A MORPHOLOGICAL STUDY AND MICROSPECTROPHOTOMETRIC ANALYSIS OF THE DNA CONTENT OF HEMOCYTES IN *Periplaneta americana* (L.)

by

Mark G. Novak

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Paul C Peterson 12/30/85
Advisor /Date

Sally M. Hotchkiss January a. 1986
Dean of the Graduate School Date

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ABSTRACT

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The DNA content of hemocytes from the American cockroach, Periplaneta americana (L.) was analyzed using Feulgen staining and the two wavelength method of microspectrophotometry. Results of this study provided evidence that polyploidy does not occur in the hemocytes of either the nymph or adult stage. A low degree of mitotic activity was indicated, and mitosis was not limited to the germinal prohemocytes. Observation of the hemocytes with light and scanning electron microscopy indicated that morphological variability is primarily dependent upon the fixation procedures used.

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INTRODUCTION

The extracellular fluid of insects functions as both a circulatory and bathing medium which is analogous to the blood and lymph of vertebrates. It is for this reason that the fluid is given the name hemolymph. The cellular components found in the hemolymph are collectively known as hemocytes and have been studied extensively since the time they were first discovered by Schwammerdam in 1758. He noted that when dissecting the abdomen of a louse, blood flowed containing "transparent globules". Since that early discovery, many physiologists, cell biologists, embryologists, and other researchers have studied insect hemocytes in detail. In spite of these extensive studies, many fundamental questions remain concerning hemocyte function and structure.

A number of functions have been attributed to insect hemocytes. Perhaps the single most important function is that of phagocytosis. Many hemocytes reportedly act in cleansing the circulatory fluid of bacteria and other microorganisms. Additionally, these cells can engulf fragments of histolyzing cells that are often present during moulting and before metamorphosis (Wigglesworth, 1972).

A second function which has been attributed to hemocytes is that of encapsulation. Two or more hemocytes may form a capsule around foreign objects too large to be phagocytized (i.e., metazoan parasites, hymenopterous parasites, etc.), effectively cutting off their food and/or oxygen supply. This function of encapsulation was experimentally induced by Grimstone et al. (1967) by inserting foreign bodies such as fragments of polymerized Araldite or parasite eggs into the insects. They observed

in larvae of the flour moth Ephestia that hemocytes clustered around foreign implants. Hemocytes were piled upon one another and became extremely flattened, forming a compact isolating tissue.

Similar accumulations of hemocytes have been reported in association with wound healing. Hemocytes are often found to aggregate at a site of injury and may form a plug which helps to seal a wound. Agglutination of these hemocytes occurs through the extension of thin strands of cytoplasmic material from one cell to another forming a net-like structure (Bursell, 1970).

Specific hemocytes may enhance wound healing by inducing coagulation of the surrounding hemolymph. This is believed to occur by the release of one or more clotting factors. Several researchers have classified these cells as coagulocytes, but other types of hemocytes have also been implicated in hemolymph coagulation. For example, Rowley and Ratcliffe (1976) reported that the granular cells of Galleria mellonella (L.) trigger the mechanism of hemolymph coagulation by releasing granules believed to contain acid mucopolysaccharides. Comprehensive studies have suggested that the ability of hemocytes to initiate or aid in coagulation depends on the insect species involved (Bursell, 1970).

Finally, hemocytes appear to play a role in the intermediary metabolism of insects, both by transporting nutrients throughout the body and by producing metabolic components such as hemolymph proteins (Bursell, 1970). Hemocytes are found to include a variety of stored materials including glycogen, neutral and acid mucopolysaccharides, phospholipids and proteins.

Morphologically, the overall shape of insect hemocytes is highly

variable. Cells that are flattened, discoid, fusiform, spherical, or amoeboid represent the majority of forms that have been described. Others are described as having a lobulate appearance with irregular margins. The size of hemocytes also varies greatly within and among insect species. Most cells range in size from three to forty-five microns with an outer plasma membrane that is roughly contoured. Most often there are small finger-like projections (filopodia, micropapillae) or lamellar extensions (lamellopodia), or other irregular processes. Indentations of the cell surface are usually confined to small infoldings and pinocytic vesicles of various kinds (Crossley, 1975).

All insect hemocytes are nucleated. The size and shape of the nuclei are rather consistent and are often used as an aid in the identification of various cell types. Binucleate and multinucleate cells do occur but are rare and do not represent any particular cell type (Bursell, 1970).

Hemocytes possess the typical eukaryotic organelles and are especially rich in mitochondria and ribosomes. Most cells are well vacuolated and possess phagosomes, lysosomes, and residual bodies of various kinds indicative of their role as phagocytes (Crossley, 1975).

One of the most basic controversies concerning insect hematology is that of classification. Many attempts to classify the various hemocyte forms have been made. These classification systems have been based on many different parameters including morphology, function, staining, and histochemical reactions. Results of these procedures have led to naming various forms of hemocytes, thereby creating many synonyms which result in a confusing terminology (Gupta, 1979a).

The earliest significant classification was proposed by Cuenot

(1896), who based his system on observations of hemocytes in several orthopteroid groups including the American roach, Periplaneta americana (L.). Cuenot determined that there was only one kind of blood cell which passed through various stages during its life. Each of these stages was identified morphologically and thought to carry out particular functions. He suggested that a hemocyte was divided into four developmental stages. Stage one was composed of small germinal elements that divided mitotically and supposedly developed directly into stage two cells. Stage two cells were phagocytic and divided mitotically. As these cells matured they enclosed acidophilic granules, became nonphagocytic, and were known as stage three cells. Cells of stages two and three degenerated gradually into stage four cells which were phagocytized and digested by young phagocytes.

Following Cuenot's publication, several other researchers, dissatisfied with Cuenot's explanation, published their own classifications. One of the most notable was Hollande (1909), who, in contrast to Cuenot, suggested the presence of several independently derived hemocyte types. Wigglesworth (1959) summarized most of the early classifications and outlined a system that was generally accepted. Studies carried out by Yeager (1945) further stimulated interest in hemocytes.

On the basis of his own observations and previous classifications, Jones (1962) distinguished nine types of hemocytes. He noted that "most workers have long agreed that insects have at least three, fairly well defined, morphologically distinct types of hemocytes": (1) prohemocytes, (2) plasmatocytes, and (3) granular hemocytes. In addition, Jones

described six other hemocyte types that "have long been recognized (e.g., Graber, 1871; Cuenot, 1896) as occurring in certain insects." Since 1962, the generally acceptable classification of insect hemocytes has been based largely on morphological characters adapted from Jones' (1962) classification.

Price and Ratcliffe (1974) published a classification scheme based on the study of fifteen insect orders. Their classification recognized the existence of a single hemocyte type and considered the others as developmental stages. The authors noted that, "due to considerable overlap in structure and the presence of numerous intermediates, the six cell types may represent different developmental and/or functional stages of one basic cell type." Specialized cells such as podocytes and vermiform cells (Jones, 1962) were considered to be variations of plasmatocytes. Additionally, adipohemocytes were regarded as free floating fat body cells.

The latest comprehensive classification scheme to be presented is that of Gupta (1979a), principally an updated revision of Jones' (1962) classification scheme. According to Gupta, only seven hemocyte types have thus far been differentiated based on ultrastructure. Like Price and Ratcliffe, Gupta regarded podocytes and vermiform cells as variations of plasmatocytes. Gupta (1979a) also discussed synonymies which have occurred. This is especially useful since the result of the previous classifications "has been the identification of over seventy different haemocyte types" (Price and Ratcliffe, 1974). A summary of the classifications presented by Jones (1962), Price and Ratcliffe (1974), and Gupta (1979a) appears in Table 1. In addition, a compilation of the

synonymies by Gupta (1979a) is presented in Table 2. These have been included to illustrate both the variability of observed hemocyte types and type descriptions.

A fundamental problem in hemocyte classification has been the inability to determine the interrelationships between the various cell types. Are they separate cell lines or are they merely different phases of one basic cell type? To examine this question, it is neccessary to understand the mechanisms of hemocyte formation.

Embryonically, hemocyte formation is thought to occur according to the median mesoderm theory. Specifically, embryonic hemocytes are formed by proliferation from mesoderm into the epineural sinus which later develops into the insect hemocoel. Postembryonically, hemocytes are formed by either hemopoietic organs, mitosis of one or more of the circulating hemocyte types, or from noncirculating accumulations of hemocytes in various parts of the hemocoel (Jones, 1962).

The presence of hemopoietic organs has been reported by several workers. Hoffman et al. (1969) reported on hemopoietic organs in Gryllus bimaculatus Degreer, and Locusta miaratoria (L.). These organs, formerly regarded as phagocytic organs, are located in the abdomen near the dorsal vessel. In Gryllus, the organ is composed of a cortical zone with reticular cell islets and a medulla which contains an accumulation of free, differentiated hemocytes. Hoffman et al. (1969) reported that hemorrhage results in the proliferation of reticular cells and subsequent hypertrophy of the hemopoietic tissue, indicating that hemocytes are produced to replace lost cells.

Nittono et al. (1964) showed that bilateral lobulation of imaginal wing

TABLE 1

Comparison of hemocyte classifications
and descriptions from Jones(19621,
Price and Ratcliffe (19741, and Gupta(1979a).

Table 1

Prohemocyte

Jones (1962)- small, pale grey, often intensely basophilic, stem cells (Yeager, 1945).

Price and Ratcliffe (1974)- small, round to oval cells (6-13um diameter), finely granular cytoplasm, nucleus 70-80%of cell volume.

Gupta (1979)- small, round, oval or elliptical cells, variable sizes (6-10um X 6-14), largenucleus almost fills cell (3-6um X 12um), homogeneous, intensely basophilic cytoplasm.

Plasmatocyte

Jones (1962)- basophilic, polymorphic cells, may send out many pseudopodia or round up in vitro.

Price and Ratcliffe (1974)- round, oval or spindle shaped cells (10-15um diameter), nucleus about 40%of cell volume, finely granular cytoplasm, cells spread out and form pseudopodia in vitro.

Gupta (1979)- small to large polymorphic cells(3.3-5um X 3.3-40um) may have irregular processes, nucleus variable in shape and size (3-9um X 4-10um), cytoplasm is abundant, granular or agranular, basophilic.

Granulocyte or Granular cell

Jones (1962)- many distinct, usually round, colorless, acidophilic granules (Hollande 1909; Jones, 1956,1959).

Price and Ratcliffe (1974)- round or oval cells (10-17um diameter); central nucleus 40-60%of cell volume, cell packed with granules, similar in size to those found in plasmatocytes.

Gupta (1979)- small to large, spherical or oval cells (10-45um X 4-32um), nucleus relatively small, round or elongate (2-8um X 2-7um), cytoplasm characteristically granular

Table 1 Continued

Cystocyte or Coagulocyte

Jones (1962)- highly unstable cells, rapidly turn into brightly hyaline forms, eject material into plasma and tend to disintegrate.

Price and Ratcliffe (1974)- round cells (9-14um diameter), central nucleus 70-75% of cell volume, release of cytoplasm forms "isles of coagulation".

Gupta (1979)- small to large cells (3-30um) spherical, hyaline, fragile, unstable, nucleus relatively small (5-11um), combines features of granulocytes and oenocytes, debatable whether different from granulocytes.

Spherule cell

Jones (1962)- round or ovoid cells with very large, distinct, generally acidophilic, colorless or pale yellow inclusions. Cells are capable of breaking down in vitro into intensely hyaline forms, can eject materials into plasma.

Price and Ratcliffe (1974)- spindle shaped or oval cells (8-16um diameter) filled with large granules or spherules (1.5-2.0um), inclusions either yellowish white or dark grey.

Gupta (1979)- ovoid or round cells (9-25 x 5-10um), nucleus generally small (5-9 x 2.5-6um) and usually obscured by intracytoplasmic spherules (1.5-Sum), spherules contain neutral and acid mucopolysaccharides and glycomucoproteins.

Oenocytoid

Jones (1962)- usually large, thick basophilic hemocytes with either elaborate canaliculi or complexly folded fine granular strands (Hollande, 1911) or with distinct plate-like, rod-1ike, or needle crystals (Cuenot, 1891).

Price and Ratcliffe (1974)- relatively large cells (up to 19um diameter) eccentric nucleus 20-40% of cell volume, cytoplasm encloses only a few granules or globules, may be confused with spherulocyte.

Table 1 Continued

Gupta (1979)- small to large, thick, oval, spherical or elongate cells (16-54um or more) nucleus relatively small (3-15um), cytoplasm homogeneous with several kinds of plate, rod, or needle-like inclusions.

Adipohemocyte

Jones (1962)- small to very large cells with refringent fat droplets and other smaller nonlipid, granular inclusions (Hollande, 1911; Ballard and Jones, 1959)

Price and Ratcliffe (1974)- not a recognized cell type. concurs with Wigglesworth (1956) in considering adipohemocytes as indistinguishable from fat body cells.

Gupta (1979)- small to large, spherical or oval cells (7-45um diameter), nucleus rather small (4-10um), cytoplasm with small to large refringent fat droplets (0.5-15um) and other non lipid granules (0.5-gum), difficult to distinguish from granulocytes.

Podocyte

Jones (1962)- very large, extremely flattened plasmatocyte-like cells with 3-8 or more long, rigid cytoplasmic extensions.

Price and Ratcliffe (1974)- regarded as a variant form of plasmatocyte.

Gupta (1979)- regarded as a variant form of plasmatocyte.

Vermiform cell

Jones (1962)- exceedingly long thread-like cells, resemble extremely elongated plasmatocyte.

Price and Ratcliffe (1974)- regarded as a variant form of plasmatocyte.

Gupta (1979)- regarded as a variant form of plasmatocyte.

TABLE *2*

List of hemocyte types

and their synonomies

adapted from Gupta (1979a)

TABLE 2

CELL TYPE	SYNONYMIES
Prohemocyte	jeune globule (Bruntz, 1908); proleucocyte (Hollande, 1911); macronucleocyte (Paillot, 1919); formative cell (Muller, 1925); smooth-contour chromophilic cell (Yeager, 1945); proleucocytoid (Yeager, 1945); jeuneleucocyte (Millera, 1947); prohaemocyte (Arnold, 1952); prohemocytoid (Jones and Tauber, 1954); plasmatocyte-like cell (Jones, 1959); young plasmatocyte (Gupta and Sutherland, 1966); young granulocyte (Francois, 1974)
Plasmatocyte	leucocyte (Kollman, 1908; Metalnikov, 1908); micronucleocyte (Paillot, 1919); plasmatocyte (Yeager and Munson, 1941); nematocyte (Rizki, 1953); vermiform cell (Lea and Gilbert, 1966); amoebocyte, lamellocyte, lymphocyte, phagocyte (many authors)
Granulocyte	amoebocyte (Cuenot, 1896); granular cell (Jones, 1946); adipohemocyte (Shrivastava and Richards, 1965; Lea and Gilbert, 1966); pycnoleucocyte (Wille and Vecchi, 1966); cystocyte (Devauchelle, 1971); spherule cell (Francois, 1975); hyaline cell, phagocyte (many authors)
Sperulocyte	spherule cell (Hollande, 1909); cellules spheruleuses (Paillot, 1919); spherocyte (Bogojavlensky, 1932); eruptive cell (Yeager, 1945); oenocytoid (Dennell, 1947); rhegmatocyte (Hrdy, 1957); hyaline cell (Whitten, 1964)
Oenocytoid	oenocytoid (Poyarkoff, 1910); Hollande 1911); cerodecyte (Hollande, 1914); non-granular spindle cell, non-phagocytic giant hemocyte (Wigglesworth, 1933, 1935); oenocyte-like cell (Yeager, 1945); crystal cell (Rizki, 1952); crystalloid and dark hyaline hemocyte (Selman, 1962); coagulocyte (Hoffman and Stoekel, 1968)
Coagulocyte	cystocyte (Yeager, 1945); coagulocyte (Gregoire, Florkin, 1950); unstable hyaline hemocyte (Gregoire, Florkin, 1950); thrombocytoid (Zachary and Hoffman, 1973)

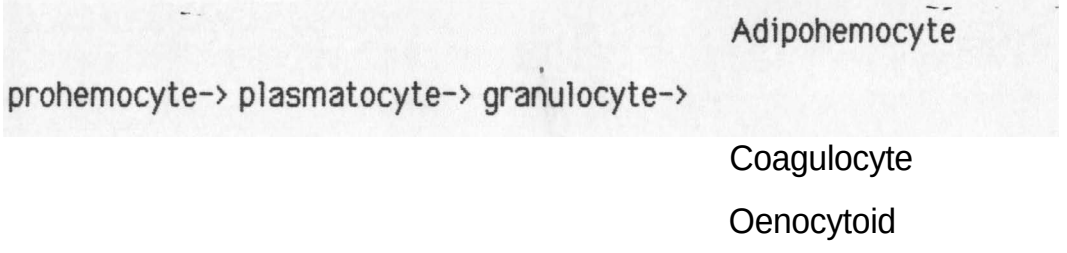
discs and surrounding tissue in Bombyx mori (L.) substantially reduced the number of circulating hemocytes. Hinks and Arnold (19771 found hemopoietic organs in association with wing discs in larvae of fifteen species of Lepidoptera. The organs contain small stem cells organized into islets which, according to the authors, generate either oenocytoids or prohemocytes and plasmatocytes. Akai and Sato (19711, using electron microscopy, revealed that several types of hemocytes were released from the hemopoietic tissues.

Mitotic activity of circulating hemocytes has been widely reported, but whether they multiply in sufficient numbers to account for observed increases or even maintain normal cell numbers is disputed. Generally, the normal mitotic index in most insects is less than one percent (Shapiro, 1968). Also in dispute are the hemocyte type, or types that are mitotically active. Hinks and Arnold (1977) reported the highest mitotic rates in Lepidoptera are for prohemocytes, granular hemocytes and spherule cells, while plasmatocyte division is rare and oenocytoid replication nonexistent. In contrast, Jones and Liu (1968) reported mitosis only in plasmatocyte-like cells and Jones (1973) emphasized that granular hemocytes never divide in Lepidoptera. Shapiro (1968) reported that prohemocytes, plasmatocytes and granular hemocytes undergo mitosis in Galleria mellonella (L). The highest mitotic activity is most consistently reported for prohemocytes.

In addition to mitotic observations, many workers have reported transitions from one cell type to another. A direct transition from germinal prohemocytes through plasmatocytes to adipohemocytes or granular hemocytes in G. mellonella was observed by Shrivastava and

Richards (1965) using autoradiographic methods. Similar observations have been reported many times in various insect orders (see Ratcliffe and Price, 1974; Landreau and Gillet, 1975) and lead one to ask whether hernocyte classes represent stages in the life of a single kind of cell or exist as stable self-sustaining cell lines within the hemocyte complex?

These conflicting viewpoints have led to the development of two different theories. The single cell theory supported by findings of Moran (1971), Scharrer (1972), Ratcliffe and Price (1974), Crossley (1975) and others posits the presence of many transitional forms of hernocytes that share characteristics of more than one type (Arnold, 1979). Moran's ultramicroscopic study, in particular, suggests considerable overlap in the fine structure of different hemocyte types. Based on experimental evidence, most of these studies suggest a transition from germinal prohemocytes to plasmatocytes to granular hemocytes and then possibly differentiating into the other hemocyte types. This differentiation pathway is represented as shown in the diagram below (from Gupta, 1979b):



In contrast, the multiple cell theory suggests development of several independent hemocyte forms. The most generally accepted variation of

this theory considers prohemocytes as the germinal entity from which all other cell lines differentiate. The differentiated cell lines grow, mature and degenerate without losing their identity (Arnold, 1979). The multiple cell theory derives from four common observations. Prohemocytes have undisputed germinal characteristics, e.g., they are mitotically active, small in size, possess a high nucleus to cytoplasm ratio, the nucleus is rich in chromatin, and the cytoplasm is rich in ribosomes and poor in mitochondria. Second, some cells appear as transitional forms between the germinal and differentiated types. Third, there are usually marked cytological differences separating mature cell types at both the gross and fine structural levels. Finally, with the exception of prohemocytes, young, mature and old cells of each type are often found in the hemolymph at all times. Studies showing distinct fine structural differences between the main types (Akai and Sato, 1973; Lai Fook, 1973; Raina, 1976) further support the multiple cell theory.

Several studies dealing specifically with cockroach hemocytes have been published and have emphasized the classification of hemocyte forms as well as total and differential hemocyte counts. Virtually all of the morphological studies suggest that a number of hemocyte types differentiate from germinal prohemocytes. However, as with other insect studies, there has been little agreement on the types of differentiated cells present or their relative proportions.

In Periplaneta americana (L.), Jones (1957) found mostly plasmatocytes and a few cystocytes in addition to prohemocytes, while Patton and Flint (1959) found only plasmatocytes and prohemocytes. A comprehensive hemocyte study of Dictyoptera: Blattaria, by Arnold (1972)

found the cockroach hemocyte complex to have two immutable categories, plasmatocytes and granulocytes, in addition to germinal prohemocytes. Spherule cells, found in some cockroach species, are believed to be differentiated from granulocytes. Arnold (1972) found no spherule cells in P. americana but calculated the relative abundance of prohemocytes (0.6-2.0%), plasmatocytes (6.0-12.0%), and granular cells (86.0-94.0%). The proportions of differentiated cells differ from other studies but most researchers agree that prohemocytes normally make up a relatively low percentage (0.5-10.0%) of the total hemocyte count. Ratcliffe and Price (1974) found five cell types in P. americana (prohemocytes, plasmatocytes, granulocytes, spherule cells, and cystocytes) but concluded that they may only be developmental stages of one basic cell type. Moran (1971) came to a similar conclusion in his ultramicroscopic study of the hemocytes of the cockroach Blaberus discoidalis Serville.

Considerable variation in total hemocyte counts has also been reported. In studies of P. americana, Smith (1938) reported average total hemocyte counts of 96,000/mm^3 for nymphs and 85,000/mm^3 for adults. Patton and Flint (1959) reported an average total hemocyte count of 50,000/mm^3 in adults. Gupta and Sutherland (1968) estimated the total hemocyte count in adult male P. americana as 18,000/mm^3. Wheeler (1963) found no significant differences in total and differential hemocyte counts between male and female P. americana.

In studies of mitotic rates of cockroach hemocytes, calculations were based on the percentage of mitotic figures found in stained blood films. Yeager and Tauber (1932) reported a rate of about 0.5% in P. fulainosa (L.) and 0.2-1.0% in P. orientalis (L.). Arnold and Salkeld (1967)

reported that cell division in cockroach hemocytes occurs most frequently in prohemocytes.

Attempts to clarify insect hematology have resulted in many discrepancies and ambiguities. One reason is that most of the studies have employed the same "classical" techniques which may not possess the power necessary to resolve many of the inconsistencies. Only a few workers have attempted to use nontraditional means (as far as insect hematology is concered) in their hemocyte studies. Examples include the autoradiographic studies of Shrivastava and Richards (1965), Krishnakumaran et al. (1966), and the microspectrophotometric work of Romer and Eisenbeis (1982). These studies have demonstrated the importance and need of approaching the problems of insect hematology with new techniques.

A primary objective of this study was to examine the DNA content of the hemocytes in the American cockroach, P. americana. Combining a Feulgen staining technique with the two wavelength method of microspectrophotometry developed by Patau (1957) and Ornstein (1957), it is possible to quantify the DNA content of individual cells. With this information it is possible to detect differences in mitotic activity, if any, between the cell types and to determine the presence of ploidy levels. In insects, where polyploid tissues have been found to be widespread, ploidy levels are often indicative of the degree of maturation in a particular cell type. Histograms based on these DNA measurements can provide data about both mitotic activity and ploidy levels. A second objective of this study was to examine the hemocytes of P. americana using light and scanning electron microscopy (SEM) to determine how different

preparation techniques affect hemocyte appearance. In addition, an SEM mapping technique, devised for the positive identification of human leukocytes (Wetzel, Erickson, Levis, 1973) was evaluated for use in the study of insect hemocytes. Since SEM cannot reveal the internal nuclear chromatin patterns and cytoplasmic granules, which are primary characteristics for identification of human leukocytes, Wetzel *et* al. (1973) devised a method that permitted identification by Giemsa staining that preserved the surface structures for SEM study. A similar problem exists in insect hematology and a successful application of this technique would be a valuable tool for recognizing hemocyte types with SEM.

MATERIALS AND METHODS

All hemocytes used in this study were acquired from adults and nymphs of the American roach, Periplaneta americana (L.). Adults were obtained from Carolina Biological Supply Company, Burlington, North Carolina. Nymphs were reared in the laboratory. All roaches were maintained at 25–29" C. and fed a diet of rat chow pellets, apples and water.

Before hemolymph was drawn, the animals were anesthetized with CO_2. Blood samples from adults were taken by amputation of a hind limb at the trochanter. Due to the nymphs' small size, two or more leg amputations were occasionally required for a sufficient hemolymph sample.

Feulgen staining and Cytochemical Methods

Cells prepared for microspectrophotometry were stained using the Feulgen nuclear reaction for specific isolation of deoxyribonucleic acid (Feulgen and Rosenbach, 1924) modified by Therrein (1966) and Bryant and Howard (1969). The quantitative basis for the Feulgen reaction is due to the stable bonds formed in stoichiometric ratio between the Schiffs reagent and the exposed aldehyde groups of hydrolyzed DNA. The new dye–DNA complex becomes a magenta color in a nonrecolorization reaction. This bond stability allows for the determination of relative DNA content in individual nuclei (Kasten, 1967; Ris and Minky, 1949). The color intensity of the reaction is dependent upon several variables including type of

fixation, hydrolysis time, pH, manufacturer of stain, time of exposure to the Schiffs reagent and thickness of cells (Olkowski, 1976). In this study, all of these factors were held as constant as possible.

Prior to staining, it was necessary to determine the optimum hydrolysis time that would yield the greatest stain intensity. A hydrolysis curve was plotted by running slides through the staining procedure while varying the hydrolysis time of each slide. Hydrolysis time intervals were 5, 15, 25, 35, 45, 65, and 90 minutes. 5N hydrochloric acid was used as the hydrolytic agent in place of the more convential hydrolysis in 1N hydrochloric acid at 60° C. in order to prolong the period of maximal Feulgen intensity (Itikawa and Ogura, 1953; Jordanov, 1963; DeCosse and Aiello, 1966).

The procedure for fixing and staining the cells was as follows:

1) Following amputation, the hemolymph was immediately mixed with 10% buffered formalin (pH=7.0) on labeled, albumenized slides.
2) The hemolymph smears were fixed in the 10% buffered formalin for 18 hours.
3) After fixation, the smears were washed twice in 70% ethanol.
4) The smears were then fixed in 70% ethanol for 12 hours.
5) The smears were rinsed in distilled water, prior to and after being hydrolyzed in 5N HCL for 30 minutes at room temperature.,
6) The hydrolyzed cells were stained one hour in freshly prepared Schiff's reagent (Harleco Fuchsin Basic E, Pure Dye Content 99%, Lot 61136), (see Table 3).
7) The smears were rinsed twice for 5 minutes each in a sulfurous

acid solution (see Table 3).

8) The smears were rinsed in distilled water and then dehydrated through a graded ethanol series (50, 75, 95, and 100%).

9) Finally, the smears were cleared in xylene and mounted in permount.

Cytophotometric Determinations:

All cytophotometric determinations were made using a Zeiss Type 01 microspectrophotometer with a Planachromat oil immersion objective, N.A. 1.30 x 100 at an optovar setting of 1.25x, and a halogen light source. A continuous interference filter monochromator was used for isolation of desired wavelength. Instrument alignment and linearity of the phototube were checked prior to use of the instrument.

The two wavelength method of microspectrophotometry was employed (Patau, 1952; Ornstein, 1952; Mendelsohn, 1961) since it provides accurate measurements of material too irregular (high distribution error) to give valid results with conventional methods (i.e., not conforming to the Beer-Lambert law). Simply stated, the shape of the object and the dye distribution have little effect on the accuracy of the measurements.

To choose the wavelengths, an absorption curve was established using a homogeneous area of a randomly selected cell from the population to be measured. Extinction coefficients ($E = \log I_0/I_S$; where, I_0=reading of background light, and I_S = light passing through the specimen) were plotted against the corresponding wavelengths. The two wavelengths were chosen so that $2E_1 = E_2$ where E_2 is the maximum extinction value. A ratio

TABLE 3

Staining Reagents

Table 3

STAINING REAGENTS

Schiff's reagent

Add 5.0g Basic Fuchsin and 11.0g K-metabisulfite to 500ml of 0.2N HCL. Stir for 2 hours and let stand overnight in a dark place. Add 1g activated charcoal (Norit A) for each 500ml of stain. Filter until solution is clear. Store refrigerated in a dark bottle. The stain is stable for approximately two weeks.

10%K-metabisulfite ($K_2S_2O_5$)

Place 10g K-metabisulfite in a flask and bring to a volume of 100ml with distilled water.

Sulfurous acid rinse

Add 10ml of 10%K-metabisulfite solution and 10ml of 1N HCL to 200ml of distilled water.

Giemsa stain (stock solution)

Add 1g Giemsa powder to 66.0 ml of glycerol and mix. Place in a 60° C. oven for 2 hours. Add 66.0 ml of methyl alcohol (acetone free) and stopper tightly.

Giemsa stain (working solution)

Add 2 ml of buffer (pH = 7.0) to every 1 ml of stock solution used.

different from 2:1 involves a mathematically more complicated procedur (Patau, 1952).

For each cell, four microspectrophotometer readings were made: I_{01} = background light at $\lambda 1$, I_{S1} = light through the specimen at $\lambda 1$, I_{02} = background light at $\lambda 2$, I_{S2} = light through the specimen at $\lambda 2$. From these measurements, nuclei transmissions (T) were calculated for each wavelength ($T_1 = I_{S1}/I_{01}$, $T_2 = I_{S2}/I_{02}$). These were transformed to $L_1 = 1 - T_1$ and $L_2 = 1 - T_2$ for determination of chrornophore (absorbing material) content (M) in a defined area (A) using the equation, $M = KAL_1C$. The absorptivity constant k was omitted since relative not absolute values were calculated. The correction factor (C) was determined by $C = (2 - Q)^{-1} \ln(Q - 1)^{-1}$ where $Q = L_2/L_1$. This factor eliminates influence of any unoccupied portions of the measured area and thus allows valid determinations of the amount of absorbing chromophores in irregular or heterogeneous nuclei.

For accuracy, all relative DNA calculations and statistical analyses were made on the main computer at Youngstown State University using a program supplied by Dr. John J. Yemma.

Five adult and five nymphal american cockroaches, P. americana, were sampled for this study. Two hundred hemocyte nuclei were read from each individual. The hemocyte nuclei were distinguished as prohemocytes or mature differentiated cells (i.e. granulocytes and plasmatocytes) on the basis of nuclear size, staining intensity and nuclear to cytoplasmic ratio. A sample size of one hundred nuclei was read for each hemocyte type. In

addition, only "ghost cells" (i.e., cells with their cytoplasmic membranes intact and faintly visible) were read. This facilitated the identification of hemocyte types. All cells read for each individual were read from the same slide.

To provide a haploid reference value for the interpretation of hemocyte DNA ploidy levels, a tissue squash was made from testes removed from an adult. This tissue was stained following the same procedure as the hemocytes. Fifty sperm nuclei were read and compared to a sample of prohemocytes (n =35) and mature hemocytes (n = 35) read from the same slide. By measuring the DNA content of the sperm nuclei, an accurate estimate of the ploidy distribution of the hemocytes was made.

It is important to note that throughout this study, DNA values were directly compared only to other values recorded from the same slide. Any attempt to directly compare values from one slide to those of another slide was avoided to prevent increased variability from extraneous sources.

All DNA readings were presented as histograms to detect changes or shifts in ploidy levels and mitotic activity.

Microscopy Procedures

Hemocytes observed with light microscopy were bled into 10% buffered formalin on a microscope slide and fixed for 30 minutes. Following a rinse in distilled water, hemocytes were stained with Giemsa reagent (Table 3) for 10 minutes. The slides were then air dried and mounted in permount. An alternative method of fixation was to bleed the hemolymph into a physiological buffer solution (pH = 7.0) and allow the hemocytes to settle for 10 minutes. Cells were then fixed in 10% formalin

for 30 minutes and prepared as above.

Hemocytes examined by scanning electron microscopy were prepared with two fixatives, glutaraldehyde and formalin which are widely used in tissue preparation. All hemolymph samples were fixed on glass coverslips. Standard SEM tissue preparation was followed, but four variations of fixation were used. in the first procedure, hemolymph was bled directly into 2%buffered glutaraldehyde and fixed for 90 minutes. With the second procedure, hemolymph was bled first into a physiological buffer solution (pH=7.0). After 10 minutes, the buffer was replaced with 2%buffered glutaraldehyde and fixed for 90 minutes. Hemolymph in the third procedure was immediately bled into 10%buffered formalin (pH=7.0) and fixed for 90 minutes. In the fourth procedure, hemolymph was bled into 10%buffered formalin and fixed for 10 minutes, after which formalin was replaced by 2% buffered glutaraldehyde and fixed for an additional 80 minutes.

Fixation *in vivo* was achieved by anesthetizing an adult cockroach with CO_2 and then injecting a 2%buffered glutaraldehyde solution into the abdomen. The animal was decapitated (to enhance penetration of the fixative) and immersed in a glutaraldehyde solution for 12 hours. Following this fixation, the animal was sectioned, and tissue samples fixed in 1% osmium tetroxide for one hour.

Following fixation, all specimens were rinsed briefly in distilled water and dehydrated through a graded ethanol series (60, 75, 95, 100%). Theywerethentransferedtoamylacetateandcriticalpointdried. After drying, slides and tissue specimens were mounted on aluminum stubs and

sputter coated with 15 angstroms of gold. All electron microscope observations were made using a Hitachi S-450 scanning electron microscope operated at 25 kv.

Hemocyte Mapping Techniaue

For positive identification of hemocytes, the following procedure adapted from Wetzel et al. (1973) was used. A small asymmetrical design was inscribed on a glass coverslip and the lower right hand corner of the slide was marked. The inscribed area of the coverslip was covered with 10% buffered formalin (pH-7.0) and hemolymph from an amputated leg was mixed into the formalin and fixed for 10 minutes. The formalin was then replaced by 2%buffered glutaraldehyde and fixed for 80 minutes. After rinsing the coverslip briefly in distilled water, the cells were stained with Giemsa stain for approximately 15 minutes. The coverslip was rinsed again in distilled water and then mounted face down on a welled microscope slide in a buffer solution (pH=7.0). The slide was scanned under a light microscope and hemocytes photographed. The coverslip was removed and washed in distilled water prior to dehydration through a graded ethanol series (60, 75, 95, and 100%). The coverslip was then transfered to amyl acetate and critical point dried. After drying, the coverslip was mounted on a specimen stub and sputter coated with 15 angstoms of gold. The photographed areas were observed with the SEM and rephotographed.

Photography

All SEM micrographs were taken with a Polaroid CU-5 land camera using Polaroid Film Land 4ax5, Polapan 52. All other micrographs were taken with a Nikon M-35 S camera mounted on a Zeiss Universai research

microscope using Kodak Plus-X pan 125 print film.

RESULTS

Results of a light microscope examination of the hemocyte complex of P. americana showed that small prohemocytes, 4–8 microns in diameter, were easily distinguished from larger cells mainly by their large nucleus to cytoplasm ratio (figs. 1A,G,H,J). Additionally, the nuclei of prohemocytes stained much darker with both Giemsa and Feulgen stains. This was probably due to chromatin being more compact than in the larger hemocytes (figs. 2C,D,E). These two characteristics were the basis for identifying prohemocytes for microspectrophotometer determination of DNA content. Prohemocytes constituted a relatively low percentage (less than 5%) of the total hemocyte complex.

The vast majority of the hemocyte complex consisted of larger cells (10–25 microns in length and 5–12 microns in width) that assumed a wide variety of shapes. In virtually all of these cells, the nuclei occupied an estimated 40–50% of the cell volume. The cytoplasm was finely granular but some cells appeared transparent while others were much darker (figs. 1B–J). From previously published definitions (Price and Ratcliffe, 1974; Gupta, 1979) most cells appeared to be plasmatocytes but many shared characteristics of granulocytes. Cells with larger granules were occasionally observed in some hemolymph preparations (figs. 1K–N) and might be identified as granulocytes, coagulocytes, or spherule cells. However, these cells were observed only when hemocytes were not immediately fixed subsequent to depositing the hemolymph on the microscope slide. Some cells, including apparent prohemocytes, appeared as if their granules were ejected into the surrounding hemolymph (fig. 1N), indicating that heavy granulation was characteristic of degrading cells

Figure 1

Light microscopy photographs of hemocytes from P. americana

A. prohemocyte (1000x)
B–F. mature hemocytes (1000x)
G,H. mature hemocytes with prohemocytes (250x)
I. mature hemocytes (250x), note varying density of cytoplasm
J. mature hemocytes with prohemocytes (250x1
K–N. hemocytes prepared with delayed fixation (450x1, note granulation of most cells and ejection of granules into the hemolymph by mature cell and prohemocyte (N)

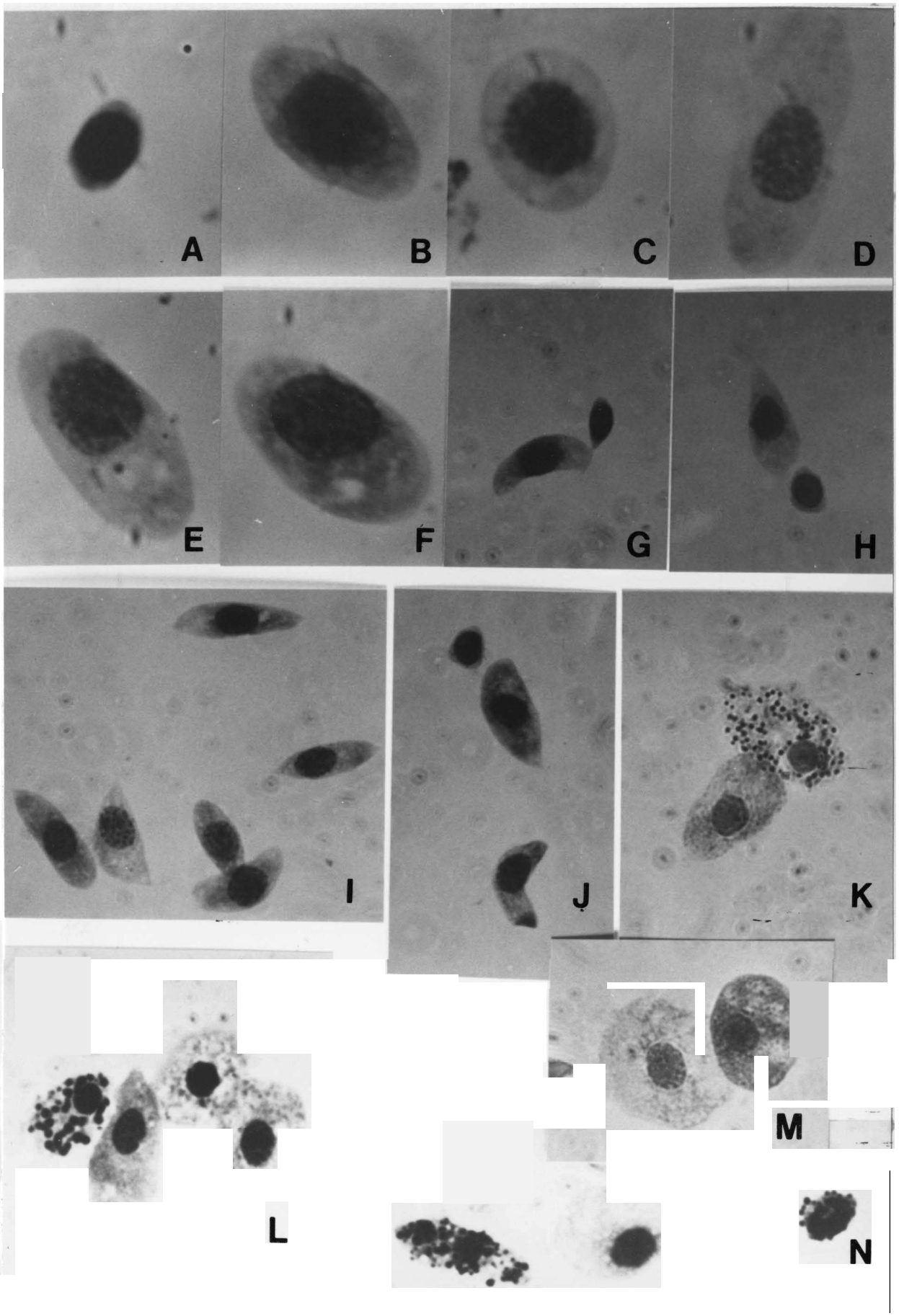
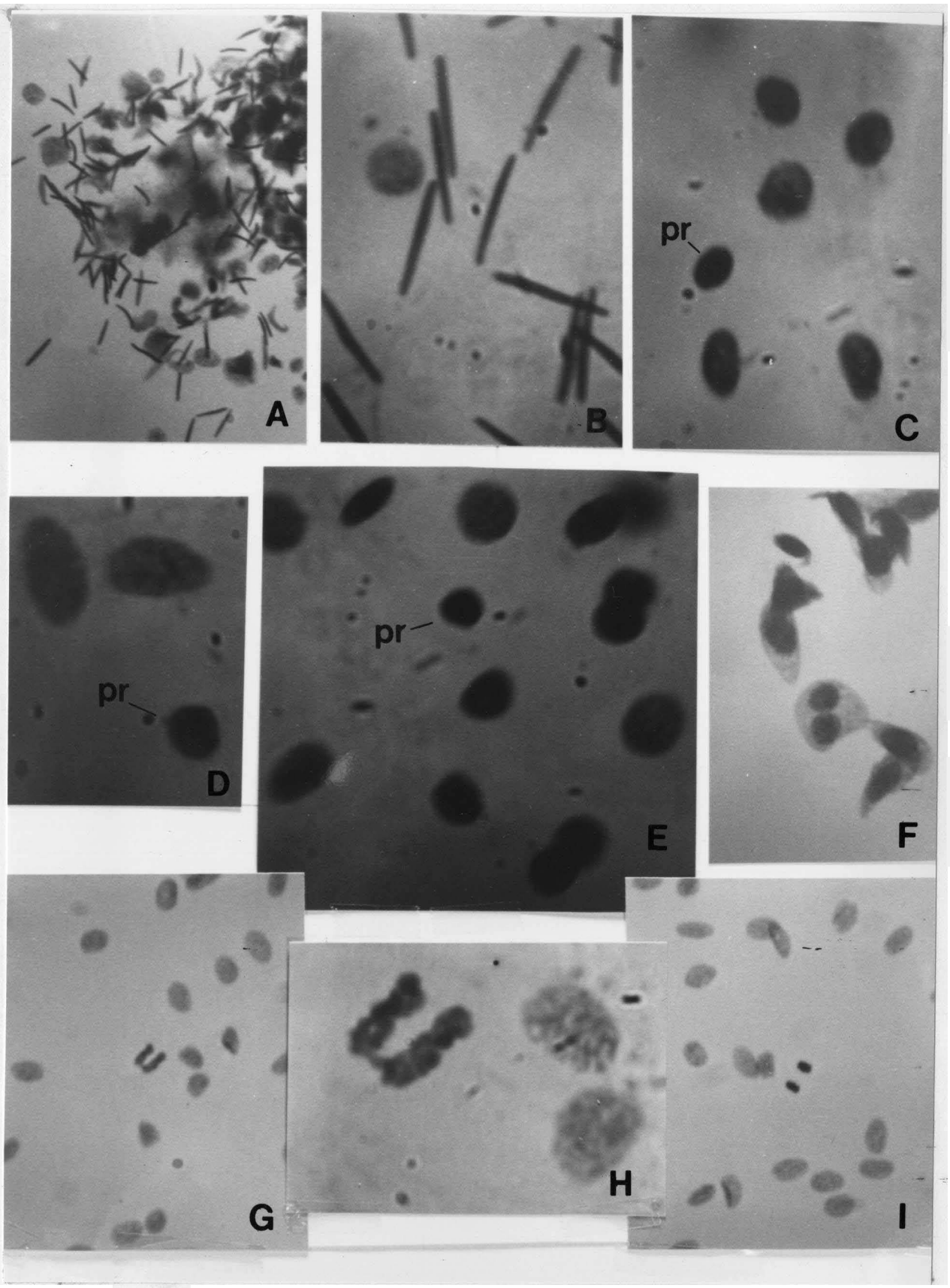

A
B
C
D
E
F
G
H
I
J
K
L
M
N

Figure *2*

Light microscopy photographs

A. testes squash (250x1
B. sperm nuclei (1000x)
C,D,E. Feulgen stained hemocyte nuclei (450x1, note smaller size and darker appearance of prohemocytes (pr)
F. binucleate hemocyte (250x1
G,H,I. Feulgen stained hemocyte nuclei (250x, H = 1000x enlargement of G), note mitotic figures


A
B
pr
C
pr
D
pr
E
F
G
H
I

rather than a specific cell type. Because of the lack of differentiation between the larger hemocytes (when fixed immediately) these cells are collectively referred to, in this study, as mature hemocytes and thus distinguished from smaller prohemocytes.

Prior to microspectrophotometric studies, absorption readings were calculated and the data plotted to determine the wavelengths of maximum (550nm) and half maximum (490nm) absorption (Fig. 3A).

Optimum hydrolysis time was determined by plotting the relative amounts of dye binding against time of hydrolysis. The resulting curve (fig. 3B) illustrates that a hydrolysis time of thirty minutes was optimal for maximal stain intensity.

The accuracy of the microspectrophotometer was determined by making 20 DNA measurements on the same cell. Results from this experiment illustrated a maximal variation of 4.6% (mean = 19.94; S.D. =0.24).

The data presented in figure 4 show the mean haploid (1C) value of sperm nuclei to equal 10.96 relative units of DNA (S.D. = 1.23). In comparison, the prohemocyte mean DNA content was 20.11 relative units (S.D. = 0.90) and the mature hemocyte mean content was 20.72 units (S.D. = 0.75). Both prohemocytes and mature hemocytes showed a mean DNA value close to the calculated diploid value of 21.92 units (2 x 10.96). The prohemocyte average was 8% smaller and the mature hemocyte value was 4% smaller than the predicted diploid value.

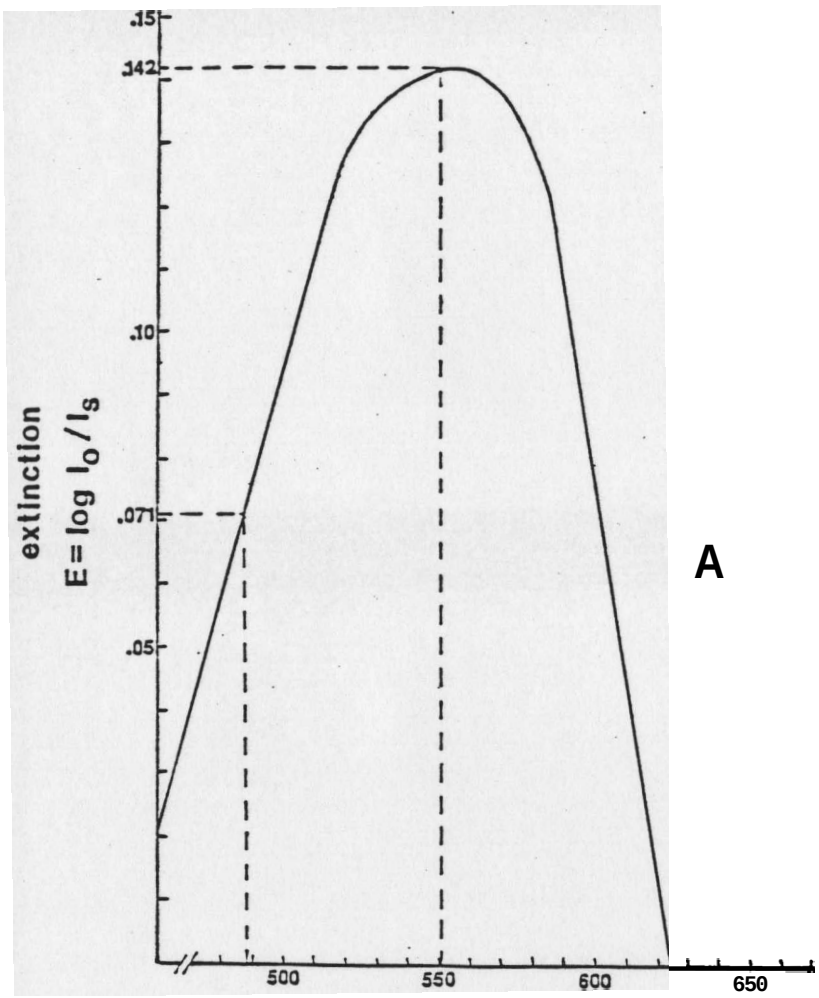
Comparison of the histograms from the larger individual samples (n = 100) (figs. 5–14) of both nymphs and adults indicated that, although the frequency distribution for all samples was unimodal, most samples of prohemocytes and mature hemocytes were skewed toward increasing DNA

Figure 3

A. absorption spectrum of Feulgen stained hemocyte nuclei of P. americana

B. hydrolysis curve representing DNA–dye binding of hemocytes at various hydrolysis times. Points represent mean values (n=20), vertical lines represent standard deviations

figure 3


.15
.142
.10
.071
.05
extinction
E = log I_o / I_s
500
550
600
650
A
wavelength (nm)

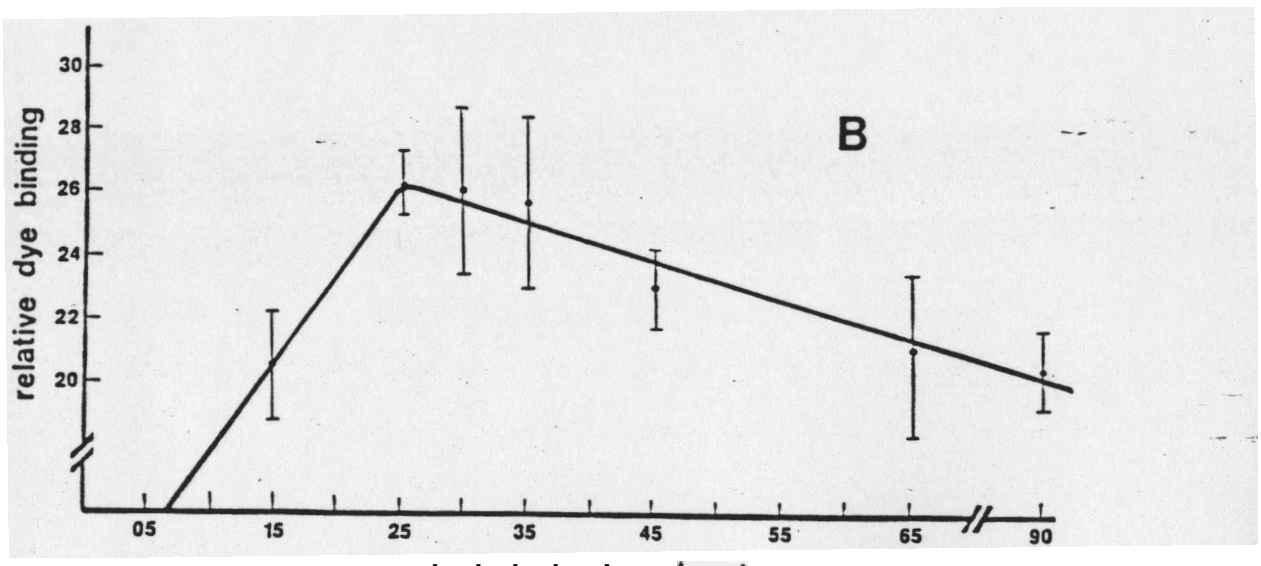

30
28
26
24
22
20
relative dye binding
B
05
15
25
35
45
55
65
90
hydrolysis time (min.)

Figure 4

Histograms representing Feulgen–DNA values for sperm nuclei (n = 50), prohemocytes (n = 35), and mature hemocytes (n = 35).

figure 4

Sperm nuclei	Prohemocytes	Mature Hemocytes
mean DNA conc. = 10.96	mean DNA conc. = 20.11	mean DNA conc. = 20.72
std. dev. = 1.23	std. dev. = 0.90	std. dev. = 0.75
std. error = 0.17	std. error = 0.15	std. error = 0.13

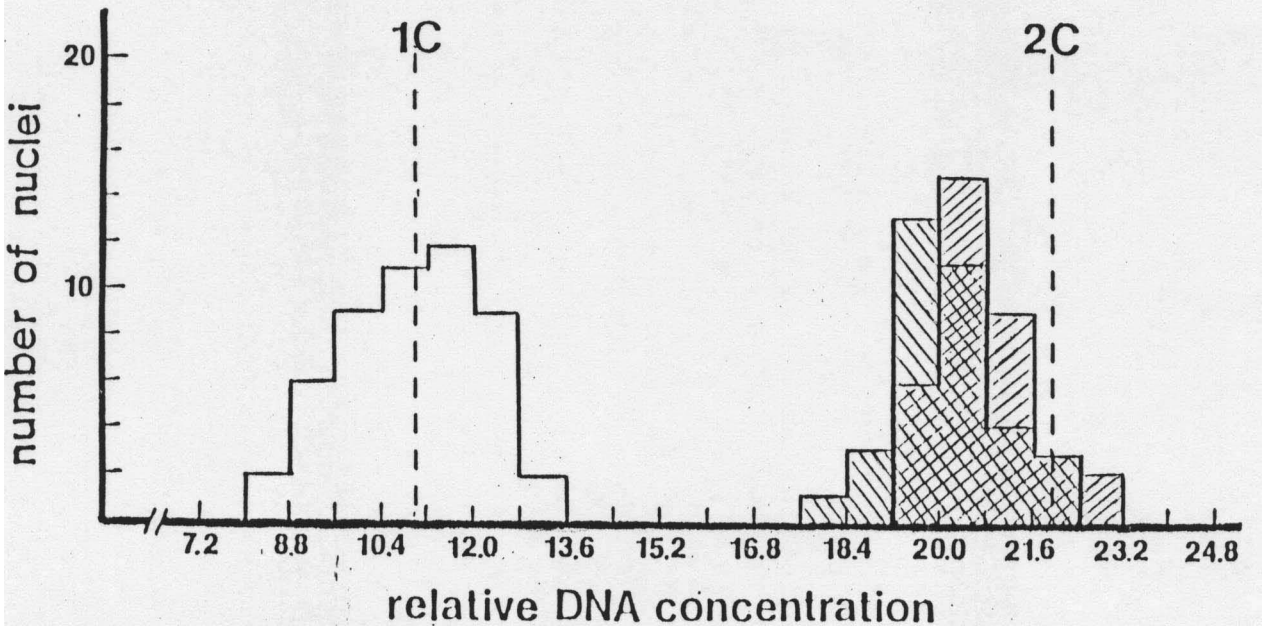
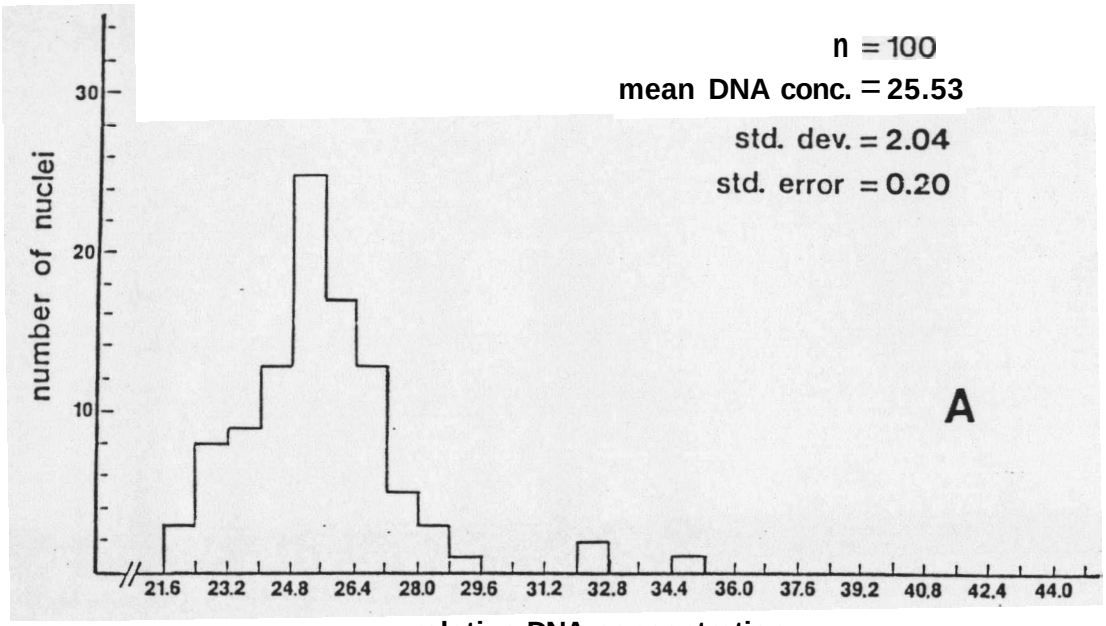


Figure *5*

A. histogram representing DNA values of nymph prohemocyte sample
B. histogram representing DNA values of nymph mature hemocyte sample

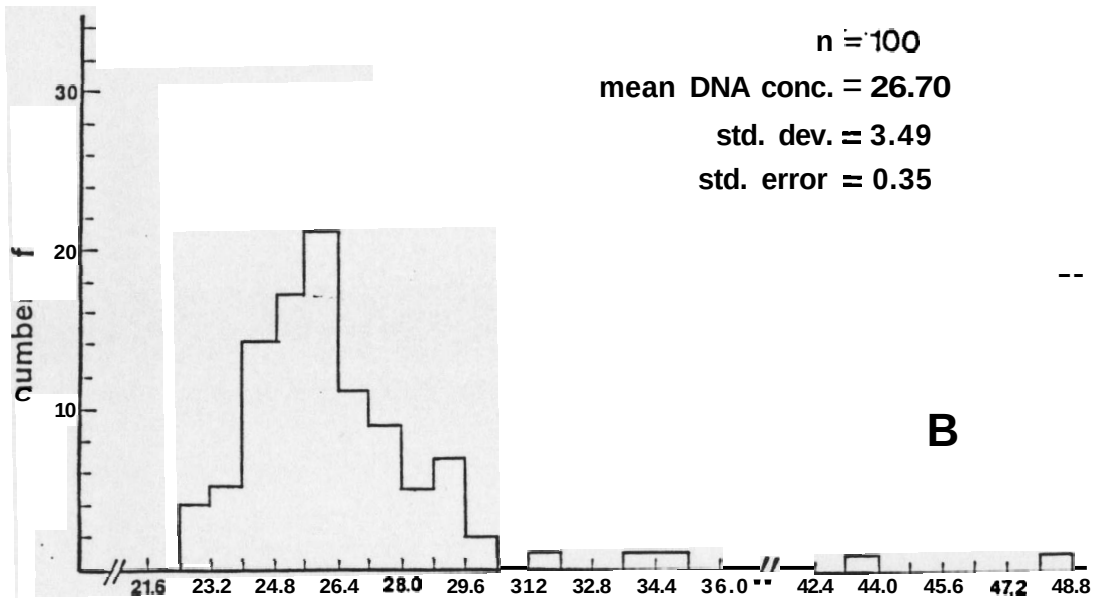
figure 5

Nymph No. 1 Prohemocytes



relative DNA concentration

Nymph No. 1 Mature Hemocytes



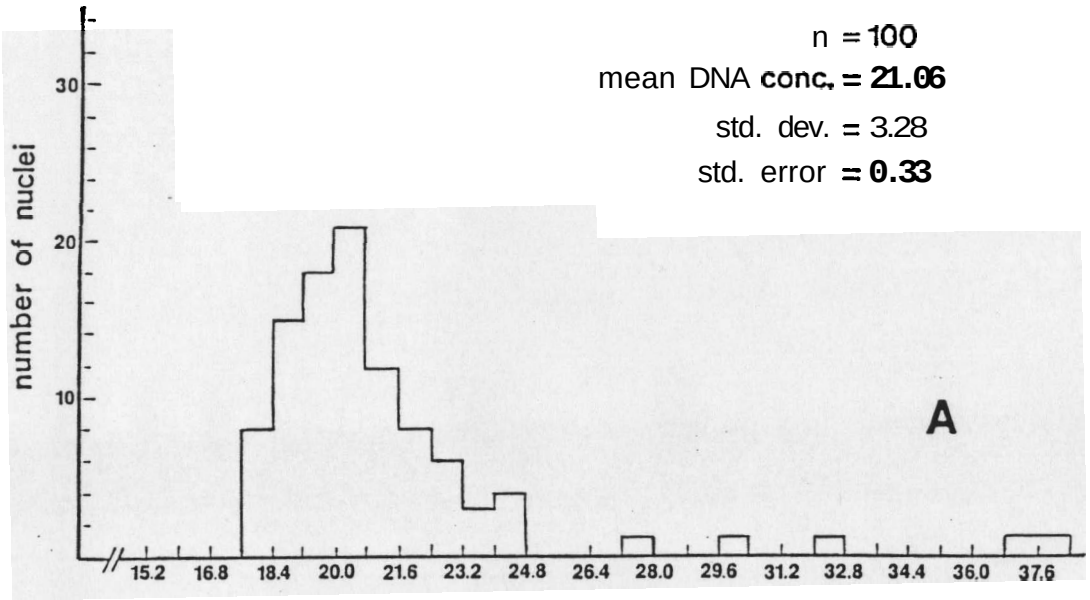
relative DNA concentration

Figure *6*

A. histogram representing DNA values of nymph prohemocyte sample
B. histogram representing DNA values of nymph mature hemocyte sample

figure 6

Nymph No. 2 Prohemocytes



Nymph No. 2 Mature Hemocytes

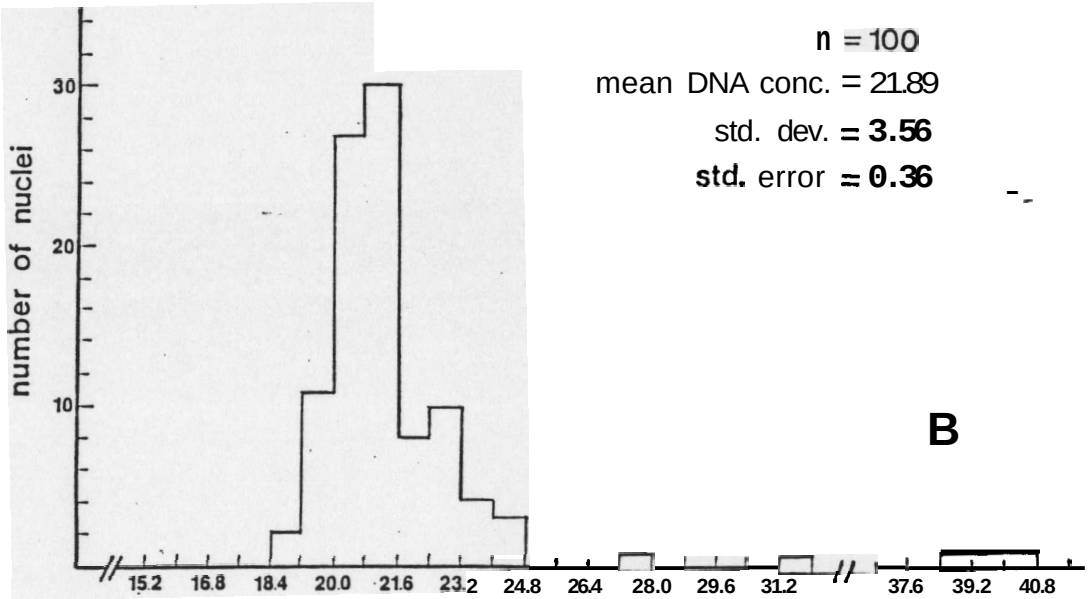
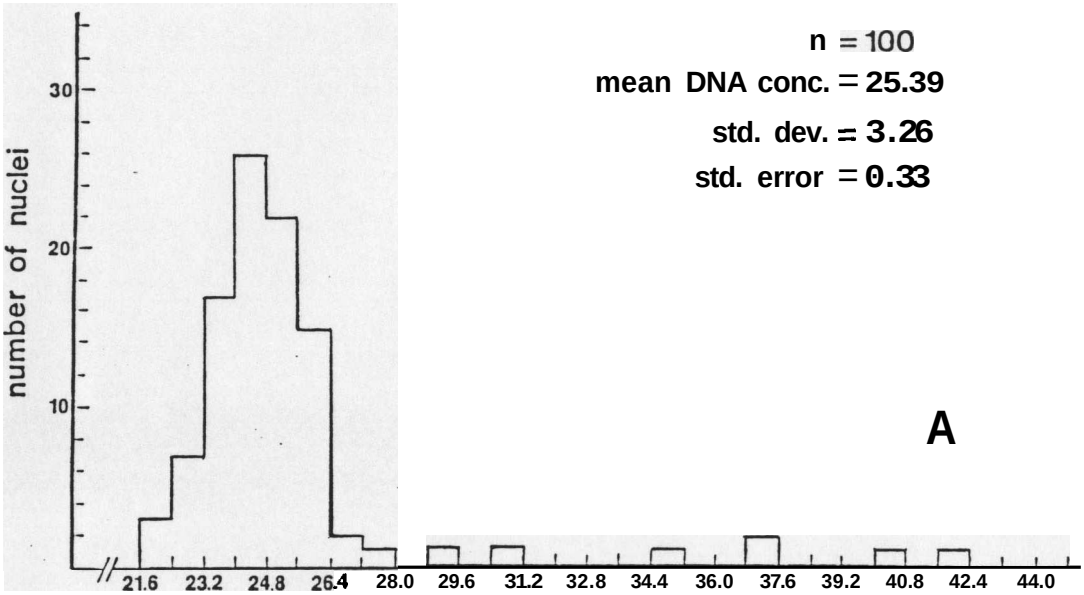


Figure 7

A. histogram representing DNA values of nymph prohemocyte sample
B. histogram representing DNA values of nymph mature hemocyte sample

figure 7

Nymph No. 3 Prohemocytes



Nymph No. 3 Mature Hemocytes

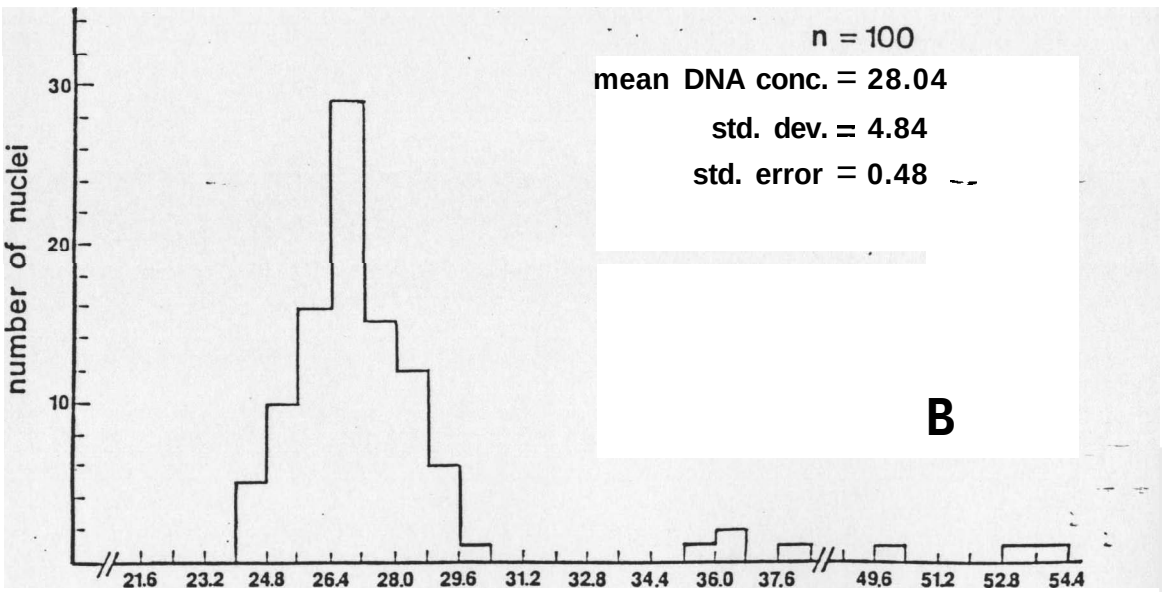
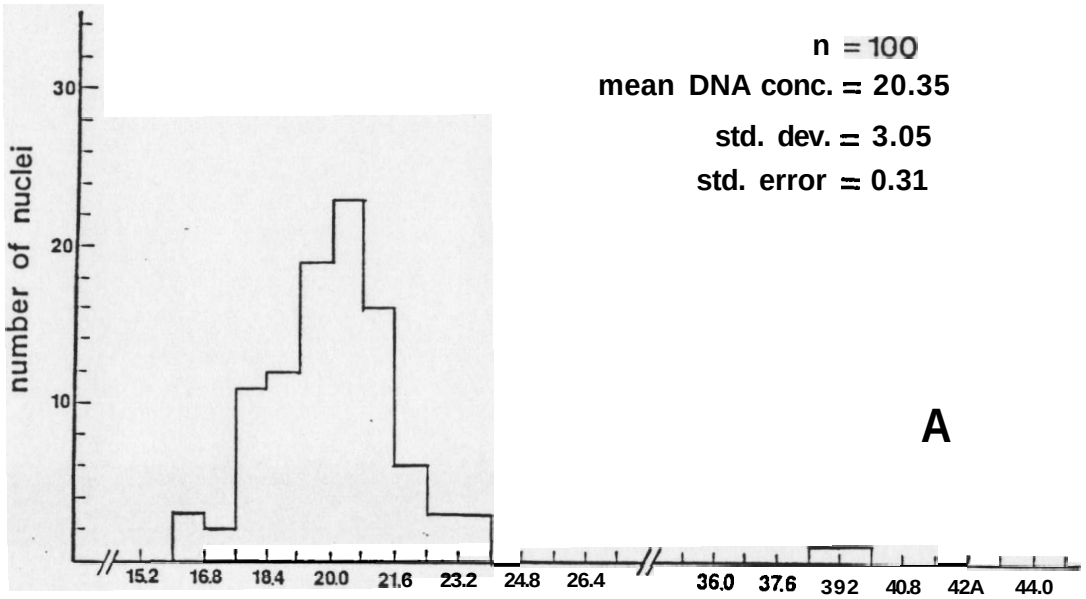


Figure 8

A. histogram representing DNA values of nymph prohemocyte sample
B. histogram representing DNA values of nymph mature hemocyte sample

figure 8

Nymph No. 4 Prohemocytes



Nymph No. 4 Mature Hernocytes

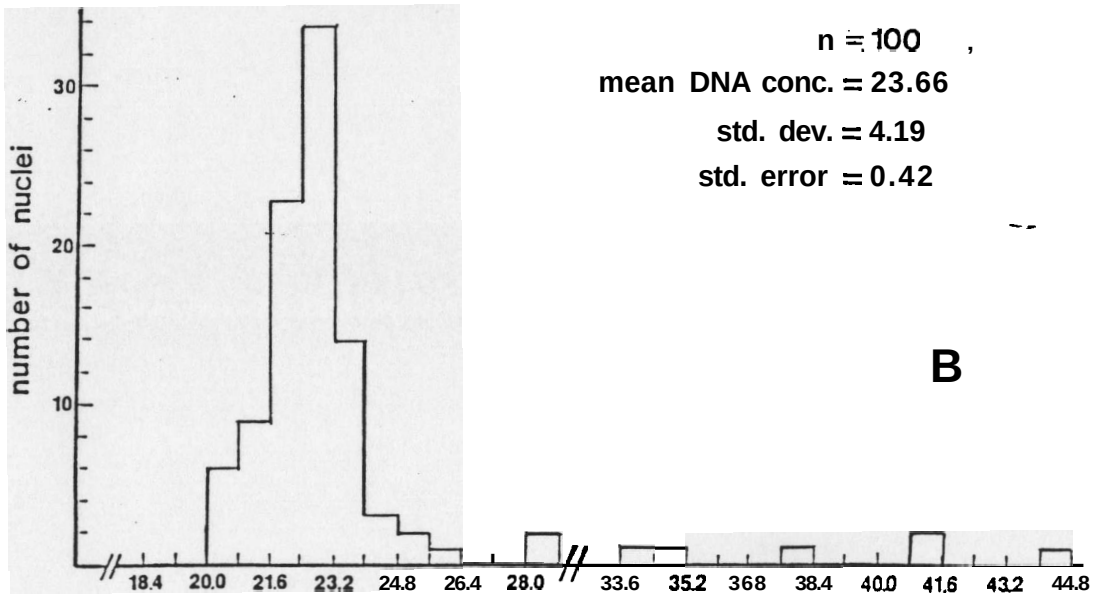
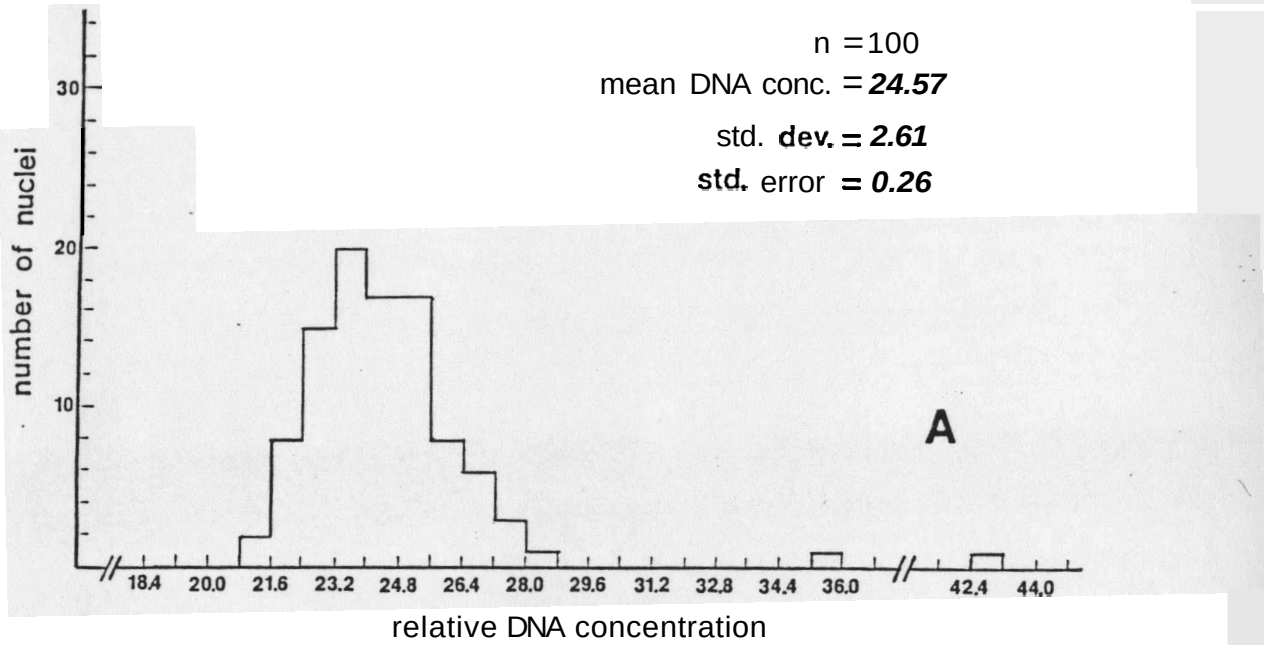


Figure 9

A. histogram representing DNA values of nymph prohemocyte sample
B. histogram representing DNA values of nymph mature hemocyte sample

figure 9

Nymph No. 5 Prohemocytes



Nymph No. 5 Mature Hemocytes

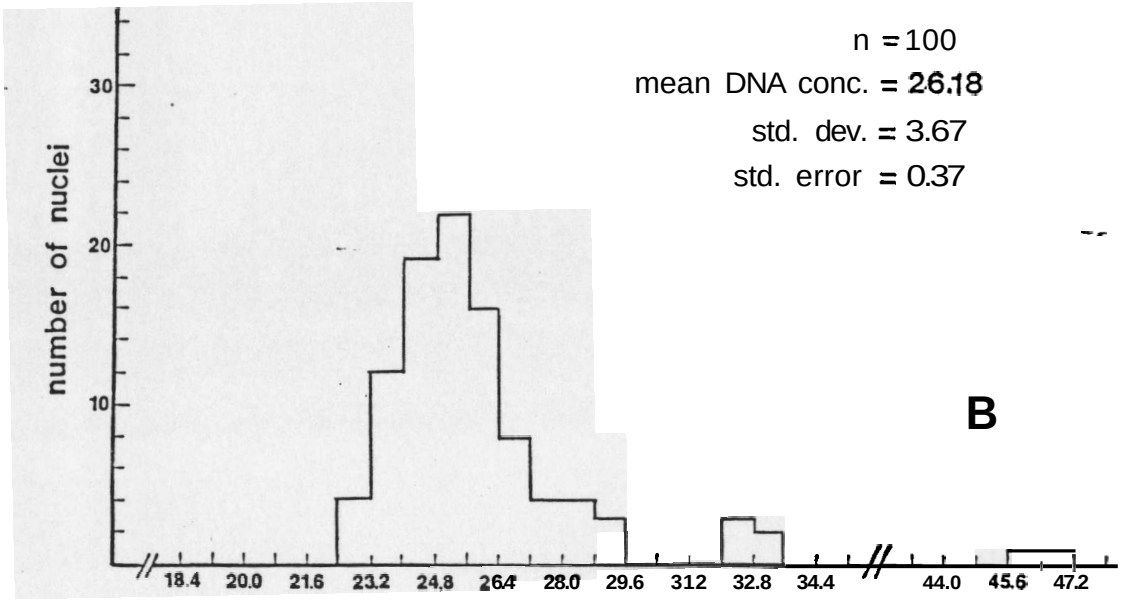
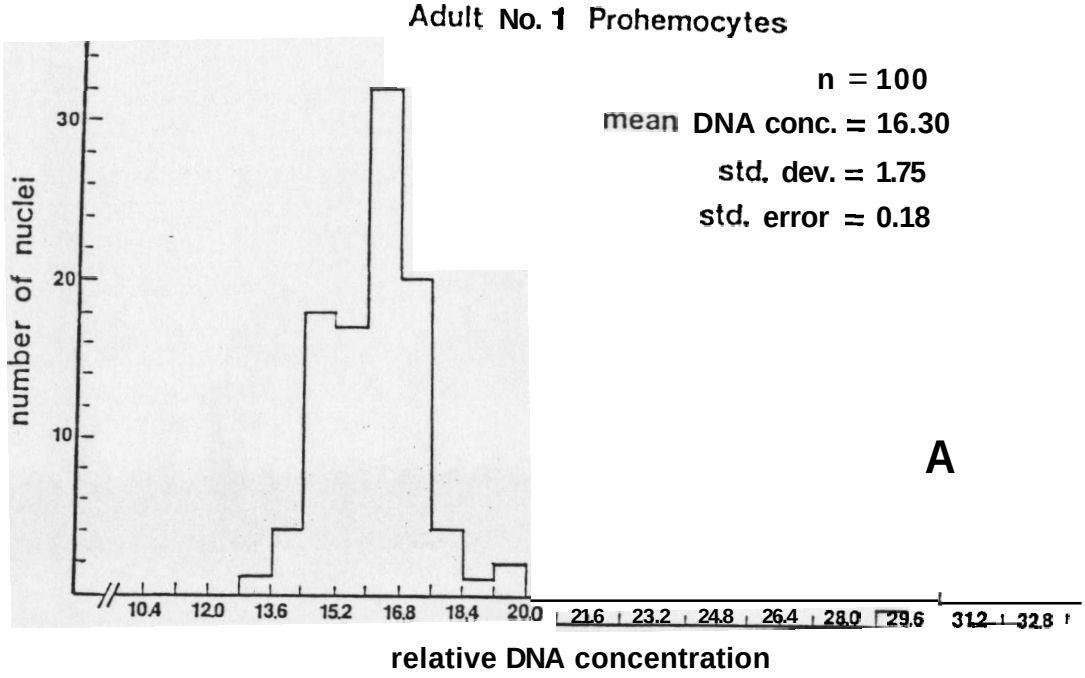


Figure 10

A. histogram representing DNA values of adult prohemocyte sample
B. histogram representing DNA values of adult mature hemocyte sample

figure 10


Adult No. 1 Prohemocytes
n = 100
mean DNA conc. = 16.30
std. dev. = 1.75
std. error = 0.18
number of nuclei
30
20
10
10.4
12.0
13.6
15.2
16.8
18.4
20.0
21.6
23.2
24.8
26.4
28.0
29.6
31.2
32.8
A
relative DNA concentration

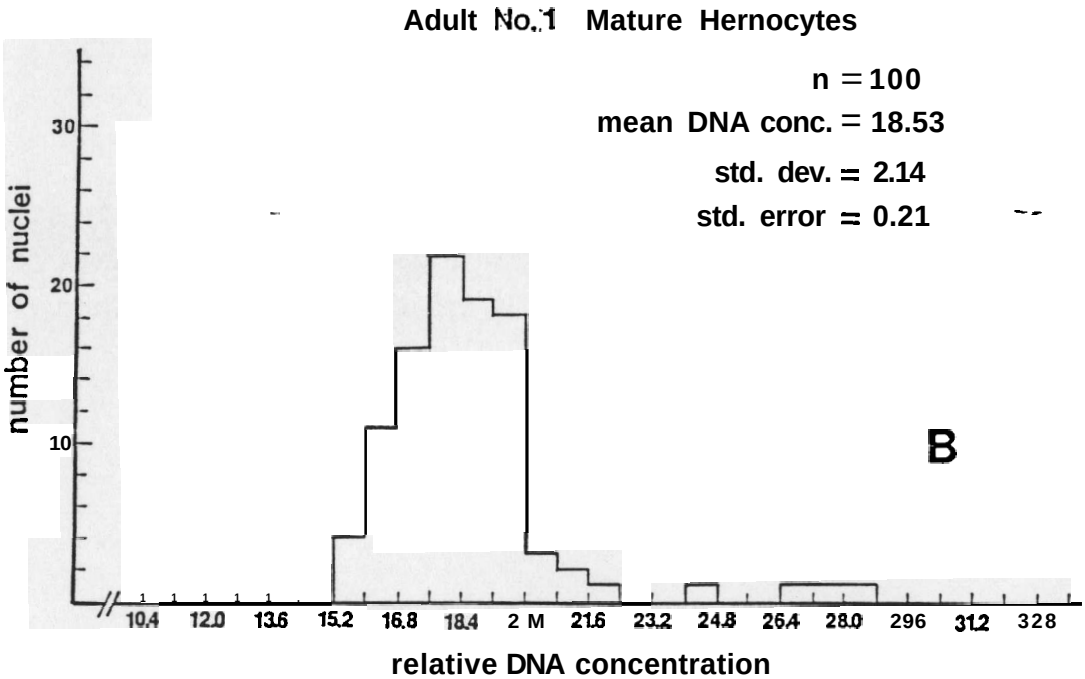
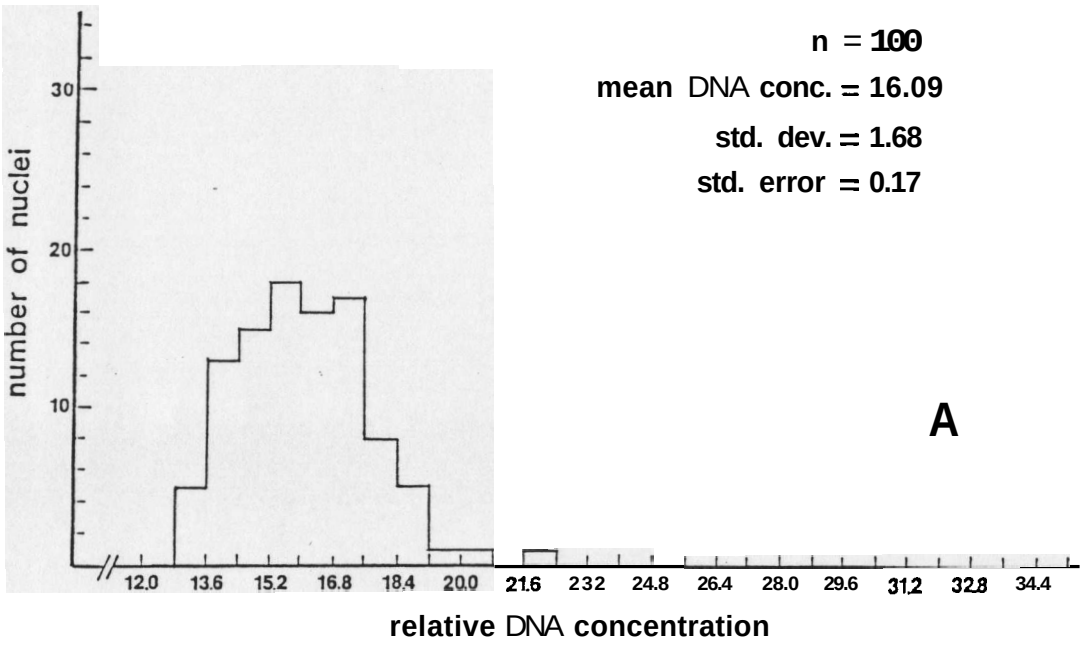

Adult No. 1 Mature Hernocytes
n = 100
mean DNA conc. = 18.53
std. dev. = 2.14
std. error = 0.21
number of nuclei
30
20
10
10.4
12.0
13.6
15.2
16.8
18.4
2 M
21.6
23.2
24.8
26.4
28.0
296
31.2
328
B
relative DNA concentration

Figure 11

A. histogram representing DNA values of adult prohemocyte sample
B. histogram representing DNA values of adult mature hemocyte sample

figure 11


Adult No. 2 Prohemocytes
n = 100
mean DNA conc. = 16.09
std. dev. = 1.68
std. error = 0.17
number of nuclei
30
20
10
A
12.0
13.6
15.2
16.8
18.4
20.0
21.6
23.2
24.8
26.4
28.0
29.6
31.2
32.8
34.4
relative DNA concentration

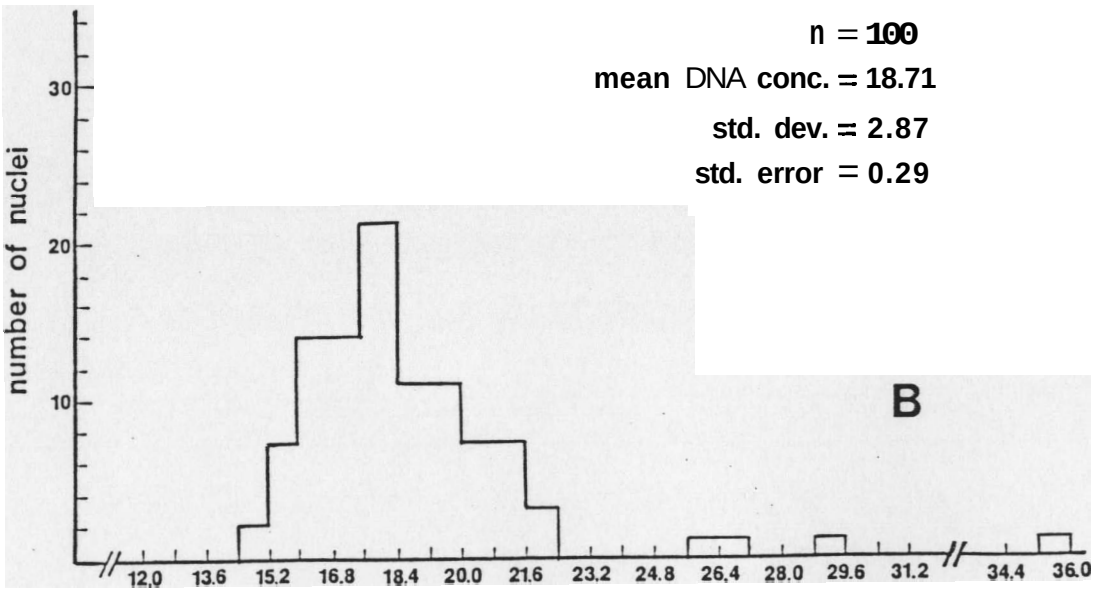
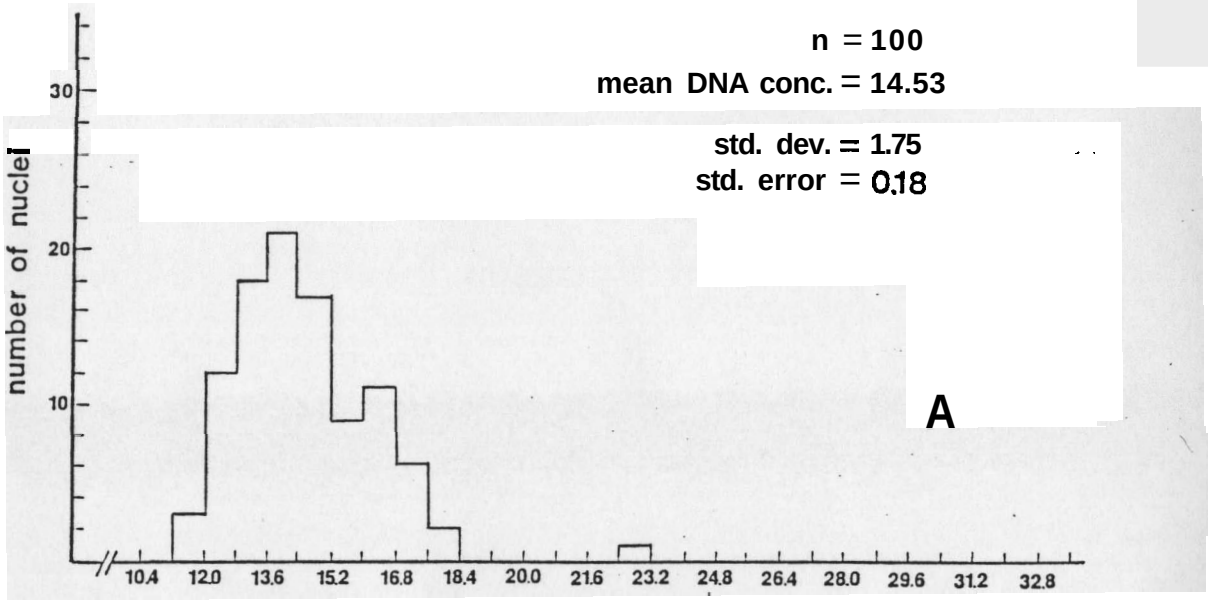

Adult No. 2 Mature Hemocytes
n = 100
mean DNA conc. = 18.71
std. dev. = 2.87
std. error = 0.29
number of nuclei
30
20
10
B
12.0
13.6
15.2
16.8
18.4
20.0
21.6
23.2
24.8
26.4
28.0
29.6
31.2
34.4
36.0
relative DNA concentration

Figure 12

A. histogram representing DNA values of adult prohemocyte sample
B. histogram representing DNA values of adult mature hemocyte sample

figure 12

Adult No. 3 Prohernocytes



Adult No. 3 Mature Hernocytes

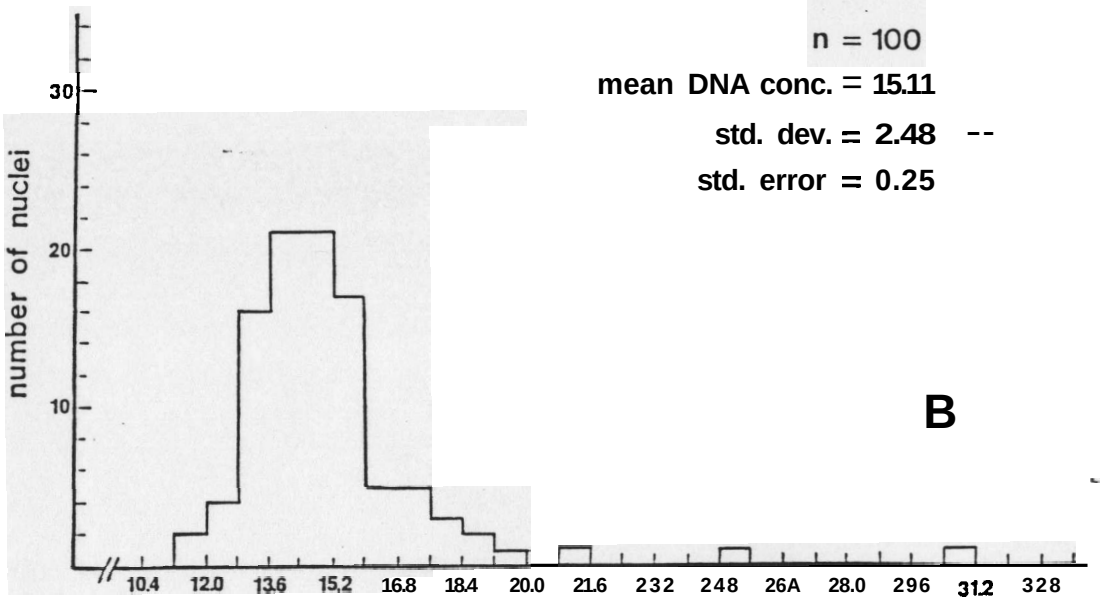
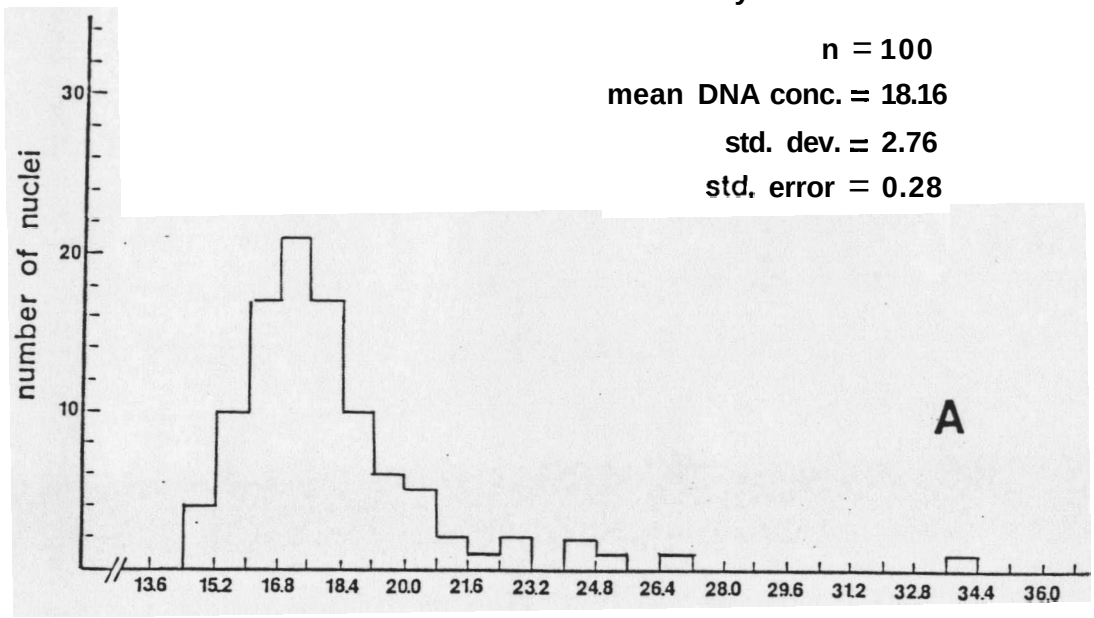


Figure 13

A. histogram representing DNA values of adult prohemocyte sample
B. histogram representing DNA values of adult mature hemocyte sample

figure 13

Adult No. 4 Prohemocytes



Adult No. 4 . Mature Hemocytes

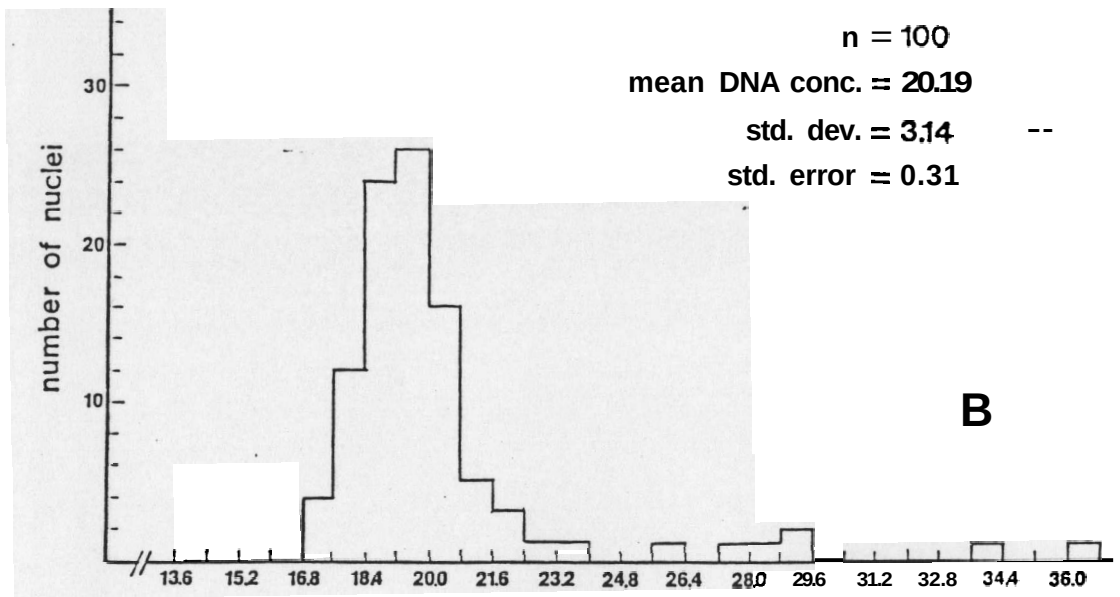
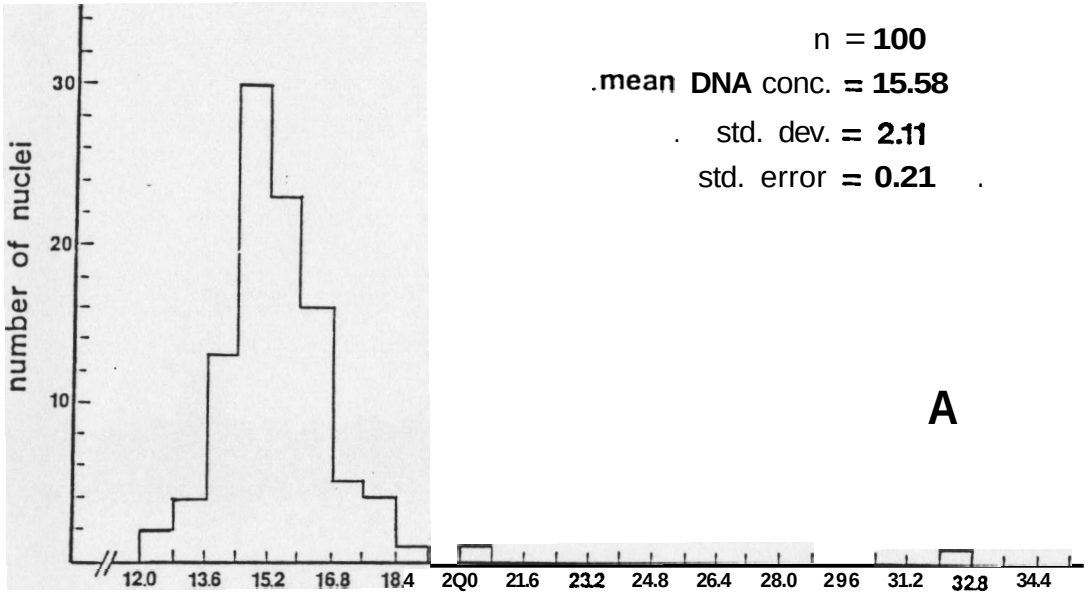


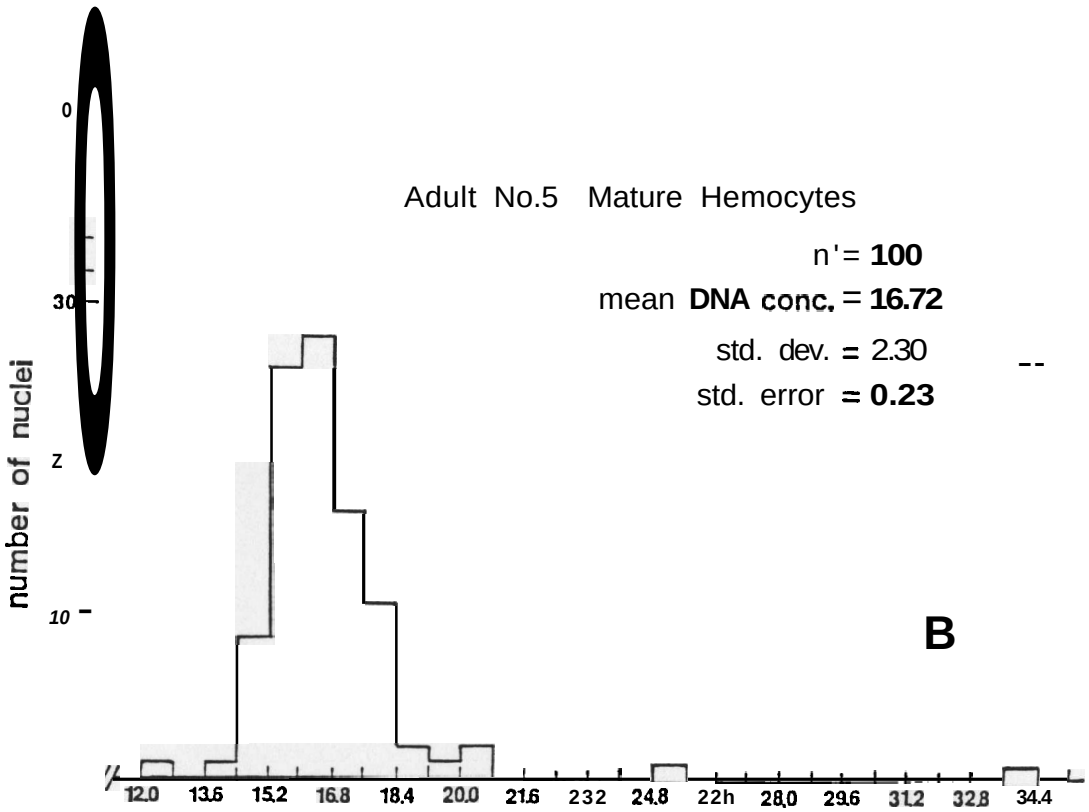
Figure 14

A. histogram representing DNA values of adult prohemocyte sample
B. histogram representing DNA values of adult mature hemocyte sample

figure 14

Adult No. 5 Prohemocytes





values. To examine the relationship between skewed and modal values and to enable the comparison of DNA levels between cells of different samples, a DNA index (DNA index = DNA value of a cell/modal DNA value of the sample) was calculated (Barlogie et al., 1978). DNA indices were determined only for cells with DNA values significantly higher than the modal sample values. Significantly higher DNA values were defined as being more than three standard deviations larger than the mode. Standard deviations of the samples were recalculated after obviously skewed values were removed to better estimate the unimodal populations. Larger values were removed until the coefficient of skewness of the sample did not deviate significantly (α= 0.05) from a normal distribution.

Values of the DNA index are presented as composite histograms (figure 15). DNA values were generally scattered between 1.15 and 2.15. In none of the groups were cells found with DNA indices markedly higher than 2.0.

Observations on the ultrastructure of the hemocytes with scanning electron microscopy emphasized the labile nature of the cells. Fixing cells *in vivo* was done to present the hemocytes in their most natural state. Examples of these cells are found in figures 16A-D. All cells were generally flattened and round, oval, or tear-drop in shape. Most were between 15-20 microns in length and 5-10 microns in width. The cell membrane had a rough or bumpy surface with few or no invaginations. Pseudopodia were usually absent but often one end of a hemocyte appeared to bestretchedout inaprobing, motilemanner, giving the cell a tear-drop appearance. Fine cytoplasmic strands occasionally interconnected groups of hemocytes.

While attempting to fix cells *in vitro*, it was found that bleeding the

Figure 15

A. histogram representing **DNA** index calculations of skewed nymph prohemocyte **DNA** measurements

B. histogram representing **DNA** index calculations of skewed nymph mature hemocyte **DNA** measurements

C. histogram representing **DNA** index calculations of skewed adult prohemocyte **DNA** measurements

D. histogram representig **DNA** index calculations of skewed adult mature hemocyte **DNA** measurements

figure 15

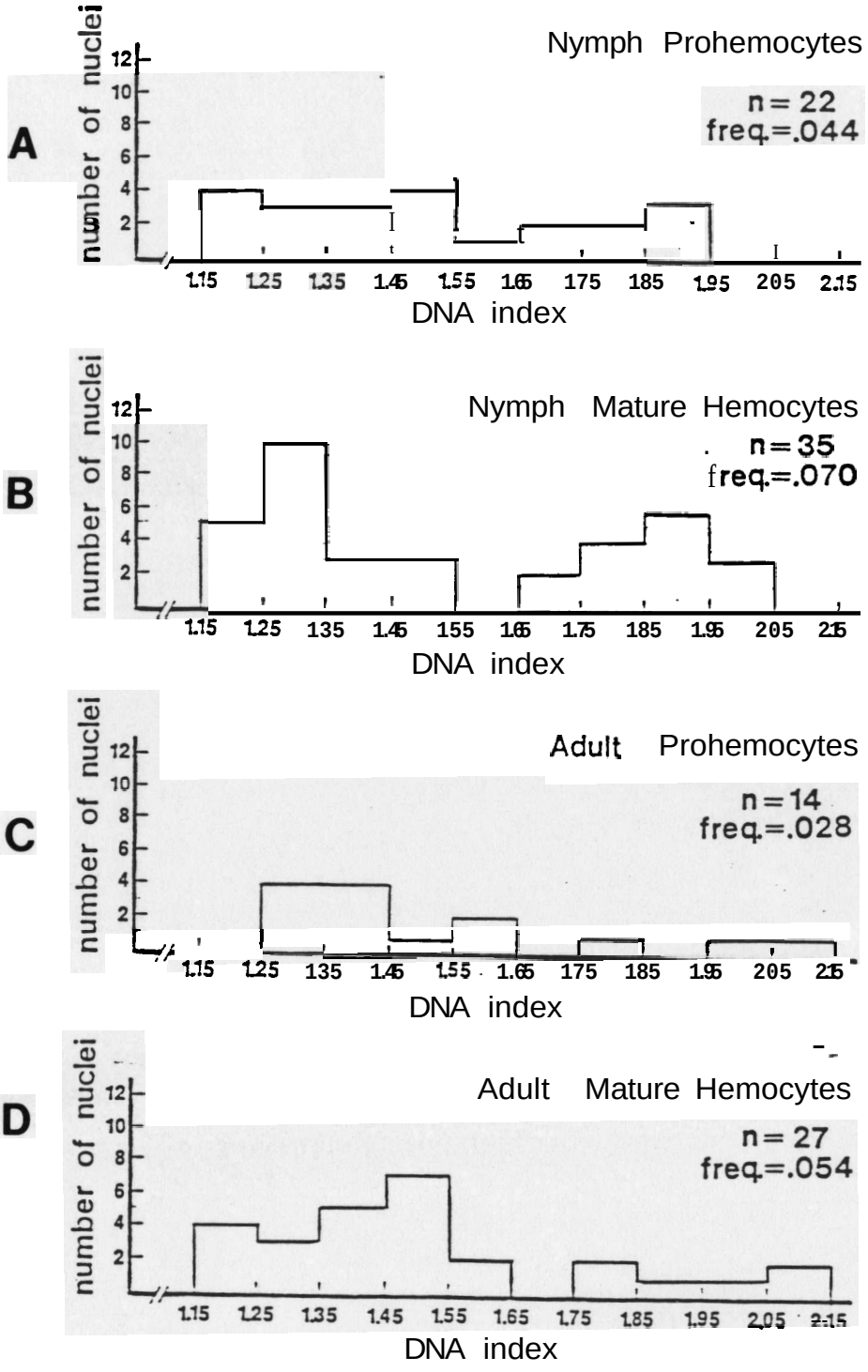

A
Nymph Prohemocytes
n = 22
freq.=.044
number of nuclei
12
10
8
6
4
2
1.15 1.25 1.35 1.45 1.55 1.65 1.75 1.85 1.95 2.05 2.15
DNA index
B
Nymph Mature Hemocytes
n = 35
freq.=.070
number of nuclei
12
10
8
6
4
2
1.15 1.25 1.35 1.45 1.55 1.65 1.75 1.85 1.95 2.05 2.15
DNA index
C
Adult Prohemocytes
n = 14
freq.=.028
number of nuclei
12
10
8
6
4
2
1.15 1.25 1.35 1.45 1.55 1.65 1.75 1.85 1.95 2.05 2.15
DNA index
D
Adult Mature Hemocytes
n = 27
freq.=.054
number of nuclei
12
10
8
6
4
2
1.15 1.25 1.35 1.45 1.55 1.65 1.75 1.85 1.95 2.05 2.15
DNA index

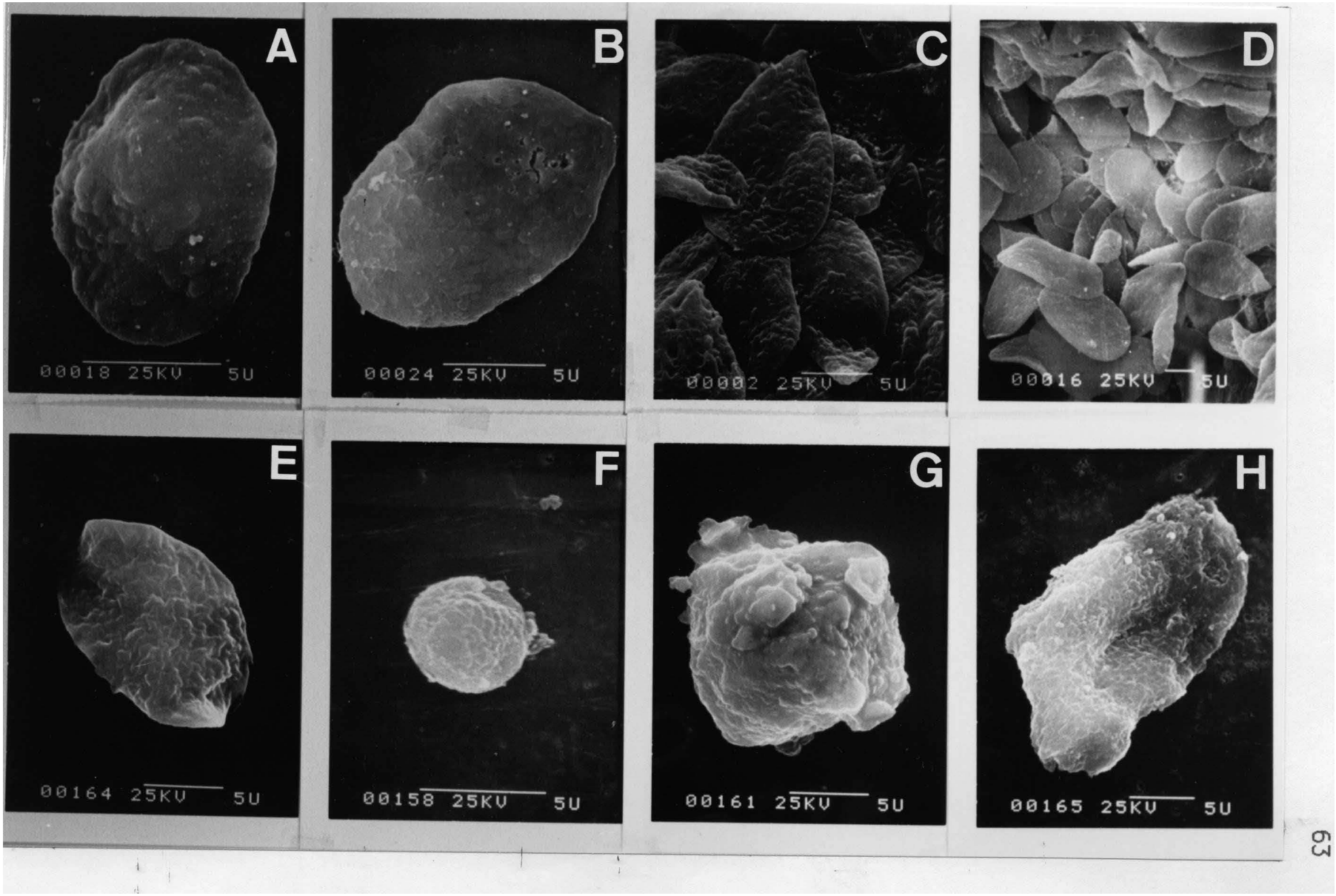
Figure 16

Scanning electron micrographs of hemocytes fixed in vivo.

A. mature hemocyte (8500x1
B. mature hemocyte (6500x1
C. aggregation of hemocytes (4500x)
D. aggregation of hemocytes (2000x1, note cytoplasmic connections

Scanning electron micrographs of hemocytes fixed in 10% buffered formalin.

E. mature hemocyte (5000x1
F. prohemocyte (8000x)
G. hemocyte with irregular surface features (5000x)
H. mature hemocyte (4000x), note membrane damage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B
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C
00002 25KV 5U
D
00016 25KV 5U
E
00164 25KV 5U
F
00158 25KV 5U
G
00161 25KV 5U
H
00165 25KV 5U

hemolymph directly into a drop of glutaraldehyde did not allow hemocytes to attatch to the microscope slide and they were washed away during subsequent staining and rinsing procedures. No hemocytes were viewed on the slides following this procedure.

Fixing hemocytes with a 10% buffered formalin solution, as for light microscopic observations, allowed the cells to settle and attatch to the glass surface. Some cells appeared to be relatively normal (figs. 16E,F) but most cell surfaces had a very uneven and irregular appearance (figs. 16G,H).

Bleeding the hemolymph into a buffer solution prior to fixation with 2% glutaraldehyde yielded a large number of attached hemocytes. However, incubation in a foreign medium without fixation resulted in a dramatic transformation of virtually all observed prohemocytes and mature hemocytes into amoeboid forms. Transformed hemocytes exhibited numerous pseudopodia and cytoplasmic extensions (figs. 17A-D), quite unlike the cells fixed *in vivo*.

In vitro fixation for a short time in 10% formalin followed by 2% glutaraldehyde produced a large number of hemocytes that appeared to be similar in size, shape and membrane appearance to cells fixed *in vivo* (figs. 17E-H, 18A-H). Occasionally, a solitary hemocyte was found with minute projections or macropapillae (fig. 18D).

Using the SEM mapping technique, Giemsa stained insect hemocytes were first observed by light microscopy. Following identification of these cells, their ultrastructural appearance was observed with SEM (figs. 19A-H). Giemsa stain was not held very long by glutaraldehyde fixed cells but restaining could be accomplished without adversely affecting the hemocytes. The size and appearance of these cells were very similar to

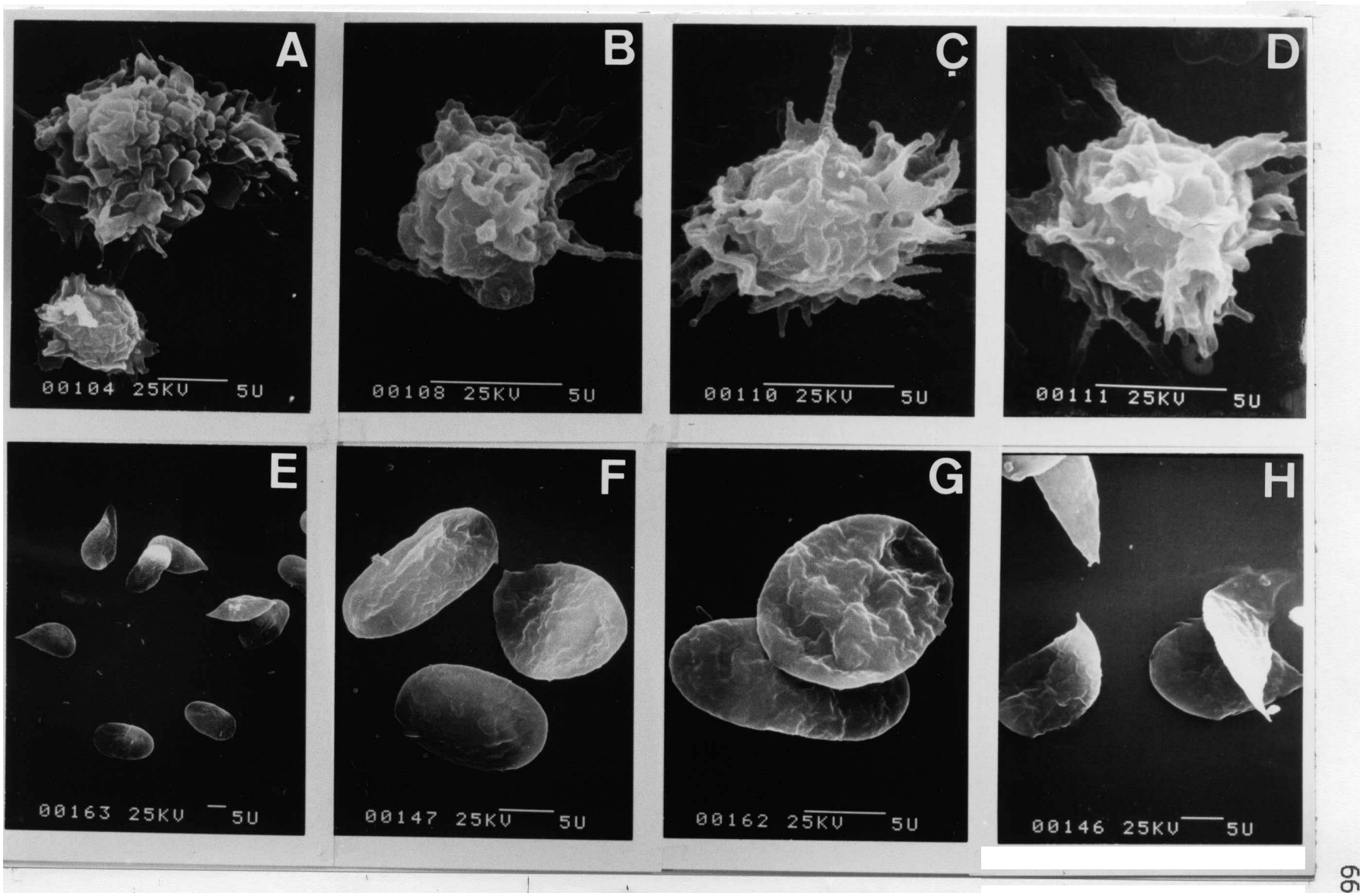
Figure 17

Scanning electron micrographs of hemocytes prepared with buffer/glutaraldehyde technique.

A. mature hemocyte and prohemocyte (4300x1, note cytoplasmic extensions
B. hemocyte with numerous pseudopodia (8000x1, note attachment to microscope slide
C. hemocyte with numerous pseudopodia (8000x1
D. hemocyte with numerous pseudopodia (8000x1

Scanning electron micrographs of hemocytes prepared with formalin/glutaraldehyde technique.

E. mature hemocytes (1000x1
F. mature hemocytes (3000x1
G. mature hemocytes (5000x1
H. mature hemocytes (1500x1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B
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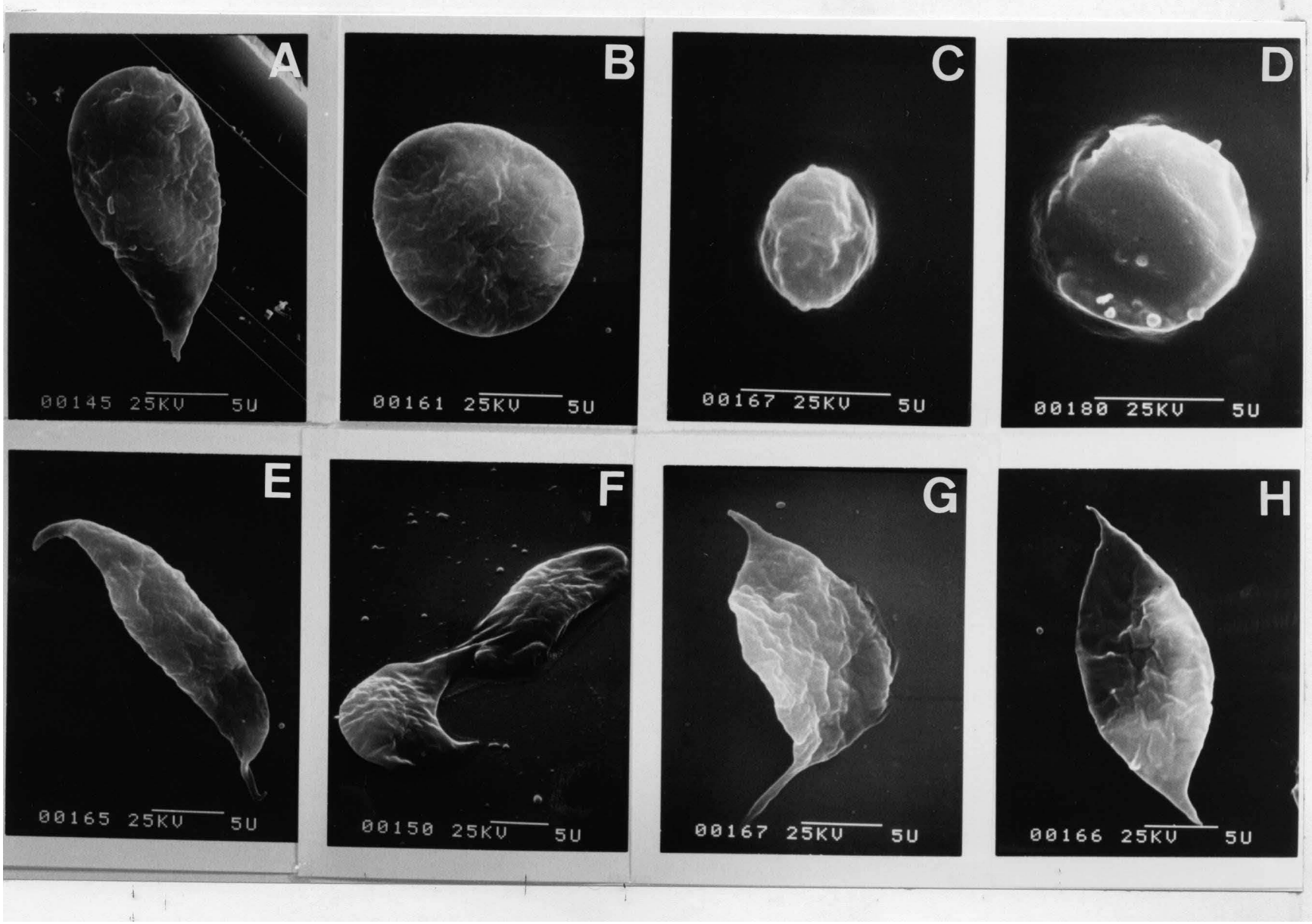
C
00110 25KV 5U
D
00111 25KV 5U
E
00163 25KV 5U
F
00147 25KV 5U
G
00162 25KV 5U
H
00146 25KV 5U

Figure 18

Scanning electron micrographs of hemocytes prepared with formalin/glutaraldehyde technique, note variations of shape.

A. mature hemocyte (5000x)
B. mature hemocyte (5000x)
C. prohemocyte (9200x)
D. prohemocyte (8000x), note papillae-like extensions
E. mature hemocyte (4500x1
F. interconnected hemocytes (3500x)
G. mature hemocyte (5000x), note cytoplasmic extension
H. extended mature hemocyte (4500x1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C
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D
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E
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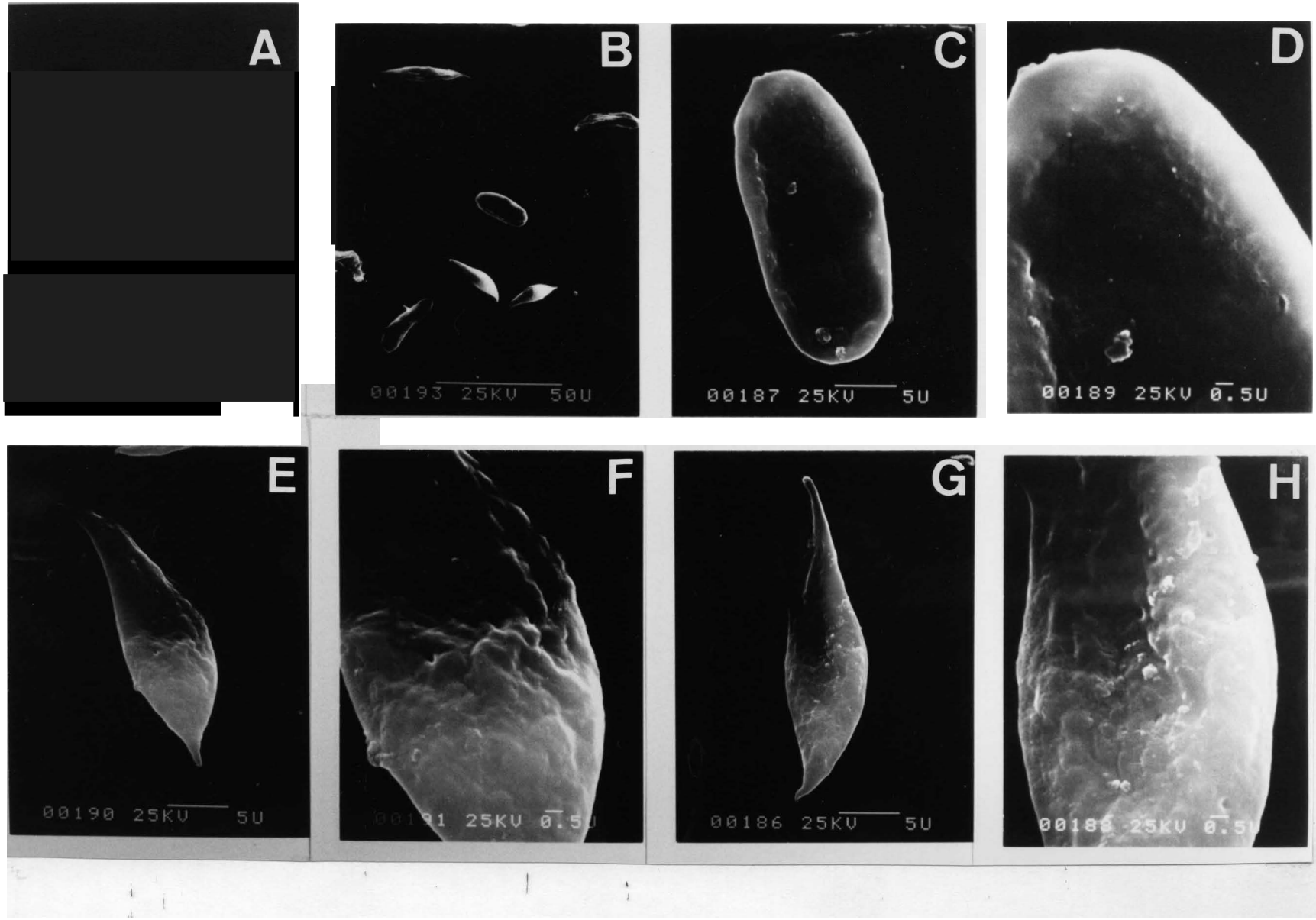
F
00150 25KV 5U
G
00167 25KV 5U
H
00166 25KV 5U

Figure 19

SEM mapping technique using formalin/glutaraldehyde preparation.

A. light microscope photograph of Giemsa stained hemocytes (450x)
B. scanning electron micrograph of Giemsa stained hemocytes (400x1
C. mature hemocyte (3700x), note small clumps of stain
D. surface of hemocyte (10,000x)
E. mature hemocyte (3700x)
F. surface of hemocyte (10,000x)
G. mature hemocyte (3700x1
H. surface of hemocyte (10,000x)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B
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C
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D
00189 25KV 0.5U
E
00190 25KV 5U
F
G
00186 25KV 5U
H

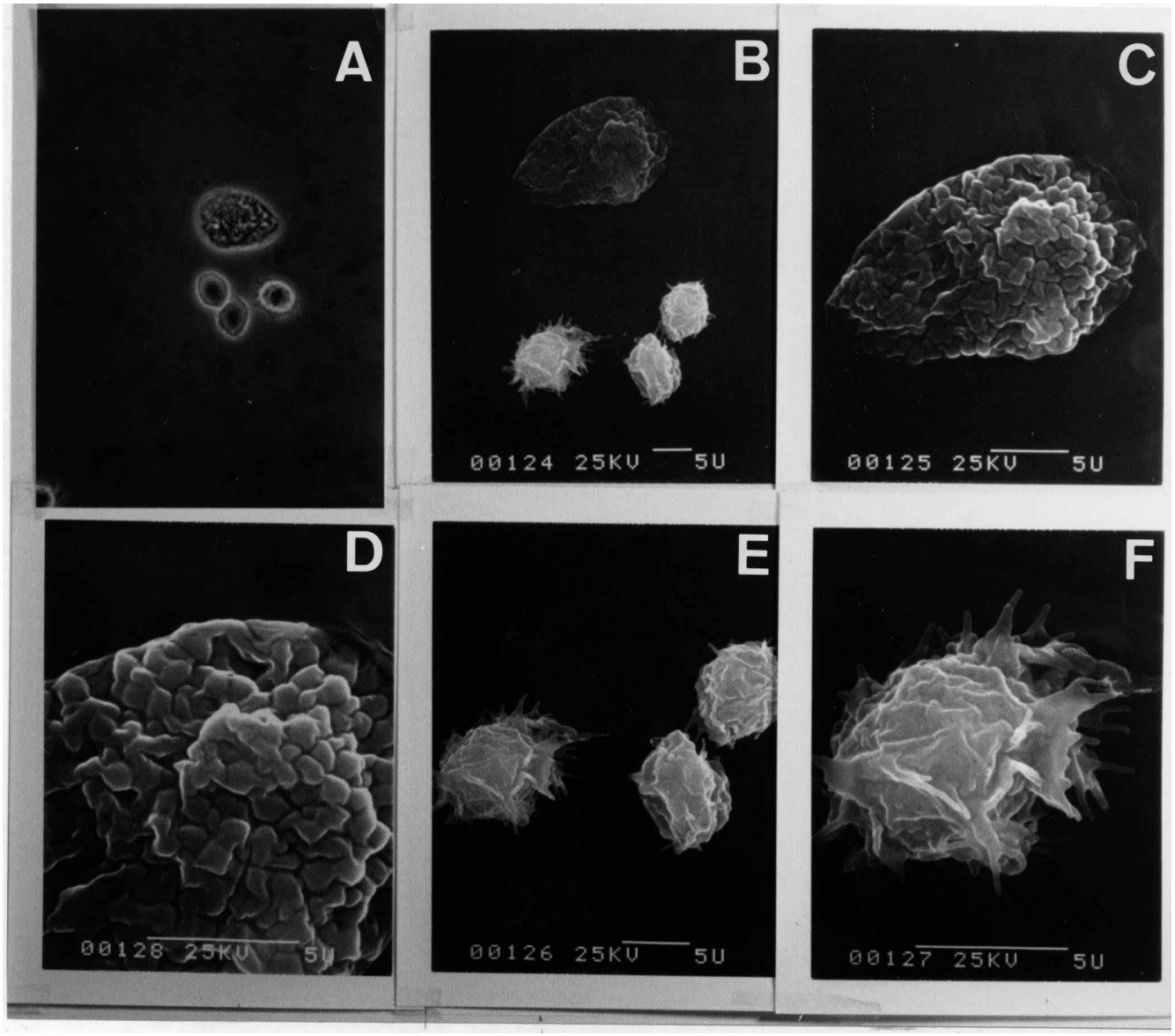
hemocytes fixed *in vivo* and cells fixed *in vitro* with formalin/glutaraldehyde but not subjected to Giemsa staining. A few artifacts which appeared to be clumps of stain were present on some cell surfaces.

Figures 20A–F illustrate how ultrastructural surface features can affect the appearance of hemocytes when observed with light microscopy. These hemocytes were first placed in a buffer solution before glutaraldehyde fixation, as described previously. When viewed by light microscopy the cytoplasm of the mature hemocyte appeared granular, virtually obscuring the centrally located nucleus. After marking the location of these cells, they were observed using SEM. The granular appearance of the mature cell was apparently caused by the very irregular cell surface and not by intracytoplasmic structures. The nuclei of the prohemocytes were also obscured by surface irregularities and pseudopodia.

Figure *20*

SEM mapping technique using buffer/glutaraldehyde fixation.

A. light microscopy photograph of Giemsa stained mature hemocyte and three prohemocytes (450x1
B. scanning electron micrograph of Giemsa stained hemocytes (1500x)
C. mature hemocyte (4000x1, note irregular surface
D. surface of mature hemocyte (8000x)
E. prohemocytes (3500x), note pseudopodia formation
F. surface of prohemocyte (8000x)


A
B
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C
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D
00128 25KV 5U
E
00126 25KV 5U
F
00127 25KV 5U

DISCUSSION

In insects, three types of tissue growth can be distinguished: (1) mitotic growth, (2) endomitotic growth, and (3) combinative growth (Romer and Eisenbeis, 1983). Unlike normal mitotic growth, endomitosis involves replication of DNA without subsequent cytokinesis. In combinative growth, mitotic and endomitotic growth are observed in the same tissue and/or cell.

The presence of somatic polyploidy (via endomitosis) in insects is a common occurrence. It was first discovered by Geitler (1939) in the water strider Gerris lateralis (L.) and high levels of polyploidy have been subsequently found in many holometabolous insects. According to Romer and Eisenbeis (1983), systematic investigations have been made in Lepidoptera, Hymenoptera, Coleoptera, Hemiptera, Nematocera, and Brachycera. Generally, tissue growth starts with a low polyploidy level and later increases during each molt of the insect. Among holometabolous insects, moderate levels of polyploidy have been recorded for hemocytes. Rasch et al. (1971) found 2C, 4C, and 8C levels in Drosophila hemocytes using microspectrophotometry and Feulgen stained cells.

Romer and Eisenbeis (1983), using cytophotometric methods, found that tissues of the cricket, Gryllus bimaculatus Degreer, reach only moderate levels of polyploidy. In Gryllus, at the completion of embryonic development the oenocytes, salivary glands, Malpighian tubules, and fat bodytissuesalreadyhaveadistinctpatternofpolyploidcells. Incontrast to holometabolous insects, these tissues do not increase their ploidy levels with each molt, but rather maintain the established ploidy pattern

throughout the organisms' life. In epidermal tissue, nerve tissue, and hemocytes, most cells were found to be diploid and undergo only mitotic growth.

The findings of Romer and Eisenbeis (1983) are not typical of all hemimetabolous insect tissues. Nair et al. (1981) found that DNA content among fat body cells of Locusta migratoria (L.) increased with age but did not undergo mitoses. Fontana (1974) reported that ploidy levels among fat body cells of Schistocerca gregaria Forskal were mostly 4C. Similar results were obtained by Besson-Lavoignet and Delachambre (1980) for epidermal cells of Tenebrio molitor (L.). In each case this was not interpreted as polyploidy but rather the result of resting G_2-phase cells which after mitosis immediately begin a new S-phase.

In this study, microspectrophotometric analysis of hemocytes was used to investigate whether growth of hemocytes in Periplaneta americana (L.) was mitotic, endomitotic or combinative. Comparison of sperm DNA content of Periplaneta with that of hemocytes, determined that the majority of hemocyte nuclei contained approximately twice the DNA content of haploid (1C) sperm nuclei and were therefore normally diploid (2C). Since the modal values of the hemocyte samples were interpreted to represent the diploid (G_1) condition, cells with significantly higher DNA values were either: (1) synthesizing (S-phase) cells, (2) post synthetic (G_2) cells, or (3) polyploid cells. DNA index histograms indicated that a small percentage (2.8-7.0%) of the nuclei measured from the hemocyte samples deviated significantly from the modal G_1 values and were

scattered between the 2C (DNA index = 1.0) and 4C (DNA index = 2.0) values, indicative of S-phase cells. Nuclei with approximately twice (1.85-2.15) the relative DNA content of G_1 cells could conceivably be either G_2 or polyploid 4C cells. These cells occurred at such a low frequency, it was inappropriate to assume they were true 4C polyploids. A more plausible conclusion was that they were post synthetic G_2-phase cells that had not entered the mitotic phase of the cycle. Values greater than 4C were not measured.

Based on data presented in the histograms, polyploids did not occur in prohemocytes or mature hemocytes of either the nymphs or adults of P. americana. Like Gryllus, hemocytes appear to remain diploid throughout the life of this organism.

Prohemocytes and mature hemocytes increased mitotically in both nymphs and adults as indicated by the presence of S-phase and G_2-phase cell populations. The relative proportion of G_1 (approx. 95%)and S-phase plus G_2-phase (2.8-7.0%) cells suggested that mitosis occurred at a low rate. Surprisingly, mature hemocytes in both nymphal and adult samples had the highest percentage of synthesizing cells. Mature nymphal hemocytes had 7.0%of their cells in the synthetic or G_2 stages, while 5.4% of the adult mature hemocytes were in the 5 or G_2 stages. Nymphal and adult prohemocyte samples showed the lowest percentage of actively synthesizing cells (4.4 and 2.8%respectively). Overall, nymphal hemocytes had a slightly higher percentage of synthesizing cells (5.7%)

than did adult hemocytes (4.1%).

No data have been published regarding ploidy levels of hemocytes in cockroaches, but several studies (Yeager and Tauber, 1932,1933; Taylor, 1935; Arnold and Salkeld, 1967) have inferred the presence and rate of mitosis from the identification of mitotic figures in hemocyte samples, i.e., the mitotic index. For cockroaches, the mitotic index was reported as 1.0% or less. Virtually all types of hemocytes undergo mitosis but prohemocytes are generally the most mitotically active cells. Data from this study indicated a slightly higher percentage of synthesizing cells in samples of mature hemocytes. This suggested that mature hemocytes contributed at least as much as prohemocytes, if not more, to the mitotic activity of the hemocyte population in both the nymphs and adults of P. americana.

Although a detailed analysis of mitotic figures was not performed, a few mitotic cells were observed in the Feulgen stained samples (figs. 2G,H,I). Since nuclear size was the basic characteristic used to separate prohemocytes from mature hemocytes in Feulgen stained samples, it was impossible to positively identify cells undergoing mitosis. The frequency of these figures was estimated to be less than five per thousand cells (<0.5%). This observation, along with similar published findings suggested that the percentage of DNA synthesizing hernocytes determined by spectrophotometry may have been too high.

Little information exists concerning the length of the cell cycle or its individual stages (G_1,S,G_2,M) in insect hemocytes. Without these data. it was impossible to establish accurate relationships or make

comparisons between individual stages. For example, an observed synthesizing frequency of 0.05 might be entirely compatible with a mitotic index frequency of 0.005 if the duration of the synthesis period lasted approximately ten times as long as the mitotic period.

In summary, the frequency of DNA synthesis, estimated by microspectrophotometry, was an indication of mitotic activity. This frequency will not necessarily be similar to that of mitotic figures present in a given hemocyte sample; however, they should be proportional to the mitotic rate.

From the hemocyte histograms, one obvious difference noted was the consistently smaller mean DNA value of prohemocytes compared to that of mature hemocytes. The most plausible explanation was that the difference was not due to an actual increase in DNA of the mature cells but rather a decrease in dye binding to the prohemocytes. Hale and Cooper (1965) reported that when human lymphocytes are transformed into blast cells, the nuclei enlarge and the apparent dye content increases before DNA synthesis begins. Using nuclear area as an index of compaction in nuclear leukocytes, Mayall and Mendelsohn (1967) observed a decrease in the apparent stain content corresponding to a decreased nuclear area. Sandritter et al. (1965) proposed that during hydrolysis, the aldehyde groups of compacted DNA are freed more slowly and thus bind a smaller amount of dye after similar hydrolysis times. As previously noted, the prohemocyte nuclei of P. americana are somewhat smaller in size than mature hemocyte nuclei (figs. 2C,D,E). As a result, the same amount of DNA is packed into a smaller area, as evidenced by the darker, denser appearance of the prohemocytes. This compaction may be responsible for

the consistently smaller DNA values of the prohemocytes rather than actual differences in DNA content.

Observation of the hemocyte complex of P. americana by light microscopy indicated that prohemocytes could be easily identified, but classification of the larger cells was in doubt. Plasmatocytes have been identified as the major component in the hemocyte complex of P. americana (Jones, 1951; Patton and Flint, 1959; Scott, 1971). Arnold (1972) reported that granulocytes constituted 85.9-93.6% of the hemocyte complex while plasmatocytes accounted for only 5.0-12.1% of the blood cells in the life stages of P. americana. Price and Ratcliffe (1974) reported that both plasmatocytes and granulocytes occur in P. americana but in many species the distinguishing characteristics between plasmatocytes and granulocytes are not well defined, i.e., "often it is impossible to distinguish the two cell types." Because of these ambiguous reports and the lack of differentiation observed in this study, the larger hemocytes were simply referred to as mature hemocytes. Generally, the mature hemocytes appeared more similar to plasmatocytes than to any other previously described cell type.

Mature hemocytes varied considerably in size and shape when prepared for observation with light microscopy. in contrast, the variations in cytoplasmic appearance, i.e., granules, spherules, etc., which serve as important classification characteristics were minor when cell fixation began immediately. When fixation was delayed *in vitro*, a wider range of intracytoplasmic variation was noted but many of these changes were observed in conjunction with cellular degredation. Why only certain cells go through this process remains to be determined, but it seems

improper to base a morphological classification system upon such variable characteristics and degrading cells.

Cellular appearance was also shown to be affected by fixation procedures for scanning electron microscopy. Glutaraldehyde, the fixative of choice for a wide variety of biological specimens, appears to be well suited for fixation of insect hemocytes and has been used in most electron microscopy studies. As demonstrated, direct exposure of hemocytes to glutaraldedhyde resulted in the loss of virtually all cells from the slide surface. Evidently this fixative acts so rapidly, hemocytes lose their typical adhesive qualities almost immediately.

Treating cells with first a weaker fixative such as 10%formalin, allowed hemocyte attatchment, but using formalin as the primary fixative was not suitable for maintaining the normal surface appearance of hemocytes examined by electron microscopy.

The best *in vitro* fixation procedure investigated was to initially fix the hemocytes with formalin, and after cell attachment replace the formalin with glutaraldehyde. The majority of these hemocytes appeared quite similar to those fixed *in vivo*. In addition, cells fixed using these procedures appeared very similar to hemocytes of the cockroach Blaberus giganteus (L.) observed by Arnold (1972) and Panesthia angustipennis (Dictyoptera) by Akai and Sato (1979).

Allowing hemocytes to settle in vitro without immediate fixation caused significant morphological changes in both prohemocytes and mature hemocytes. In response to the introduction into a foreign medium, the normally flattened cells send out numerous pseudopodia and cytoplasmic extensions. Whether this represents motility or is a phagocytic response

is unknown, but the cells' morphological appearance is reminiscent of human phagocytic white blood cells observed by SEM. It would be interesting to determine whether this morphological change could be manifested in vivo in response to the introduction of foreign substances.

Olson and Carlson (1974) found formation of pseudopodia in only a minority of P. americana hemocytes when observed by SEM. Hemocytes in their study were incubated for three hours in a buffer solution prior to glutaraldehyde fixation. Most of these cells were ellipsoid or spherical with small evaginations and indentations. However, the irregular appearance and unusually small size of their cells (6-8 microns) was indicative of poor fixation and/or preparation. Pseudopodia formation by hemocytes has been reported in a number of transmission electron microscopy studies such as Moran's (1971) observations of the cockroach Blaberus discoidalis Serville. In this case the entire leg was immediately immersed in glutaraldehyde for two hours and then rinsed overnight in buffer.

The use of the SEM mapping technique adapted from Wetzel et. al. (1973) has proven successful in the study of insect hemocytes. Little difference in the structural appearance of the hemocytes was detected between cells fixed *in vivo* and cells stained with Giemsa when using the formalin/glutaraldehyde technique. This method could, and should be used in future studies as an aid to interpreting variations in cells or types. in this study, the mapping procedure suggested that characteristics attributedtointernalstructuresofthehemocyteswhenobservedwith light microscopy may actually be the effect of fixation techniques on the cellular membrane. Figures 20A-F demonstrated how a mature hemocyte

might have been mistakenly identified as a granulocyte or spherule cell using light microscopy. With electron microscopy it was evident that the granular appearance of the mature cell was caused by irregular surface features and not intracytoplasmic inclusions.

In conclusion, the objective of this study was to look at some of the problems of insect hematology using microspectrophotometry and scanning electron microscopy. Using microspectrophotometric analysis of DNA content, it was determined that the hemocytes of P. americana are normally diploid in both nymphal and adult stages. No evidence of polyploidy was found. Additionally, the data indicated that a low degree of mitotic activity occurs but in contrast to some previously published studies, prohemocytes were not found to be the predominant mitotically active cell type. Observations of the hemocytes of P. americana with light and scanning electron microscopy generally indicated only minor variations in hemocyte appearance when cells were fixed immediately. However, the appearance of hemocytes can change significantly depending on the fixatives and preparation techniques used. It is felt that variations in these procedures are the predominant reasons for the abundance of described cell "types". Finally, it should be questioned whether -- morphological characteristics represent a valid basis for a useful classification system. When dealing with such labile cells as insect hemocytes, a more useful parameter might be determined and more properly applied.

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