. Free ribosomes are observed throughout the cytoplasm of these cells, in many cases forming small clusters or rosettes (Plate VII).

Although electron dense bodies are rare in these cells, large bodies, with low electron density content, are observed quite frequently in the periphery of the cytoplasm of these cells. The larger of these electron light bodies are seen to have an internal structure of alternating electron light and electron dense bands (Plate VIII).

In cross sections and under higher magnification, these alternating electron dense bands appear to be tubular in nature (Plate VIII, figure b). The diameter of these electron light bodies ranges from 1 to 2 microns.

Vacuoles limited by a single membrane with periodic ribosome like granules lining the inner surface of this membrane have been found within the cytoplasm of some coagulocytes (Plate IX). Mitochondria and the vacuoles
with flocculent content are also observed in the cytoplasm of these cells but are less ubiquitous than in the cell types previously described.

The coagulocytes sediment last in pellet during the preparatory hemolymph centrifugation, therefore, requiring large amounts of sectioning of the imbedded material as well as careful examination of the sectioned material in the electron microscope for these cells to be observed. In relation to the relative abundance of the different hemocyte types described, the coagulocytes appear to be the least abundant.
DISCUSSION

The present study of the ultrastructure of hemocytes of the fifth nymphal stage of *Melanoplus differentialis* (Thomas) permits the classification of these cells into four main groups. Following the prevailing terminology encountered in the literature, the groups are: nucleocytes, plasmatocytes, granulocytes and coagulocytes.

The nucleocytes are large round cells with a prominent nucleus that occupies the greater portion of the cytoplasm. The narrow cytoplasm surrounding the nucleus is rich in free ribosomes and mitochondria. The endoplasmic reticulum is well defined in the perinuclear region. The electron dense as well as the electron light bodies are rarely found in the cytoplasm of these cells. Vacuoles with a flocculent material are more abundant toward the periphery of the cytoplasm than in the perinuclear region.

The plasmatocytes are elongated cells, presenting frequent digitations on their cell surface. The nucleus of these cells occupies a smaller area of the cytoplasm than its counterpart in the nucleocytes. The nuclear membrane presents deep invaginations, the outline of which are followed by membranes of the endoplasmic reticulum. The nuclear chromatin is characteristically compact and closely associated with the nuclear membrane. As in
nucleocytes, the cytoplasm of these cells is rich in free ribosomes and mitochondria. Microtubules are observed to be oriented in the direction of the longer axis of the cell, suggesting a skeletal or supportive role for the cell. Electron dense and electron light bodies, as well as vacuoles with flocculent content, are generally observed to be located toward the periphery of the cell cytoplasm. The Golgi apparatus, with numerous small vesicles in its vicinity is observed occasionally in these cells.

The third type of cell, the granulocytes, were the most frequently observed. These cells are characterized by numerous electron dense bodies, found throughout their cytoplasm. The nucleus occupies a smaller portion of the cytoplasm in comparison to its counterpart in the nucleocytes and plasmatocytes. Electron light bodies and vacuoles with flocculent content are fewer in number when compared to the abundant electron dense bodies. The endoplasmic reticulum is well defined in the periphery of the nucleus but only short strands of cysternae are observed interspersed among the electron dense bodies. Horseshoe shaped double membrane structures, that appear to be engulfing groups of cytoplasmic ribosomes, are observed in the cytoplasm of these cells.

The fourth group of cells distinguished, are the coagulocytes. These cells are characterized by the expanded cysternae of their endoplasmic reticulum. In
these cells the membranes of the endoplasmic reticulum are seen to be continuous with the outer nuclear membrane, and both being studded with ribosomes. Free ribosomes are observed throughout the cytoplasm, in many cases forming small clusters or rosettes. Electron dense bodies are rare in this type of cell, but large electron light bodies, one to two microns in diameter, are frequently found occupying the cytoplasmic space between the nucleus and the cell membrane. Some of these large electron light bodies are observed to have an internal structure consisting of alternating electron dense and electron light bands. When observed in cross section and under higher magnification, the alternating electron dense bands appear to be tubular in nature. No reference to the possible role or function of these large cytoplasmic bodies has been found in the literature. Another unusual structure found in the cytoplasm of these cells (coagulocytes) are the large vacuoles, limited by a single membrane with small electron-dense granules on the inner surface of this limiting membrane. The presence of electron-dense granules, similar to ribosomes in size and electron density, on the inner surface of the membrane, raises the possibility that these vacuoles may be an intra-vacuolar synthesizing machinery not previously described. Whether this is the case, it remains to be experimentally established by radioautographic and histochemical studies. Mitochondria and smaller
vacuoles with flocculent content are also present in the cytoplasm of these cells but are less ubiquitous than in the three cell types described above.

The size of the hemocytes was found to vary within each group and could not be used as a criteria to aid in their classification. From the electron micrographs presented in this work (see results), it becomes evident that the plasmatocytes, nucleocytes and coagulocytes contain electron dense bodies similar to those found in granulocytes, though their number is much smaller in their plasmatocytes, nucleocytes and coagulocytes than in the granulocytes.

Functionally, plasmatocytes of the Egyptian cotton worm have been shown by Harpaz et al. (1969) to be involved in defense mechanisms by being capable of phagocitize infective virus particles (Harpaz, 1969; Kislev et al., 1969).

When the hemocytes, in the present study, were tested for acid phosphatase, the electron dense bodies found in the cytoplasm of plasmatocytes and granulocytes were found to give a positive reaction by lead phosphate precipitate. These results are in agreement with those of Harpaz et al. (1969) for Spodoptera littoralis.

Crossley (1964), working with Calliphora erythrocephala has shown that the hemocytes responsible for the engulfment of histolyzing prepupal larval tissue also

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give positive acid phosphatase reaction. Although Crossley chose to call these phagocytic hemocytes F-type, the electron micrographs presented in his paper show that the ultrastructure of this hemocyte corresponds to that of a granulocyte type cell described in the present study. This indicates that some of the hemocytes of different groups of insects have similar ultrastructural characteristics with possible common function. Barra (1969) working with Colembola insects, presents electron micrographs that demonstrate the presence of granulocytes in the exuvial fluid, between the old and new cuticle. The fine structure of this cell is also found to be analogous to that of the granulocytes of Melanoplus differentialis described in the present study, as well as to the granulocytes of Locusta migratoria described by Hoffmann (1968b).

Neither the manner in which the granulocytes of Colembola transverse the hypodermis of this insect, nor their function in the exuvial fluid is known according to Barra (1969). He observed, however, that the presence of granulocytes in the exuvial fluid, corresponds to that of the beginning of the molting cycle, suggesting that these cells may participate either in the breakdown of the old cuticle, the formation of the new one, or both. Whichever is the case, it has not yet been experimentally established, but the role of granulocytes in different functions becomes evident.
The nucleocyte's fine structure, as described in the present study, reveals that the cytoplasm of these cells contains vacuoles with flocculent content, similar to hemolymph with respect to their electron density, suggesting a phagocytic role for these cells. The nucleocyte ultrastructure, as described in the present work parallels in every respect the ultrastructure of the "reticular cells" described by Hoffmann (1970), in the hemocytopoietic organs of Locusta migratoria and Gryllus bimaculatus. These "reticular cells", according to Hoffmann, are capable of phagocytosis as well as being capable of differentiating into plasmatocytes, granulocytes or coagulocytes. The cytoplasmic projections or pseudopodia observed in the nucleocyte cell surface (see Plate I) also lends support for a phagocytic role of this cell.

The role of coagulocytes in blood coagulation have been extensively studied in Periplaneta americana by Gregoire (1951, 1953, 1957, and 1959). In the process of coagulation, the coagulocytes are said to become distorted in morphology and break down to release clouds of cytoplasmic material into the hemolymph around them (Gregoire, 1953). As a result, islands of coagulation, consisting of coagulocytes and hemolymph, are formed (Gregoire, 1959). These studies of insect blood coagulation have been carried out with the aid of phase
contrast microscopy under which the coagulocytes are described as hyaline hemocytes because of their appearance under this instrument.

At the outset of coagulation of the hemolymph in *Locusta migratoria*, Hoffmann et al. (1968b) described the coagulocyte ultrastructure as undergoing the following modifications: absorption of the hyaloplasm; vesiculation of the endoplasmic reticular cysternae, the content of which becomes very dense; condensation of nuclear chromatin conferring on the nucleus a pinocytotic appearance; the mitochondria conserve their dense matrix; the electron dense bodies do not show any evident changes in their structure and conserve their marginal position in the cytoplasm. The cell surface, according to this author, is now in contact with fibrillar material precipitated around the coagulocyte. The source of the fibrillar material is not established by this author.

The hemocytes described as coagulocytes in the present study correspond in every ultrastructural detail to the granulocytes of *Locusta migratoria* as described by Hoffmann et al. (1968b) and Hoffmann (1970). Thus, it appears to be fairly certain that the coagulocytes, as their name implies, function as cells associated with the coagulation of insect hemolymph.

The ultrastructure and classification of the hemolymph of *Melanoplus differentialis* as described in the
present study is found to be in agreement with that
employed by Hoffmann in describing the hemocytes of
Locusta migratoria and Gryllus bimaculatus (Hoffmann,

Although electron microscopy gives a static view
of the hemocytes due to the methods involved in the
preparation of the material, nevertheless an attempt to
correlate their ultrastructure with their possible func­
tion under normal physiological conditions, has been made.

The diverse classification systems were no doubt due
to the methods of fixation and staining employed by dif­
ferent workers involved in the study and classification
of these cells. These problems are not encountered in
electron microscopical studies where the classification of
these cells is based on their ultrastructural and not
staining characteristics.

Although any classification system is to a certain
degree arbitrary and subjective to the worker studying
these cells, a system of this sort, based on the ultra­
structure of these cells, is useful in order to ascribe
the different hemocyte functions to a specific type of
cell.

The origin of insect hemocytes has been a source
of controversy among insect physiologists that has pre­
vailed until today (see introduction). Recently, however,
Hoffmann (1970) has shown conclusively that the
Orthopterans *Gryllus bimaculatus* and *Locusta migratoria* possess hemocytopoietic organs. Within these organs, located in the dorsal diaphragm, the different types of hemocytes are shown to differentiate from reticular cells of mesodermic origin. The differentiation of hemocytes takes place in the form of isogenic cell islets as is the case in vertebrates. Thus, the hypothesis that hemocytes originate by mitosis of freely circulating cells (Jones, 1962) seems no longer tenable. Hoffmann (1970) has also demonstrated that after experimentally induced bleeding, poorly differentiated cells (reticular cells) capable of undergoing mitotic divisions, appear in circulation. Therefore, it seems reasonable to conclude that the hemocytes in circulation that have been observed undergoing mitosis is due to either the method employed in their study or due to a normal release of undifferentiated cells from the hemocytopoietic organs (see Rizki 1957, Jones 1962, Avery 1963, and Hoffmann 1970). In any case, the mitotic index of circulating hemocytes has never accounted for the changes in total hemocyte counts at different stages of development (Rizki 1962, Avery 1963).

In the present study, of circulating hemocytes of the *Melanoplus differentialis* nymph, no hemocyte was observed to be undergoing mitosis. Although the number of hemocytes examined in the course of the study may be statistically too small for a definite conclusion, it nevertheless
supports the idea that the method used in obtaining the hemolymph may produce a premature or precocious release of undifferentiated cells, in the process of mitosis from the hemocytopoietic organs that under normal physiological conditions would be limited to these organs and not found in circulation.
SUMMARY

The hemocytes of the fifth instar of the grasshopper Melanoplus differentialis (Thomas) based on the fine structure of these cells, have been found to constitute four main types:

1. Nucleocytes; large ovoid cells with a prominent nucleus surrounded by a narrow cytoplasm. The cell membrane occasionally is observed to form finger-like projections or pseudopodia. The nuclear membrane follows the general outline of the cell membrane. The chromatin within the nucleus is condensed and in association with the nuclear membrane. The cytoplasm is rich in free and membrane-bound ribosomes. The endoplasmic reticulum is generally located in the perinuclear region. Mitochondria of variable size and shape, are found throughout the narrow cytoplasm. Microtubular structures are found in the peripheral cytoplasm among mitochondria. Electron dense and electron light bodies are observed in the cytoplasm of these cells but are not as abundant as in the plasmatocytes and granulocytes in which they occupy large portions of the cytoplasm. Vacuoles of flocculent content, of presumed pinocytotic origin are present in the peripheral cytoplasm.

2. Plasmatocytes; the cells are characteristically elongated with frequent digitations or small pseudopodia
on their cell membrane. The nucleus is smaller than that of nucleocytes and present invaginations in which membranes of the rough endoplasmic reticulum are frequently observed. Chromatin is compact and associated with the nuclear membrane as in nucleocytes. Free and bound ribosomes are observed in the cytoplasm of these cells. Microtubules are observed to run in the direction of the longer axis of these cells. Mitochondria of various sizes and shapes are found throughout the cytoplasm. Electron dense bodies are more abundant in the cytoplasm of these cells than in the nucleocytes. Electron light bodies and vacuoles with flocculent material are generally located toward the periphery of the cytoplasm. The Golgi apparatus and associated vesicles are well defined.

3. Granulocytes; these cells present a wide cytoplasmic area surrounding the relatively small nucleus. The abundance of electron dense bodies that practically fill the cytoplasm, is the most distinctive characteristic of these cells. Few electron light bodies and vacuoles with flocculent material are also observed. Among the ubiquitous dense bodies, mitochondria and free ribosomes are observed. Endoplasmic reticulum membranes are preferentially located in the perinuclear region. Horseshoe shaped double membrane structures surrounding ribosome clusters are occasionally present in the cytoplasm of these cells which is mostly filled by electron
dense bodies.

4. Coagulocytes; the characteristic structural feature of these cells is the extensive network of endoplasmic reticulum with expanded cisternae. Free ribosomes or clusters of ribosomes abound in the cytoplasm. Unique in this type of cell are large membrane-bound bodies with an internal structure of alternating electron light and electron dense bands. The content of these structures in certain sections appear to be tubular in nature. Another unique structure of these cells are single membrane-bound vacuoles studded by ribosome-like particles on the inner surface of the limiting membrane. Electron dense bodies, mitochondria are also present in the cytoplasm of these cells but do not constitute a characteristic feature for this type of cell.
### SUMMARY OF DISTINGUISHING MORPHOLOGICAL CHARACTERISTICS OF THE FOUR TYPES OF HEMOCYTES OF *MELANOPLUS DIFFERENTIALIS*

<table>
<thead>
<tr>
<th>Hemocyte Type</th>
<th>Nucleus</th>
<th>Cytoplasm</th>
<th>Special Feature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleocyte</td>
<td>A large nucleus, the outer membrane of which is studded with ribosomes.</td>
<td>Cytoplasm is rich in free ribosomes, rough endoplasmic reticulum and mitochondria. Few electron dense and electron light bodies present. Also seen are vacuoles containing flocculent material suggestive of a phagocytic origin.</td>
<td></td>
</tr>
<tr>
<td>Plasmatocytes</td>
<td>Vacuoles with flocculent contents are present in the cytoplasm. Electron dense bodies are more frequent than in nucleocytes, and are found to be dispersed throughout the cytoplasm. The rough endoplasmic reticulum is less extensive than in nucleocytes.</td>
<td>Cells are characteristically elongated with finger-like projections or pseudopodia extending from their surface.</td>
<td></td>
</tr>
<tr>
<td>Hemocyte Type</td>
<td>Distinguishing Morphological Characteristics</td>
<td></td>
<td></td>
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<tr>
<td>---------------</td>
<td>---------------------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nucleus</td>
<td>Cytoplasm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Granulocytes</td>
<td>Occurrence of numerous electron dense bodies that fill most of the cytoplasm is the main characteristic of this cell type. Short strands of rough endoplasmic reticulum, free ribosomes and mitochondria can be observed among the abundant electron dense bodies in the cytoplasm of these cells.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coagulocytes</td>
<td>The membranes of the endoplasmic reticulum are clearly observed to be continuous with the outer membrane. Expanded cisternae of the rough endoplasmic reticulum is the distinctive feature of these cells. The cytoplasm also contains large membrane bound bodies that present an internal structure consisting of alternating electron dense bands. Vacuoles filled with a flocculent electron dense material are occasionally found. The inner surface of the limiting membrane of these vacuoles is observed to be studded with ribosome-like particles.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Plate I

Nucleocyte, (block 350) characterized by its large nucleus (N), smooth cell membrane with occasional pseudopodia (X). Note the dense chromatin material closely associated with the nuclear membrane. Cytoplasm rich in free ribosomes (r), rough endoplasmic reticulum (EL), mitochondria (m), microtubules (mt); electron dense body (ED), electron light body with granular content (el), vacuole with flocculent material (V), (mag. 28,000X).
Plate II

Nucleocyte (block 347) under lower magnification. Nucleus (N), cell membrane pseudopodium (X), rough endoplasmic reticulum (er), mitochondria (m) poorly defined as in Plate I.

Electron light bodies (EL) do not show granular content as in Plate I; vacoule (V). Microtubules and electron dense bodies are not visible in this electron micrograph, (Mag. 17,000X).
Plate III

Plasmatocyte (block 349) showing nuclear membrane invagination followed by ribosome studded membranes of the endoplasmic reticulum (er). Electron dense bodies (ED) of various shapes and sizes; Golgi complex (G); vacuole (V) with granular content. (See description in text) Nucleus (N) (Mag. 17,000X).
Plate IV

Plasmatocyte (block 349) with characteristic elongated shape and pseudopodia (X) on its cell surface. Note that the flocculent content of the vacuoles (v) is of similar density and appearance of that of the surrounding material, suggesting their phagocytic origin (see discussion). Nucleus (N), Mitochondria (m), (Mag. 22,000X).
Plate V

Granulocytes (block 349) characterized by the numerous electron dense bodies (ED) found throughout the cytoplasm. Vacuoles (v) of small size are observed in the cytoplasm of this cell. Mitochondria (m), nucleus (N). (Mag. 17,000X)
Plate VI

Figure a: granulocyte (block 347) showing horseshoe shaped double membrane (X) surrounding a group of free ribosomes. This grid was stained with uranyl acetate alone to leave glycogen granules unstained and the micrograph printed on low contrast photographic paper (Mag. 25,500X).

Figure b: granulocyte (block 350) also showing the horseshoe shaped double (X) membrane surrounding a group of free ribosomes. Staining procedures as for figure a but printed on high contrast photographic paper. Vacuole (v) with flocculent material is seen in the periphery of the cytoplasm. Electron dense bodies (ED) in figures a and b have a grayish appearance because of staining procedure. Short strands of endoplasmic reticulum (er) are seen in the vicinity of mitochondria and electron dense bodies (Mag. 25,500X).
Plate VII

Coagulocyte (block 349) with the characteristically expanded endoplasmic reticulum (er), membranes of which are observed to be continuous with the outer nuclear membrane (arrow). Mitochondria (m), large electron light body (EL). Cytoplasm rich in free ribosomes (r). (Mag. 25,500X).

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Plate VIII

Figure a: portion of a coagulocyte (block 349) showing a very expanded endoplasmic reticulum (er), continuous with the outer nuclear membrane (arrow). Free ribosomes in cytoplasm forming rosettes (r). Microtubules (mt), vacuole with flocculent material (V). (Mag. 25,500X).

Figure b: (block 349) portion of another coagulocyte illustrating the expanded endoplasmic reticulum (er) continuous with the outer nuclear membrane. Note the outer nuclear membrane as well as the membranes of the endoplasmic reticulum are studded with ribosomes. Free ribosomes forming rosette (r) patterns as in figure a (Mag. 25,500X).
Plate VIII

fig.a

fig.b
Plate IX

Figure a: (block 349) coagulocyte with large electron light bodies (el) in its cytoplasm. The electron light body (Y) shows internal organization of alternating electron light and electron dense bands. Small vesicles (v) adjacent to an electron light body (EL). Nucleus (N). (Mag. 42,500X).

Figure b: (block 349) large electron light (el) body in the cytoplasm of a coagulocyte. Note the alternating electron light and electron dense bands. In cross section, the electron dense bands of these bodies appear to be tubular (circle). Electron dense body (ED) adjacent to the large structural electron light body. Nucleus (N) (Mag. 42,500X).
Plate X

Figure a: (block 349) three different size membrane bound vacuoles (V) in the cytoplasm of a coagulocyte (Mag. 25,500X).

Figure b: higher magnification of figure a, printed in high contrast photographic paper to enhance the limiting membrane surrounding these vacuoles (V). Note the ribosome like particles on the inner surface of this membrane. Cell membrane (cm). (Mag. 61,200X).
REFERENCES


