Insect Hemocytes

Development, forms, functions, and techniques

Edited by

A. P. Gupta

Professor of Entomology
Rutgers University

Cambridge University Press

Cambridge
London New York Melbourne

91.

4 Hemocyte types: their structures, synonymies, interrelationships, and taxonomic significance

A. P. GUPTA

Department of Entomology and Economic Zoology, Rutgers University, New Brunswick, New Jersey 08903, U.S.A.

Contents

4.1. Introduction *page* 86
4.2. Main hemocyte types 88
4.2.1. Prohemocyte (PR)
Structure
Synonymies
Interrelationship with other types
4.2.2. Plasmatocyte (PL)
Structure
Synonymies
Interrelationship with other types
4.2.3. Granulocyte (GR)
Structure
Synonymies
Interrelationship with other types
4.2.4. Spherulocyte (SP)
Structure
Synonymies
Interrelationship with other types
4.2.5. Adipohemocyte (AD)
Structure
Synonymies
Interrelationship with other types
4.2.6. Oenocytoid (OE)
Structure
Synonymies
Interrelationship with other types
4.2.7. Coagulocyte (CO)
Structure
Synonymies
Interrelationship with other types
4.3. Other hemocyte types 113
4.3.1. Podocyte (PO)
Structure
Synonymies
Interrelationship with other types
4.3.2. Vermicyte (VE)
Structure

Synonymies
Interrelationship with other types
4.3.3. Additional miscellaneous hemocyte types
4.4. Hemocyte types in various orders 116
4.5. Plesiomorphic hemocyte and its differentiation into other types 116
4.6. Phylogenetic significance of hemocyte types 117
4.7. Summary 120
Acknowledgments
References

4.1. Introduction

Hemocytes of arthropods and several other invertebrate groups have been studied (see Gupta, 1979). Among arthropods, they have been most extensively studied in insects, followed by crustaceans, arachnids, and myriapods. Hemocytes of a few onychophorans also have been described. It is not surprising, therefore, that the need for a reliable, uniform classification of various hemocyte types has been felt more keenly by insect hematologists than by those of other arthropod groups. Fortunately, a generally acceptable hemocyte classification in insects, based largely on morphological characteristics, now exists.

Hemocyte classifications both in insects and other arthropods have been variously based on morphology, functions, and staining or histochemical reactions of hemocytes. Thus, it is not unusual to find the same hemocyte type or its various forms being referred to by different names in various arthropods, by different authors – a situation that has inevitably resulted in a confusing mass of terminology. Consequently, it becomes very difficult to compare hemocytes of one species with those of others. This has particularly hindered any phylogenetic consideration of the evolution of hemocyte types in various arthropod groups and the Onychophora. Clearly, there is a need for a uniform hemocyte classification for insects as well as other arthropod groups. The insect hemocyte classification that is generally used has evolved over more than half a century. According to Millara (1947), Cuénot (1896) was the first to classify insect hemocytes into four categories and was later followed in this attempt by Hollande (1909, 1911) and others. Wigglesworth (1939) summarized most of the earlier classifications and presented a classification that was widely accepted. He modified it later (Wigglesworth, 1959). On the American side, Yeager's (1945) work stimulated considerable interest in the study of hemocytes. Jones (1962) revised and greatly improved Yeager's classification.

In order to adopt a uniform hemocyte classification for discussing hemocytes and their physiological significance in various insects, it is necessary to homologize terminologies used by different authors on the bases of descriptions, observed functions, line drawings, and mi-

Table 4.1. Summary of hemocyte types in various taxa (orders), based on published and unpublished information and personal observation

Taxa	Prohemocyte (PR)	Plasmatocyte (PL)	Granulocyte (GR)	Spherulocyte (SP)	Adipohemocyte (AD)	Coagulocyte (CO)	Oenocytoid (OE)
Collembola	—	—	GR	—	—	—	—
Thysanura	—	PL	GR	SP	—	CO	—
Ephemeroptera	PR	PL	GR	—	—	—	OE
Odonata	—	PL	GR	—	—	—	—
Orthoptera (= Cursoria)	PR	PL	GR	SP[a]	—	—	—
Dermaptera	PR	PL	GR	SP	—	CO	—
Blattaria	PR	PL	GR	SP	AD	CO	OE
Mantodea	PR	PL	GR	SP	—	—	—
Plecoptera	PR	PL	GR	SP	—	CO	—
Hemiptera	PR	PL	GR	—	AD	—	OE
Hymenoptera	PR	PL	GR	—	AD	—	OE
Coleoptera	PR	PL	GR	SP	AD	CO	OE
Megaloptera	PR	PL[a]	GR	—	—	CO	OE
Neuroptera	PR	PL	GR[a]	SP	AD	—	OE
Trichoptera	PR	PL	GR	—	—	—	—
Lepidoptera	PR	PL	GR	SP	AD	CO	OE
Diptera	PR	PL	GR	SP	AD	CO	OE

[a] These terms were not used by the original authors, but have been adopted by me after scrutiny of the original micrographs and figures.

crographs of hemocytes studied by those authors. A summary of the seven main hemocyte types in various insect orders is presented in Table 4.1. The three terms indicated by the table's footnote were not used by the original authors, but have been adopted by me after scrutinizing original descriptions and figures. Hemocytes categorized as amoebocytes and/or phagocytes by the original authors have been assigned mostly to the category of plasmatocyte (PL), although they could be included in granulocyte (GR), spherulocyte (SP), and/or adipohemocyte (AD), inasmuch as these last three forms also are supposedly phagocytic in certain insects.

4.2. Main hemocyte types

There is disagreement among insect hematologists about the number of hemocyte types in various insects. From one or a few to as many as nine or more types have been described, particularly by light microscopy. Ultrastructurally, however, only seven types have so far been identified in various insects: prohemocyte (PR), plasmatocyte (PL), granulocyte (GR), spherulocyte (SP), adipohemocyte (AD), oenocytoid (OE), and coagulocyte (CO). Of these seven, AD has been reported only by Devauchelle (1971) and CO by Goffinet and Grégoire (1975) and Ratcliffe and Price (1974). Podocyte (PO) and vermicyte (VE) have not been recognized as distinct types in electron microscopic studies so far, primarily because ultrastructurally they appear similar to PLs (Devauchelle, 1971). A general description of various hemocyte types, based on both light and electron microscopic studies, their synonymies, and interrelationships is presented below. I must emphasize that although I am including PO and VE in the following description, I do not regard them as distinct types. Furthermore, I believe that description of "new" hemocyte types, based on superficial dissimilarities, should be avoided (see also Chapters 8, 10).

4.2.1. *Prohemocyte* (PR)

Structure

PRs are small round, oval, or elliptical cells with variable sizes (6–10 μm wide and 6–14 μm long). The plasma membrane is generally smooth (Fig. 4.1A), but may show vesiculation (Fig. 4.3A). The nucleus is large, centrally located, and almost filling the cell; nuclear size is variable (3.6–12 μm) in various insects; several nuclei and nucleoli may be present. A thin or dense, homogeneous and intensely basophilic layer of cytoplasm surrounds the nucleus, the nucleocytoplasmic ratio being 0.5–1.9 or more. The cytoplasm mav contain granules, droplets, or vacuoles (Fig. 4.3A).

The laminar nature of the plasma and nuclear membranes may not

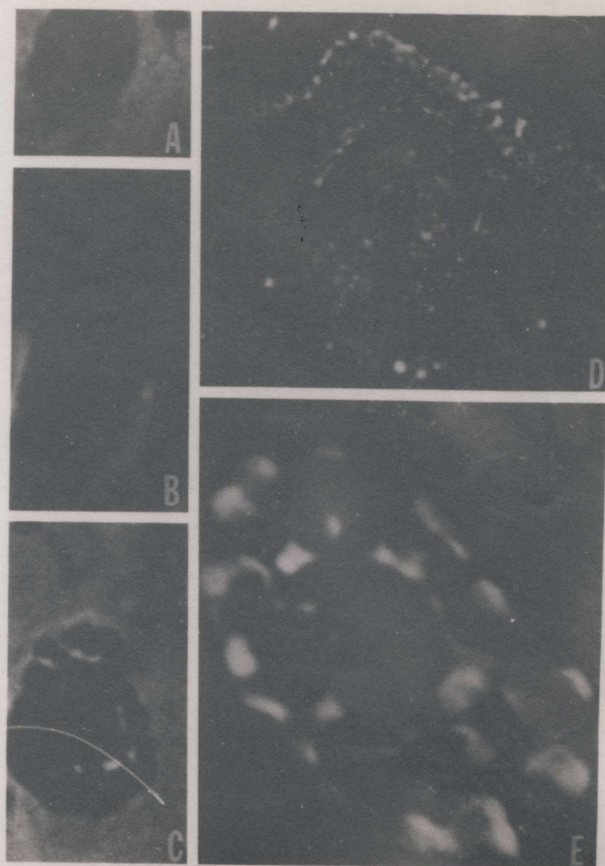


Fig. 4.1. **A.** Prohemocyte of *Periplaneta americana*. Ca. × 8,000. **B.** Plasmatocyte of *P. americana*. Ca. × 10,000. **C.** Spherulocyte of *Nauphoeta cinerea*. Ca. × 8,500. **D.** Granulocyte of *Locusta migratoria*. **E.** Spherulocyte of *N. cinerea*. Ca. × 25,000. (**C** and **E** from Gupta and Sutherland, 1967; **D** from Costin, 1975)

be evident. The cytoplasm generally contains a low concentration of endoplasmic reticulum (ER), mitochondria, and Golgi bodies. However, free ribosomes, rough endoplasmic reticulum (RER), and even mitochondria may be numerous. Centrioles – indicating the mitotic nature of PRs – and microtubules have been observed in the cytoplasm.

PRs are generally found in groups and appear indistinguishable from young or small PLs. They may be numerous, rare, or absent, depending on the developmental and physiological state of the insect at the time of observation. PRs are seldom seen in vivo.

Synonymies

The term that has survived to date with little or no change since its adoption by Hollande (1911) is "proleucocyte." Yeager (1945) used the term "proleucocytoid" and Jones and Tauber (1954) "prohemocytoid." I believe Arnold (1952) was the first to use the term "prohaemocyte." Other synonyms for PR are "macronucleocyte" (Paillot, 1919); "formative cell" (Müller, 1925); "jeune globule" (Bruntz, 1908); "smooth-contour chromophilic cell" (Yeager, 1945); "jeune leucocyte" (Millara, 1947); "plasmatocytelike cell" (Jones, 1959); "young plasmatocyte" (Gupta and Sutherland, 1966; Gupta, 1969); "young granulocyte" (François, 1974); and "proleucocyte" (many authors).

Interrelationship with other types

The controversial questions often raised regarding PRs are: (1) are they the stem cells that transform into other hemocytes? and (2) if they are, are they the main postembryonic source of hemocytes? Although there are substantiating reports that PRs do transform into at least a few other hemocyte types, evidence on their being the main postembryonic source is inconclusive. The term "prohemocyte" suggests that these cells give rise to other types, but it has not yet been demonstrated conclusively that all hemocyte types are derived from PRs. The most generally accepted view is that PRs transform into PLs (Yeager, 1945; Arnold, 1952, 1970, 1974; Jones, 1954, 1956, 1959; Shrivastava and Richards, 1965; Mitsuhashi, 1966; Wille and Vecchi, 1966; Beaulaton, 1968; Devauchelle, 1971; Lai-Fook, 1973; Beaulaton and Monpeyssin, 1976, 1977). Several authors have suggested that PRs transform into other types as well (Muttkowski, 1924; Bogojavlensky, 1932; Yeager, 1945; Arvy and Lhoste, 1946; Ashhurst and Richards, 1964). Arnold (1952) stated that "haemocytes, with the possible exception of the Oenocytoids, apparently develop originally from a common source, the prohaemocytes," but has now changed his mind. Wille and Vecchi (1966), however, suggested that PR can give rise to OE. Arnold (1970), in *Diploptera punctata*, stated that PRs are likely stem cells for PLs, GRs, and SPs, but the direction of differentia-

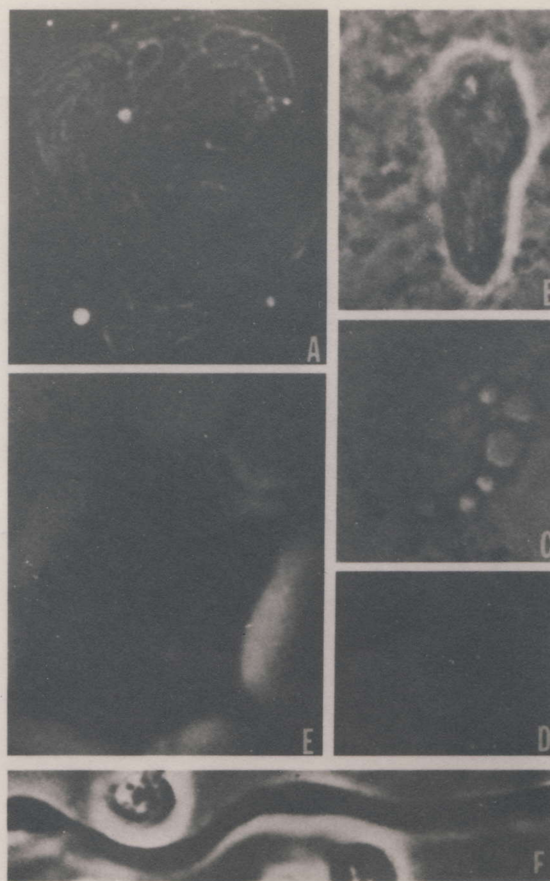


Fig. 4.2. **A.** Oenocytoid of *L. migratoria*, showing cytoplasmic filaments. **B.** Oenocytoid of *P. americana*. Ca. × 6,800. **C.** Adipohemocyte of *P. americana* nymph. Ca. × 7,225. **D.** Coagulocyte of *Epicauta cinerea*. Ca. × 6,800. **E.** Podocyte of *P. americana* nymph. Ca. × 7,225. **F.** Vermicyte of *P. americana*. Ca. × 1,190. Costin, 1975; **C** from Gupta and Sutherland, 1966; **D–F** from Gupta, 1969)

tion is determined early, perhaps in the hemopoietic tissue. Yeager (1945) and Jones (1959) reported that PRs can give rise to SPs and ADs. Devauchelle (1971) reported that PRs, PLs, GRs, and ADs are derived from each other. François (1974) found that PRs transform into GRs. Recently, Sohi (1971) indicated by subculturing that PRs are the germinal cells from which other categories develop, while Arnold and Sohi (1974) indicated two cell lines in subculture. Some authors (Gupta and Sutherland, 1966; Hoffmann et al., 1968; Akai, 1969; Gupta, 1969; Zachary and Hoffmann, 1973) did not recognize the existence of PRs (see also Chapters 2, 8).

As I stated earlier, the evidence on whether PRs constitute the main postembryonic source of hemocytes is inconclusive. There is growing evidence that PRs reside in the hemopoietic organs (Hoffmann et al., 1968; Arnold, 1970; Akai and Sato, 1973; Zachary and Hoffmann, 1973; François, 1974; Hinks and Arnold, 1977) and differentiate into other hemocyte types. Hoffmann (1967), Arnold (1974), and Beaulaton and Monpeyssin (1976) stated that PRs are germinal cells. Earlier (1970), Arnold stated that PRs appear in the hemolymph only intermittently and often in groups, suggestive of their release from hemopoietic tissue. Wille and Vecchi (1966) reported that PRs are abundant in newly emerged bees, but rare in old ones. Gupta and Sutherland (1968) reported an increase in PLs, GRs, SPs, and COs (= CYs) in *Periplaneta americana* following treatment with sublethal doses of chlordane.

4.2.2. *Plasmatocyte* (PL)

Structure

PLs are small to large, polymorphic cells with variable sizes (3.3–5 μm wide and 3.3–40 μm long). The plasma membrane may have micropapillae, filopodia, or other irregular processes, as well as pinocytotic or vesicular invaginations (Figs. 4.1*B*; 4.3*B*,*C*). The nucleus may be round or elongate and is generally centrally located. It may be lobate (Fig. 4.3*C*), vary in size (3–9 μm wide and 4–10 μm long) in various insects, and appear punctate. Scattered chromatin masses may be present along with the nucleolus (Fig. 4.3*C*). Occasionally, binucleate PLs may be found.

The laminar nature of the plasma and nuclear membranes may or may not be visible. The cytoplasm is generally abundant and may be granular or agranular; it is basophilic and rich in organelles. Generally, there is well-developed and extensive RER (Fig. 4.4*B*), which may form greatly distended cisternae or a vacuolar system. Golgi bodies (= dictyosomes = golgiosomes or internal reticular apparatus) (Fig. 4.4*A*) and lysosomes (membrane-bounded, electron-dense bodies, 0.1–1.30 μm in size) may be numerous; lysosomes can be

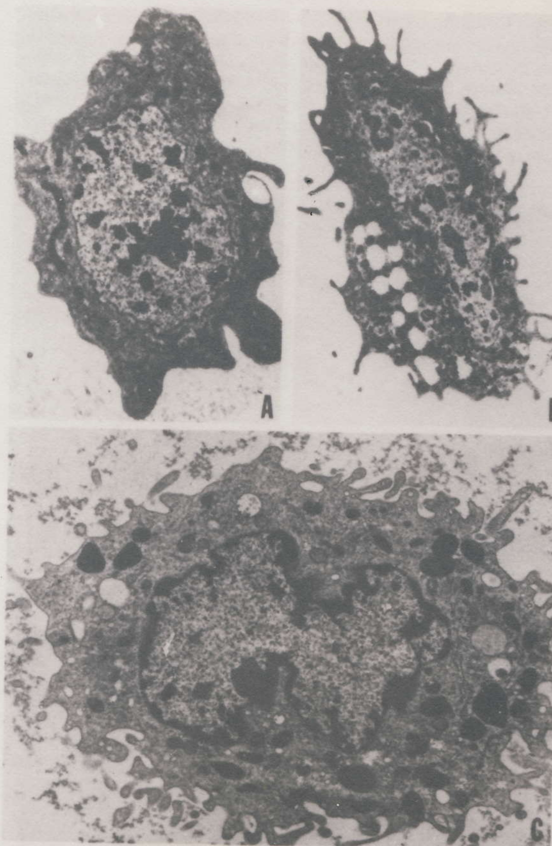


Fig. 4.3. A. Prohemocyte of *Pectinophora gossypiella*. Ca. × 8,760. **B.** Plasmatocyte of *P. gossypiella*, showing micropapillae. Ca. × 10,950. **C.** Plasmatocyte of *Carausius morosus*, showing micropapillae and lobate nucleus. × 6,570. (A and B from Raina, 1976; C from Goffinet and Grégoire, 1975)

identified by the presence in them of the reaction products of the hydrolytic marker enzymes, acid phosphatase and thiamine pyrophosphatase (Scharrer, 1972), and are often associated with the RER or the vacuolar system. The Golgi bodies produce the electron-dense granules (generally 0.5 μm in diameter) that one observes in the PLs. Microsomes and cisternae of the ER (= "ergastoplasme" of French authors) may be present. Free ribosomes (polysomes or polyribosomes)

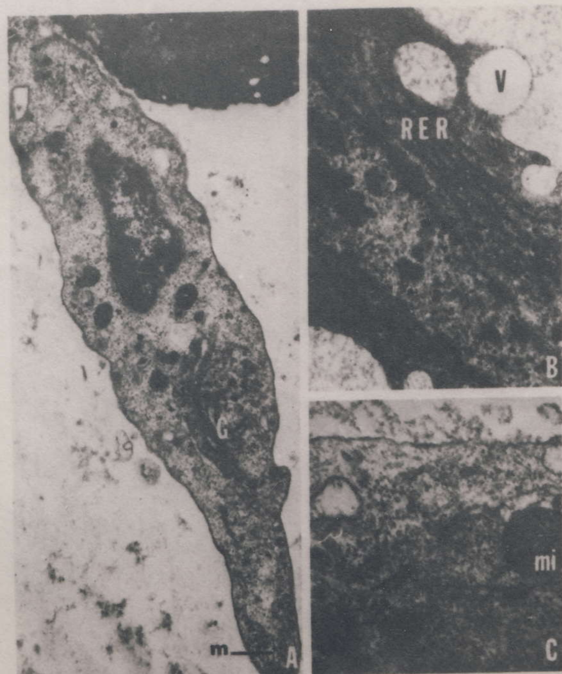


Fig. 4.4. A. Plasmatocyte of *Melolontha melolontha*, showing Golgi (*G*) and intracytoplasmic microtubules (*m*). × 12,600. **B.** Portion of plasmatocyte of *P. gossypiella*, showing rough endoplasmic reticulum (*RER*) and vacuoles (*V*). Ca. × 21,350. **C.** Portion of plasmatocyte of *Ephestia kühniella*, showing mitochondrion (*mi*) and intracytoplasmic microtubule (*m*). Ca. × 44,100. (A reinterpreted from Devauchelle, 1971; B from Raina, 1976; C reinterpreted from Grimstone et al., 1967)

or those attached to microsomes or RER may be present; intracytoplasmic microtubules are present, sometimes arranged in bundles (Fig. 4.4*A*,*C*).

PLs are generally abundant and in some insects may be indistinguishable from PRs and GRs. Several types (most often the transitional forms) of PLs have been described on the bases of their sizes and shapes.

Synonymies

Yeager and Munson (1941) first introduced the term "plasmatocyte." Some of the commonly used synonyms of PL are "leucocyte" (Kollman, 1908; Metalnikov, 1908); "micronucleocyte" (Paillot, 1919); "phagocyte," "amoebocyte," and "lymphocyte" (many authors), "podocyte" (Devauchelle, 1971); and "vermiform cell" (Lea and Gilbert, 1966; Devauchelle, 1971). PLs also include the "lamellocyte" of some authors and the "nematocyte" of Rizki (1953).

Interrelationship with other types

The first real problem one encounters with PLs is that of distinguishing them, particularly the so-called young PLs, from the PRs. This situation is further complicated by the presence of many transitional forms between these two types. The distinction between PRs and PLs is generally based on the relative cell and nuclear sizes, intensity of cytoplasmic basophilia, and the extent and development of the intracellular organelles.

The question that is often raised regarding PLs is whether they are the primary cells that give rise to other forms by secondary transformation. Taylor (1935) claimed that amoebocytes (= mostly PLs), and not chromophils (= PRs), are the basic types. Gupta and Sutherland (1966) and Gupta (1969) supported Taylor's claim and considered PRs as young PLs. Direct transformation of PLs into GRs (Yeager, 1945; Jones, 1956; Gupta and Sutherland 1966; Hoffmann, 1967; Devauchelle, 1971; Beaulaton and Monpeyssin, 1977), SPs (Devauchelle, 1971; Breugnon and Le Berre, 1976; Beaulaton and Monpeyssin, 1977), ADs (Yeager, 1945; Shrivastava and Richards, 1965; Gupta and Sutherland, 1966; Raina, 1976), COs (Gupta and Sutherland, 1966; Devauchelle, 1971), OEs (Gupta and Sutherland, 1966; Hoffmann, 1967; Beaulaton and Monpeyssin, 1977), VEs (Tuzet and Manier, 1959; Gupta and Sutherland, 1966; Lea and Gilbert, 1966; Devauchelle, 1971; François, 1974, 1975), and POs (Gupta and Sutherland, 1966; Nappi, 1970; Devauchelle, 1971; François, 1974, 1975) has been reported, but not substantiated. Devauchelle (1971) considered VEs and POs ultrastructurally similar to PLs. That it is the PL which transforms into other types is indicated also by the corresponding decrease of PLs and increase of other types in differential hemocyte counts. For

example, in *Prodenia*, when PLs fall in number, spheroidocytes (= ADs) increase (Yeager, 1945); in *Drosophila melanogaster*, when POs increase, PLs decrease (Rizki, 1962); and in *P. americana*, within 4 hr of antennal hemorrhage, GRs increase, while PLs decrease (pers. observ.).

It has also been suggested (Gupta and Sutherland, 1966; Moran, 1971; Scharrer, 1972; Price and Ratcliffe, 1974; Beaulaton and Monpeyssin, 1976) that insects have only one basic type of hemocyte and that the commonly recognized types of hemocytes are merely different physiological manifestations of the same type, depending on the physiological needs of the insect at different times. Although the PL has been regarded as the primary type in insects, a survey of the hemocyte types in other arthropod groups reveals that the GR, not the PL, is the basic type (Gupta, 1979) (see also Chapter 8).

4.2.3. *Granulocyte* (GR)

Structure

GRs are small to large, spherical or oval cells (Figs. 4.1*D*; 4.5*A*,*B*; 4.6*A*,*B*) with variable sizes (10–45 μm long and 4–32 μm wide, rarely larger). The plasma membrane may or may not have micropapillae, filopodia, or other irregular processes. The nucleus may be relatively small (compared with that in the PL), round or elongate, and is generally centrally located. Nuclear size is variable (2–8 μm long and 2–7 μm wide).

The laminar nature of the plasma and nuclear membranes may not be visible. The cytoplasm is characteristically granular (Figs. 4.1*D*; 4.5*A*,*B*; 4.6*A*). Several types of membrane-bounded granules have been described in the GRs of various insects (Figs. 4.6*A*,*B*; 4.7*A*–*C*; 4.8*A*,*B*). Recently, Goffinet and Grégoire (1975) summarized and synonymized various types of granules into three categories, based on their observations in *Carausius morosus*. The following summary and synonymy of granules are based on these and other works:

1. Structureless, electron-dense granules: = unstructured inclusions (type 1) of Baerwald and Boush (1970); melanosome-like granules of Hagopian (1971); opaque body of Moran (1971); type 2 bodies of Scharrer (1972); and electron-dense granules of Raina (1976) and others
2. Structureless, thinly granular bodies: = type 3 of Scharrer (1972); and electron-lucent granules of Raina (1976)
3. Structured granules: = "globules" or "granules multibullaires" of Beaulaton (1968); "grains denses structures" in the AD of Devauchelle (1971); and Landureau and Grellet (1975); "corpus fibrillaires" of Hoffmann et al. (1968, 1970); cylinder inclusions (type 2), regular-packed inclusions (type 3), and inclusions with bandlike units (type 4) of Baerwald and Boush (1970); "Granula mit tubularer Binnenstruktur" of Stang-Voss

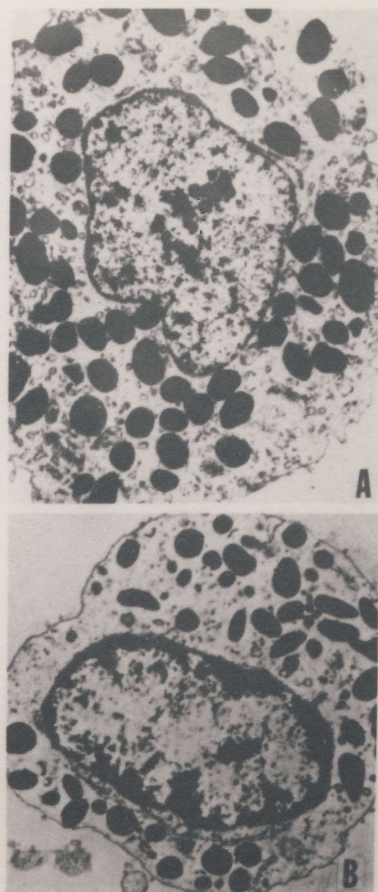


Fig. 4.5. A. Granulocyte of *M. melolontha*. *N* = nucleus. × 6,300. **B.** Granulocyte of *Thermobia domestica*. × 9,400. (**A** courtesy of Dr. G. Devauchelle; **B** from François, 1975)

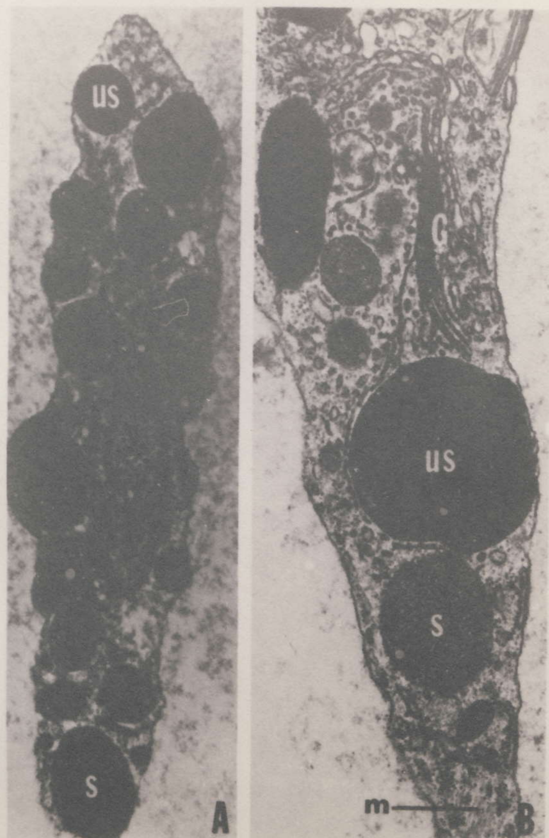


Fig. 4.6. A. Granulocyte of *P. americana*, showing structured (*s*) and unstructured (*us*) granules. Ca. × 16,000. **B.** Portion of granulocyte of *Leucophaea maderae*, showing derivation of structured (= premelanosome-like) granule (*s*) from Golgi (*G*), structureless or unstructured (= melanin-like) granule (*us*), and intracytoplasmic microtubules (*m*). × 3,000. (**A** reinterpreted from Baerwald and Boush, 1970; **B** reinterpreted from Hagopian, 1971)

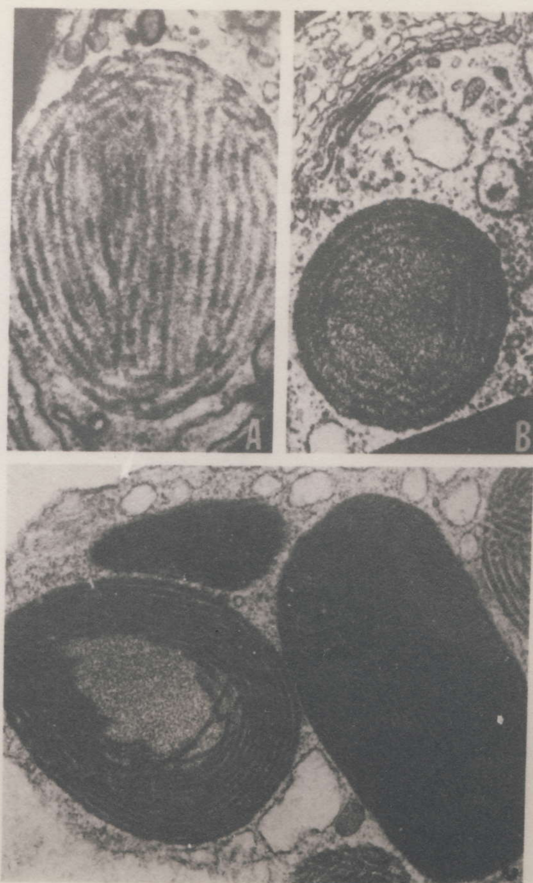


Fig. 4.7. A. Structured granule from granulocyte of *L. maderae*, showing internal microtubules. × 40,000. **B.** An earlier stage of development of internal microtubules. × 50,000. **C.** Section of a structured granule showing concentric arrangement of internal microtubules. × 38,000. (**A**–**C** from Hagopian, 1971)

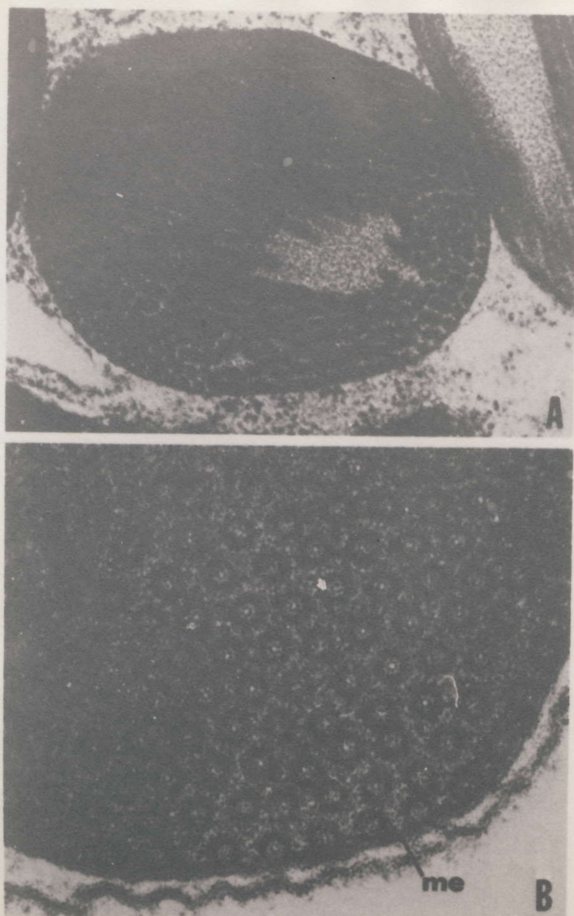


Fig. 4.8. A. Section of structured granule of a granulocyte of *L. maderae*, showing arrangement of microtubules about 25 nm in diameter. × 63,000. **B.** Highly magnified view of microtubules of structured granules. Note micro-microtubules (5 nm in diameter, arrow) within microtubules and limiting membrane (*me*) of granule. × 240,000. (**A** and **B** from Hagopian, 1971)

(1970); premelanosome-like granules of Hagopian (1971); tubule-containing bodies or TCB of Moran (1971); type 1 of Scharrer (1972); and granules with a microtubular structure of Ratcliffe and Price (1974).

The length or the diameter of the structureless granules varies from 0.15 to 3 μm or more in various insects, while that of the structured granules varies from 0.5 to 2 μm. The shape of the granules may be spherical, ovoid, elongate, or irregularly polygonal (Figs. 4.5*A*,*B*; 4.6*A*,*B*; 4.7*B*,*C*; 4.8*A*). The diameter of the microtubules within the structured granules varies from 15 to 80 nm) in various insects. Internally, the microtubules may show micro-microtubules about 5 nm in diameter (Hagopian, 1971) (Fig. 4.8*B*). Akai and Sato (1973) also have described "subunits of fibrils" in their so-called secretory vesicles. The number of microtubules per granule may vary from 9 to 80. From the accounts provided by Hagopian (1971), Scharrer (1972), Akai and Sato (1973), and François (1975), it appears that the granules are derived from the Golgi bodies (Fig. 4.6*B*), the microtubules developing during the later stages of morphogenesis. It is conceivable that the structureless, electron-dense granules represent the final stage of development of these granules in which the structured nature becomes obliterated. Supposedly, the granules are eventually released into the hemolymph. Histochemical analysis shows that most of the granules contain sulfated, periodate-reactive sialomucin and other glycoproteins or neutral mucopolysaccharides (François, 1974, 1975; Costin, 1975). Occasionally, lipid droplets may be present, especially in older GRs.

In addition to the structureless and structured granules, the cytoplasm is rich in free ribosomes (polysomes), Golgi bodies, both ER and RER, and lysosomes. Mitochondria are generally few in number. Marginal bundles of intracytoplasmic microtubules are also present (Fig. 4.6*B*).

Synonymies

Jones (1846) first established the category of granular cells, and later Cuénot (1896) mentioned "amoebocytes" with finely granular cytoplasm. Shrivastava and Richards (1965) and Lea and Gilbert (1966) treated GRs as ADs. The so-called "pycnoleucocytes" of Wille and Vecchi (1966) are probably GRs. Recently, Devauchelle (1971) synonymized GRs with cystocytes (= COs), and François (1975) did with SPs. GRs have been referred to as "phagocytes," "amoebocytes," and "hyaline cells."

Interrelationship with other types

GRs have been widely misinterpreted and confused with PLs, SPs, ADs, and COs (= cystocytes). François (1975) considered the SPs, described by Gupta and Sutherland (1966) and Price and Ratcliffe

(1974), as GRs. Goffinet and Grégoire (1975) reported separate categories of GRs and COs in *Carausius morosus*. As a matter of fact, the separate existence of the GR (not to be confused with PL, SP, AD, and CO) is now recognized by most authors, although Devauchelle (1971) has included both GR and CO in his type III.

How are GRs formed? Are they derived from PRs or PLs? Both sources of origin have been suggested (Arnold, 1974). Gupta and Sutherland (1966) indicated that the derivation of GR from PL is a short step. Takada and Kitano (1971) reported that GRs showed a trend to increase and PLs to decrease with time. Are GRs capable of transforming into other types of hemocytes? There are reports that indicate that GRs do indeed give rise to SPs, ADs, and COs (Gupta and Sutherland, 1966). Arnold (1974) has suggested that GRs "might be considered basic units from which more precisely structured and functioning classes of cells have developed." This is supported by my survey of hemocyte types in Arthropoda (Gupta, 1979). Hinks and Arnold (1977), however, have suggested separate origins of GRs and SPs.

The presence of microtubules in the granules and in the cytoplasm of the GR also has caused debate. According to Crossley (1975), the microtubules of the granules do not have the "dimensions of typical cytoplasmic microtubules (24–27 nm diameter), nor have been demonstrated to be sensitive to colchicine or vinblastine . . . and therefore they should not be called microtubules." According to him, only in *Leucophaea* are the dimensions of the inclusion tubules (25 nm) comparable to those of the intracytoplasmic microtubules.

The intracytoplasmic microtubules have been described in several insects (Grimstone et al., 1967; Baerwald and Boush, 1970, 1971; Devauchelle, 1971; Hagopian, 1971; Scharrer, 1972; to mention a few investigators) and are generally narrower in diameter than the microtubules of the granules. These intracytoplasmic microtubules may be arranged into marginal bundles (Hagopian, 1971) or may be randomly distributed in the cytoplasm (Devauchelle, 1971) and supposedly are found in all hemocyte types, except OEs (Devauchelle, 1971), although Raina (1976) has described them in OEs.

4.2.4. *Spherulocyte* (SP)

Structure

SPs are ovoid or round cells (Figs. 4.1*C*,*E*; 4.9*A*,*B*) with variable sizes (9–25 μm long and 5–10 μm wide) and usually larger than GRs. The plasma membrane may or may not have micropapillae, filopodia, or other irregular processes. The nucleus is generally small (5–9 μm long and 2.5–6 μm wide), central or eccentric, rich in chromatin bodies, and generally obscured by the membrane-bounded, electron-dense, intracytoplasmic spherules that are characteristic of these cells.

The number of the spherules may vary from few to many, and the diameter from 1.5 to 5 μm. The spherules contain granular, fine-textured, filamentous, or flocculent material (Raina, 1976). The granules within the spherules may vary from 15 to 17 nm in diameter (Akai and Sato, 1973). In addition to the spherules, the cytoplasm contains polyribosomes (Fig. 4.10*C*), Golgi bodies (moderately to well developed) (Fig. 4.10*A*), membrane-bounded vacuoles (= lysosomes) (Fig. 4.9*C*), numerous randomly distributed microtubules, elongated mito-

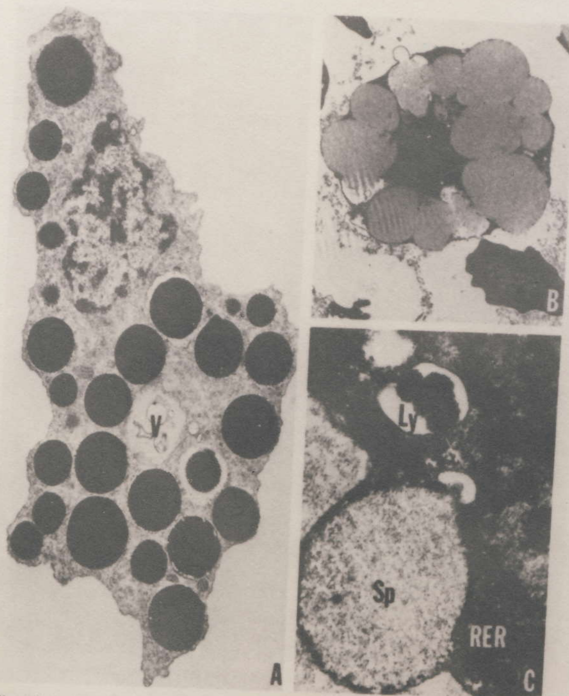


Fig. 4.9. A. Spherulocyte of *M. melolontha*, showing eccentric nucleus (*N*) and numerous spherules and a vacuole (*V*). × 9,450. **B.** Spherulocyte of *Bombyx mori*. Ca. × 7,000. **C.** Portion of spherulocyte of *P. gossypiella*, showing rough endoplasmic reticulum (*RER*), spherule (*Sp*) with granular contents, and lysosome (*Ly*). Ca. × 21,000. (**A** from Devauchelle, 1971; **B** from Akai and Sato, 1973; **C** from Raina, 1976)

chondria, and RER (Figs. 4.9C, 4.10A,C). Devauchelle (1971) has also described a more or less loose network of fibrils in the cytoplasm (Fig. 4.10B). SPs release the material in their spherules into the hemolymph by exocytosis.

Histochemically, the spherules have been reported to contain neu-

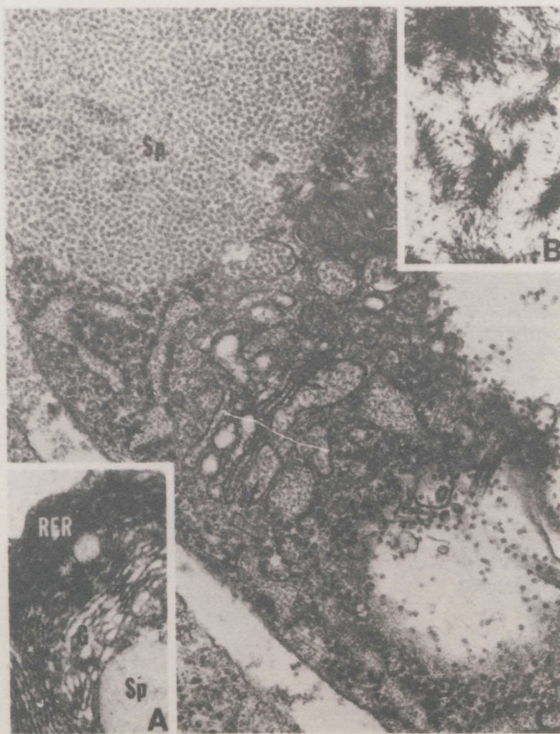


Fig. 4.10. A. Portion of spherulocyte of *P. gossypiella*, showing rough endoplasmic reticulum (*RER*) and Golgi (*G*) involved in formation of spherule (*Sp*). Ca. × 15,750. B. Portion of spherulocyte of *M. melolontha*, showing loose network of intracytoplasmic fibrils. C. Portion of spherulocyte of *B. mori*, showing spherule (*Sp*) with fine granules, rough endoplasmic reticulum containing fibrous material in its cisternae (arrows), and ribosomes. × 50,400. (A from Raina, 1976; B from Devauchelle, 1971; C from Akai and Sato, 1973)

tral or acid mucopolysaccharide and glycomucoproteins by several authors (Vercauteren and Aerts, 1958; Nittono, 1960; Ashhurst and Richards, 1964; Gupta and Sutherland, 1967; Costin, 1975; Beaulaton and Monpeyssin, 1977). Much earlier, Hollande (1909) reported that the spherules contain "lipochrome" (a kind of carotenoid lipid). The presence of tyrosinase has been reported by Dennell (1947), Jones (1956), and Rizki and Rizki (1959). Most recently, Costin (1975) reported the presence of nonsulfated sialomucin, in addition to glycoproteins and neutral mucopolysacchrides.

Synonymies

Hollande (1909) was the first to use the term "spherule cells," which is now generally used by most workers. Other terms that have been used by various authors are "cellules sphéruleuses" or "cellules à sphérules" (Paillot, 1919; Paillot and Noel, 1928); "spherocytes" (Bogojavlensky, 1932); "eruptive cells" (Yeager, 1945); "oenocytoids" (Dennell, 1947); "rhegmatocytes" (?)(Hrdý, 1957); and "hyaline cells" (Whitten, 1964, her Fig. 1R). Harpaz et al. (1969) classified SPs as ADs.

Interrelationship with other types

The main controversies about SPs concern the transformation of these cells into other types, formation of the spherules, and the functions of these cells. The transformation of SPs into other types is not well documented. Gupta and Sutherland (1966) suggested that SPs are capable of transforming into ADs and COs (= cystocytes) and that SPs are themselves derived from GRs. Millara (1947) and Arnold and Salkeld (1967) also considered the SP as a phase in the life of a GR. Later, Arnold (1974) stated that "they seem to be another specialized cell within the granular hemocyte complex." Beaulaton (1968) has suggested that SPs are degenerated OEs. Hinks and Arnold (1977) consider SPs as separate types with mitotic capabilities.

Little information is available on the formation of the spherules. According to Akai and Sato (1973), the material in the spherules is first observed in enlarged cisternae of the RER, then transferred into the Golgi complex, where it is packaged into the membrane-bounded spherules.

The role of SPs is highly controversial. Hollande (1909) considered these cells respiratory in function because of the presence of the so-called lipochrome. It has been demonstrated by Åkesson (1945), Ashhurst and Richards (1964), Arnold and Salkeld (1967), Gupta and Sutherland (1967), and Costin (1975) that the material contained in the spherules is neutral or acid mucopolysaccharide, not a carotenoid lipid. Hollande (1909) stated also that these cells contained an oxidase. Dennell (1947), Jones (1959), and Rizki and Rizki (1959) re-

ported tyrosinase in the spherules of various Diptera, but Gupta and Sutherland (1967) found no tyrosinase in the SPs of cockroaches. Gupta and Sutherland (1965) were supposedly the first to report SPs in cockroaches. Whitten (1964) suggested that SPs (= her hyaline cells) may play a role in the darkening of the puparium in some cyclorrhaphous Diptera. Pérez (1910) reported that SPs took part in histolysis. Although this has been disputed by Åkesson (1945), the histolytic role of SPs should not be surprising, considering the fact that before and after molting several SPs are observed to congregate on histolyzing tissue. Gupta (1970) has suggested the probable histolytic or phagocytic functions of SPs. It is probable that SPs both histolyze and phagocytize tissues in at least a few insects. The phagocytic function was reported by Kollman (1908), Cameron (1934), and Åkesson (1945), but further work is needed to demonstrate clearly the histolytic role of SPs. Raina (1976) found no evidence of their role in phagocytosis. Metalnikov and Chorine (1929) and Metalnikov (1934) found that the SPs in *Galleria* are related to bacterial immunity. Nittono (1960) stated that strains of silkworm larvae that lacked SPs completely or incompletely tended to produce relatively smaller quantities of silk. Wigglesworth (1959) suggested that SPs are involved in the uptake and transport of other substances, such as hormones. Akai and Sato (1973) suggested that SPs are sources of some blood proteins.

4.2.5. *Adipohemocyte* (AD)

Structure

ADs are small to large, spherical or oval cells (Fig. 4.2C) with variable sizes (7–45 μm in diameter). The plasma membrane may or may not have micropapillae, filopodia, or other irregular processes. The nucleus is relatively small (compared with that in a PL or SP), round or slightly elongate, and centrally or eccentrically located. Nuclear size is variable (4–10 μm in diameter). The nucleus may appear concave, biconvex, punctate, or lobate.

The laminar nature of the plasma and nuclear membranes may not be visible. The cytoplasm contains characteristic small to very large refringent fat droplets (0.5–15 μm in diameter) and other nonlipid granules (0.5–9 μm in diameter) and vacuoles, which, according to Arnold (1974), become filled with lipids under certain conditions. In addition, the cytoplasm contains well-developed Golgi bodies, mitochondria, and polyribosomes.

Histochemically, ADs are reported to contain PAS-positive substance in the granules (Ashhurst and Richards, 1964; Lea and Gilbert, 1966). Costin (1975) did not recognize ADs as a type in her study.

Synonymies

Hollande (1911) first introduced the term "adipoleucocyte," although Kollman (1908) had earlier used the term "adipo-spherule cell" for some hemocytes of invertebrates. Other terms used for ADs are "spheroidocytes" (Yeager, 1945; Arnold, 1952; Rizki, 1953; Jones, 1959); "later stages of spherules" (Whitten, 1964); and "adipocytes" (Wigglesworth, 1965) (see also Chapter 8).

Interrelationship with other types

The main controversy about ADs concerns their identity as a distinct category of hemocytes. Scrutiny of the literature leads one to believe that they are not a distinct type. Several authors have reported that it is difficult to distinguish them from GRs (Jones, 1970; Arnold, 1974), and many others did not recognize the category of ADs in their studies (Wittig, 1968; Akai and Sato, 1973; Costin, 1975; François, 1975; Goffinet and Grégoire, 1975; Boiteau and Perron, 1976; Raina, 1976; Beaulaton and Monpeyssin, 1977; to mention a few recent ones). Raina (1976) noted a progressive accumulation of lipid drops in GRs and on that basis considered ADs as functional stages of GRs. Gupta and Sutherland (1966) also have reported the transformation of GRs into ADs. Only one of the ultrastructural studies (Devauchelle, 1971) of hemocytes includes ADs as separate category. However, his micrographs (his Figs. 20, 21, 24) are strikingly similar to the GRs (cf. Fig. 4.11 with Fig. 4.6B) described by Hagopian (1971) and other authors.

On the basis of the histochemical nature of these cells also it is difficult to justify the term, and hence the category, of ADs. According to Crossley (1975), "ultrastructural studies of so-called adipohaemocytes include cells which contain no reported lipid (Devauchelle, 1971, Fig. 18–22), lipid of doubtful authenticity (Pipa and Woolever, 1965) or material believed to be mucoprotein or mucopolysaccharide (Beaulaton, 1968, Fig. 7)." Costin (1975) also did not recognize the AD on the basis of the histochemistry of hemocytes in *Locusta migratoria*.

The resemblance of ADs to fat body cells is also confusing and adds to the difficulty of identifying ADs in fresh hemolymph samples. For example, one may find all gradations between small ADs and fat body cells (Wigglesworth, 1955). Jones (1965) suggested that hemocytes "with excentric nucleus and many brilliant fatlike droplets should be termed ADs only when they can be clearly distinguished from fat body cells." According to him (Jones, 1975), ADs are at least 10 times smaller than fat body cells.

Gupta and Sutherland (1966) suggested that under certain conditions, such as chilling, starvation, and diapause (periods of nonfeeding and reduced metabolic rate), the PLs respond by changing into ADs

with "lipid" droplets. This was based on their observation that mealworm larvae, when chilled for 20–24 hr at 5 °C, showed numerous "lipid" droplets and ADs, other types of hemocytes being rare. Such larvae subsequently recovered. Ludwig and Wugmeister (1953) noted an increased amount of free fats in the hemolymph of the starving Japanese beetle, *Popillia japonica*, and Clark and Chadbourne (1960) reported a greater number of ADs (= their spheroidocytes) in diapausing larvae of the pink bollworm, *Pectinophora gossypiella*. Thus, it

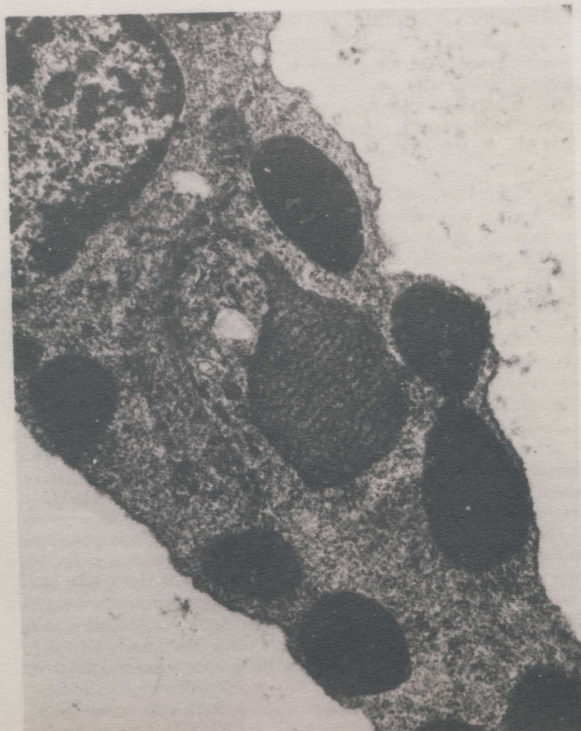


Fig. 4.11. Supposed adipohemocyte of *M. melolontha*. But note that it looks like a granulocyte. × 41,500. (From Devauchelle, 1971)

seems very likely that the appearance (or transformation from PLs) of ADs in the hemolymph at certain times is governed by the physiological state of the insect.

4.2.6. *Oenocytoid* (OE)

Structure

OEs are small to large, thick, oval, spherical, or elongate cells (Figs. 4.2*A*,*B*; 4.12*A*,*B*) with widely variable sizes (16–54 μm or more) and shapes. The plasma membrane is generally without micropapillae, filopodia, or other irregular processes. The nucleus is generally small, round or elongate, and generally eccentrically located (Figs. 4.2*B*, 4.12*A*). Nuclear size may vary (3–15 μm). Occasionally, two nuclei may be present.

The laminar nature of the plasma and nuclear membranes may not be visible. The cytoplasm is generally thick and homogeneous and has several kinds of plate-, rod-, or needlelike inclusions. According to Costin (1975), the OE is distinguished by an elaborate system of filaments that fills the cytoplasm (Figs. 4.2*A*; 4.12*C*,*D*) and is visible under a phase-contrast microscope. Histochemically, the filaments resemble the cytoplasm and hence are not visible in stained preparations. Hoffmann (1966) and Hoffmann et al. (1968) also have reported such filaments. In addition to the above intracytoplasmic inclusions and filaments, a few electron-dense spherules may be present in the cell periphery (Devauchelle, 1971). With the exception of polyribosomes and the abundant, large mitochondria, which are conspicuous, other organelles, such as ER and Golgi, are poorly developed. Supposedly, lysosomes are absent.

Histochemically, OEs are reported to contain tyrosinase (Dennell, 1947), protein (Akai and Sato, 1973), and PAS-positive-only granules, indicating the presence of glycoproteins or neutral mucopolysaccharides and sulfated, periodate-reactive sialomucin (Costin, 1975).

One peculiarity of OEs seems to be their highly labile nature. They are particularly fragile in vitro and lyse quickly, ejecting material in the hemolymph. They are nonphagocytic.

Synonymies

No two terms have caused as much confusion as "oenocytes" and "oenocytoids." Oenocytes differ from oenocytoids in that they are ectodermal in origin, usually segmentally arranged, yellow in color, and not hemocytes. Poyarkoff (1910) first introduced the term OE, followed by Hollande (1911). In order to avoid any confusion between oenocytes (proposed by Wielowiejsky, 1866) and oenocytoids, Hollande (1914) proposed to replace the term oenocyte by "cerodecytes." It is not surprising that several earlier authors mistook oenocytes for

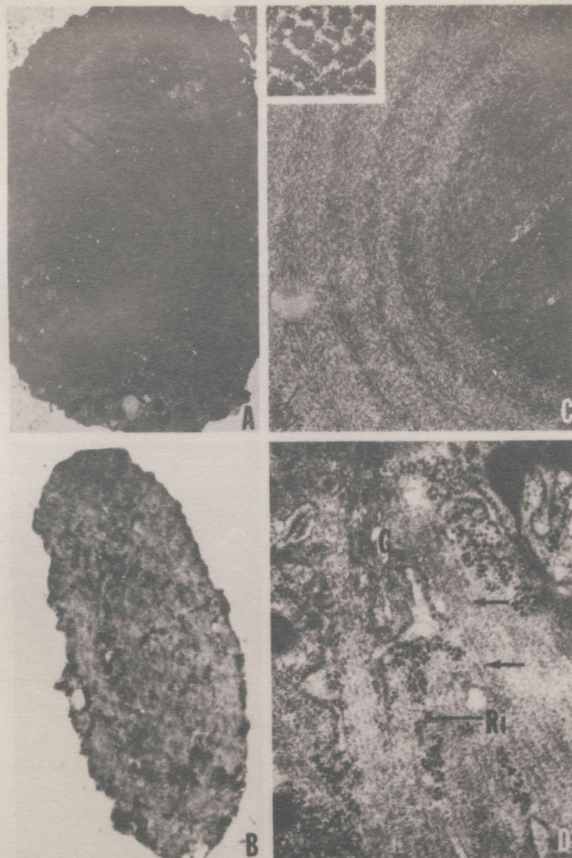


Fig. 4.12. A. Oenocytoid of *B. mori*, showing concentric arrangement of intracytoplasmic fibrils (arrows) and eccentric nucleus (*N*). × 3,000. **B.** Oenocytoid of *P. gossypiella* × 6,600. **C.** Portion of oenocytoid of *B. mori*, showing highly magnified view of concentric rings of intracytoplasmic fibrils. Note also unoriented fibrils (arrows). Ca. × 30,000. **Inset** (ca. × 225,000) shows fibrils in cross section. **D.** Portion of oenocytoid of *P. gossypiella*, showing longitudinally arranged intracytoplasmic microtubules (arrows), Golgi (*G*), and ribosomes (*Ri*). × 35,475. (**A** and **C** from Akai and Sato, 1973; **B** and **D** from Raina, 1976)

OEs. Even after Hollande's (1920) detailed description of OEs, several authors (Metalnikov and Gaschen, 1922; Müller, 1925; Tateiwa, 1928; Metalnikov and Chorine, 1929; Bogojavlensky, 1932; and Cameron, 1934) used the term oenocyte instead of OE in their respective works.

Other synonyms used for OEs are "oenocyte-like cells" (Yeager, 1945); large "non-granular spindle cells" and "non-phagocytic giant hemocytes" (Wigglesworth, 1933, 1955; see discussion in Jones, 1965); "crystalloid" and "dark hyaline hemocytes" (Selman, 1962); "crystal cells" (Rizki, 1953, 1962; Nappi, 1970); and COs (Hoffmann and Stoekel, 1968).

Interrelationship with other types

The main controversy about OEs concerns their identity as a separate category, particularly their distinction from COs. The view that OEs are part of the CO complex has received support owing to the observation by some authors (Lea and Gilbert, 1966) that OEs are unstable in vitro and that they undergo rapid and drastic transformation into hyaline cells (= COs). These authors report that in *Hyalophora cecropia*, OEs begin to transform within 15–30 sec, and fully transformed OEs are found within 15 min. Jones (1959) and Nittono (1960) also have reported such transformation of OEs in *Prodenia* and *Bombyx*, respectively. Coupled with these observations are the reports by many authors that OEs are either found in very small numbers or are absent. This may partly explain why several authors either have not reported OEs in their studies or do not recognize OEs as a distinct category. Crossley (1975), however, stated that ultrastructurally OEs and COs are different, and indeed some authors (Hoffmann et al., 1968) have described both OEs and COs in their ultrastructural studies. The ultrastructural identity of OEs is also supported by the fact that although these cells eject material into the hemolymph as COs do, this does not result in plasma gelation (Arnold, 1974).

The origin or derivation of these cells is also controversial. Gupta and Sutherland (1966) and Beaulaton and Monpeyssin (1977) suggested that OEs are differentiated from PLs. Devauchelle (1971) has indicated that OEs might be derived from PRs. Arnold (1974) stated that "the cells seem to be allied with the complex of granular cells, but their origins and relationships are not understood." Hinks and Arnold (1977) found them originating in the hemopoietic tissue.

4.2.7. *Coagulocyte* (CO)

Structure

COs are generally small to large (3–30 μm long), spherical, hyaline, fragile, and unstable cells, combining the features of GRs and OEs

(Arnold, 1974). The plasma membrane is generally without any micropapillae, filopodia, or other irregular processes. The nucleus is relatively small (5–11 μm long), generally eccentric, oval, sharply outlined, and under phase-contrast may appear cartwheel-like owing to the arrangement of the chromatin in that fashion (Fig. 4.2*D*). According to Goffinet and Grégoire (1975), there is a pronounced perinuclear cisterna (Fig. 4.13*A*), which, together with microruptures in the plasma membrane, supposedly distinguishes these cells from other types.

The laminar nature of the plasma and nuclear membranes may not be visible. The plasma membrane may show microruptures. The cytoplasm is hyaline and rich in polyribosomes, but has fewer mitochondria and moderately developed ER. In addition, the cytoplasm has some spherical or elongate granular inclusions, about 1 μm in diameter (Fig. 4.13*A*). François (1975) has described four types of such granules in *Thermobia domestica:* (1) electron-dense, homogeneous granules, generally resembling those in GRs; (2) moderately electron-dense, homogeneous granules; (3) heterogeneous granules with a central or lateral dense zone, the remaining portion being homogeneously granular; and (4) structured granules, with internal microtubules (15 nm in diameter), arranged in a parallel fashion and 40 nm apart. Goffinet and Grégoire (1975) also described structural granules in the COs of *C. morosus*. It is obvious that there is a very close resemblance between GRs and COs.

Histochemically, COs are clearly distinguishable from PLs and GRs according to the periodic acid–Schiff (PAS) test (Costin, 1975). According to her, "compared with the cytoplasm of the other types of blood cells, that of coagulocyte has much reduced basophilia." It is very weakly PAS-positive.

Synonymies

Grégoire and Florkin (1950) for the first time introduced the term "coagulocyte" or "unstable hyaline hemocyte" in *Gryllulus* and *Carausius*. Earlier, Yeager (1945) for the first time used the term "cystocyte" for cells with cystlike inclusions. Jones (1950) used that term for "coarsely granular haemocytes," and later (Jones, 1962) suggested that the "term coagulocyte for these cells may be preferred to cystocyte because these cells are only identified by their function." Wigglesworth (Chapter 11) synonymized "thrombocytoids" of Zachary and Hoffmann (1973) with COs.

Interrelationship with other types

The main controversies about COs concern their identity, function, and origin. It is still debatable whether the COs are ultrastructurally different from GRs. Devauchelle (1971) found them indistinguishable

from GRs and synonymized them with the latter. Moran (1971) found a type of cell in *Blaberus discoidalis* (frequently in newly molted, untanned adults) with membrane-bounded, tubule-containing bodies (TCB) filled with rows of 34-nm tubules, which are quite different from the intracytoplasmic microtubules. He suggested that these cells are equivalent to COs (cystocytes). Ratcliffe and Price (1974), however, have identified COs (their cystocyte) in their work. Most recently, Goffinet and Grégoire (1975) and Grégoire and Goffinet (Chapter 7) claimed a separate identity for them. According to them, the perinuclear cisternae of the COs are much more pronounced than those of PRs, PLs, and GRs, and their plasma membrane is ruptured during coagulation, whereas those in PRs, PLs, and GRs remain intact.

The role of the COs in hemolymph coagulation is generally accepted and has been recently reconfirmed by Grégoire (1974), François (1975), Goffinet and Grégoire (1975), and Grégoire and Goffinet (Chapter 7). Gupta and Sutherland (1966), however, have suggested that COs are the effect rather than the cause of coagulation on the basis of their observation that as soon as coagulation starts, several PLs transform into COs. This view, however, is not accepted by Grégoire. Supposedly, COs also contain phenol-oxidizing enzymes (Crossley, 1975). It must be mentioned here that in several arthropod groups coagulation of the hemolymph is caused by the GR (Gupta, 1979), and evidence is accumulating that this is also true in some insects (Rowley and Ratcliffe, 1976; Rowley, 1977).

The origin of COs is still debatable. Grégoire and Goffinet (Chapter 7) and Hinks and Arnold (1977) have suggested that these cells originate in the hemopoietic organs. However, if we accept the premise that hemocytes respond to bodily injury in the insect, it is conceivable that either the injury itself would induce the production of COs or some other type of hemocyte would produce them by transformation. For more details on the structure, functions, and origin of COs, the reader is referred to Chapter 7.

4.3. Other hemocyte types

4.3.1. *Podocyte* (PO)

Structure

These hemocytes have not been recognized as a separate category in any ultrastructural study and are not ordinarily observed in the hemocyte samples under the light microscope. They should be regarded as a variant form of PL. According to Arnold (1974), they have been correctly identified only in *Prodenia* (Yeager, 1945; Jones, 1959). However, I (Gupta, 1969) have observed them in *P. americana*

nymphs. More often than not, radiate PLs with pseudopodia are mistaken for POs.

These hemocytes are very large (Fig. 4.2*E*), extremely flattened PL-like cells with several cytoplasmic extensions (Fig. 4.13*B*). The nucleus is generally large and centrally located and may appear punctate.

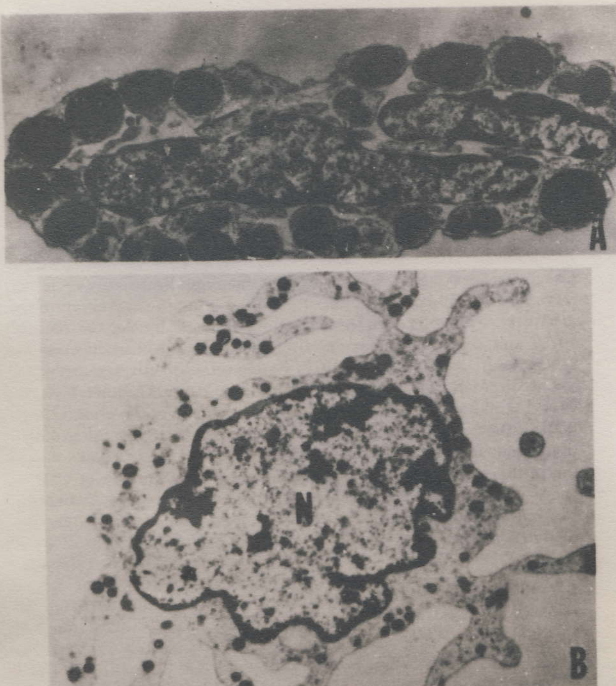


Fig. 4.13. A. Coagulocyte, showing perinuclear cisternae (arrows) and those of endoplasmic reticulum (*er*). Note presence of electron-dense (structureless) granules such as are found in granulocytes. × 20,000. B. Podocyte, showing pseudopodia. Note resemblance to prohemocyte or young plasmatocyte. *N* = nucleus. × 9,500. (A and B courtesy Dr. G. Devauchelle)

Synonymies

Yeager (1945) introduced the term "podocyte." Graber's (1871) "star-shaped amoebocytes" and Lutz's (1895) radiate cells may have included POs.

Interrelationship with other types

POs are derived from PLs. Gupta and Sutherland (1966) have suggested their transformation from PLs. This seems to be supported by Rizki's (1962) observation that in *D. melanogaster* when POs increase in differential counts, PLs decrease. Rizki's (1953) POs appear to be PLs. Whitten (1964) questioned the concept of POs, and Devauchelle (1971) considered them variant forms of PLs.

4.3.2. *Vermicyte* (VE)

Structure

This form is generally called vermiform cell and should not be regarded as a separate category. As the name suggests, these are extremely elongated cells with slightly granular or agranular cytoplasm. The nucleus may be located centrally or eccentrically (Fig. 4.2*F*).

Synonymies

Yeager (1945) introduced the term "vermiform cell," but the term "vermicyte" is more appropriate. Tuzet and Manier (1959) used the term "giant fusiform cells" for VEs.

Interrelationship with other types

The origin of VEs is unknown. However, it is conceivable that they are derived from PLs, as has been suggested by Gupta and Sutherland (1966). Lea and Gilbert (1966) considered them a variant form of PLs. According to Arnold (1974), "they seem to occur mainly just prior to pupation, but never in large numbers."

4.3.3. Additional miscellaneous hemocyte types

In addition to the above nine hemocyte types, several authors have, from time to time, reported hemocytes, many or all of which have not been generally accepted – for example: "haemocytoblast" of Bogojavlensky (1932); "leucoblast" of Arvy and Gabe (1946) and Arvy (1954); "proleucocytoid" and "prohaemocytoid" of Yeager (1945) and Jones (1950), respectively. Yeager also introduced the term "nematocyte." Rizki (1962) in his works used the terms "lamellocyte" and "crystal cell." The latter was adopted also by Whitten (1964). According to Arnold (1974), crystal cells and lamellocytes are considered variants of

OEs and PLs, respectively. Gupta (1969) also suggested that the crystal cell is probably an OE. Terms such as "seleniform cell" (Poyarkoff, 1910); "miocyte" (Tillyard, 1917); "splanchnocyte" (Muttkowski, 1924); "teratocyte" (Hollande, 1920); "pycnonucleocyte" (Morganthaler, 1953; Wille and Vecchi, 1966); "nucleocyte" and "rhegmatocyte" (Hrdy, 1957) are rarely encountered in the literature; most likely several of these cells are not even hemocytes. Jones (1965) introduced the term "granulocytophagous" cell in his work on *Rhodnius prolixus*, and Ritter (1965) and Scharrer (1965) "anucleate crescent body" and "crescent cell," respectively, in the cockroach, *Gromphadorhina portentosa*. Zachary and Hoffmann (1973) described the hemocyte "thrombocytoid" that takes part in encapsulation in *Calliphora erythrocephala* (Zachary et al., 1975).

Several years ago, I (Gupta, 1969) reported that the hemocytes in several orders of insects have not been studied. For example, as of that year, among Apterygota, only Thysanura had been studied. Among the orthopteroid groups, Isoptera and Embioptera awaited studies. In the hemipteroid complex, no account of hemocytes was available in Zoraptera, Phthiraptera, Corrodentia, and Thysanoptera; and finally in the neuropteroid group, hemocytes were yet to be studied in Raphidiodea, Mecoptera, and Siphonaptera. It seems that situation has changed very little since then, for hemocytes in most of the above groups are still awaiting studies.

4.4. Hemocyte types in various orders

In terms of the number of species studied in various orders, Lepidoptera, Hymenoptera, Coleoptera, and Diptera appear to be the most extensively studied groups. In addition to the Heteroptera, Homoptera, and Odonata, of which only a few species have so far been studied, Dermaptera, Plecoptera, Trichoptera, and Thysanoptera are the most poorly studied groups. Hemocytes of several insect orders are unknown.

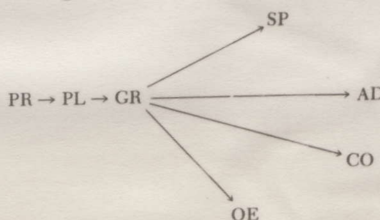
With the exception of the GR and possibly also PLs, all other types of hemocytes are not present in all insect orders (see Table 4.1). According to Arnold (1974), all hemocyte types have been reported only in *Prodenia* (Yeager, 1945; Jones, 1959). Most insects seem to possess PRs, PLs, and GRs (see also Chapter 8).

4.5. Plesiomorphic hemocyte and its differentiation into other types

I have reported elsewhere (Gupta, 1979) that the granulocyte (GR) is the plesiomorphic hemocyte, and it is the only hemocyte type that has been reported in all major arthropod groups, including all studied insect orders, and the Onychophora. The following account of the presence of GR in Insecta is based on Arnold's (1974) reinterpretation of

various works, my own (Gupta, 1969) survey of the hemocyte literature in many insects, and the most recent transmission electron microscopic (TEM) studies of hemocytes. As far as is known, only Bruntz (1908), Millara (1947), Barra (1969), Gupta (1969), and François (1974, 1975) have worked on the hemocytes of the Apterygota; and on the basis of these works, both Collembola (it is controversial whether they should be included in Apterygota) and Thysanura possess GRs. All higher orders of insects possess GRs (see Table 4.1). Some of the most recent TEM studies (Hoffmann et al., 1968, 1970; Baerwald and Boush, 1970; Hagopian, 1971; Moran 1971; Scharrer, 1972; Ratcliffe and Price, 1974; Goffinet and Grégoire, 1975; Beaulaton and Monpeyssin, 1976; Brehélin et al., 1976; Ratcliffe et al., 1976; Rowley and Ratcliffe, 1976; Rowley, 1977; Schmit and Ratcliffe, 1977) have reported (or can be interpreted to show) GRs in various insects. The most highly specialized neuropteroid orders also possess GRs.

Since it has been reported by several authors that one hemocyte type can and does differentiate into another type, it is conceivable that during evolution the plesiomorphic GR differentiated into other hemocyte types. It can be postulated also that the GR originates from the so-called prohemocyte (PR) or stem cell and goes through the plasmatocyte (PL) stage before becoming a distinct GR type. In taxa in which only GRs have been observed (e.g., Xiphosura), the PR and PL are merely evanescent stages and have not achieved distinctness as types. In taxa that are reported to possess other types besides PR, PL, and GR, the last perhaps further differentiated into SP, AD, CO, and OE, not necessarily in that order. The post-GR differentiation is generally accompanied by distinct PRs and PLs. Furthermore, in the more highly evolved taxa any of the types may be suppressed. The main differentiation pathways as postulated above may be represented as shown in the diagram.



4.6. Phylogenetic significance of hemocyte types

The prospect of using variations in hemocyte types in various insect orders for phylogenetic considerations is severely limited owing to (1)

lack of uniform terminology, and hence the difficulty of establishing comparisons, and (2) paucity of comprehensive studies of hemocyte types in large numbers of species within various orders to enable one to draw meaningful conclusions on the basis of important variations. However, hemocyte types and their numerical variations in many insects orders are known. Does the number of hemocyte types in various orders have any phylogenetic significance? Arnold (1972a,b, 1976) and Arnold and Hinks (1975) have used hemocytes in insect taxonomy, and several authors have suggested (see Gupta, 1979) that some phylogenetic relationship among various arthropod groups can be demonstrated on the basis of the occurrence of the GR and other hemocyte types.

A review of the insect hemocyte literature indicates some phylogenetic trends in the diversity of the hemocyte types in Insecta as a group as the evolutionary ladder is ascended (Fig. 4.14). This was observed also by Arnold (1974), although he attributed this diversity more to "shifts in the emphasis on certain functions or in the assignment of functions to different tissues" than to phylogeny. And at least in certain instances he may be right.

As far as is known, only Bruntz (1908), Millara (1947), Barra (1969), Gupta (1969), and François (1974, 1975) have worked on the hemocytes of the Apterygota; and on the basis of these works, Collembola possess only the plesiomorphic GR, whereas the Thysanura (Lepismatidae) seem to possess PL, GR, SP, and CO. Assuming that the Thysanura originated from Symphyla-like ancestors, and that the latter had hemocyte types comparable to those of *Scutigerella* (Gupta, 1968), we find that a reduction from six (PR, PL, GR, SP, AD, and CO) in the symphylan ancestor to four in the Thysanura has occurred. I have no evidence to suggest whether this is a secondary suppression and/or reduction or, as Arnold (1974) suggested, attributable to shifts in functions. It is also possible that future studies will reveal more types than are presently known.

According to Carpenter (1976), the derivation of the Pterygota from the apterygote Thysanura is almost universally accepted. It is also generally believed that the pterygotes evolved along four evolutionary lines: paleopteroid, orthopteroid, hemipteroid, and neuropteroid. It is interesting to note (Fig. 4.14) that in the Palaeoptera, although the number of hemocyte types has not increased from the ancestral thysanuran number, the OE has already made its appearance in the very beginning of the evolution of winged insects. In addition, the PR has achieved distinctness, and the CO is either suppressed or its function is taken over by the GR, which is generally the case in some other arthropods (aquatic Chelicerata and some Crustacea).

It is also evident from Fig. 4.14 that beyond the paleopteroid line the number of hemocyte types increased, and all the six or seven types

were realized in the orthopteroid, hemipteroid, and neuropteroid lines. It seems that by the time the orthopteroid line evolved, the plesiomorphic GR had already differentiated into all the distinct types presently known in pterygote insects, and that no further evolution in the hemocyte types occurred beyond that point. It is doubtful whether within the orthopteroid group any phylogenetic significance of the hemocyte types exists. The number of hemocyte types reported in various orders of this group (see Gupta, 1969; Arnold, 1974) is so variable that it is very difficult to discern any phylogenetic trends. On the basis

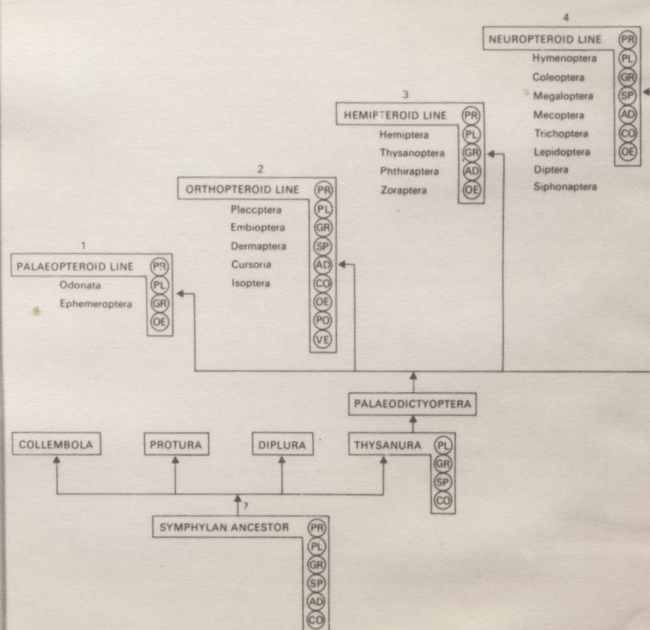


Fig. 4.14. Diagram showing distribution of various hemocyte types in four evolutionary lines (groups) and in symphylan ancestor of Insecta. Note that listed orders under each evolutionary line are only some examples of representative orders, not necessarily those in which hemocyte types designated under each evolutionary line are present. *AD* = adipohemocyte; *CO* = coagulocyte; *GR* = granulocyte; *OE* = oenocytoid; *PL* = plasmatocyte; *PO* = podocyte; *PR* = prohemocyte; *SP* = spherulocyte; *VE* × vermicyte. (From Gupta, 1979)

of the information presently available, the hemocyte types vary from three to eight or nine by light microscope, and seven (PR, PL, GR, SP, CO, AD, OE) types have been demonstrated by TEM. Unfortunately, hemocytes of several hemipteroid orders have not been studied (Gupta, 1969; Arnold, 1974), most of the work being confined to a few species in the order Hemiptera (Poisson, 1924; Hamilton, 1931; Khanna, 1964; Wigglesworth, 1955, 1956; Jones, 1965; Lai-Fook, 1970; Zaidi and Khan, 1975). Five (PR, PL, GR, AD, OE) types by light microscopy and four (PR, PL, GR, OE) by TEM studies have been identified. The apparent absence of SP and CO in the hemipteroid group as a whole is probably attributable to lack of enough studied species. It is difficult to imagine that these two types have been suppressed in the hemipteroid orders.

In the neuropteroid group, we find the seven major types (PR, PL, GR, SP, AD, CO, OE), although not all these types have been reported from each of the orders in this group (Gupta, 1969), and not all are recognized as types by all authors. As a matter of fact, the types of hemocytes reported vary widely even within an order in this group. For example, two to seven types have been reported in Lepidoptera, Coleoptera, and Diptera; five types in Hymenoptera, Neuroptera, and Megaloptera; and only three (PR, PL, GR) in Trichoptera (see Gupta, 1969, for various listings; Arnold, 1974). Since all the orders are highly evolved, it is quite conceivable that most or all major types would be found in all the orders as more studies become available.

4.7. Summary

Among arthropods, hemocytes have been most extensively studied in insects. A uniform hemocyte classification is still lacking for insects as well as for other arthropod groups. There is disagreement among insect hematologists about the number of hemocyte types in various insects. From one or a few to as many as nine or more types have been described, particularly by light microscopy. Ultrastructurally, however, only seven types have so far been identified in various insects: prohemocyte (PR), plasmatocyte (PL), granulocyte (GR), spherulocyte (SP), adipohemocyte (AD), oenocytoid (OE), and coagulocyte (CO). Of these seven, AD and CO have been described by only a few authors. All seven types have been described under various other names, and these synonymies are mentioned. It is suggested that of the seven types of hemocytes, the GR is the plesiomorphic hemocyte type and has evolved into other hemocyte types. I have postulated that the GR originates from the PR and, goes through the PL stage before becoming a distinct GR type. In taxa in which only GRs have been found, the PR and PL are merely evanescent stages and have not achieved distinctness as types. In taxa that are reported to

possess other types besides PR, PL, and GR, the last further differentiates into SP, AD, and OE, not necessarily in that order. This post-GR differentiation is generally accompanied by distinct PRs and PLs. Furthermore, in more highly evolved taxa, as well as in some lower ones, any of the types may be suppressed.

A review of the insect hemocyte literature indicates some phylogenetic trends in the diversity of the hemocyte types as the evolutionary ladder is ascended in Insecta.

Acknowledgments

The material presented in this chapter is adopted and modified from Gupta (1979). Without the published illustrations I have included, this review would not have been possible. For providing me with prints or negatives and allowing me to reproduce their published illustrations, I am most indebted to Drs. H. Akai, R. J. Baerwald, N. M. Costin, G. Devauchelle, J. François, G. Goffinet, Ch. Grégoire, A. V. Loud (for the late Dr. M. Hagopian), A. K. Raina, G. Salt, and S. Sato. I am grateful to Dr. J. W. Arnold for his comments and suggestions. The entire credit and my deep appreciation for preparing all the illustrations of this review go to Dr. Y. T. Das and Mr. S. B. Ramaswamy. I sincerely appreciate the secretarial assistance of Mrs. Joan Gross.

References

Akai, H. 1969. Ultrastructure of haemocytes observed on the fat-body cells in *Philosamia* during metamorphosis. *Jpn. J. Appl. Entomol. Zool. 13:*17–21.

Akai, H., and S. Sato. 1973. Ultrastructure of the larval hemocytes of the silkworm, *Bombyx mori* L. (Lepidoptera: Bombycidae). *Int. J. Insect Morphol. Embryol.* 2(3):207–31.

Åkesson, B. 1945. Observations on the haemocytes during the metamorphosis of *Calliphora erythrocephala* (Meig.). *Ark. Zool.* 6(12):203–11.

Arnold, J. W. 1952. The haemocytes of the Mediterranean flour moth, *Ephestia kühniella* Zell. (Lepidoptera: Pyralididae). *Can. J. Zool. 30:*352–64.

Arnold, J. W. 1970. Haemocytes of the Pacific beetle cockroach, *Diploptera punctata. Can. Entomol. 102*(7):830–5.

Arnold, J. W. 1972a. Haemocytology in insect biosystematics: The prospect. *Can. Entomol. 104:*655–9.

Arnold, J. W. 1972b. A comparative study of the haemocytes (blood cells) of cockroaches (Insecta: Dictyoptera: Blattaria), with a view of their significance in taxonomy. *Can. Entomol. 104:*309–48.

Arnold, J. W. 1974. The hemocytes of insects, pp. 201–54. *In* M. Rockstein (ed.). *The Physiology of Insecta*, Vol. 5, 2nd ed. Academic Press, New York.

Arnold, J. W. 1976. Biosystematics of the genus *Euxoa* (Lepidoptera: Noctuidae). VIII. The hemocytological position of *E. rockburnei* in the "declarata group." *Can. Entomol. 108:*1387–90.

Arnold, J. W., and C. F. Hinks. 1975. Biosystematics of the genus *Euxoa* (Lepidoptera: Noctuidae). III. Hemocytological distinctions between two closely related species, *E. campestris* and *E. declarata. Can. Entomol. 107;*1095–1100.

Arnold, J. W., and E. H. Salkeld. 1967. Morphology of the haemocytes of the giant cockroach, *Blaberus giganteus*, with histochemical tests. *Can. Entomol. 99:*1138–45.

Arnold, J. W., and S. S. Sohi. 1974. Hemocytes of *Malacosoma disstria* Hübner (Lepidoptera: Lasiocampidae): Morphology of the cells in fresh blood and after cultivation *in vitro. Can. J. Zool.* 52(4):481–5.

Arvy, L. 1954. Présentation de documents sur la leucopoïèse chez *Peripatopsis capensis* Grube. *Bull. Soc. Zool. Fr. 79:*13.

Arvy, L., and M. Gabe. 1946. Identification des diastrases sanguines chez quelques insectes. *C. R. Soc. Biol. (Paris) 140:*757–8.

Arvy, L., and J. Lhoste. 1946. Les variations du leucogramme au cours de la métamorphose chez *Forficula auricularia* L. *Bull. Soc. Zool. Fr. 70:*114–48.

Ashhurst, D. E., and A. G. Richards. 1964. Some histochemical observations on the blood cells of the wax moth, *Galleria mellonella* L. *J. Morphol. 114:*247–53.

Baerwald, R. J., and G. M. Boush. 1970. Fine structure of the hemocytes of *Periplaneta americana* (Orthoptera: Blattidae) with particular reference to marginal bundles. *J. Ultrastruct. Res. 31:*151–61.

Baerwald, R. J., and G. M. Boush. 1971. Vinblastine-induced disruption of microtubules in cockroach hemocytes. *Tissue Cell 3*(2):251–60.

Barra, J. A. 1969. Tégument des Collemboles: Présence d'hémocytes à granules dans le liquide exuvial au cours de la mue (Insectes, Collemboles). *C. R. Acad. Sci. Paris 269D:*902–3.

Beaulaton, J. 1968. Etude ultrastructurale et cytochimique des glandes prothoraciques de vers à soie aux quatrième et cinquième âges larvaires. I. La *tunica propria* et ses relations avec les fibres conjonctives et les hémocytes. *J. Ultrastruct. Res. 23:*474–98.

Beaulaton, J., and M. Monpeyssin. 1976. Ultrastructure et cytochimie des hémocytes d'*Antheraea pernyi* Guér. (Lepidoptera, Attacidae) au cours du cinquième âge larvaire. I. Prohémocytes, plasmatocytes et granulocytes. *J. Ultrastruct. Res.* 55(2):143–56.

Beaulaton, J., and M. Monpeyssin. 1977. Ultrastructure et cytochimie des hémocytes d'*Antheraea pernyi* Guér. (Lepidoptera, Attacidae). II. Cellules à sphérules et oenocytoïdes. *Biol. Cell.* 28(1):13–8.

Bogojavlensky, K. S. 1932. The formed elements of the blood of insects. *Arch. Russ. Anat. Hist. Embryol. 11:*361–86. (In Russian.)

Boiteau, G., and J. M. Perron. 1976. Etude des hémocytes de *Macrosiphum euphorbiae* (Thomas) (Homoptera: Aphididae). *Can. J. Zool.* 54(2):228–34.

Brehélin, M., D. Zachary, and J. A. Hoffmann. 1976. Fonctions des granulocytes typiques dans la cicatrisation chez l'orthoptère *Locusta migratoria* L. *J. Microsc. Biol. Cell.* 25(2):133–6.

Breugnon, M., and J. R. Le Berre. 1976. Fluctuation of the hemocyte formula and hemolymph volume in the caterpillar *Pieris brassicae. Ann. Zool. Ecol. Anim.* 8(1):1–12. (In French.)

Bruntz, L. 1908. Nouvelles recherches sur l'excrétion et la phagocytose chez les Thysanoures. *Arch. Zool. Exp. Gén. 38:*471–88.

Cameron, G. R. 1934. Inflammation in the caterpillars of Lepidoptera. *J. Pathol. Bacteriol. 38:*441–66.

Carpenter, F. M. 1976. Geological history and evolution of the insect. *Proc. 15th Int. Congr. Entomol. 1976:*63–70.

Clark, E. W., and D. S. Chadbourne. 1960. The hemocytes of non-diapause and diapause larvae and pupae of the pink bollworm. *Ann. Entomol. Soc. Amer. 53:*682–5.

Costin, N. M. 1975. Histochemical observations of the haemocytes of *Locusta migratoria. Histochem. J.* 7:21–43.

Crossley, A. C. 1975. The cytophysiology of insect blood. *Adv. Insect Physiol. 11:*117–221.

Cuénot, L. 1896. Etudes physiologiques sur les Orthoptères. *Arch. Biol. 14:*2[illegible].

Dennell, R. 1947. A study of an insect cuticle. *Proc. R. Soc. Lond.* (*B*) *134:*79–110.

Devauchelle, G. 1971. Etude ultrastructurale des hémocytes du Coléoptère *Melolontha melolontha* (L.). *J. Ultrastruct. Res. 34:*492–516.

François, J. 1974. Etude ultrastructurale des hémocytes du Thysanoure *Thermobia domestica* (Insecte, Aptérygote). *Pedobiologia 14:*157–62.

François, J. 1975. Hémocyte et organe hématopoïètique de *Thermobia domestica* (Packard) (Thysanura: Lepismatidae). *Int. J. Insect Morphol. Embryol.* 4(6):477–94.

Goffinet, G., and Ch. Grégoire. 1975. Coagulocyte alterations in clotting hemolymph of *Carausius morosus* L. *Arch. Int. Physiol. Biochim.* 83(4):707–22.

Graber, V. 1871. Ueber die Blutkorperchen der Insekten. *Sitzb. Akad. Math.-Nat. Wiss. Wien 64:*9–44.

Grégoire, Ch. 1974. Hemolymph coagulation, pp. 309–60. *In* M. Rockstein (ed.). *The Physiology of Insecta*, Vol. 5, 2nd ed. Academic Press, New York.

Grégoire, Ch., and M. Florkin. 1950. Blood coagulation in arthropods. I. The coagulation of insect blood, as studied with the phase contrast microscope. *Physiol. Comp. Oecol.* 2(2):126–39.

Grimstone, A. V., S. Rotheram, and G. Salt. 1967. An electron-microscope study of capsule formation by insect blood cells. *J. Cell Sci.* 2:281–92.

Gupta, A. P. 1968. Hemocytes of *Scutigerella immaculata* and the ancestry of Insecta. *Ann. Entomol. Soc. Amer. 61*(4):1028–9.

Gupta, A. P. 1969. Studies of the blood of Meloidae (Coleoptera). I. The haemocytes of *Epicauta cinerea* (Forster), and a synonymy of haemocyte terminologies. *Cytologia 34*(2):300–44.

Gupta, A. P. 1970. Midgut lesions in *Epicauta cinerea* (Coleoptera: Meloidae). *Ann. Entomol. Soc. Amer. 63:*1786–8.

Gupta, A. P. 1979. Arthropod hemocytes and phylogeny, pp. 669–735. *In* A. P. Gupta (ed.). *Arthropod Phylogeny.* Van Nostrand Reinhold, New York.

Gupta, A. P., and D. J. Sutherland. 1965. Observations on the spherule cells in some Blattaria (Orthoptera). *Bull. Entomol. Soc. Amer. 11:*161.

Gupta, A. P., and D. J. Sutherland. 1966. *In vitro* transformations of the insect plasmatocyte in certain insects. *J. Insect Physiol. 12:*1369–75.

Gupta, A. P., and D. J. Sutherland. 1967. Phase contrast and histochemical studies of spherule cells in cockroaches. *Ann. Entomol. Soc. Amer. 60*(3):557–65.

Gupta, A. P., and D. J. Sutherland. 1968. Effects of sublethal doses of chlordane on the hemocytes and midgut epithelium of *Periplaneta americana. Ann. Entomol. Soc. Amer. 61*(4):910–18.

Hagopian, M. 1971. Unique structures in the insect granular hemocytes. *J. Ultrastruct. Res. 36:*646–58.

Hamilton, M. A. 1931. The morphology of the water scorpion, *Nepa cinerea* Linn. *Proc. Zool. Soc. Lond. 193:*1067–1136.

Harpaz, F., N. Kislev, and A. Zelcer. 1969. Electron-microscopic studies on hemocytes of the Egyptian cottonworm, *Spodoptera littoralis* (Boisduval) infected with a nuclear-polyhedrosis virus, as compared to noninfected hemocytes. I. Noninfected hemocytes. *J. Invertebr. Pathol. 14:*175–85.

Hinks, C. F., and J. W. Arnold. 1977. Haemopoiesis in Lepidoptera. II. The role of the haemopoietic organs. *Can. J. Zool.* 55(10):1740–55.

Hoffmann, J. A. 1966. Etude des oenocytoïdes chez *Locusta migratoria* (Orthoptère). *J. Microsc. (Paris)* 5:269–72.

Hoffmann, J. A. 1967. Etude des hémocytes de *Locusta migratoria* L. (Orthoptère). *Arch. Zool. Exp. Gén. 108:*251–91.

Hoffmann, J. A., A. Porte, and P. Joly. 1970. On the localization of phenoloxidase activity in coagulation of *Locusta migratoria* (L.) (Orthoptera). *C. R. Hebd. Séances Acad. Sci. Paris 270D:*629–31.

Hoffmann, J. A., and M. E. Stoekel. 1968. Sur les modifications ultrastructurales des coagulocytes au cours de la coagulation de l'hémolymphe chez un insecte Orthopteroïde: *Locusta migratoria C. R. Séances Soc. Biol. Strasbourg 162:*2257–9.

Hoffmann, J. A., M. E. Stoekel, A. Porte, and P. Joly. 1968. Ultrastructure des hémocytes de *Locusta migratoria* (Orthoptère). *C. R. Hebd. Séances Acad. Sci. Paris 266:*503–5.

17 Identification key for hemocyte types in hanging-drop preparations

A. P. GUPTA

Department of Entomology and Economic Zoology, Rutgers University, New Brunswick, New Jersey 08903, U.S.A.

Contents

17.1. Introduction — page 527
17.2. Selected species for examination — 527
17.3. Procedure for hanging-drop preparation — 529
17.4. Summary — 529
References

17.1. Introduction

The identification key presented as Table 17.1 is for the novice who is studying insect–or for that matter any other arthropod–hemocytes for the first time. It is often very frustrating for a beginner to try to identify various types of hemocytes in a hemolymph sample or film under light microscope. This key should be helpful in guiding the beginner to become acquainted with the seven main types of hemocytes described in Chapter 4. Because not all hemocyte types are readily observed in any one species, at all developmental stages, and under all physiological conditions, it is important to examine hemolymph samples from different species at various developmental stages and under different physiological conditions. The method of study is no less important. The key is based on hemocyte observations under a phase-contrast microscope in hanging-drop preparations of fixed or unfixed hemolymph from various insects (Gupta and Sutherland, 1966, 1967; Gupta, 1968, 1969).

17.2. Selected species for examination

Although the key can be used to identify hemocytes from any species, I suggest the following insects be used in the beginning: adult *Blaberus* spp. for typical prohemocytes (PRs), plasmatocytes (PLs), granulocytes (GRs), and spherulocytes (SPs); larvae of *Galleria mellonella* and *Porthetria dispar* for typical PRs, PLs, oenocytoids (OEs), SPs, and adipohemocytes (ADs), particularly in larvae about to molt, which

Table 17.1. Identification key for insect hemocyte types

1.	Nucleus compact, large in relation to cell size, centrally located, almost filling the cell; very thin, peripheral layer of homogeneous cytoplasm around the nucleus; cells may be round, oval, or elliptical, but always small (compared with other cells in sample) (Figs. 4.1A, 13.8)	*Prohemocyte (PR)*
1′.	Nucleus not compact, generally small in relation to cell size, not nearly filling the cell	2
2 (1′).	Nucleus with chromatin arranged in cartwheel-like fashion, generally eccentric, oval, and sharply outlined; cytoplasm hyaline, generally scant, may contain some spherical or elongate granular inclusions; cell sometimes with cystlike blebs in process of exocytosis (Figs. 4.2*D*, 7.5, 10.2*C*, 13.17) (Beware! COs may be confused with OEs and with GRs in some insects)	*Coagulocyte (CO)*
2′.	Nuclear chromatin not arranged in cartwheel-like fashion, nucleus eccentric or central; cytoplasm not hyaline, abundant, homogeneous, and without any plate-, rod-, or needlelike inclusions and filaments	3
3 (2′).	Cytoplasm generally agranular or slightly granular; nucleus round or elongate and central, and may or may not appear punctate; cells polymorphic and variable in size in various insects (Figs. 4.1*B*, 13.10)	*Plasmatocyte (PL)*
3′.	Cytoplasm generally agranular, thick, and homogeneous with or without several kinds of plate-, rod-, or needlelike inclusions and filaments; "vacuoles" may or may not be present; nucleus generally small, round or elongate, and generally eccentric; cells variable in size and shape, generally lyse quickly in vitro, ejecting material into the hemolymph (Figs. 4.2*A*,*B*, 8.14, 10.1*E*, 13.23)	*Oenocytoid (OE)*
4 (3′).	Cytoplasm distinctly granular	4′
4′.	Cytoplasm prominently and characteristically granular; granules may or may not be numerous; nucleus comparatively small (compared with that in plasmatocyte) and compact, round or elongate, and generally central (Figs. 4.1*D*, 13.14) (Note that GRs in lower orders are generally larger than in higher orders.)	*Granulocyte (GR)*
5 (4′).	Granules in cytoplasm considerably enlarged and appear as distinct spherules or droplets	5′
5′.	Spherules nonrefringent, generally obscuring the nucleus, number of spherules varying from few to many; nucleus rather small, central or eccentric; cells ovoid or round with variable sizes, usually larger than granulocytes, and may be observed releasing material from spherules into hemolymph by exocytosis (Figs. 4.1*C*,*E*, 13.19)	*Spherulocyte (SP)*
6 (5′).	Spherules or droplets refringent owing to presence of lipid; nucleus relatively small (compared with that in plasmatocytes or spherulocytes), round or slightly elongate, central or eccentric, and may or may not appear concave, biconvex, punctate, or lobate; cytoplasm may contain other nonlipid granules (Figs. 4.2*C*, 10.1*C*, 10.4 (larger cell)	*Adipohemocyte (AD)*

is when GRs accumulate lipids and appear as ADs; and larval *Tenebrio molitor* for ADs, particularly after the larvae have been chilled at 5 °C for 20–24 hr.

17.3. Procedure for hanging-drop preparation

1. Take a square coverslip and put a tiny drop of saline-versene (NaCl, 0.9 g; KCl, 0.942 g; $CaCl_2$, 0.082 g; $NaHCO_3$, 0.002 g; distilled water, 100 ml + 2% versene).
2. Cut the tip of the antenna or leg (or proleg) and let a drop of hemolymph flow into the saline-versene drop.
3. Carefully turn the coverslip upside down and place it over the depression of a depression or cavity slide. Seal the sides of the coverslip with petroleum jelly.
4. Examine the hanging-drop preparation under the phase-contrast microscope.
5. You will notice that the hemocytes are more evenly distributed near the periphery than at the center of the hemolymph drop. It will, therefore, be easier to focus them sharply near the periphery.
6. Make a fresh preparation for examination every 8–10 min because hemocytes begin to deteriorate after bleeding.
7. For longer-lasting preparations, you may try hemolymph from an insect that has been heat-fixed in water at 60 °C for about 5 min.

17.4. Summary

A beginner's identification key for seven main hemocyte types (prohemocytes, plasmatocytes, granulocytes, spherulocytes, adipohemocytes, coagulocytes, and oenocytoids) is presented. It is suggested that hanging-drop preparations of hemolymph be used to identify these hemocytes under phase-contrast microscope.

References

Gupta, A. P. 1968. Hemocytes of *Scutigerella immaculata* and the ancestry of Insecta. *Ann. Entomol. Soc. Amer. 61*(4):1028–9.

Gupta, A. P. 1969. Studies of the blood of Meloidae (Coleoptera). I. The haemocytes of *Epicauta cinerea* (Forster), and a synonymy of haemocyte terminologies. *Cytologia 34*(2):300–44.

Gupta, A. P., and D. J. Sutherland. 1966. *In vitro* transformations of the insect plasmatocyte in certain insects. *J. Insect Physiol. 12:*1369–75.

Gupta, A. P., and D. J. Sutherland. 1967. Phase contrast and histochemical studies of spherule cells in cockroaches. *Ann. Entomol. Soc. Amer. 60*(3):557–65.

IV. Techniques

18 Insect hemocytes under light microscopy: techniques

J. W. ARNOLD AND C. F. HINKS
Biosystematics Research Institute, Research Branch, Agriculture Canada, Ottawa, Ontario K1A 0C6, Canada

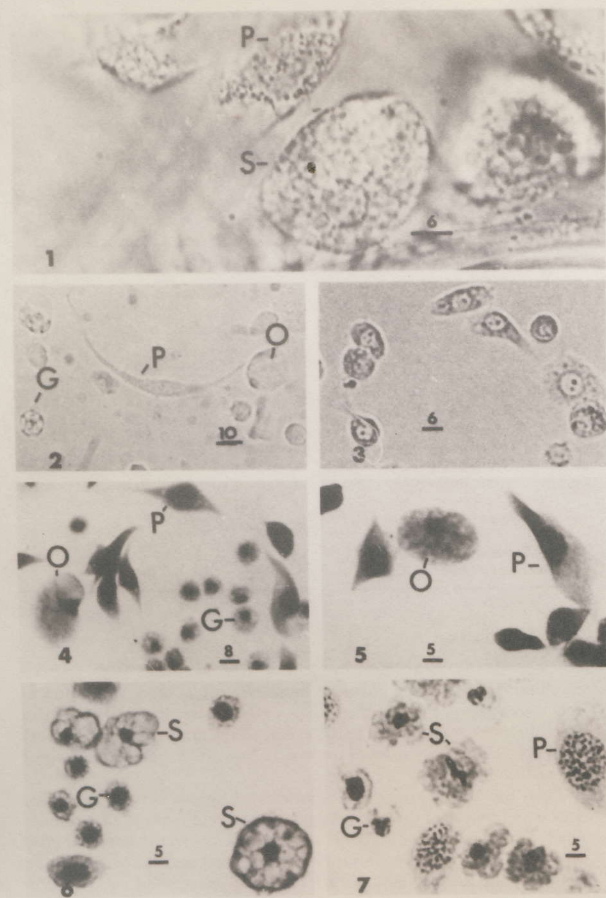
Contents

18.1. Introduction — *page* 531
18.2. In vivo procedure — 533
18.3. In vitro procedure — 534
18.4. Blood film preparation — 534
  18.4.1. Fixation and slide preparation
  18.4.2. Staining
    Rapid Giemsa staining
    Full Giemsa staining
      Comments
    Hematoxylin–eosin–alcian blue staining
      Comments
    Acetocarmine staining
      Comments
    Acridine orange staining
      Comments
18.5. Summary — 537
References

18.1. Introduction

In general, the techniques used in vertebrate hematology must be modified for the study of insect hemocytes. The same principles of technique apply, and the insect hemocytes can be observed in vivo, in vitro, in living culture, and in blood films fixed and stained in a variety of ways. The procedures described below are a small sampling of these techniques.

Each procedure provides a somewhat different view of the cells, and it is often useful to employ more than one method, if possible. Such a combination of methods at best includes an in vivo or in vitro technique as a basis for interpretation of fixed and stained cells. To some extent, all the methods are empirical because of variability in the character of both hemolymph and hemocytes in different species. Most of the following techniques have been found suitable for a wide variety of insects, but there are preferred methods for certain species



Figs. 18.1–18.7 Hemocytes of insects as seen with various techniques. Magnification as indicated, in microns. **18.1** Hemocytes of *Blaberus giganteus* (Dictyoptera) in vivo, in a wing vein. **18.2.** Hemocytes of *Euxoa declarata* (Lepidoptera) in vitro, as a wet film. **18.3.** Hemocytes of *Malacosoma disstria* (Lepidoptera) in vitro, in subcul-

and for different purposes, and the in vivo procedure is obviously limited to species with transparent areas of the body.

18.2. In vivo procedure

A relatively clear and sometimes excellent view of living hemocytes can be obtained with transmitted light through transparent regions or appendages of certain insects. The procedure is similar to the one long used by vertebrate hematologists to demonstrate living blood cells in thin tissues, such as the ear membrane of the rabbit or the toe membrane of the frog. Simply, it involves immobilization of the insect so that the transparent structure can be manipulated under the compound microscope. Wings are most suitable for this purpose, and for best results they are sandwiched in glycerol or refined immersion oil between thin glass coverslips to reduce diffraction at the cuticle-air interface (Arnold, 1959). The wings of orthopteroid insects are best by far, as the blood circulates freely there in thin veins and sinuses, and the hemocytes are large. Particularly among the large blaberoid cockroaches, the hemocytes can be observed here with great clarity at high magnification (Fig. 18.1), circulating freely in the blood or moving ameboidally in regions out of the main flow (Arnold, 1961). Not all clear-winged insects are so satisfactory, for a variety of reasons (Arnold, 1964), including the small size of the hemocytes (e.g., among Hemiptera), the extreme thickness of the vein walls (e.g., Odonata), the near occlusion of veins by tracheae (e.g., Lepidoptera), the poor circulation of blood in the wings (e.g., Diptera), or the extremely rapid circulation of blood in the wings (e.g., Hymenoptera). Nevertheless, with ingenuity and patience many such insects can be used to gain an impression of the real size and form of the living hemocytes before resorting to techniques that involve injury or sacrifice of the insect or denaturation of the hemocytes. Similarly, with ingenuity and patience other appendages and clear regions of the insect body can serve as windows to the blood. The legs, prothoracic extensions, caudal cerci, and/or respiratory structures of some aquatic insects can be useful, and the dorsum of the abdomen sometimes provides a view of hemocytes in the dorsal sinus or heart. In these cases too, clarity is improved by covering the structure or area with saline (with a trace of wetting agent) or glycerol or immersion oil under a coverslip. Al-

(continued from facing page)
ture. **18.4.** Hemocytes of *E. declarata* after rapid Giemsa staining. **18.5.** Hemocytes of *E. declarata* after full Giemsa staining. **18.6.** Hemocytes of *E. declarata* after hematoxylin–eosin–alcian blue staining. Note very selective staining of spherulocytes. **18.7.** Hemocytes of *E. declarata* after acetocarmine staining. Note distinctions between granulocytes and spherulocytes. Note mitosis in the latter. *S* = spherulocyte; *G* = granulocyte; *O* = oenocytoid; *P* = plasmatocyte.

though phase-contrast microscopy can be used here, it is often less effective than standard light microscopy with the condenser manipulated to improve contrast and depth of field (see also Chapter 20).

18.3. In vitro procedure

This procedure includes a variety of techniques that involve the transfer of blood from the insect to glass without or with little exposure to air. There are two common ones: blood under oil and as a wet film under a coverglass. Perhaps the simplest technique is to sever the antenna under mineral oil on a glass slide. Blood issues from the cut end and forms a discrete globule on the slide, protected from the air by the inert oil. Under oil immersion, or at lower magnification with an immersion adapter, the hemocytes can be observed in their normal form as they flow from the antenna and in gradually changing form on the glass surface.

The more common technique, the wet film, involves directly transferring a drop of blood to the microscope slide and covering it immediately with a coverslip ringed with petroleum jelly to exclude air. The hemocytes are seen here clearly (Fig. 18.2), both in suspension for a short time and attached to the glass. They slowly alter their form and size under these conditions and should be examined immediately. It is here that the explosive so-called coagulocytes may be identified best. The view of cells in culture is somewhat similar to this (Arnold and Sohi, 1974), through the flat surface of the standard tissue culture flask (Fig. 18.3), but the typical hemocyte forms are not maintained in subcultures.

The technique for wet films can be used effectively also with treated blood. Heat-fixed blood can be used directly, or living blood can be diluted in fluids that suppress coagulation, such as Turk's diluting fluid (Fisher) or dilute solutions of sequestering agents such as tetrasodium ethylenediaminotetraacetate. Here the form of the hemocytes is retained for longer periods than in wet films of living blood.

18.4. Blood film preparation

Although vertebrate blood can be prepared for microscopy by placing a drop directly on a microscope slide and drawing it out with a coverslip to dry quickly in air, insect blood treated in this way will usually agglutinate before the film can be made or else will show much cellular distortion. For this reason, it is best to fix insect blood before preparing the film. This is accomplished in two ways: by a suitable degree of heat fixation or by dilution of the blood in a fixative that does

not immediately cause gelation of the hemolymph. Heat fixation can be followed by other types of fixation if desired, after the film has dried on the slide.

The vertebrate technique, using live blood directly on the slide, can serve in special preparations where cell form is less important than nuclear integrity. In such case, a bead of fresh blood is exposed immediately to acetic acid vapor for rapid fixation. This procedure, as detailed below, is effective for demonstrating mitosis in hemocytes, using nuclear stains.

18.4.1. Fixation and slide preparation

Heat can serve to fix hemocytes very rapidly within the insect, without appreciable change to their shape or size, and at the same time prevent coagulation of the hemolymph. Consequently, after heat fixation, the blood can be withdrawn from the insect and spread easily on a microscope slide without clumping or distortion of the cells. At the same time, the film of blood and its cells adheres evenly to the glass without need for adhesives. Heat fixation is accomplished by plunging the insect into water held at approximately 60 °C for a short period. The temperature can be varied within 5 °C, depending on the size of the insect, and the exposure varied from 1 to 10 min on the same basis. Within these limits, the temperature and time are not critical, but should be regulated for particular species by empirical testing. Slide preparation with heat-fixed blood is a simple process of placing a drop on the slide and touching it with the edge of a coverslip, which is then drawn along the slide with the drop trailing behind. The resulting blood film is air-dried (preferably on a slide warmer at about 32 °C) before staining.

Dilution of living blood directly into a water-soluble chemical fixative also accomplishes rapid fixation of the hemocytes with little coagulation of the hemolymph or cell distortion. It is the preferred method for some insects and for some purposes, especially where a succession of blood samples is required from the same insect. Dilute solutions of formalin (5%) or glutaraldehyde (0.4 M) are recommended. The procedure involves the pooling of some of the fixative solution on the microscope slide, immersing an appendage such as the antenna in it, and severing the appendage so that the blood flows directly into the solution without contacting the air. Blood from other types of wounds can also be dropped from above directly into the fixative so that exposure to air is very brief. In either case the mixture is stirred immediately to prevent clumping of the cells and can be spread on the slide if desired. The mixture is then air-dried completely on the slide and rinsed to remove traces of fixative before staining.

18.4.2. Staining

For most purposes, hemocytes are stained in one of the Romanowsky preparations, which depend on the formation of azure and other oxidation products of methylene blue, usually in combination with eosin (Humason, 1967). With these preparations, variation of the buffer level toward the acid side increases the precision of nuclear staining and decreases cytoplasmic basophilia; the reverse increases the amount of blue in various elements. One can, therefore, alter the effect of the stain for different purposes. We find that the Giemsa preparation is most reliable and use it almost exclusively. It can be used directly for rapid staining or with differentiation for more elegant results (see also Chapter 20).

Rapid Giemsa staining (Fig. 18.4)
Solution of Giemsa (Fisher): 1 drop of concentrate per milliliter distilled water in a Stender dish. Place air-dried slides directly in the solution or after 1 min in absolute methyl alcohol. Inspect slides for depth of staining after 3 min (max. 5 min). Rinse in distilled water for 1 min. Blot-dry using Kodak lens-cleaning tissue. Mount permanent slides in Canada balsam; temporary slides in glycerol or immersion oil.

Full Giemsa staining (Fig. 18.5)
Immerse air-dried films of heat-fixed hemolymph in Giemsa solution (1 drop of concentrate per milliliter distilled water) for 20 min to 2 hr. Rinse in distilled water; then immerse briefly in distilled water to which a few drops of lithium carbonate have been added (to differentiate red-staining structures). Rinse in distilled water, then immerse briefly in distilled water to which a few drops of dilute hydrochloric acid have been added (to differentiate blue-staining structures). Rinse in distilled water and examine. Repeat differentiation if staining is too dense. Blot dry using Kodak lens-cleaning tissue. Mount in Canada balsam.

Comments. Cells are better differentiated than when rapid Giemsa method is used; excellent for photography.

Hematoxylin–eosin–alcian blue staining (Fig. 18.6)
Immerse air-dried films of heat-fixed hemolymph in 15% acetic acid in methanol for 20 min. Hydrate through graded alcohols to distilled water. Immerse in 1% alcian blue 8GX in 0.1 N HCl for 20 min. Rinse in distilled water and immerse in Harris's hematoxylin for 20 min. Rinse in distilled water and differentiate in acid alcohol; then blue in Scott's solution. Examine and repeat differentiation if necessary. Dehydrate to 90% alcohol and immerse in 1% eosin in 90% alcohol for 3 min. Rinse off excess stain in 90% alcohol, transfer to absolute alcohol, then to xylene; mount in Canada balsam.

Comments. Mitotic cells are easier to identify than in Giemsa-stained preparations. Spherulocytes are very distinct, retaining pale blue, whereas the cytoplasm of all other cell types stains pink to pinkish purple.

Acetocarmine staining (Fig. 18.7)
Express small bead of fresh hemolymph from a CO_2-narcotized insect onto the center of a round coverglass and invert over a vial of glacial acetic acid for 90 min. Reverse coverglass with hemolymph uppermost in a solid watch glass and cover with acetocarmine (Humason, 1967) for 90 min. Blot off excess stain carefully and squash the preparation gently onto a microscope slide in a drop of Venetian turpentine.

Comments. Cell types are identifiable; mitotic cells very clear in all cell types; excellent for photography.

Acridine orange staining
Express a small drop of fresh hemolymph from a CO_2-narcotized insect onto a quartz microscope slide. Mix with an equal volume of acridine orange solution, 0.1 mg/ml in 0.9% NaCl. Place coverglass over hemolymph preparation and immediately examine with fluorescence microscope.

Comments. The inclusions of spherulocytes are rapidly and specifically stained to give an intense orange fluorescence. Nuclei of all hemocytes give a yellowish green fluoroscence.

18.5. Summary

Techniques for light microscopy of insect hemocytes are modifications of those used in vertebrate hematology and include in vivo and in vitro procedures as well as methods of fixation and staining. The in vivo procedures utilize transparent regions of the insect body, preferably the wings sandwiched in an inert solution under glass. The in vitro procedures include the examination of blood issuing from a wound under oil, of the cells in culture, or more commonly of preparations of covered wet films of living blood ringed with inert oil to exclude air. Preparation of insect blood for standard histological staining should be preceded by the killing of the insect in hot water to fix the cells and prevent hemolymph coagulation or by dilution of the living

19 Techniques for total and differential hemocyte counts and blood volume, and mitotic index determinations

M. SHAPIRO

Gypsy Moth Methods Development Laboratory, U.S. Department of Agriculture, Otis Air Force Base, Massachusetts 02542, U.S.A.

Contents

19.1. Introduction — page 539
19.2. Total hemocyte count — 539
19.3. Differential hemocyte count — 541
19.4. Blood volume — 542
19.5. Reliability as influenced by internal and external factors — 544
19.5.1. Internal factors
19.5.2. External factors
19.6. Summary — 546
References

19.1. Introduction

The three most common measurements made to describe the blood picture of a given insect at a given time or from one time to another are total hemocyte count (THC), differential hemocyte count (DHC), and blood volume (BV). The purpose of this chapter is to examine the methods used to obtain these values and their reliability.

19.2. Total hemocyte count

The first study of THCs in insects was made by Tauber and Yeager (1934). In 1935, they studied Orthoptera, Odonata, Hemiptera, and Homoptera. A year later, these same authors extended their study to include Neuroptera, Coleoptera, Lepidoptera, and Hymenoptera. The insects were heat-fixed (60 °C for 5–10 min) and bled from a proleg, after which the blood sample was diluted with physiological saline. The THC (i.e., the number of circulating hemocytes per cubic millimeter) was determined by the method employed for mammalian blood counts. This work represents an outstanding contribution and has served as a model for subsequent investigations.

Tauber-Yeager (1935) fluid (NaCl, 4.65 g; KCl, 0.15 g; $CaCl_2$,

0.11 g; gentian violet, 0.005 g; and 0.125 ml acetic acid/100 ml) was used also by Fisher (1935), Smith (1938), and Shapiro (1967, 1968). Other physiological saline solutions were utilized by Rosenberger and Jones (1960), Collin (1963), and Gupta and Sutherland (1968). Acetic acid, a component of the Tauber-Yeager fluid, was used for *Galleria* (Stephens, 1963; Shapiro, 1966; Jones, 1967a) and for *Heliothis* (Shapiro et al., 1969; Vinson, 1971). Turk's solution (1–2% glacial acetic acid, slightly colored with gentian violet) was used for *Pectinophora* (Clark and Chadbourne, 1960) and for *Euxoa* (Arnold and Hinks, 1976). Patton and Flint (1959) reported that Turk's solution did not prevent coagulation in hemolymph samples of *Periplaneta*. Versene (1–2% tetrasodium EDTA) was superior to Turk's solution and oxalate and was routinely used. Wittig (1966), studying phagocytosis in *Pseudaletia* larvae, also found versene (2% plus a trace of methylene blue) to be superior to glacial acetic acid. Formalin (10% in 0.85% NaCl) has also been employed as a diluting fluid (Jones, 1956). Physiological saline did not prevent cell agglutination, but the addition of acetic acid (1%) or formaldehyde reduced clumping.

In making total counts from *Pieris*, the first and second drops of hemolymph were utilized (Kitano, 1969). The first drop of hemolymph in unfixed and unfed *Rhodnius* contained more hemocytes than the second drop (Jones, 1962). The number of hemocytes was also reduced from three successive drops of *Bombyx* hemolymph (Matsumoto and Sakurai, 1956). On the other hand, Wittig (1966) found no significant differences in DHCs taken from the first and second drops of *Pseudaletia* hemolymph. Jones (1956) used the first drop of *Sarcophaga* hemolymph for DHCs. The rest of the hemolymph was placed on a second slide, and a portion was used for THCs. Some 3 or 4 drops of *Prodenia* hemolymph were allowed to flow on a glass slide. A portion of the blood was drawn into a Thoma white blood cell pipette, diluted, and counted (Rosenberger and Jones, 1960). After the hemolymph was diluted in a pipette, the first 3 or 4 drops were discarded (Jones, 1967a; Shapiro, 1967, 1968).

In many instances, hemolymph was drawn into a Thoma white blood cell pipette, diluted, and counted in a hemacytometer. The hemolymph dilution ranged from 1:20 (Rosenberger and Jones, 1960; Wittig, 1966) to 1:50 (Clark and Chadbourne, 1960; Shapiro, 1967) to 1:100 (Fisher, 1935; Gupta and Sutherland, 1968). Wittig (1966) adjusted the dilution of hemolymph so that a suspension contained between 800 and 1,600 cells/mm² area counted. This adjustment could not be made when the amount of blood available or the number of hemocytes per cubic millimeter was low.

Fisher (1935) found that the standard white cell pipette required too much hemolymph from *Periplaneta*. A micropipette was made that required only 1.7 μl and a final dilution of 1:44. Patton and Flint (1959) also used a special micropipette for *Periplaneta* in which 1 μl of hemolymph could be drawn and diluted 1:100.

THCs are counted in a standard hemacytometer according to the formula (Jones, 1962):

$$\frac{\text{hemocytes in } x \text{ 1-mm squares} \times \text{dilution} \times \text{depth of chamber}}{\text{number of 1-mm squares counted}}$$

Kitano (1969) counted the number of hemocytes in the smallest square (0.00025 mm²), counted 80 squares, and multiplied by a factor of 4,000 to give THC. Fisher (1935) counted hemocytes from three of the four white cell squares in each of the two chambers. Wittig (1966) calculated the THC per cubic millimeter by counting cells in four 1-mm² areas in each of the two chambers. Hemocytes from five 1-mm² squares (the four corner and central squares) were counted by Rosenberger and Jones (1960), Jones (1967a), Shapiro (1966, 1967), and Gupta and Sutherland (1968). White cells in all nine 1-mm² squares were counted by Clark and Chadbourne (1960).

In counting the hemocytes, a variation is to be expected. But when the distribution of the cell count was uneven and clumping was observed, the counts were discarded (Rosenberger and Jones, 1960; Shapiro, 1967; Kitano, 1969). Stephens (1963) questioned whether it was reasonable to discard counts when the means of the two chambers differed greatly. Wittig (1966) felt that such a rejection was justified if it was done on the basis of percent of the mean instead of the number of cells per volume, "for the weight of a number is different for high and low means."

19.3. Differential hemocyte count

In general, the method used by Shapiro (1966) may be considered typical. Larvae were submerged in a hot-water bath (56–58 °C for 1–2 min), and a proleg on the sixth abdominal segment was cut with fine scissors. The hemolymph was allowed to fall on a clean, grease-free microscope slide, and a smear was made in the conventional manner, by drawing a second slide across the first one at a 45 ° angle. The smear was allowed to air-dry and was stained by a modified Pappenheim-panoptic method (Pappenheim, 1914). The details of the method are as follows: (1) flood air-dried smear with May-Grünwald solution and allow to remain on slide for 3 min; (2) add distilled water so that a layer is formed and allow to stand for 2 min; (3) discard the May-Grünwald-water layer; (4) flood slide with Giemsa solution (1 part concentrated Giemsa to 40 parts distilled water) and allow to dry; (5) wash slide in running tap water and allow to dry.

The smear is examined under oil immersion, and 200 cells per slide are differentiated. By this method of staining, azurophilic material appears purple red; chromatin, reddish violet; and basic protoplasm, blue (Pappenheim, 1914).

Using the Yeager (1945) classification system as modified by Jones (1959), the following types of hemocytes were counted: prohemocytes (PRs), plasmatocytes (PLs), granulocytes (GRs), adipohemocytes (ADs), spherulocytes (SPs), podocytes (POs), and oenocytoids (OEs). Degenerating cells and cells in mitosis were also counted. In addition, hemocytes were found that could not be placed in the preceding classes with certainty; these cells were designated as unclassified cells. Jones (1962) recommended that a minimum of five insects of a given stage and physiological status be used. Whenever possible, a minimum of 200 cells should be classified per insect.

In *Rhodnius*, 100 hemocytes were classified from each of five insects as either PRs, PLs, GRs, or OEs. Mitotically dividing cells were also counted (Jones, 1967b). Vinson (1971) examined stained blood films from a minimum of five *Heliothis* larvae per time period. A minimum of 150 cells per larva was classified. A minimum of 200 and a maximum of 5,000 cells were counted in *Tenebrio* larvae (Jones and Tauber, 1954). Jones (1967a) counted 200–1,000 cells in each *Galleria* larva. Whenever possible, 200 cells were counted in *Sarcophaga* (Jones, 1956), in *Periplaneta* (Gupta and Sutherland, 1968), and in *Drosophila* (Nappi and Streams, 1969). Arnold and Hinks (1976) examined 200 hemocytes per smear of the noctuid, *Euxoa*. Five smears were examined per instar.

From 15 to 20 sections of each face fly larva, *Orthellia*, were examined, and a minimum of 100 cells was counted per section (Nappi and Stoffolano, 1972). Wittig (1966) counted 350–400 hemocytes for each DHC from *Pseudaletia*. Yeager (1945) attempted to count at least 400 cells in each DHC from *Prodenia*, but could not from young larvae, old pupae, and adults. Differential counts from *Anagasta* larvae were obtained by classifying 500 cells per smear. At least 10 larvae from each larval stage were used (Arnold, 1952a). In a subsequent study on the effects of fumigants on the hemocytes of *Anagasta*, Arnold (1952b) made DHCs from 20 larvae per time period; 500 cells were also counted in blood smears of *Blaberus* (Arnold, 1969).

19.4. Blood volume

BV is a little used but important value. It has been reported that an inverse relationship exists between the THC and BV in *Bombyx* (Nittono, 1960), in adults of *Locusta* (Webley, 1951), and in last-stage nymphs and adults of *Periplaneta* (Wheeler, 1962, 1963). In addition,

Wheeler (1963) found that the absolute number of circulating hemocytes per cubic millimeter, obtained by multiplying the BV and the THC, was relatively constant, notwithstanding changes in both the BV and the THC.

In a few instances, BVs were determined by weighing insects, removing as much hemolymph as possible, and reweighing the insects (Richardson et al., 1931; Arnold and Hinks, 1976). This exsanguination method appears crude, as more sophisticated methods are available. Smith (1938) employed the cell dilution method of Yeager and Tauber (1932). The following formula was used:

$$V_o = \frac{d \cdot c_1}{co - c_1} + a;$$

where V_o = total blood volume; d = amount of dilution fluid in cubic millimeters; co = original cell count per cubic millimeter; c_1 = diluted blood cell count per cubic millimeter; and a = volume of blood drawn in making the original count.

A dye solution method (Yeager and Munson, 1950) was used for *Sarcophaga* (Jones, 1956), *Tenebrio* (Jones, 1957), and *Galleria* (Shapiro, 1966; Jones, 1967a). An 0.2% amaranth red dye in 0.85% NaCl was injected into *Sarcophaga* larvae and pupae, 5% of body weight (Jones, 1956). *Galleria* larvae were injected with 10 μl of 1% amaranth red in saline per gram body weight. The dye was allowed to circulate within the hemocoel for 3–5 min. Then hemolymph was drawn, and the intensity of color was compared to a series of standards. Hemolymph volume percent were converted into microliters (Jones, 1967a).

Shapiro (1966) investigated the BV of *Galleria* larvae. The dye method employed was essentially that used by Yeager and Munson (1950) and modified by Lee (1961) with further modifications by Martignoni and Milstead (pers. comm.). Each larva was weighed and injected with a 1% aqueous amaranth solution. The volume injected was equal to 5% of the insect's body weight. After the amaranth was injected, the larva was placed in a shell vial (1.5 × 6.4 cm), and the dye was allowed to circulate within the hemocoel. After 10 min, a proleg on the sixth abdominal segment was cut, and the blood was collected in a capillary tube (Kimax No. 34500) that had been flooded with pure nitrogen to retard melanization. The blunt end of the tube was sealed on an alcohol burner, and the tube was refrigerated (4 °C) for several minutes and centrifuged (3,100 rpm for 10 min). The tube was recooled, and the portion of the tube containing sedimented hemocytes was cut off and discarded. The plasma was drawn into a disposable Drummond micropipette (10-μl capacity) and diluted in 1 ml Aronsson's buffer (0.995 M). This solution and the standard (Aronsson's buffer) were placed in separate 1.0-ml cuvettes (10-mm light path) and

the relative absorbances were determined with a Beckman DB spectrophotometer at 515 mm, the wavelength at which the maximum absorbance of amaranth occurred.

Previously, the absorbances of known concentrations of amaranth had been determined, and, in accordance with Beer's law, the absorbance was proportional to the concentration. The absorbance value of the test sample was plotted against the concentrations of known samples, and the concentration of the test sample was thus obtained. Once the concentration of amaranth in the test sample had been determined, the blood volume was calculated by the following formula:

$$V = \frac{d(c' - c'')1}{(c'')}$$

where V = blood volume in microliters; d = volume of dye injected in microliters; c' = original concentration in percent; c'' = concentration of dye after circulation in percent. In order to obtain the blood volume, V is divided by the body weight of the larva.

19.5. Reliability as influenced by internal and external factors

Reliability of data is influenced by two factors, acting separately or in concert: (1) internal factors, which are related to real differences between insects, and/or (2) external factors, caused by problems in techniques. These two areas will be discussed and assessed.

19.5.1. Internal factors

Some of the variations between similar insects have been shown to be related to real factors in physiology (Arnold, 1974). Patton and Flint (1959) observed that blood cell counts of *Periplaneta* varied significantly intraspecifically and with the stage of development. The mean cell count was 50,400/mm³ with a range of 21,000–99,000 and a standard deviation of 21,000. The authors concluded that a norm must be established for each insect before the THC can be employed as a measure of the physiological state. Collin (1963) showed that the variability of the total count in *Melolontha* was about 30%. He felt that the great variability in the relative proportions of hemocytes (DHC) made it difficult to use that measure as an index of the physiological or pathological state of the insect. Jones (1967b) found the great variability of unfixed DHCs of *Rhodnius* was not attributable to great variations between sequential 100-cell counts per sample or to great differences between the sexes.

Although total counts from insects were variable, the range obtained was "quite comparable to the range obtained by other investigators from mammals" (Tauber and Yeager, 1935). Yeager (1945)

tested the reliability of the DHC by examining smears from 100 insects and determining the larval stages of the donors. Some 76% of the smears were identified correctly as to larval stage. Moreover, 82% of these positives were identified correctly to within 48 hr of the true age. In *Galleria*, the ratio of round to fusiform PLs could be utilized as an index of larval weight of insects within the rearing colony (Shapiro, 1966).

Insects used for hematological studies should be as uniform as possible; of the same stage, instar, age, size (Wittig, 1966), and possibly sex. In last-stage armyworms, even among an apparently homogeneous group, individual THCs ranged from 75% to 140% of the mean of the group. The mean of a treated group is related to the average count from a group of untreated insects not only at a given time, but also during a particular time frame in insect development, when cell population changes may occur (Wittig, 1966).

Because of inherent variations, replications must be made. In *Pseudaletia*, more replications of THCs were required than of DHCs, because of greater variabilities in the former. Severe treatments, which might lead to overt disease or death, would be expected to result in hematological changes (Wittig, 1966). Jones and Tauber (1954) noted that normal or high THCs among mealworms had little significance, but greatly reduced THCs accompanied by abnormal hemocytes signified morbidity and eventual mortality. Rosenberger and Jones (1960) believed that the THC in *Prodenia* was not a good indication of health, as normal counts were obtained in both healthy and unhealthy insects. When an insect can recover from a given treatment or condition, however, changes in the blood picture will probably be slight and well within the range of variations found among untreated insects (Wittig, 1966).

19.5.2. External factors

In general, the external factors may be (1) inherent problems of the method itself and/or (2) failure of the investigator to utilize a given method correctly. It is quite possible that two methods, both used to obtain the same data for a given parameter, may have differences in sensitivity. For example, exsanguination of insects for BV measurements is less precise than the dye dilution method. Heat fixation would be useless for studying coagulation in insect hemolymph, as coagulocytes can be recognized only in unfixed preparations. The choice of a given method is dependent upon the types of data required. Differences in total counts between heat-fixed and unfixed hemolymph from stoneflies were felt to be attributable primarily to technical difficulties (Arnold, 1966).

When a given technique is selected, it must be followed precisely.

Even small differences in sampling and counting procedures could lead to large differences in the data produced. The "technique should not be varied in its details during a test, unless it has been established that such changes do not affect the result" (Wittig, 1966). Because it is often difficult to obtain consistent results owing to variations within the insect population, it becomes important not to add more variability to the system. It would be beneficial to the field of insect hematology if workers using the same species of test insect would use the same techniques, so that variations attributable to different techniques could be minimized.

19.6. Summary

Techniques are available to investigate changes in hemocyte populations regarding cell numbers (THCs), cell types (DHCs), and blood volumes. Moreover, these parameters can be combined to determine the absolute numbers of hemocytes within an insect at a given time or through time. Unfortunately, many studies have involved a single parameter. The reliability of these techniques must be critically evaluated so that only the best available ones are used in hematological studies. We must answer the question: Does heat fixation preserve the hemocyte population in situ or does it artifactually cause large numbers of hemocytes to enter the circulation. Perhaps, each method should be defined as to its best usage, so that a mosaic of techniques might be utilized to best advantage.

Once a method is selected for use, it must be followed precisely. Variations in a method might produce variations in results. Assuming that a given method is carried out well, more attention must be given to the test insect. The population should be defined as to age, stage, size, and sex in order to minimize inherent variations. If periodicity is a problem as far as reproducibility of data is concerned, then tests must be carried out at the same time. Is periodicity of hemocyte populations a problem? The growth and development of the insects should be optimal under laboratory conditions. The use of semisynthetic artificial diets and improved rearing techniques should improve the synchrony of growth and minimize the variability of food materials.

References

Arnold, J. W. 1952a. The haemocytes of the Mediterranean flour moth, *Ephestia kühniella* Zell. (Lepidoptera: Pyralididae). *Can. J. Zool.* 30:352–64.

Arnold, J. W. 1952b. Effects of certain fumigants on haemocytes of the Mediterranean flour moth, *Ephestia kühniella* Zell. (Lepidoptera: Pyralididae). *Can. J. Zool.* 30:365–74.

Arnold, J. W. 1966. An interpretation of the haemocyte complex in a stonefly, *Acroneuria arenosa* (Plecoptera: Perlidae). *Can. Entomol.* 98:394–411.

Arnold, J. W. 1969. Periodicity in the proportion of haemocyte categories in the giant cockroach, *Blaberus giganteus*. *Can. Entomol.* 101:68–77.

Arnold, J. W. 1974. The hemocytes of insects. *In* M. Rockstein (ed.). *The Physiology of Insecta*, 2nd ed. Academic Press, New York.

Arnold, J. W., and C. F. Hinks. 1976. Haemopoiesis in Lepidoptera. I. The multiplication of circulating haemocytes. *Can. J. Zool.* 54:1003–12.

Clark, E. W., and D. S. Chadbourne. 1960. The haemocytes of nondiapause and diapause larvae and pupae of the pink bollworm. *Ann. Entomol. Soc. Amer.* 53:682–5.

Collin, N. 1963. Les Hémocytes de la larve de *Melolontha melolontha* L. (Coleoptère Scarabaeidae). *Rev. Pathol. Veg. Entomol. Agric. Fr.* 42:161–7.

Fisher, R. A. 1935. The effect of acetic acid vapor treatment on the blood cell count in the cockroach, *Blatta orientalis* L. *Ann. Entomol. Soc. Amer.* 28:146–53.

Gupta, A. P., and D. J. Sutherland. 1968. Effects of sublethal doses of chlordane on the hemocytes and midgut epithelium of *Periplaneta americana*. *Ann. Entomol. Soc. Amer.* 61:910–18.

Jones, J. C. 1956. The hemocytes of *Sarcophaga bullata* Parker. *J. Morphol.* 99:233–57.

Jones, J. C. 1957. DDT and the hemocyte picture of the mealworm, *Tenebrio molitor* L. *J. Cell. Comp. Physiol.* 50:423–8.

Jones, J. C. 1959. A phase contrast study of the blood cells in *Prodenia* larvae (Order Lepidoptera). *Q. J. Microsc. Sci.* 100:17–23.

Jones, J. C. 1962. Current concepts concerning insect hemocytes. *Amer. Zool.* 2:209–46.

Jones, J. C. 1967a. Changes in the hemocyte picture of *Galleria mellonella* (Linnaeus). *Biol. Bull. (Woods Hole)* 132:211–21.

Jones, J. C. 1967b. Normal differential counts of haemocytes in relation to ecdysis and feeding in *Rhodnius*. *J. Insect Physiol.* 13:1133–41.

Jones, J. C., and O. E. Tauber. 1954. Abnormal hemocytes in mealworms (*Tenebrio molitor* L.). *Ann. Entomol. Soc. Amer.* 47:428–44.

Kitano, H. 1969. On the total hemocyte counts of the larva of the common cabbage butterfly, *Pieris rapae crucivora* Boisduval (Lepidoptera: Pieridae) with reference to the parasitization of *Apanteles glomeratus* L. (Hymenoptera: Braconidae). *Kontyû* 37:320–6.

Lee, R. M. 1961. The variation of blood volume with age in the desert locust (*Schistocerca gregaria* Forsk.). *J. Insect Physiol.* 6:36–51.

Matsumoto, T., and M. Sakurai. 1956. On the density of haemocytes in the blood bled from a wound in *Bombyx mori* L., *J. Seric. Sci. Jpn.* 25:147–8. (In Japanese, English summary.)

Nappi, A. J., and J. G. Stoffolano, Jr. 1972. Haemocytic changes associated with the immune reaction of hematode-infected larvae of *Orthellia caesarion*. *Parasitology* 65:295–302.

Nappi, A. J., and F. A. Streams. 1969. Haemocyte reactions of *Drosophila melanogaster* to the parasites *Pseudocoila mellipes* and *P. bochei*. *J. Insect Physiol.* 15:1551–66.

Nittono, Y. 1960. Studies on the blood cells in the silkworm, *Bombyx mori* L. *Bull Seric. Exp. Stn. (Tokyo)* 16:171–266.

Pappenheim, A. 1914. *Clinical Examination of the Blood and Its Technique*. Wright, Bristol.

Patton, R. L., and R. A. Flint. 1959. The variation in the blood cell count of *Periplaneta americana* (L.) during a molt. *Ann. Entomol. Soc. Amer.* 52:240–2.

Richardson, C. H., R. C. Burdette, and C. W. Eagleson. 1931. The determination of the blood volume of insect larvae. *Ann. Entomol. Soc. Amer.* 24:503–7.

Rosenberger, C. R., and J. C. Jones. 1960. Studies on total blood counts of the southern armyworm larva, *Prodenia eridania* (Lepidoptera). *Ann. Entomol. Soc. Amer.* 53:351–5.

Shapiro, M. 1966. Pathologic changes in the blood of the greater wax moth. *Galleria mellonella* (Linnaeus), during the course of starvation and nucleopolyhedrosis. Ph.D. thesis, University of California. Berkeley, California.

Shapiro, M. 1967. Pathologic changes in the blood of the greater wax moth, *Galleria mellonella*, during the course of nucleopolyhedrosis and starvation. I. Total hemocyte count. *J. Invertebr. Pathol.* 9:111–13.

Shapiro, M. 1968. Pathologic changes in the blood of the greater wax moth. *Galleria mellonella*, during nucleopolyhedrosis and starvation. II. Differential hemocyte count. *J. Invertebr. Pathol.* 10:230–4.

Shapiro, M., R. D. Stock, and C. M. Ignoffo. 1969. Hemocyte changes in larvae of the bollworm, *Heliothis zea*, infected with a nucleopolyhedrosis virus. *J. Invertebr. Pathol.* 14:28–30.

Smith, H. W. 1938. The blood of the cockroach *Periplaneta americana* L.: Cell structure and degeneration, and cell counts. *New Hampshire Agric. Exp. Stn. Tech. Bull.* 71:1–23.

Stephens, J. M. 1963. Effects of active immunization on total hemocyte counts of larvae of *Galleria mellonella* (Linnaeus). *J. Insect Pathol.* 5:152–6.

Tauber, O. E., and J. F. Yeager. 1934. On the total blood (hemolymph) cell count of the field cricket, *Gryllus assimilis pennsylvanicus* Burm. *Iowa State Coll. J. Sci.* 9:13–24.

Tauber, O. E. and J. F. Yeager. 1935. On total hemolymph (blood) counts of insects. I. Orthoptera, Odonata, Hemiptera, and Homoptera. *Ann. Entomol. Soc. Amer.* 28:229–40.

Tauber, O. E., and J. F. Yeager. 1936. On the total hemolymph (blood) all counts of insects. II. Neuroptera, Coleoptera, Lepidoptera, and Hymenoptera. *Ann. Entomol. Soc. Amer.* 29:112–18.

Vinson, S. B. 1971. Defense reaction and hemocytic changes in *Heliothis virescens* in response to its habitual parasitoid *Cardiochiles nigriceps*. *J. Invertebr. Pathol.* 18:94–100.

Webley, D. P. 1951. Blood cell counts in the African migratory locust (*Locusta migratoria migratorioides* Reiche and Fairmaire). *Proc. R. Entomol. Soc. (Lond.)* A26:25–37.

Wheeler, R. E. 1962. Changes in hemolymph volume during the moulting cycle of *Periplaneta americana* L. (Orthoptera). *Fed. Proc.* 21:123.

Wheeler, R. E. 1963. Studies on total hemocyte count and hemolymph volume in *Periplaneta americana* (L.) with special reference to the last moulting cycle. *J. Insect Physiol.* 9:223–35.

Wittig, G. 1966. Phagocytosis by blood cells in healthy and diseased caterpillars. II. A consideration of the method of making hemocyte counts. *J. Invertebr. Pathol.* 8:461–77.

Yeager, J. F. 1945. The blood picture of the southern armyworm (*Prodenia eridania*). *J. Agric. Res.* 71:1–40.

Yeager, J. F., and S. C. Munson. 1950. Blood volume of the roach *Periplaneta americana* determined by several methods. *Arthropoda* 1:255–65.

Yeager, J. F., and O. E. Tauber. 1932. Determinations of total blood volume in the cockroach, *P. fuliginosa*, with special reference to method. *Ann. Entomol. Soc. Amer.* 25:315–27.