CELLULAR ELEMENTS WITHIN THE BLOOD OF THE NEWFOUNDLAND BAIT SQUID ILLEX ILLECEBROSUS (LESUEUR, 1821) (CEPHALOPODA: COLEOIDEA): A STUDY IN FUNCTIONAL MORPHOLOGY

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CELLULAR ELEMENTS WITHIN THE BLOOD OF THE NEWFOUNDLAND BAIT SQUID ILLEX ILLECEBROSUS (LESUEUR, 1821) (CEPHALOPODA: COLEOIDEA): A STUDY IN FUNCTIONAL MORPHOLOGY

by

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A Thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

Department of Biology
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March, 1975

St. John's Newfoundland

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... when I in awesome wonder consider

all the worlds Thy hands have made ...

Stuart K. Hine

It is to my mother and father, and to Harold Johnson, that I would like to dedicate this effort, should it contain anything of merit or if it expresses any of the wonder of squid and the worlds created therein.

Abstract

The oegopsid squid Illex illecebrosus was selected for consideration by reason of the limited knowledge which surrounds the cellular elements of its blood and the potential significance these answers might have on its unique phylogeny.

Standard blood smear preparations were utilized to define a working image of those elements, later to be described as eosinophilic granulocytes. Incorporated within this study was the application of Villanueva stain which, having imparted greater contrast between nucleus and cytoplasm, made microvideomat analysis possible. Relationships between selected nuclear configurations and their areas, selected nuclear configurations and the associated cytoplasmic areas, and thirdly, between the nuclear and cytoplasmic areas were derived from such data.

Living preparations stained supravitally provided correlative information as to the nature and/or presence of cell organelles observed at the levels of light and electron microscopy.

Experiments were devised to establish the existence of a phagocytic mechanism and, if successful, the optimum concentration of carmine solution necessary to ellicit such a response. Favorable results prompted additional studies implemented with time-lapse

photography. The functional aspects of amoeboid movement and agglutination were elucidated by these means.

Further investigations employing electron microscopy thus completed this initial inquiry into the nature of cellular elements within the blood of the Newfoundland bait squid.

Data compiled from the foregoing research methods indicate that these circulating cells are eosinophilic granulocytes. The prominent nucleus which may assume one of six different configurations exhibits fine structure typical of mammalian organization, as do the other organelles. Mitochondria, granular endoplasmic reticulum, multivesicular bodies, vacuoles, pinocytic vesicles, and entities resembling lysosomes constitute the normal array of cellular components. Dispersed ribosomes are present as well. The possible role of multivesicular bodies in the pinocytic process are noted.

The ability of these leucocytes to phagocytize foreign material and to attain different positions or locations by means of amoeboid movement were verified through time-lapse photography.

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INTRODUCTION

A study of the circulatory system is, in truth, the study of a mechanism, for under its influence the activities of many bodily functions are co-ordinated; their products being distributed to all regions by means of the blood. One such product, a derivative of myeloid tissue, is the white blood cell. The reader must remember, however, that to stress this point of nomenclature (denoted by "blood cell") is to emphasize the technicalities proposed by semantics. Actually, leucocytes are not cells of the blood as terminology suggests, because their presence is merely of an opportunistic nature. Rather, they utilize the circulating fluid only as a means for getting where they are needed most. As a result, their importance as a defensive agent lies beyond the boundaries of their communication's network; the exception being any of the blood-oriented infections. Of interest are the cell organelles whose interrelationship with function make the protective role possible.

Such an overview defines, although rather briefly, the existing situation within typically mammalian species. Upon the author's good fortune of attending a Comparative Invertebrate class instructed by Memorial University's Dr. F. A. Aldrich and, later, the reading of a Ph.D. thesis by Dr. Helen Bradbury, a desire to investigate similar adaptations in the local squid became apparent.

This interest stems from learning that cephalopods have gone out on an "evolutionary limb". In fact, all molluscan groups have developed along somewhat isolated, highly specialized pathways. What is significant is the particular direction travelled.

That cephalopods are the most advanced mollusc, if not invertebrate, can be well documented. These animals have stimulated interest because of their unique adaptations, their importance to the economic scene and, quite naturally, their value to scientific endeavors related to ecology, physiology, embryology, paleontology and ethology.

Such an evolved organism is not without faults, however. Possibly the two most influential restrictions are to be found in the excretory and respiratory systems. The low blood pressure at which the renal organs work could not maintain the rapid filtration necessary for the successful osmoregulation in a more dilute environment (Wells, 1962, p. 150). Their commendable attempt to establish a successful respiratory system falls short when one considers it is always functioning at its physiological maximum. These deficiencies, together with the limited availability of copper (serving as the oxygen carrier) in the world's oceans, have prevented the development of a system adaptable to other niches, such as those found in fresh water.

The sad part of the cephalopod dilemma is that these animals appear to represent an evolutionary dead end. Even if more copper

were obtainable, the amount required would increase blood viscosity to a level detrimental for the established principles of blood pressure and velocity. With a sense of awe and pity, one cannot help but wonder if there are not other lessons to be learned from these unique, highly sophisticated animals.

Therefore, it is the purpose of this thesis to elucidate the morphology and correlative function(s) of hemocytes within the squid Illex illecebrosus (LeSueur, 1821), as they relate to the lifestyle of this exceptional organism.

However, before continuing, it would seem appropriate to review the classification of this species under examination. The outline proposed by Bradbury (1970, pp. 6-7) will familiarize the reader with upcoming morphological considerations and terminology.

Phylum Mollusca

Class Cephalopoda (Cuvier, 1797)

Subclass Coleoidea (Bather, 1888) Characterized by a single pair of gills and an internal shell considerably reduced (absent in some species of the subclass).

Order Teuthoidea Teuthidida (Naef, 1916) Internal shell restricted to rudimentary phragmacone; rostrum and pro-ostracum absent.

Suborder Oegopsida (d'Orbigny, 1839) Open eye (that is, without a cornea and directly bathed by sea water). The eye is closed by an eyelid, however.

Family Ommastrephidae (Steenstrup, 1857) (a) Presence of an inverted T-shaped hyponomal locking cartilage which is strongly developed. (b) Suckers of the sessile arms are biserial in

arrangement whereas those of the tentacular manus and dactylus are tetraserial, with the exception of those of the dactylus of the genus Illex. (c) Buccal membrane connectives of the arms attached to the arms in the formula D:D:V:D:, as first described by Verrill in 1880. (d) Anterior to the hyponomal locking cartilage, a muscular bridge passes from the hyponome to the ventrum of the head. (e) The caudal fin is less than 60% of the mantle length (Roper, et al., 1969):

Subfamily Illicinae (Posselt, 1890) (a) The hyponomal groove is smooth (Steenstrup, 1880), that is, it lacks both central and lateral foveolae, or pockets. (b) Photophores are lacking (Roper, et al., 1969).

Genus Illex (Steenstrup, 1880) There are four rows of suckers on the manus and eight rows of suckers on the dactylus of the tentacular arm (Ferussac and d'Orbigny, 1835-1848).

Species I. illecebrosus (LeSueur, 1821) (a) The hectocotylus is distinct, but less well developed than in the other three species of the genus, I. coindetii (Verany, 1837), I. argentinus (de Castellanos, 1960) and I. oxygonius (Roper, et al., 1969) (Aldrich and Lu, 1968; Roper, et al., 1969; Mangold, et al., 1969). (b) No tentacular locking or fixing apparatus present (Steenstrup, 1880).

Figures 1 and 2 supplement the above description.

Throughout those articles which comprise a historical review of the literature, there exists a great discrepancy with regards to the nomenclature of blood cells. In fact, nine different connotations are represented. For the sake of clarity and accuracy, then, this author has retained the original descriptive term proposed by each writer. Common synonyms like hemocyte, blood cell, corpuscle or cellular element were substituted where no such specification was given.

As early as three centuries before Christ, a desire to understand and appreciate the biology of cephalopods was expressed through the observations of Aristotle. Since that time, the volume of related

FIGURE I. A photograph illustrating the external anatomy of *Illex illecebrosus* (Lesueur, 1821)

(a) Preserved male specimen; ML 230 mm (ventrum)

(b) Preserved female specimen; ML 270 mm (dorsum)

1. Hyponomal Valve
2. Hyponome
3. Nuchal's Cartilage
4. Chromatophore
5. Tentacle
6. Sessile Arm
7. Eye
8. Mantle
9. Mid-dorsal Band of Pigmentation
10. Caudal Fin

Photography relied upon available preserved material, therefore, the difference in size between specimens is purely coincidental and not based upon sexual dimorphism.

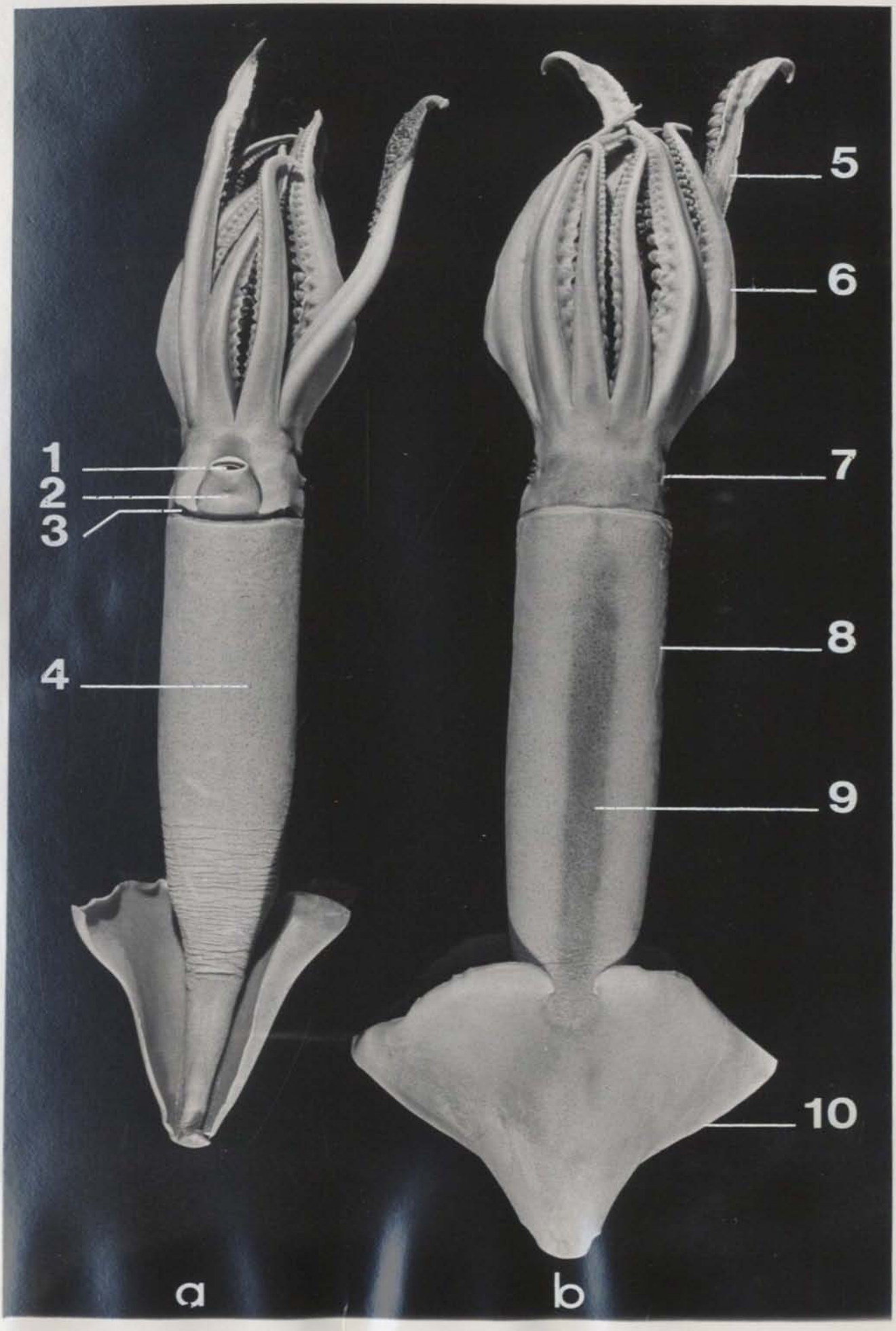
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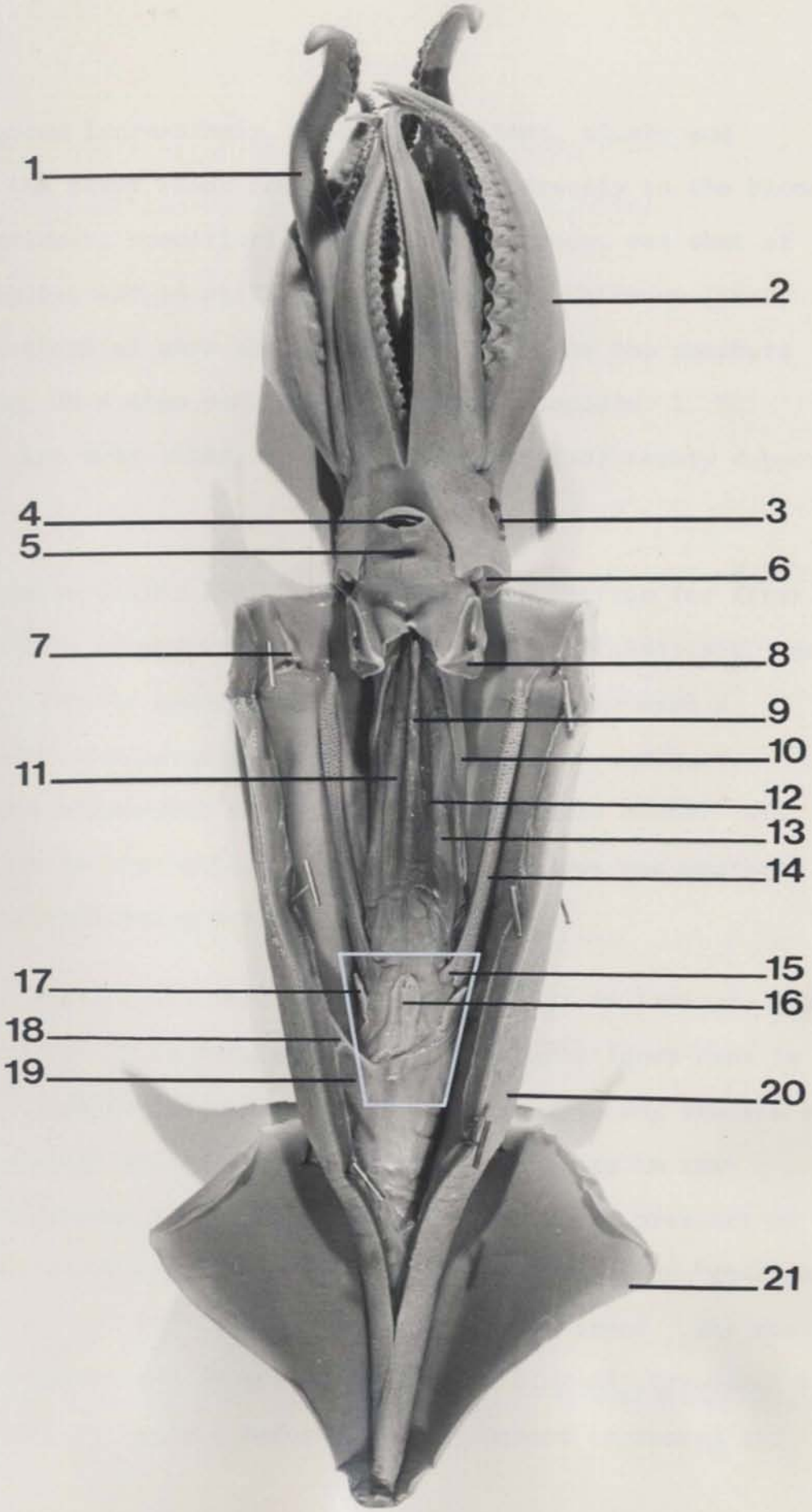
FIGURE 2. Internal anatomy of the Newfoundland bait squid *Illex illecebrosus* (Lesueur, 1821)

Preserved female specimen; ML 270 mm (ventrum)

1. Tentacle
2. Sessile Arm
3. Eye
4. Hyponomal Valve
5. Hyponome
6. Nuchal's Cartilage
7. Mantle Cartilage
8. Hyponomal Cartilage
9. Rectum
10. Hyponomal Retractor Muscle
11. Anterior Vena Cava
12. Ink Sac
13. Hepatic Gland
14. Ctenidium
15. Ctenidial Vein
16. Nidamental Gland
17. Oviduct
18. Anterior Mantle Artery
19. Mid-pallial Mesentery
20. Mantle
21. Caudal Fin

Anatomical details concerning that portion of the circulatory system enclosed by white lines are given in Figure 3.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literature has grown increasingly, although at times, slowly and inadvertently. The first study found pertaining directly to the blood cells of these animals, specifically the coleoid group, was that of Leydig (1857, species not identified). Quoting from Cattaneo (1889, p. 8), ". . . he (Leydig) says that in some cephalopods the amoeboid cells are colored; they also possess ramifying pseudopodia. . .". The work of Vogt and Yung (1888, species not identified) merely supported these findings.

It is Cattaneo (1889; 1891) who deserves recognition for first noting the phenomenon of agglutination within Sepia officinalis L. and Sepiola vulgaris (species questionable). Furthermore, he made a distinction between true pseudopodia that resulted from amoeboid activity, and those cytoplasmic expansions that precluded death. His attention was drawn to the bilobed nature of the nucleus and whether it predisposed multiplication by direct division.

Cephalopod blood cells were described by Cuénot in 1891 and 1897. His treatise, "Études sur le Sang et les Glandes Lymphatiques dans la Série Animale, 2e partie: Invertébrés", is well known to any student of invertebrate hematology for it spans this taxonomic range in its entirety. With reference to S. officinalis, he noted the presence of but one cell type; amoebocytes, measuring 15 μ and displaying "granules de ferment". Short, pointed pseudopodia were also observed. The nuclei were round, horseshoe-shaped or trilobed. Within Eledone aldrovandi Raf. this change of shape or nuclear deformation, as Cuénot expressed it,

continues until the division of the nucleus. Comments regarding these successive stages of amoebocyte degeneration were explained.

Knoll (1893) offers merely a summation of the results presented by these pioneer workers, with the concluding remark that he, too, supported their observations. His study included Eledone moschata Lam., Octopus tetracirrhus delle Chiaje, 1830, Octopus vulgaris L., and Sepia sp. Of particular interest are his colorful hand-drawn sketches which accompany the article.

The same year, Faussek made the statement that such nuclear configurations (as those previously mentioned by Cuénot) were artifacts; the true shape of the nuclei being influenced by the action of reagents. This opinion was formulated as a result of work involving three apparently undetermined species of the genera Loligo, Octopus and Sepia.

While investigating the anatomy of the octopod Opisthoteuthis depressa Ijima and Ikeda, Meyer (1907) disclosed the presence of "diffused" blood corpuscles within the animal's urine. He indicated these cells to be small and generally circular, with "clearly discernible spherical nuclei" (p. 210). Many granules of varying sizes were contained within the cytoplasm. It was noted that these structures impart a purple hue to corpuscles aggregated in concentrated masses.

The next major contribution is that of Kollmann (1908) who dealt with E. aldrovandi, E. moschata, O. vulgaris, S. officinalis and S. elegans d'Orb. Here interest centered around the hemopoietic

mechanism which, as a result, involved the white body and the subsequent cells that differentiated from it. The latter were described as acidophilic granulocytes possessing "simple" and polymorphic nuclei. At times "double" nuclei were observed. Familiar as he was with the various cellular elements represented throughout the different phyla, it is both interesting and surprising to note that Kollmann (1908, p. 70) found those of the Cephalopoda to be unique, saying:

> "Only one type of leucocyte exists within the cephalopods that I have studied. All are granular elements. This fact is worthy of remark. It is the only example with which I am acquainted. In all the other cases, one finds at least a certain number of hyaline elements interdispersed with the granular elements."

Following closely is the work by Isgrove (1909). She characterized the blood corpuscles of E. cirrosa Leach as being small, colorless, amoeboid, and very granular. These corpuscles measured 15μ in diameter and exhibited rounded or slightly curved nuclei.

Lange (1920) investigated the functional role of cephalopod hemocytes during her regeneration experiments on the appendages of O. vulgaris, E. moschata, and S. officinalis. It appears that wound healing was initiated by the agglutination of these leucocytes covering the exposed surface of the incision. Pseudopod formation was not encountered during this process, however the disappearance of the intracellular granules was noted. Originally distributed throughout

the cytoplasm, these latter structures were seen to "gather around the nucleus and along the cell wall and finally disappear" (Lange, 1920, p. 17). Accompanying the hyalinosis was a progressive elongation of both the cell and nucleus. Eventually, this transformed mass of agglutinated blood corpuscles will contribute to the building of new connective tissue.

Of importance to this area of molluscan cytology is Jullien's thesis published in 1928. Examining the cuttlefish S. officinalis, he cited the presence of round or oval cells, 7-8μ in diameter. The bilobed nucleus was excentrically placed within a cytoplasmic mass containing small eosinophilic granulations. Such was characteristic of cells transported by the circulating blood; the structural make-up undergoing change only within the connective tissue.

Kiyono (1929; from Ohuye, 1938, p. 608) reiterates the existence of this horseshoe or bilobed configuration attributed to the nucleus. No reference was made to the actual species observed other than it was an octopod.

In 1932, Sereni and Young noticed the great infiltration of octopod (O. vulgaris) amoebocytes to regions of nerve degeneration. These amoebocytes appeared to be engaged in active phagocytosis. Cellular division was also frequently encountered.

A comparative study of the leucopoietic organ within several members of the class Cephalopoda was undertaken by Noel and Jullien in

1933. It was found that the mature blood cells of S. officinalis possessed eosinophilic granulations and a polymorphic nucleus. The amount of cytoplasm varied; those cells with bilobed nuclei having much more than those with rounded nuclei. Furthermore, it seems that a direct relationship existed between the abundance of cytoplasm and the quantity of granulations. Mitotic figures were never observed.

As with the cuttlefish, the "corps blanc" of O. vulgaris was acknowledged as being the area of leucocyte production. However, the differentiated cells that represented the formed elements were not the same. Hemocytes exhibiting few, non-refractile, rather basophilic granules typified this octopod. Also deviating from that model recognized as "cephalopod" or, more accurately, "coleoid", were the blood cells of E. moschata. In this species, the granulations were very small and exclusively fuchsinophilic.

Last to be considered was the squid Loligo vulgaris Lam. Closely resembling Sepia, this animal had corpuscles which contained numerous acidophilic granules. The granules might differ in size, but all were highly refractile. Nuclei were relatively small and devoid of nucleoli. Generally, the chromatin was confined to an oval or spherical area, although a very clearly defined indentation was observed occasionally. The true polymorphic condition as expressed by the presence of two or more nuclear lobes was not evident.

Yeager and Tauber (1935) briefly discussed the total hemolymph cell count of the squid Loligo pealii LeSueur. After sampling nine animals (13 individual counts) a value of $7.0 \pm 3.6 \times 10^3/mm^3$ was obtained.

Three years later, Ohuye elaborated on the cell concept put forth by previous writers. From standard blood smears treated with Giemsa stain (prepared from Idiosepius paradox Ortmann and Polypus dofleini Wulker), he observed basophilic granulocytes in addition to the already established presence of the eosinophilis. On occasion, cells were noted to display both types of granules. Granulocytes with numerous basophilic rodlets were seen as well. A cellular diameter of 8-15μ was recorded along with strong amoeboid and phagocytic activity. The nuclei of these granulocytes were round, oval or reniform, however at times there existed a polymorphic condition. The author continues by saying, "Such a polymorphic nucleus is found also in the hyaline amoebocytes" (Ohuye, 1938, p. 608).

A mere reference to the existence of amoebocytes in the circulating blood was given by Tomsett (1939) in his monograph on S. officinalis.

Bolognari (1950; 1951; 1952) revived interest in the blood cell morphology of dibranchiates (S. officinalis and O. vulgaris) once again with his presentation of hand-drawn sketches similar to those of previous workers. His description of the mature leucocyte is worthy of

note in that it supports the findings of Ohuye (1938) e.g., the presence of basophilic granulations. Additionally, he made reference to the works of (a) Carazzi (1901) on O. vulgaris, O. de filippi Verany, S. elegans, S. officinalis, and Sepiola rondeletii Ststr., (b) Thore (1936) on O. vulgaris, and (c) Cazal and Bogorage (1943) on O. vulgaris. These researchers stated that the white body produces cells similar, if not identical, to those of the circulating blood.

Arvy (1960) spoke of the scant number of leucocytes present within the digestive tracts of the Octopoda, specifically O. vulgaris, and questioned their importance as "functional agents".

Due to the scarcity of available literature, special effort was made to secure any relevant source, and it is for this reason that Kawaguti's 1963 article on Sepia esculenta Hoyle, "Electron Microscopy on the Heart Muscle of the Cuttlefish" was included. For, although no formal description was given (his primary interest being the fine structure of muscle fibers) there is one transmission electron micrograph of a coleoid blood cell; the first published photograph of its type.

Studies into the nature of the phagocytic system within the octopods E. cirrosa, E. moschata, and Octopus sp. were made by Baginski (1965a). Upon injection of a trypan blue solution he found no cells containing grains of the dye. He concluded, therefore, that cephalopods were deprived of a phagocytic mechanism. In addition, he felt that these animals lacked true "blood" because the circulating fluid transported but one type of cell.

In another paper (1965b) he dealt with the parabranchial gland and its role in amoebocytopoiesis. Small cells, 5-7μ in diameter, possessing round nuclei and lightly acidophilic cytoplasm were assumed to be true amoebocytes. Baginski proposed a cellular life cycle which terminated upon their transformation into connective tissue.

Barber and Graziadei (1965) inadvertently captured an amoebocyte while investigating fine structure of the vascular system in O. vulgaris and S. officinalis. Cytoplasmic organelles which normally occur throughout the cell were noted as being (a) a system of rough endoplasmic reticulum, (b) small granules that appeared to be free ribosomes, (c) large (0.5μ) membrane-bound granules, and (d) numerous mitochondria. Also to Barber's credit (1968, in association with Martin and Boyde) is a scanning electron micrograph depicting a small portion of a vena cava from O. vulgaris and the spherical amoebocyte that rests on its inner surface.

Stuart (1968) proposed to elucidate what structures, if any, constitute the reticulo-endothelial apparatus of E. cirrosa. He found numerous phagocytes localized in the gills, salivary glands and the white body, along with individual leucocytes of the blood. All displayed abundant cytoplasm, eosinophilic granules and a diameter of 12-15μ. Their nuclear shape suggested the appearance

of human monocytes, as his accompanying light micrograph tends to support. Later, Stuart (1970) expanded his ideas to encompass the phagocytic cells of other invertebrate species and their responses to injury and the introduction of foreign materials.

Bradbury (1970) presented a comprehensive study of the Newfoundland bait squid, Illex illecebrosus. With regard to the formed elements of the blood, she distinguished but one cell type, displaying large, irregularly-shaped nuclei and limited amounts of cytoplasm. However, it is her photograph (light microscopy) which stands as the only one from the teuthoids.

The ability of cephalopod amoebocytes to pass through intercellular spaces (diapedesis) was noted by Young (1970) while investigating the neurovenous tissues of O. vulgaris, E. cirrosa and E. moschata.

Current literature, with its benefits of technological advancements, has brought forth several articles of peripheral interest. Owing to the very nature of the circulatory system, one can easily perceive the involvement of isolated cells in studies relating to well vascularized tissues or, indeed, the arteries and veins, themselves. In addition to the several papers already mentioned, the following are such examples.

A reconsideration of the leucopoietic organs of O. vulgaris by Cowden (1972) yields additional information. He employed irregular nuclear shape as the criterion for distinguishing mature leucocytes. Other features associated with this level of maturation were the presence of "basophilic, granular, cytoplasmic inclusions" (Cowden, 1972, p.114); a positive, cytoplasmic response to the PAS reaction; condensation of nuclear chromatin and the absence of a nucleolus. As before, one cell type prevailed; the eosinophilic components not having been preserved by the Zenker-formal fixation. An analogy to the vertebrate monocyte was proposed.

One year later (1973), in collaboration with Curtis, he extended these findings with observation of the properties of living cells dissociated from white body tissue of Octopus briareus Robson. Utilizing phase, differential interference, and fluorescence microscopy, they were able to clearly define but a single function - the capability to form "cell-to-cell associations", or cell aggregates. Also, emphasis was shifted from phagocytosis to the surrounding of and probably neutralization of foreign materials or invaders.

Recently, the team of Witmer and Martin (1973) have submitted a comprehensive account of these amoebocytes and the organelles contained within them. The cells, observed in blood sinuses of the branchial heart appendage of Octopus dofleini

martini (Pickford), had a diameter of 5μ-15μ and displayed both cellular and nuclear polymorphism. Present in the cytoplasm were vesicles and vacuoles measuring up to 2.2μ; highly osmiophilic (electron dense) granules, 0.1μ-1μ; electron light granules, 0.1μ-0.77μ; lysosomes, mitochondria, although rare, and a perinuclear arrangement of endoplasmic reticulum and free ribosomes.

This history of the elucidation of the characterization of cephalopod hemocytes is partially governed by two prevailing themes. First, there is a predominance of octopod and sepioid species having been studied, and, second, there is an inconsistent usage of terminology or nomenclature for the cells being studied. The former results from an inability to maintain pelagic teuthoid species in captivity for any length of time. Consequently, only five studies relate specifically to blood cells within such teuthoids (i.e., squid) (Faussek, 1893; Noel and Jullien, 1933; Yaeger and Tauber, 1935; Ohuye, 1938; and Bradbury, 1970). Also, apparent discord among these researchers as to how to name the cell or cell type arises from individual personal preferences and, most probably, the prevalent scientific style or usage of their day.

For these reasons, the author has endeavored to bring order into this confusing state of affairs by preparing the following table.

TABLE 1. Summary of data within references comprising a historical review of the literature.

Researcher(s)	Animal(s) Studied	Specific Area of Observation	Designated Cell Type	Cell Characteristics
Leydig (1857)	Not Identified	Not Identified	Amoeboid Cell	Colored; ramifying pseudopodia
Vogt & Yung (1888)	Not Ident.	Not Ident.	Amoebocyte	Irregular globules; some pseudopodia
Cattaneo (1889;1891)	S. officinalis	Blood from Branchial hearts	Amoebocyte	Round; 12-20μ; generally granulated; nucleus, 3-4μ; in some cells with larger volume, the nucleus is divided; here and there, pairs of adhering cells
	Sepiola vulgaris	"	"	Round or oval; 10-18μ; resembling those of Sepia with smaller, fewer granules
Cuénot (1891;1897)	S. officinalis	Branchial heart Blood from Branchial vessel	Amoebocyte	15μ; displaying "granules de ferment"; short, pointed pseudopodia; polymorphic nucleus
	E. aldrovandi	"	"	Presence of two nuclei
Knoll (1893)	E. moschata O. tetracirra O. vulgaris Sepia (sp. ?)	Heart	Leucocyte	Endorsed the findings of Cattaneo and Cuenot
Faussek (1893; 1901)	Loligo (Sp. ?) Octopus " Sepia "	White body	Not Ident.	Nuclear configurations (of Cuenot) artifacts; true shape influenced by action of reagents
Carazzi (1901)	O. De Filippi O. vulgaris S. elegans S. officinalis Sepiola rondeletii	White body	Not Ident.	Cells similar to those of circulating blood
Meyer (1907)	Opisthoteuthis depressa	Peripheral blood Branchial heart Urine	Blood Corpuscle	Small; generally circular; spherical nucleus; granules of varying sizes which impart purple hue to cells aggregated in concentrated masses
Kollmann (1908)	E. aldrovandi E. moschata O. vulgaris S. elegans S. officinalis	Blood from heart and/or branchial vessels	Leucocyte	Acidophilic granulocytes with "simple" and polymorphic nuclei; sometimes "double" nuclei
Isgrove (1909)	E. cirrosa	Blood from efferent artery at base of gill; anterior aorta; anterior vena cava	Corpuscle	15μ; colorless; amoeboid; very granular; round; slightly curved nucleus
Lange (1920)	E. moschata O. vulgaris S. officinalis	Blood vessels at site of incision (appendages)	Blood Corpuscle Leucocyte	Agglutinated cells cover incision; pseudopod formation not encountered; disappearance of intracellular granules noted; followed by progressive elongation of cell and nucleus; eventually transformed mass of cells contributes to building of new connective tissue

Jullien (1928)	S. officinalis	Blood Mantle connective tissue	Blood Cell	Round or oval; 7-8μ; eosinophilic granules; bilobed nucleus; ability to transform into connective tissue cells
Kiyono (1929)	An octopod	Circulating Blood	Not Ident.	Re-states occurrence of horseshoe or bilobed configuration of nucleus
Sereni & Young (1932)	O. vulgaris	Connective tissue	Amoebocyte	Infiltrates area of nerve degeneration; engage in active phagocytosis
Noel & Jullien (1933)	E. moschata	White body	Not Ident.	Very small; exclusively fuchsinophilic granules
	Loligo vulgaris	"	"	Numerous acidophilic granules that were highly refractile; nucleus small, devoid of nucleoli; round or oval; at time, indented
	O. vulgaris	"	"	Few, non-refractile, rather basophilic granules
	S. officinalis	"	"	Polymorphic nucleus; cells with bilobed nuclei exhibit more cytoplasm than those in which the nucleus is round; direct relationship between abundance of cytoplasm and quantity of granules
Yeager & Tauber (1935)	Loligo pealii	Circulatory system	Hemolymph cell	$7.0 \pm 3.6 \times 10^3$ /mm³ (cell count)
Thore (1936)	O. vulgaris	White body	Not Ident.	Cells similar to those of circulating blood
Ohuye (1938)	Idiosepius paradoxa Polypus dofleini	Peripheral blood	Granulocyte	8-15μ; basophilic, eosinophilic or combination of both granules; basophilic rodlets; strongly amoeboid and phagocytic; nucleus round, oval, reniform; at times, polymorphic
			Hyaline amoebocyte	Polymorphic nucleus
Tompsett (1939)	S. officinalis	Peripheral blood	Amoebocyte	Mentioned occurrence, only
Cazal & Bogorage (1943)	O. vulgaris	White body	Not Ident.	Cells similar to those of circulating blood
Bolognari (1950; 1951; 1952)	O. vulgaris S. officinalis	White body	Leucocyte	Round; 7-9μ; eosinophilic granules, sometimes in association with basophilic granules; polymorphic nucleus; nucleolus absent
Arvy (1960)	Octopoda (O. vulgaris)	Lower Digestive tract	Leucocyte	Questioned functional role in digestion/assimilation processes
Kawaguti (1963)	S. esculenta	Lumen of ventricle	Blood cell	Transmission electron micrograph

Baginski (1965;a,b)	E. moschata E. cirrosa Octopus (Sp. ?)	Parabranchial gland Peripheral blood Connective tissue	Amoebocyte	5-7μ; lightly acidophilic cytoplasm; round nucleus; ability to transform into connective tissue cells
Barber & Graziadei (1965)	O. vulgaris S. officinalis	Peripheral blood vessel	Amoebocyte	System of rough endoplastic reticulum; small granules, thought to be free ribosomes; 0.5μ membrane-bound granules; numerous mitochondria
Martin, Barber & Boyde (1968)	O. vulgaris	Inner surface vena cava	Amoebocyte	Scanning electron micrograph
Stuart (1968)	E. cirrosa	Gills Peripheral blood Salivary glands White body	Phagocyte Leucocyte	12-15μ; abundant cytoplasm, eosinophilic granules; nuclear shape similar to human monocytes
Bradbury (1970)	Illex illecebrosus	Peripheral blood	Formed element Blood cell	Large, irregularly shaped nucleus; limited amounts of cytoplasm
Young (1970)	E. cirrosa E. moschata O. vulgaris	Capillaries	Amoebocyte	Ability to pass through intercellular spaces (diapedesis)
Cowden (1972)	O. vulgaris	Blood from branchial hearts White body	Leucocyte	Basophilic granular inclusions; positive, cytoplasmic response to the PAS reaction; irregular nuclear shape; condensation of nuclear chromatin; nucleolus absent
Cowden & Curtis (1973)	O. briareus	White body	Leucocyte Hemocyte	Capacity to form cell-to-cell associations
Witmer & Martin (1973)	O. dofleini martini	Blood sinuses of branchial heart appendage	Amoebocyte	5-15μ; cellular and nuclear polymorphism; vesicles and vacuoles up to 2.2μ; electron dense granules 0.1-1.0μ; electron light granules 0.1-0.77μ; lysosomes; few mitochondria; perinuclear arrangement of endoplasmic reticulum and free ribosomes

METHODS AND MATERIALS

General Procedure for Squid Collection

Squid were collected between the months of July and September, 1972-1973, during which time they came inshore at points near Holyrood and Portugal Cove, Conception Bay. The traditional Neyle's patent hand-line jigger and the mechanized Japanese model (Quigley, 1964) were employed for the actual capture. Once secured, the animals were placed in a large, plastic container in which the sea water was periodically changed until the boat docked. At this time the squid were transferred to circular tanks of 275 gallon capacity and quickly transported by truck to the Marine Sciences Research Laboratory at Logy Bay. Here, they were maintained in similar tanks supplied with cold (9-12°C) circulating sea water. Dead capelin (Mallotus villosus Müller) suspended from a mono-filament line, as first perfected by Bradbury and Aldrich, 1969, were approached by these teuthoids with varying results. However, this food would be ignored should it be taken from the line during feeding or dropped to the tank bottom for other reasons.

Extraction of Blood

Due to the fact that major vessels lie protected within the mantle cavity, dissection is required when obtaining blood samples. A 2% solution of ethanol in sea water recommended by

Mangold (personal communication) was used as a general anesthetic. On several occasions, methanol was substituted with equally good results. Flaccidity and chromatophore contraction (paling) denote submission.

A ventral, mantle-length incision was made with Lister's bandage scissors; the lower blade of which terminates bluntly. Such a precaution avoids piercing the delicate tissues of the viscera. Plastic, 5 cc syringes fitted with disposable 23 gauge needles were utilized to obtain blood from the posterior venae cavae, the ctenidial veins and, less frequently, the branchial hearts (Figure 3).

The choice of plastic instruments over those of glass was due to several advantages; the foremost being that their surface reduces the possibility of cell clumping (Stuart, 1968, p. 402). The amount of blood extracted varied from 1 cc to 6 cc depending upon the animal's size and/or degree of caecal distension.

Light Microscopy

Stained Preparations. The author followed standard laboratory techniques used in the preparation of thick and thin blood smears, the only deviation involved the substitution of a modified slide when making thin smears according to the two slide method (Cinchona Products Institute, Inc., 1948, pp. 1-3). Such

FIGURE 3. Generalized diagram of squid ctenidia and associated vascular structures, with sites of blood sampling indicated. (Modified from Beck and Braithwaite, 1962, p. 187). Black squares denote sampling points.

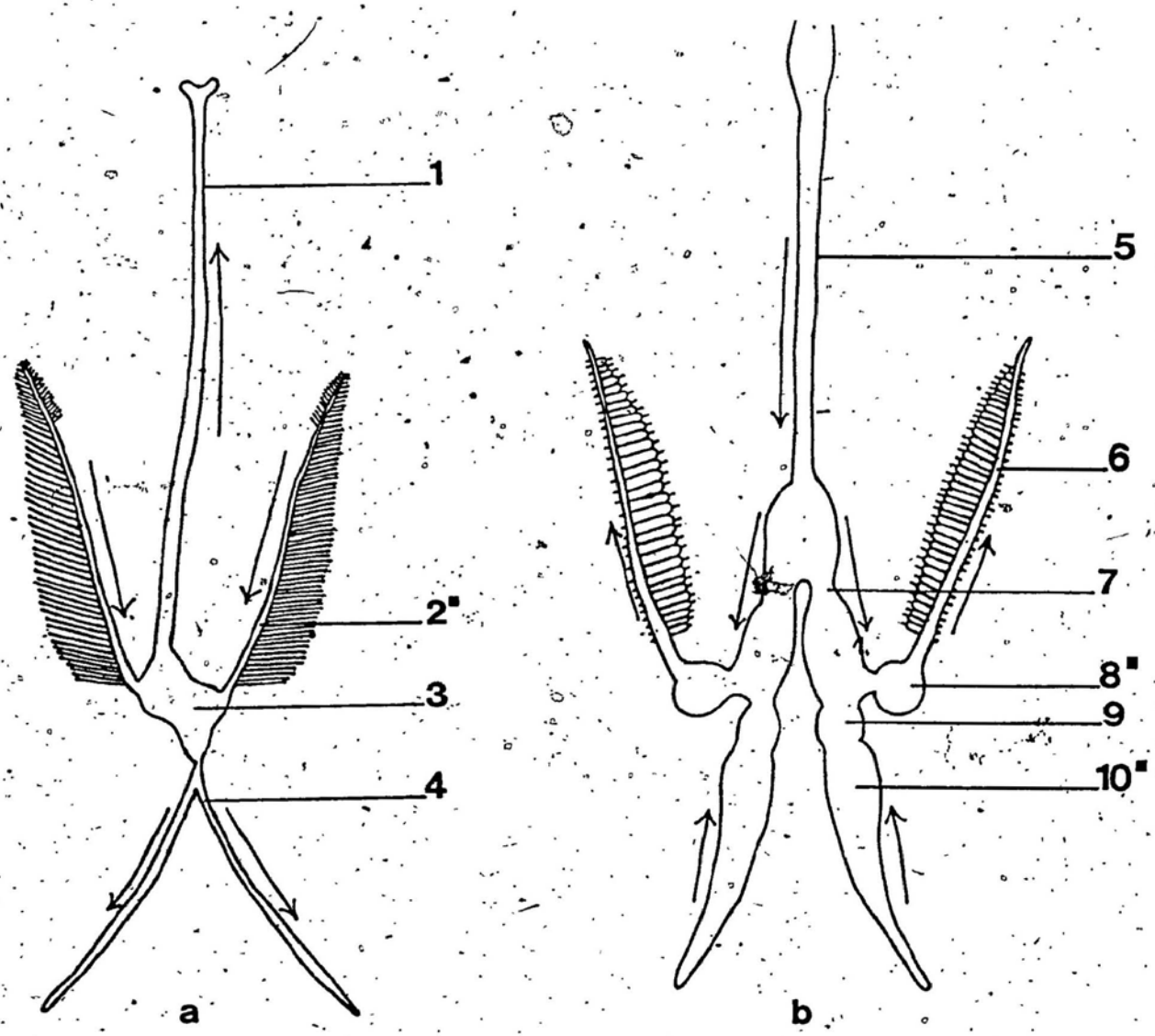
a. Arterial system

1. anterior aorta
2. ctenidial vein
3. systemic heart
4. lateral mantle artery

b. Venous system

5. anterior vena cava
6. ctenidial artery
7. renal portion of anterior vena cava
8. branchial heart
9. renal portion of posterior vena cava
10. posterior vena cava

Arrows indicate direction of blood flow.


1
2"
3
4
a
5
6
7
8"
9
10"
b

a tool is easily constructed by scoring across one corner of a slide (Figure 4.1) and breaking this fracture under water with the aid of pliers. The resulting smear is then contained within certain boundaries (Figure 4.3), unlike the routine practice illustrated in Figure 4.4. This procedure is especially helpful when examining parasites or the blood corpuscles of marine invertebrates because it concentrates the small quantity of material into a specific area, making its examination much easier. However, even this measure proved to be unsuitable for the hemocytes of I. illecebrosus, therefore the author resorted to thick smears which were successful.

While most preparations were allowed to air dry, some were fixed at arbitrary (5, 7-8, and 10 minute) intervals with a 10% solution of formalin in sea water. The latter was absorbed by use of paper toweling attached to the underside of an inverted petri dish. Realizing that physical distortions occur during drying, it was felt that immediate fixation might reveal a truer picture of the cells being studied.

Further variations included the utilization of a circular, 18 mm plastic retainer which had been affixed to a slide with vaseline. The well, 14 mm x 2 mm, was filled with several drops of squid blood after which the entire setup was placed in a refrigerator and maintained at 4°C. It was hoped that the

FIGURE 4. Preparation and use of "spreader" when making thin blood smears by the two slide method

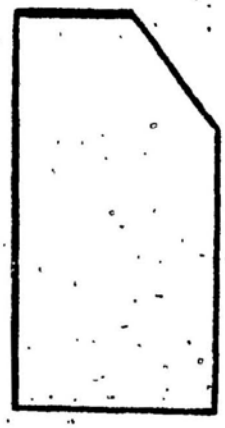
1. Scoring of upper right corner to facilitate fracture.

2. Working implement, i.e., the "spreader".

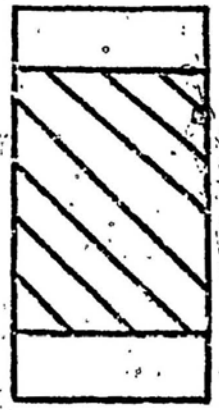
3. Blood smear prepared by a standard (unmodified) slide.

4. Blood smear prepared by use of "spreader" as shown in 2.

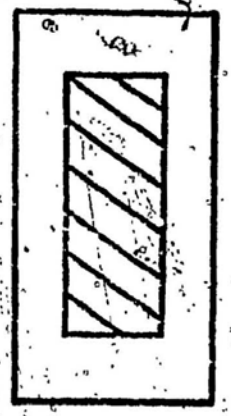
1



2



3



4

intervening time spent in this cold, simulated environment would permit the corpuscles to settle out and, subsequently, form pseudopodia. As a means of comparison, this technique was repeated at room temperature. These slides were also air dried prior to staining.

All preparations were then stained following the widely used and recommended treatments of Giemsa, Leishman, and Wright (Carleton and Drury, 1962). The recently developed stain Villanueva (Villanueva, 1970) proved to be an excellent supplement. Finally, a lasting record was made by applying Permount and coverslips.

Most generalizations regarding cellular morphology were formulated upon examination of blood preparations such as these. However, the information for cellular analysis through microvideomat observations was to be provided only by those slides stained with Villanueva's mixture. This particular compound imparted greater contrast between the cytoplasm and nucleus, thereby producing a clearer image necessary for work of this nature.

A Zeiss photomicroscope equipped with a 35 mm camera was employed when taking photographs of this material. Images enhanced with a green filter and magnified up to 1000x were recorded on black and white panchromatic film, Kodak Plus-X Pan. Kodak photomicrography film 2483 (blue filter) was used for color prints. Standard methods established for the development and printing of such film were followed.

Microvideomat Studies. This instrument computes area (in this case, nuclear and cytoplasmic dimensions) by measuring the amount of light which falls on a photosensitive screen. It is comprised of three interworking parts: a Zeiss photomicroscope, adaptable to phase contrast microscopy and equipped with a 35 mm camera; a video monitor; and a digital readout device.

To operate, one places a slide on the specimen stage and focuses an image at the desired magnification. As with any such observation, it is best to concentrate on randomly dispersed cells that are both complete (whole, not in a process of lysis) and single (not agglutinated). Having chosen the appropriate cells, one opens the camera stop fully which transfers the original image to the viewing screen. Now, one is able to discriminate the region to be measured by superimposing a white counterpart or likeness. All surroundings not included for analysis are then eliminated by blackening the remaining portion of the screen. The digital readout immediately issues a raw value indicating the area of that isolated, white "shape" on the monitor. For final tabulation, one multiplies this number by the calibration appropriate to the magnification used.

Due to the heterogeneous staining properties of both the nucleus and cytoplasm, discrepancies result which interfere with total discrimination of the desired portion to be studied.

However, this phenomenon involves each observation so that a consistent method of evaluation should eliminate or at least neutralize such errors.

One hundred blood cells were processed by the microvideomat method in the hope that a system of classification for hemocytes might be based on nuclear and cytoplasmic areas.

Living Preparations: 1. Supravital Staining. Supravital staining augments any research of living cells *in vitro* through the application of selected, relatively non-toxic dyes. This method was therefore employed to supplement previously acquired knowledge (from fixed and stained preparations mentioned earlier), regarding the complement of organelles possessed by the hemocytes of *I. illecebrosus*.

A prescribed technique using potassium dichromate in sulfuric acid was followed to insure chemically clean, dust- and grease-free glassware. Stock solutions of neutral red and Janus green B were prepared by standard methods (Doan and Ralph, 1964). These dyes were then applied to glass slides either separately or conjointly. Once made and allowed to dry, the dye films may be stored for later examination, when a few drops of fresh squid blood were placed on one of the slides and a coverslip added. Within five to ten minutes, internal cellular structures could be distinguished. As in the previously indicated procedures, microscopic observation was made at magnifications up to 1000x.

2. Phagocytosis Experiment. Carmine was selected for making a suspension of particulate matter. Solutions of 0.01, 0.1, and 1% were prepared with sterile, distilled water; any large granules being removed by coarse filtration. This approach was favored over the use of sea water for it provided a more inert and therefore compatible medium.

Test tubes in the actual experiment contained equal volumes of blood (1 cc) and one drop of a specific particulate suspension. Blood was delivered in measured quantities by a 5 cc plastic syringe fitted with a 21 gauge needle, while the carmine solutions were added with a 1 ml serological pipet. Each tube was then sealed with Parafilm, to restrict air contact, and placed in a 4°C refrigerator.

After one hour, standard wet mount preparations were made for examination. Favorable results together with the apparent normality of these blood cells suggested further study involving time-lapse photography.

3. Time-Lapse Observations. This attempt to elucidate cellular function was greatly facilitated by the means of time-lapse photography. A Nikon inverted phase contrast microscope, model M, equipped with a Vinten scientific camera (200 foot magazine)

and the Vinten D.C. control intervalometer was used throughout the various studies. It should be noted that both microscope and camera were situated on a board which, in turn, rested upon six tennis balls to minimize vibration. Furthermore, simultaneous viewing of the field being photographed was made possible by a Wild side tube viewer that transmitted 25% of the light.

Regular procedures outlined for squid dissection and blood extraction were followed when obtaining material for these wet mount preparations. The latter were then sealed with liquid paraffin and examined for hemocyte motility and agglutination. Observations regarding phagocytosis were made from squid blood samples retained within a 35 mm Teflon ring; the actual dimensions of the well being 20 mm x 5 mm. A "window-pane" coverslip, 45 mm x 50 mm, was added so that the 1% carmine suspension could be introduced when desired.

Each sequence was photographed at room temperature using black and white 16 mm film exposed at 4-second intervals. Examination of the processed film was greatly assisted through the use of a Bell and Howell directamotion which enabled frame-by-frame viewing. For the purpose of printing a photographic succession illustrating amoeboid movement or the engulfment of particles, negative release prints (not timed) were made from the original footage.

Electron Microscopy

Cell studies pertaining to the hemocytes of this ommastrephid, Illex illecebrosus, were concluded with a brief consideration of ultrastructure. Four different approaches to the methodology employed during tissue preparation were followed.

Initially, whole squid blood was spun down at room temperature for 25 minutes at setting number 7 on an International Clinical bench-size centrifuge, Model CL. The white, sheet-like precipitate (approximately 2 mm) was cut into small pieces and then fixed with such standard agents as Karnovsky, 2.5% gluteraldehyde or 10% formalin in sea water over a period of 1.5 hours or 2 hours at 4°C. The fixative was then removed and a 0.1 M solution of sodium cacodylate buffer (pH 7.4) added and left overnight. Osmium tetroxide made up as a 1% solution with s-Collidine buffer was used to postfix the "pellet" for 30 minutes before block staining with uranyl acetate. The latter was a saturated, aqueous solution in which the tissue mass was immersed for 30 minutes. Dehydration through a series of graded ethanol followed. One hour within the clearing agent, acetone, preceded a 50:50 mixture of the same and Spurr, the embedding resin. Lastly, the hemocyte material was transferred to a mold or capsule filled with the full-strength resin and placed in an oven for 24 hours at 60°C. Tissue samples from the gills, branchial hearts,

liver, and mantle were processed in the same manner.

A second approach involved immediate fixation, the agent having been drawn into the syringe before blood extraction. Centrifugation followed as did the remaining sequence outlined previously. However, this schedule was greatly accelerated through the employment of a rapid method developed by Rowden in 1973.

Alternately, a 35 mm Teflon ring (20 mm x 5 mm well) was affixed with vaseline to a plastic-covered slide. Fresh squid blood used to fill the well was obtained following regular procedures. This setup was then placed in a refrigerator for 20 minutes and maintained at 4°C. It was felt that such a technique would retrieve hemocytes while by-passing the complications associated with centrifugation. At the appropriate time, the fluid portion of the blood was removed; the cellular elements having attached themselves to the plastic substrate. A 10% solution of formalin in sea water and 2.5% gluteraldehyde were employed (separately) as fixatives with further processing according to the slow or regular method cited earlier. Embedding required that the hemocyte-containing area of plastic be cut from the original piece before subdividing and transferring it to the embedding capsule.

Finally, this same approach was used with the exception that the blood sample remained at room temperature. For the purpose

of studying phagocytosis at high resolution a 1% solution of carmine was added during the interval allocated for hemocyte settling.

Polymerized resin blocks, containing material processed according to these four methods, were then trimmed and sectioned on a Huxley microtome using glass knives. Silver sections (500-600 Å) were picked up on Formvar-coated, 200 mesh copper grids and stained with lead citrate (Venable and Coggeshall, 1965). A Philips 300 electron microscope equipped with a 35 mm camera was employed to view these preparations, all photographs being taken with Kodak fine grain positive safety film or Kodak electron image plates.

RESULTS

Light Microscopy

Stained Preparations. As indicated previously, the following morphological description was based upon random observations utilizing stained blood cell preparations. Only two restrictions were stipulated throughout this study; that the hemocytes be intact and singular. Further examination of the phenomena that surround agglutination will ensue.

The blood cells of *Illex illécebrosus* are leucocytes and have a cellular diameter which may vary from 16μ to 24μ; the average being 20μ to 22μ. Table 2 outlines the distribution of this range. Generally, these cells are round or oval in shape although, infrequently, protoplasmic extensions are seen. The latter appear to be initial stages in the formative process of pseudopodia.

Each nucleus is relatively large (Figures 7, 9) in comparison to the mass of surrounding cytoplasm and exhibits an intense pinkish-purple coloration. Also typical is the highly vacuolated nature of this area which imparts to the chromatin a coarse, network-like appearance. It (the nucleus) is polymorphic with the particular degree of nuclear indentation, that gives rise to the number of lobes, seemingly deriving its origin from

TABLE 2. Diameter tabulations for the cellular and nuclear components of 100 blood cells randomly selected from a blood smear preparation from Illex illecebrosus.

CELLULAR DIAMETERS (μ)	FREQUENCY OF OCCURRENCE
16	1
17	1
18	1
19	9
20	25
21	25
22	27
23	10
24	1

NUCLEAR DIAMETERS (μ)	FREQUENCY OF OCCURRENCE
15	4
16	15
17	26
18	30
19	20
20	4
21	1

the nuclear "face". The opposing region of nuclear periphery is rounded, conforming to the cell membrane along which it is closely associated. The majority of hemocytes possess exceptionally dark concentrations along this "rounded" portion and, occasionally, the existing lobes are defined. Nucleoli were not visible.

The cytoplasm is basophilic, forming a homogeneous matrix of coarsely granular composition. Vacuoles constitute one of the normally occurring organelles. They measure 1μ in diameter. Large, oval and elongated refractile bodies are often seen as well, being located free within the cytoplasm or, more generally, encroaching the nucleus. Although usually present as single entities they may exist in pairs. Their dimensions span a range from 2μ x 1μ to 5μ x 6μ in diameter. Occasionally one may observe basophilic rods and spheres. The former (approximately 0.7μ) shadow the rounded portion of the nucleus while the latter (diameter: 1μ) appear in the cytoplasm or over the nucleus. The leucocytes of I. illecebrosus also possess eosinophilic granulations which measure 1μ in diameter. It is interesting to note that two variations may exist; an indiscernible mass assuming the form of a pink haze and clusters or concentrations which measure up to 3μ in diameter. From these three conditions the following combinations have been observed: (1) concentrations, individual granules, haze; (2) concentrations, individual granules; (3) individual granules, haze; (4) individual granules, solely.

Figure 5 is a composite display illustrating the various morphological characteristics attributed to these coleoid hemocytes. Included is a representative photograph of cells found within the central region of a blood smear. They exhibit strange qualities in that their cytoplasm and cellular inclusions stain according to those cells along the smear periphery, however the nucleus is always devoid of its typical coloration, forming an eerie contrast of white.

Microvideomat Studies. Data collected through microvideomat analysis have been tabulated in Table 3; the nuclear and cytoplasmic diameters of each hemocyte being included as supplementary information. These values become meaningful, in terms of establishing a system for blood cell classification, when correlated with the corresponding nuclear configuration. Therefore, it became important to categorize each chromatin mass according to the number of lobes displayed.

Of the one hundred cells observed, 85 could be assigned to one of six different categories. Fifteen remain undefined, although suggested groupings have been offered for ten. The criterion upon which the above categories were based was the degree of nuclear lobulation (Figure 6). This variation in shape may be displayed as: (1) the nucleus being a compact oval or spherical mass; (2) a slight indentation; (3) an increasingly prominent indentation; (4) an accentuated reniform, this bilobed state occasionally exhibited as ʒ-shaped; (5) a trilobed configuration;

FIGURE 5: Composite photograph illustrating the general morphology of cellular elements within the blood of Illex illecebrosus.

1. Nuclear configuration type 4a (bilateral indentation), eosinophilic granule within upper portion of cell is the "concentrated" variation.

2. Note especially the large refractile organelles (on either side of nucleus) defined from electron micrographs as multivesicular bodies. A single eosinophilic granule is situated between the nucleus and the refractile body to its right.

3. Exemplifies the staining properties of hemocytes found within the central portion of a blood smear and additionally, the nuclear configuration designated as type 5b (trilobate).

4. The extension of a lobate pseudopodium. The pinkish area between this extension and the nucleus represents the "haze" type of eosinophilic granulation.

5. Linear arrangement of agglutinated blood cells.

(Villanueva 1,210x)

1

2

3

4

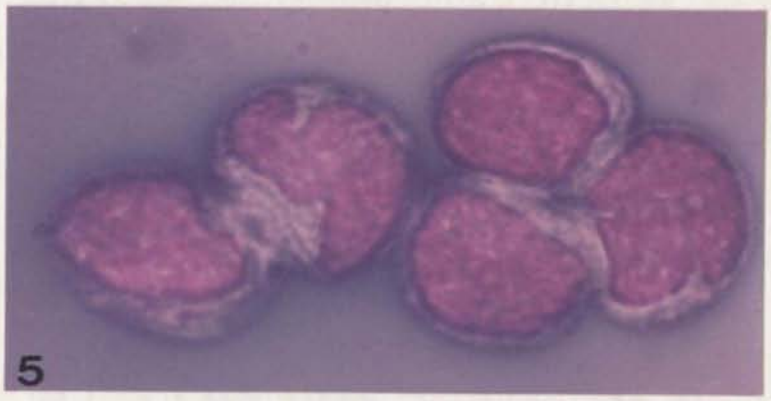

5

TABLE 3. Microvideomat analysis of 100 blood cells randomly selected from a smear preparation having required the fresh blood of Illex illecebrosus.

An asterisk accompanying a number which refers to the type of nuclear configuration (column 2) denotes a suggested grouping for that particular chromatin mass.

A blank denotes the nucleus remains undefined.

Cell No.	Type of Nuclear Configuration	Nuclear Diameter (μ)	Nuclear Area ($x10^{-4}$ mm^2)	Cell Diameter (μ)	Cytoplasmic Area ($x10^{-4}$ mm^2)
1	5	17	4.96	22	.96
2	4	19	4.88	22	.84
3	1	18	3.68	22	.80
4	5	18	5.84	22	.92
5	1	18	4.76	21	.60
6	3	20	4.40	22	1.20
7	2	17	3.72	20	.56
8	4	18	4.56	21	1.64
9	5	16	4.00	19	.76
10	1	18	6.12	22	.48
11	2*	19	4.12	23	1.00
12	1	18	4.44	23	2.36
13	4	17	4.00	21	1.28
14	3	18	3.96	22	1.84
15	2	17	4.48	21	1.72
16	4	16	4.72	20	.52
17	5	18	4.60	22	.92
18	2	15	4.80	21	.80
19	2	17	5.32	20	.64
20	4	19	5.36	21	.64
21	2*	17	5.56	20	.48
22	2*	18	5.20	20	.64
23	1	19	5.24	22	.60
24	3	17	4.32	19	.64
25		15	4.24	19	.56
26	5*	20	5.16	22	.56
27		19	5.32	23	1.28
28	2	19	5.12	21	.96
29	5	17	4.36	20	.84
30	3	17	4.72	21	.48
31	1	16	3.88	17	.64
32	3	18	5.64	21	.64
33	2	17	5.52	22	1.20
34		19	5.12	21	.40
35	2*	21	6.56	24	.36
36	1	17	5.28	20	.36

(continued)

Table 3 continued

37	1	19	6.92	22	.56
38	2	17	4.76	19	.20
39	2*	19	5.12	22	.68
40	3	16	3.92	19	.20
41	1	18	5.76	21	.36
42	2	18	5.36	21	.56
43	3	18	5.00	21	.56
44	5	17	5.04	20	.60
45	5*	18	5.04	22	.80
46	5	17	4.68	20	.84
47	2	19	5.52	22	1.44
48	5	18	5.56	22	1.16
49	5	18	4.32	23	1.56
50	2	18	4.68	23	1.24
51	3	19	5.48	21	.32
52	2	17	4.68	21	.96
53	3	18	5.68	21	.64
54	2	16	3.92	19	.76
55	3	17	4.72	21	.76
56	3	17	4.36	20	.68
57	2*	19	5.32	22	1.00
58	5	18	6.00	22	1.12
59	2	15	1.36	17	.32
60	1	18	4.80	20	.52
61	3	16	3.96	20	.40
62	1	18	5.60	20	.72
63	3	20	6.28	22	.84
64	3	17	4.72	20	.68
65	1	17	5.60	20	.28
66	4	18	7.40	22	1.20
67	6	18	7.12	23	.84
68	1	18	4.92	22	1.00
69	4	18	4.84	21	.76
70	1	19	5.92	22	1.12
71	2*	19	6.12	22	1.08
72		16	4.64	20	.76
73	3	17	3.80	19	.44
74	2	16	4.20	20	.48

(continued)

Table 3 continued

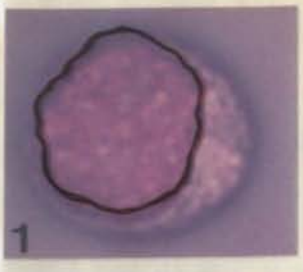
75	2	18	4.48	21	.56
76	3	18	4.72	22	.64
77	2	17	4.56	19	.56
78	4	18	4.84	21	.64
79	1	16	4.32	20	.48
80	1	19	6.00	22	.68
81	1	17	3.76	20	1.00
82	1	19	5.60	23	1.52
83	3	17	3.88	21	.60
84	6	18	6.32	23	1.08
85	3	19	5.28	21	.88
86	3	20	3.84	23	.88
87	4	17	5.16	22	1.40
88	2	16	4.56	20	.92
89		16	4.32	20	.52
90	2	16	3.76	19	.76
91	2	17	4.60	20	.64
92	1	16	3.72	20	.44
93	3	17	4.44	21	1.20
94	2	19	4.76	21	.48
95	2	16	4.08	18	.68
96	5	19	5.60	23	.64
97	1	15	4.60	20	1.12
98	5	16	4.08	20	.40
99	5	19	5.88	21	.72
100	2*	18	4.80	22	.48
		Avg. = 17.63	Avg. = 489.04	Avg. = 20.96	Avg. = 79.48

FIGURE 6. A criterion for the classification of leucocytes within *Illex illecebrosus*: nuclear lobulation.

(1) Type one - round or oval nucleus
(2) Type two - slight indentation
(3) Type three - progressive indentation
(4) Type four - bilobate nucleus
(5) Type five - trilobate nucleus
(6) Type six - multilobate nucleus

(Villanueva, 1,210x)

(a) and (b) in Type four denote differing nuclear configurations of the bilobate condition, while in Type five differing nuclear configurations in the trilobate condition.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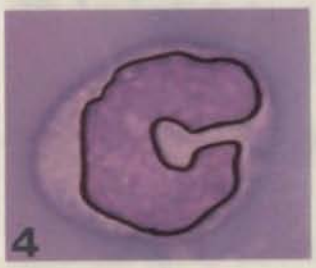
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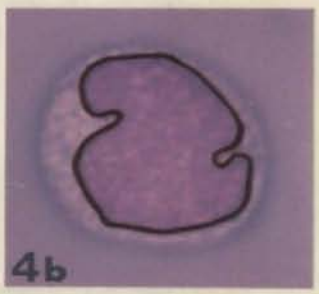
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5a

5b

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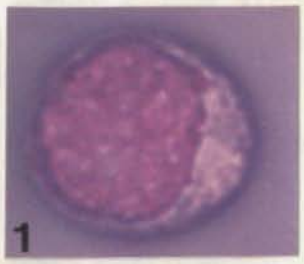
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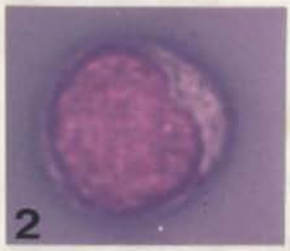
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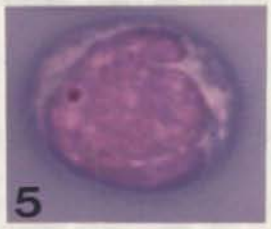
4a

4b


5

5a

5b

6

and (6) the multilobed condition. Nuclear lobes as expressed within these last two categories may be indistinct, like budding outgrowths of the original (Figure 6 - 5a), or distinct entities. The latter are not separated like those characteristic structures of human neutrophils but rather are joined by thick chromatin strands. The frequency of occurrence for each of the designated categories was: Type 1, having 20; Type 2, 21; Type 3, 20; Type 4, 9; Type 5, 13; and Type 6, 2.

The following graphs (Figures 7-9) serve to illustrate the proposed relationships of these nuclear shapes to their respective areas, the cytoplasmic mass which surrounds them and of the nuclear areas to those of the cytoplasm. Further analysis of such data reveals the distribution of nuclear and cellular diameters of hemocytes within the different categories (Table 4).

Living Preparations: I. Supravital Staining. When stained supravitally with Janus green B, the mitochondria appear as bluish-green rods and spheres. These organelles tend to concentrate around the nucleus and along the cell periphery. Granules, measuring approximately 1μ, are revealed upon their exposure to neutral red. Characterized as intensely red spheres, they are seen near and/or superimposing the nuclear chromatin. Neutral red vacuoles were not observed, however, due to their progressive enlargement with time it would seem probable that formative stages (indistinguishable at this point) were dispersed among the granules mentioned previously.

FIGURE 7. The relationship between selected nuclear configurations as in Figure 6 and associated nuclear areas in blood cells of the ommastrephid *Illex illecebrosus*.

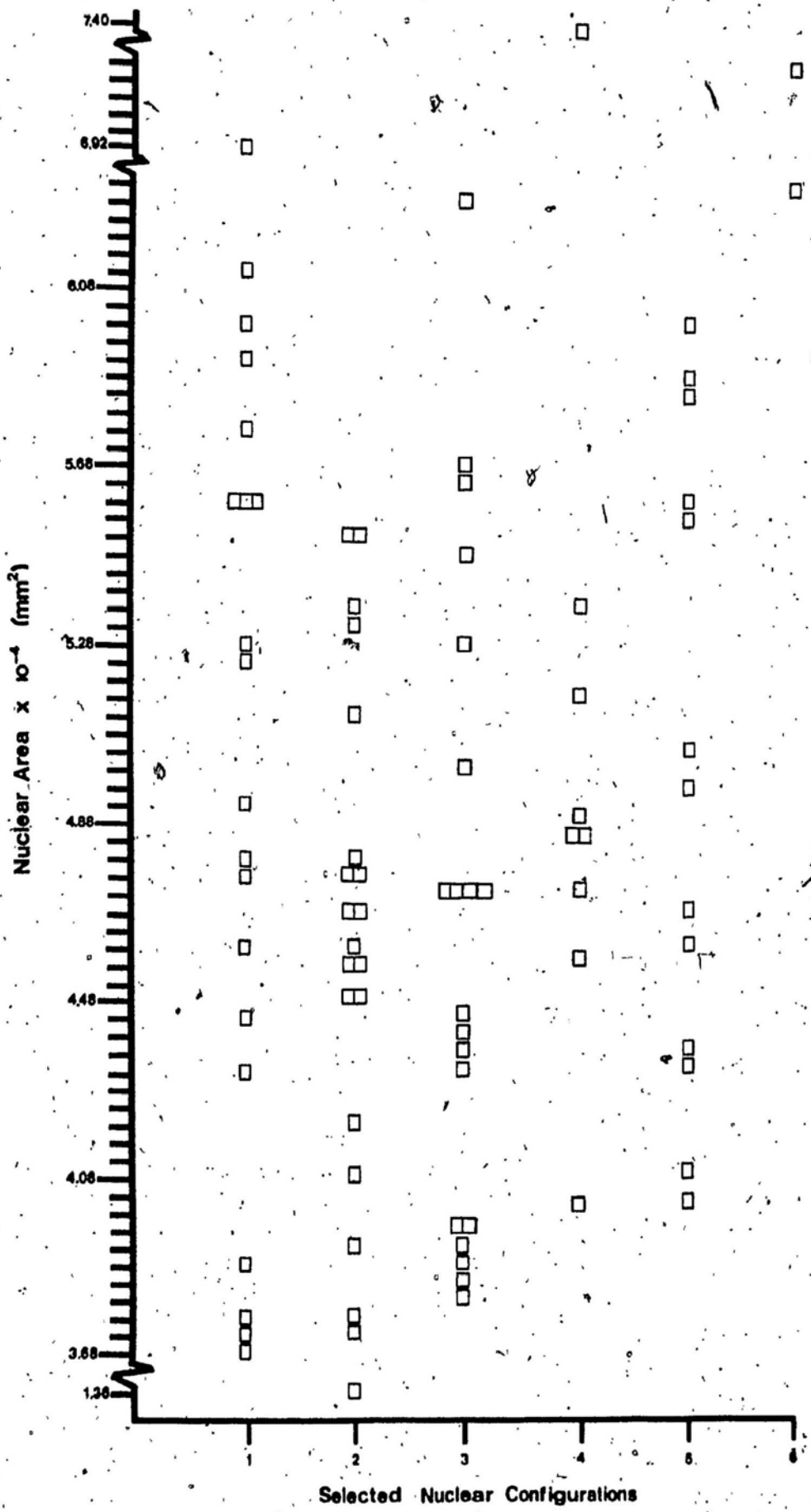

7.40
6.92
6.08
5.68
5.28
4.88
4.48
4.08
3.68
1.36
Nuclear Area x 10⁻⁴ (mm²)
1
2
3
4
5
6
Selected Nuclear Configurations

FIGURE 8. The relationship between selected nuclear configurations as in Figure 6 and the cytoplasmic area in blood cells of *Illex illecebrosus*.

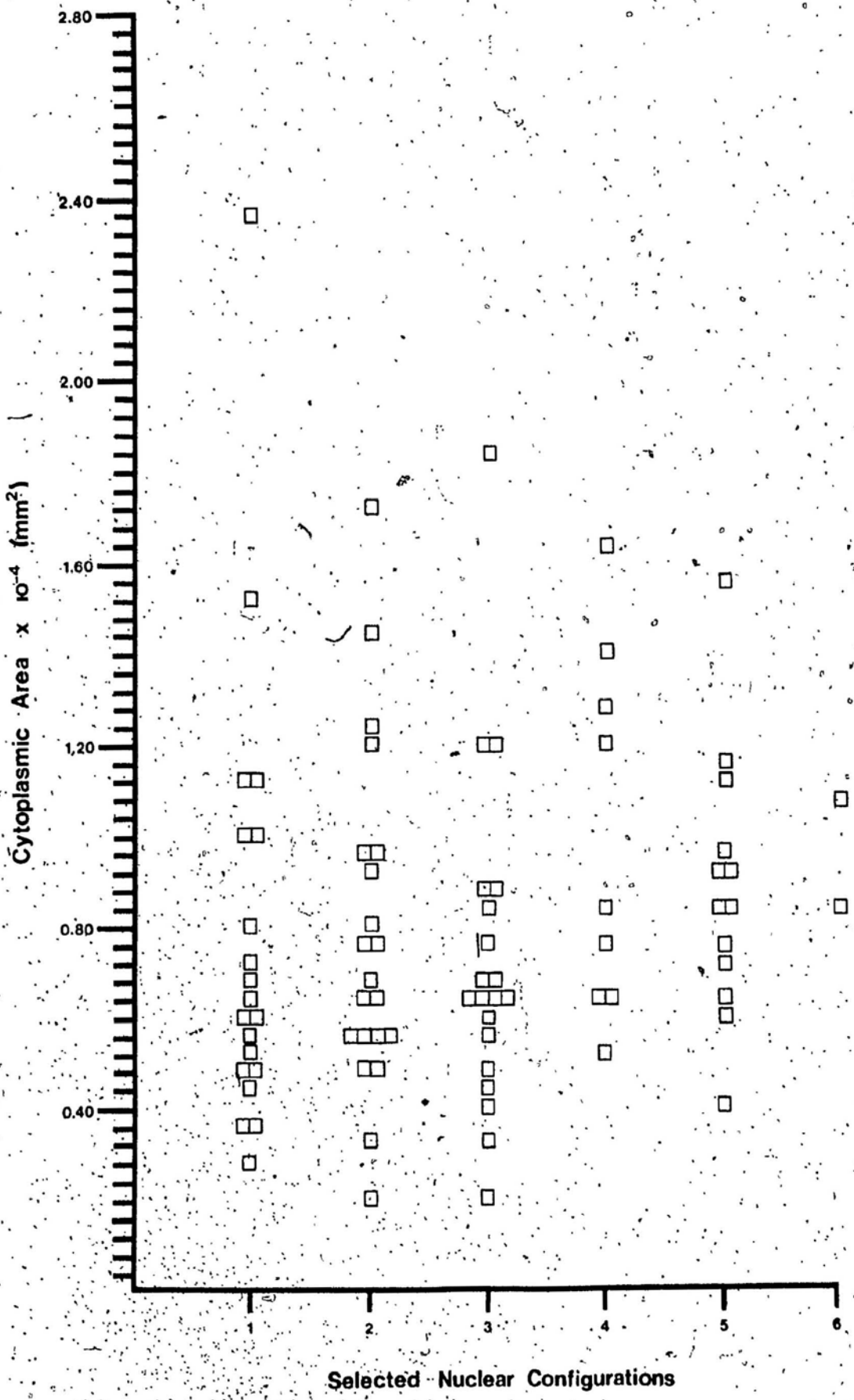

2.80
2.40
2.00
1.60
1.20
0.80
0.40
Cytoplasmic Area x 10^-4 (mm^2)
1
2
3
4
5
6
Selected Nuclear Configurations

FIGURE 9. The relationship between the nuclear and cytoplasmic areas of blood cells of *Illex illecebrosus*.

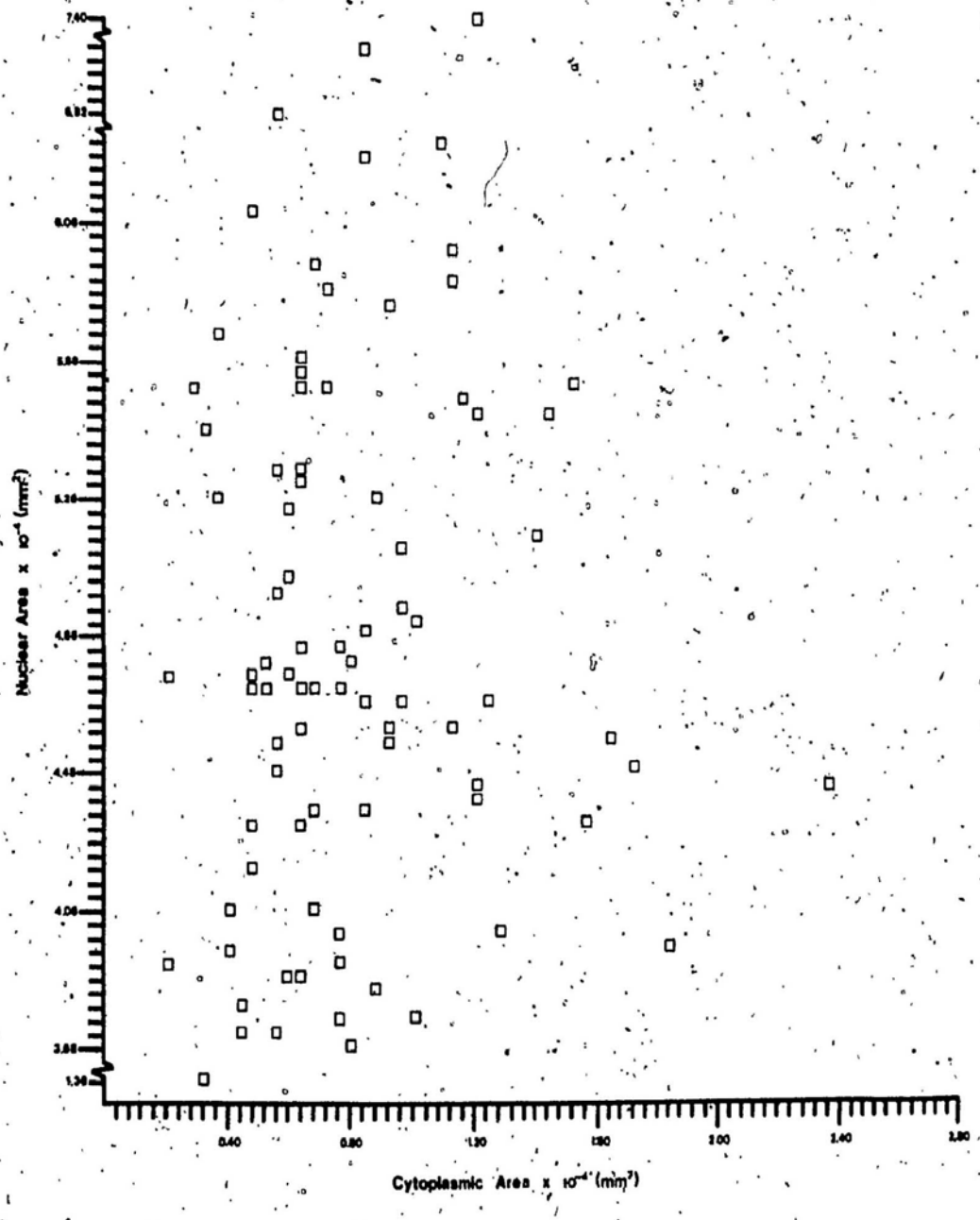

Nuclear Area x 10⁻⁴ (mm²)
Cytoplasmic Area x 10⁻⁴ (mm²)
0.40
0.80
1.20
1.60
2.00
2.40
2.80

TABLE 4. Relationships between the cell dimensions and nuclear configurations of 100 blood cells randomly selected from a blood smear preparation obtained from *Illex illecebrosus*.

TYPE #1	Nuclear Diameter (μ)	Frequency of Occurrence	Cellular Diameter (μ)	Frequency of Occurrence
	15	1	16	1
	16	3	17	
	17	3	18	
	18	8	19	
	19	5	20	8
			21	2
			22	7
			23	2
TYPE #2				
	15	2	17	1
	16	5	18	1
	17	8	19	4
	18	3	20	5
	19	3	21	7
			22	2
			23	1
TYPE #3				
	16	2	19	3
	17	8	20	3
	18	5	21	9
	19	2	22	4
	23	3	23	1
TYPE #4				
	16	1	20	1
	17	2	21	5
	18	4	22	3
	19	2		
TYPE #5	16	2	19	1
	17	4	20	4
	18	5	21	1
	19	2	22	5
			23	2
TYPE #6	18	2	23	2

2. Phagocytosis Evaluation. Preliminary experiments designed to determine the existence of a phagocytic response and, if successful, the optimum concentration of carmine required to illicit such behavior were examined. Intracellular granules possessing size, pigmentation and refractile qualities similar to those of free-floating carmine particles remaining in the fluid surroundings were considered one in the same.

An incubation period of one hour under 4°C conditions preceded analysis of the 1% suspension. The other samples (0.1% and 0.01%) remained at this temperature until counting could be initiated; an interval of 45 minutes between each sample. Twenty consecutive cells all intact and occurring singly were studied.

As Table 5 indicates, hemocytes within the 1% carmine suspension exhibited the highest degree of phagocytosis. More importantly, these blood cells displayed normal appearances, implying that this preparation of dye was both compatible and non-toxic. Such knowledge prompted the incorporation of time-lapse photography in order that a fuller appreciation of such behavior might be obtained.

3. Time-Lapse Observations. Each of the three time-lapse films showed, quite clearly, that amoeboid movement, phagocytosis, and agglutination are integral parts of this hemocyte's behavioral responses. Individual consideration follows.

1% Carmine Solution - 1 Hour Incubation Period	
Cell Number	Number of Granules Phagocytized by Cell
1	4
2	7
3	4
4	1
5	0
6	0
7	4
8	2
9	1
10	1
11	0
12	1
13	3
14	6
15	4
16	3
17	*
18	2
19	2
20	5

*concentrated mass, individual granules could not be perceived clearly.

continued

TABLE 5. The degree of phagocytosis occurring *in vitro* by blood cells from *Illex illecebrosus* following the introduction of 0.01, 0.1, and 1% carmine solutions.

Table 5 continued

0.1% Carmine Solution - 1 Hour 45 Minutes Incubation Period	
Cell Number	Number of Granules Phagocytized by Cell
1	1
2	4
3	5
4	1
5	8
6	1
7	0
8	2
9	0
10	1
11	0
12	2
13	0
14	2
15	2
16	3
17	0
18	0
19	1
20	2

(continued)

Table 5 continued

0.01% Carmine Solution - 2 Hours 30 Minutes Incubation Period	
Cell Number	Number of Granules Phagocytized by Cell
1	0
2	0
3	0
4	0
5	1
6	3
7	2
8	1
9	4
10	0
11	2
12	3
13	1
14	0
15	4
16	0
17	3
18	3
19	4
20	4

When floating freely within the bloodstream, these corpuscles of the teuthoid cephalopod, I. illecebrosus, are round (Figure 10-1). Internal organization, including the prominent nucleus, is difficult to discern. Eventually, exploratory pseudopodia emerge (Figure 10-7). Only one projection was observed initially, however before the cell's actual adherence to the slide, up to four pseudopodia could be seen. These protoplasmic extensions are blade-like in nature and protrude a distance which just exceeds the cellular diameter. They appear to sweep around the hemocyte, although in truth, this is probably an illusion; the cell really exhibiting a whirling or spiral movement.

Pre-attachment activity proceeds once the exploratory pseudopodia at the leading edge make contact with the substrate surface (glass slide); distending and/or coalescing to form a fan (Figure 10-19). This area can be distinguished by its pale coloring and the manner in which it forms a margin about the cell's anterior. Simultaneously, from the particular hemocyte being observed, two pale, short, pointed pseudopodia appear at the posterior region (Figure 10-20). Reasons for designating these anatomical regions as "front" and "back" will become evident upon further examination of the movement sequence. At this point, the cell elongates slightly; the advancing edge going out of focus and the pseudopodia at the rear coming together as one large, dark extension (Figure 10-24). An abrupt relocation of internal structures followed as

In Figures 10 and 11 are presented several phenomena as exhibited by time-lapse photography. These are (1) free-floating cells, pseudopodia formation, and attachment, and (2) locomotion on a surface and subsequent detachment.

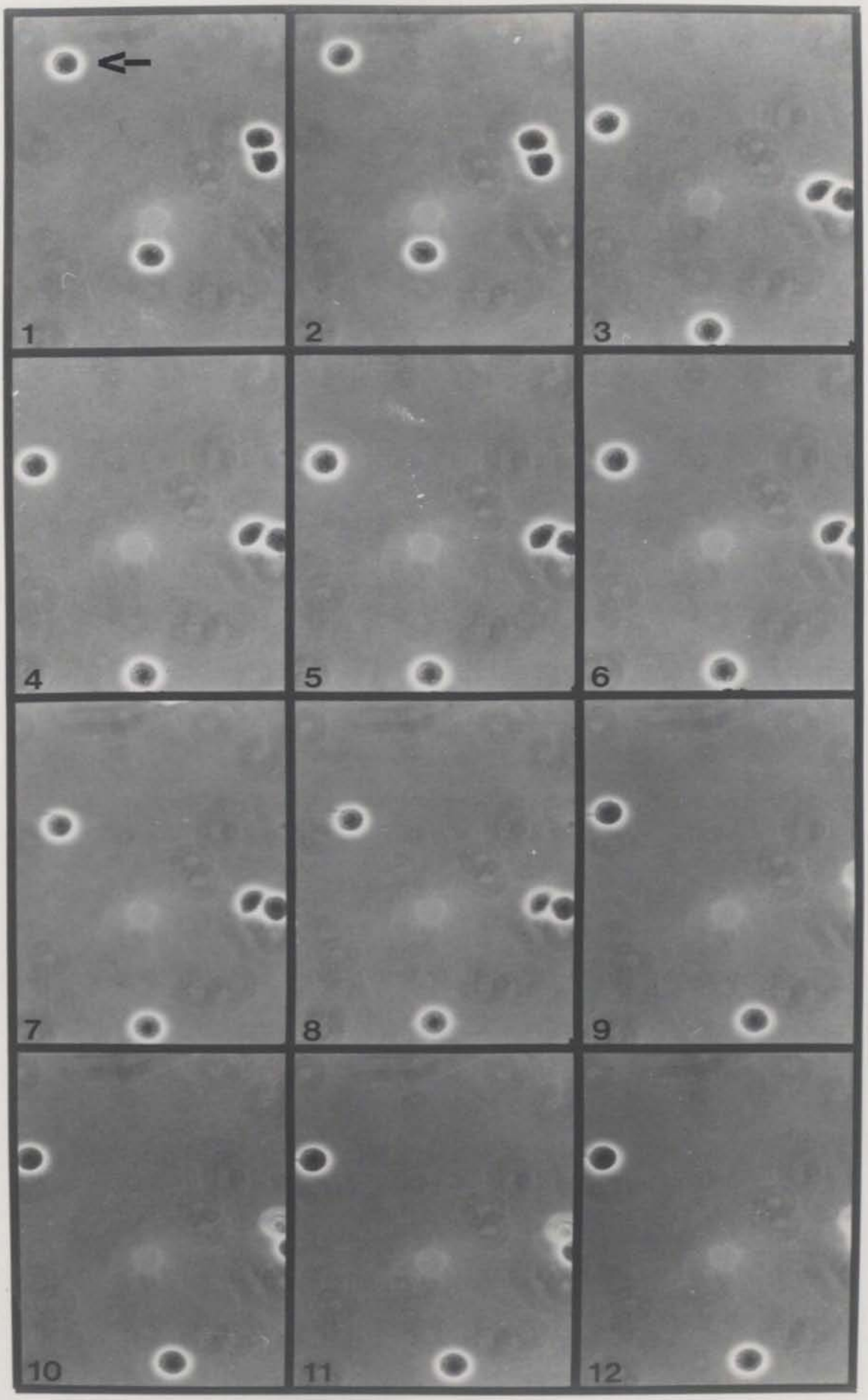
The first of these is to be seen in Figure 10.

FIGURE 10. A time-lapse progression illustrating the amoeboid movement of cellular elements from the blood of Illex illecebrosus. (238x)

Free-floating cells to their subsequent attachment to a substratum.

An arrow denotes the hemocyte selected for consideration.

Sequences filmed at room temperature.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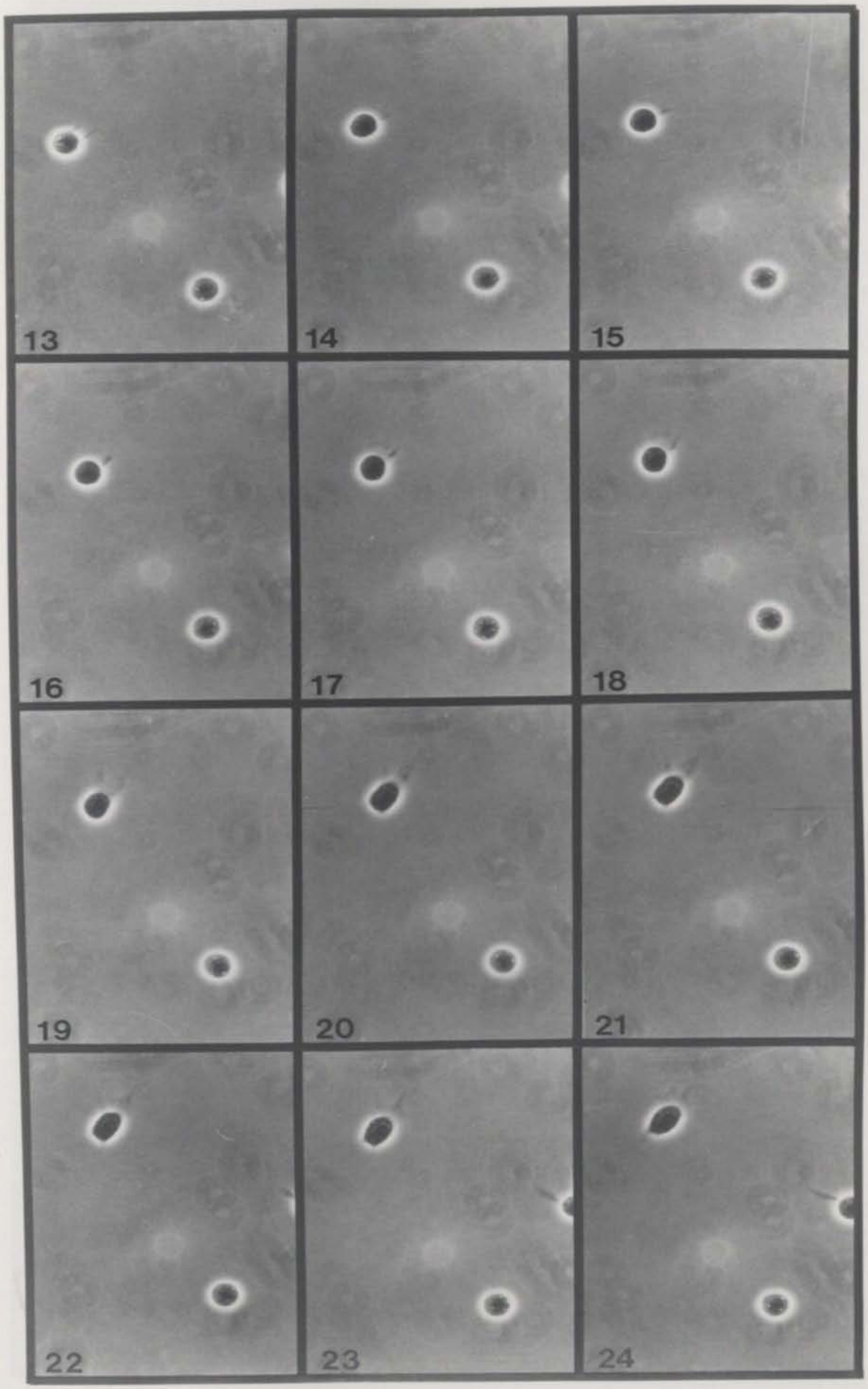
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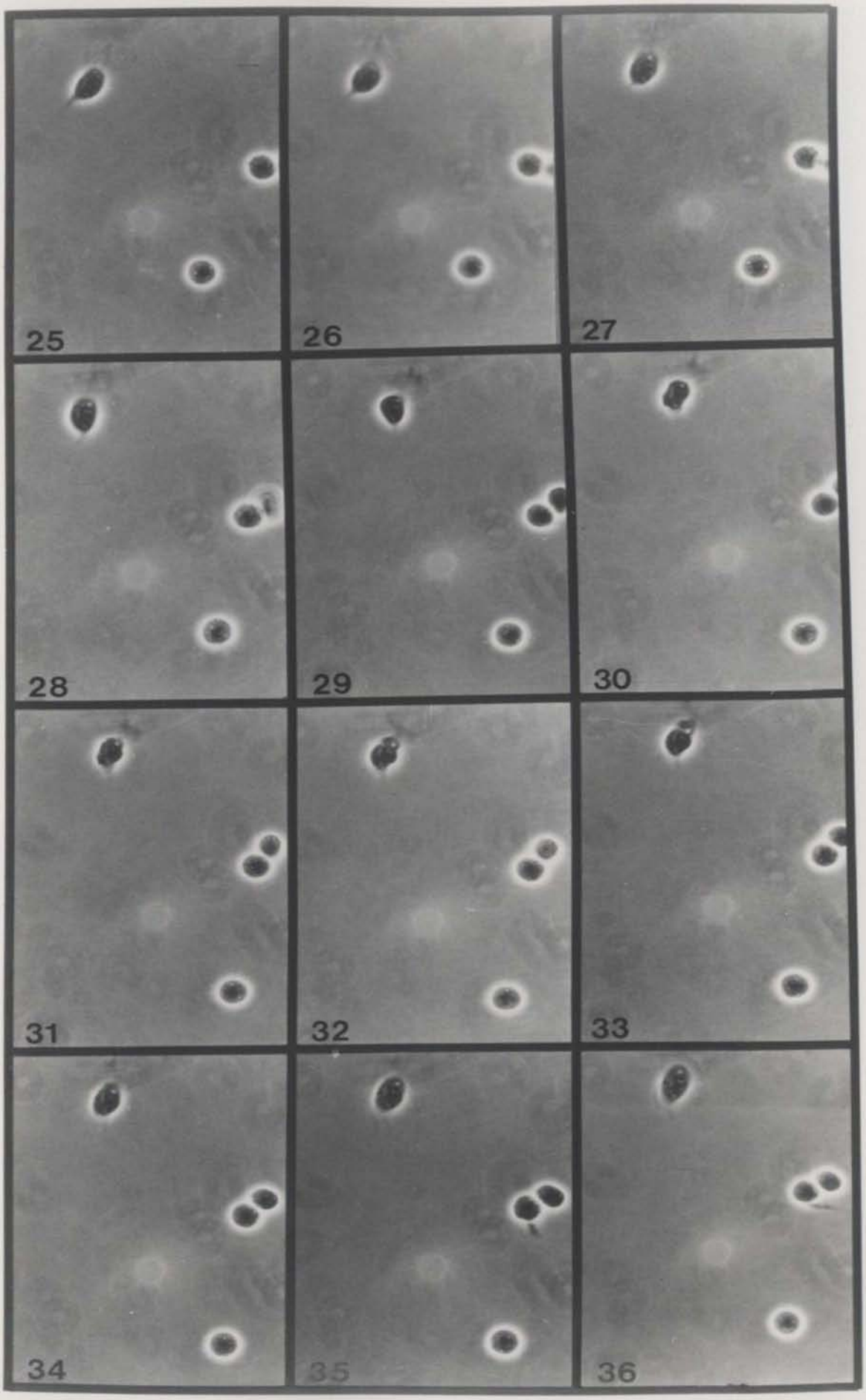
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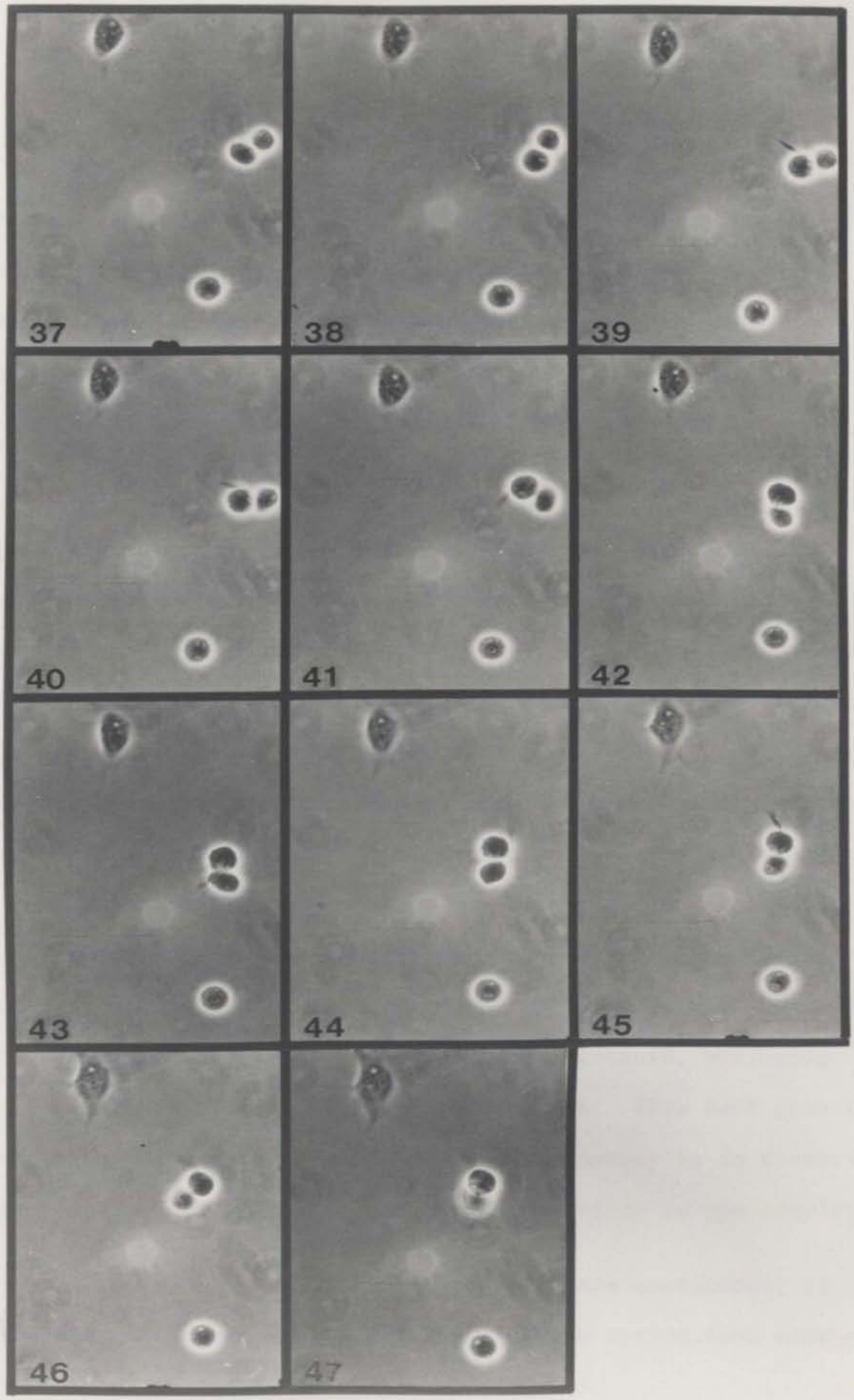
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noted by further expansion and a "ruffling" or undulation along one side of the corpuscle (Figure 10-31). Such movement appears to originate anteriorly, almost reminiscent of peristaltic waves. Just as one side of this cellular element was thrown into waves, so the same occurred on the opposing side. Yet here, a single large bulge was seen instead of the small, numerous ones previously mentioned (Figure 10-32).

Protoplasmic re-absorption transforms the posterior pseudopodium into a former state; although still pointed, it is now slender and pale (Figure 10-32). Later, it seemingly disappears with several veil-like expansions being formed in its place (Figure 10-36). However, closer observation reveals very long, indistinct pseudopodia which become visible only upon the undulating movement of these cellular projections. The entire cell then went out of focus but, subsequently, was noticed to flatten while oscillating gently, a motion solely describable as "nestling" (Figure 10-46). The nucleus, itself, was visible although specific details regarding lobation remained indistinct. One refractile granule, measuring 1.5μ - 2.0μ, was contained within the cytoplasm. Five dark granules all concentrated over the nucleus were approximately 1μ in diameter (Figure 10-47). Attachment to the microscope slide is now completed.

Because the processes of a living cell are continuous, it is difficult to isolate events, delineating one motion from another.

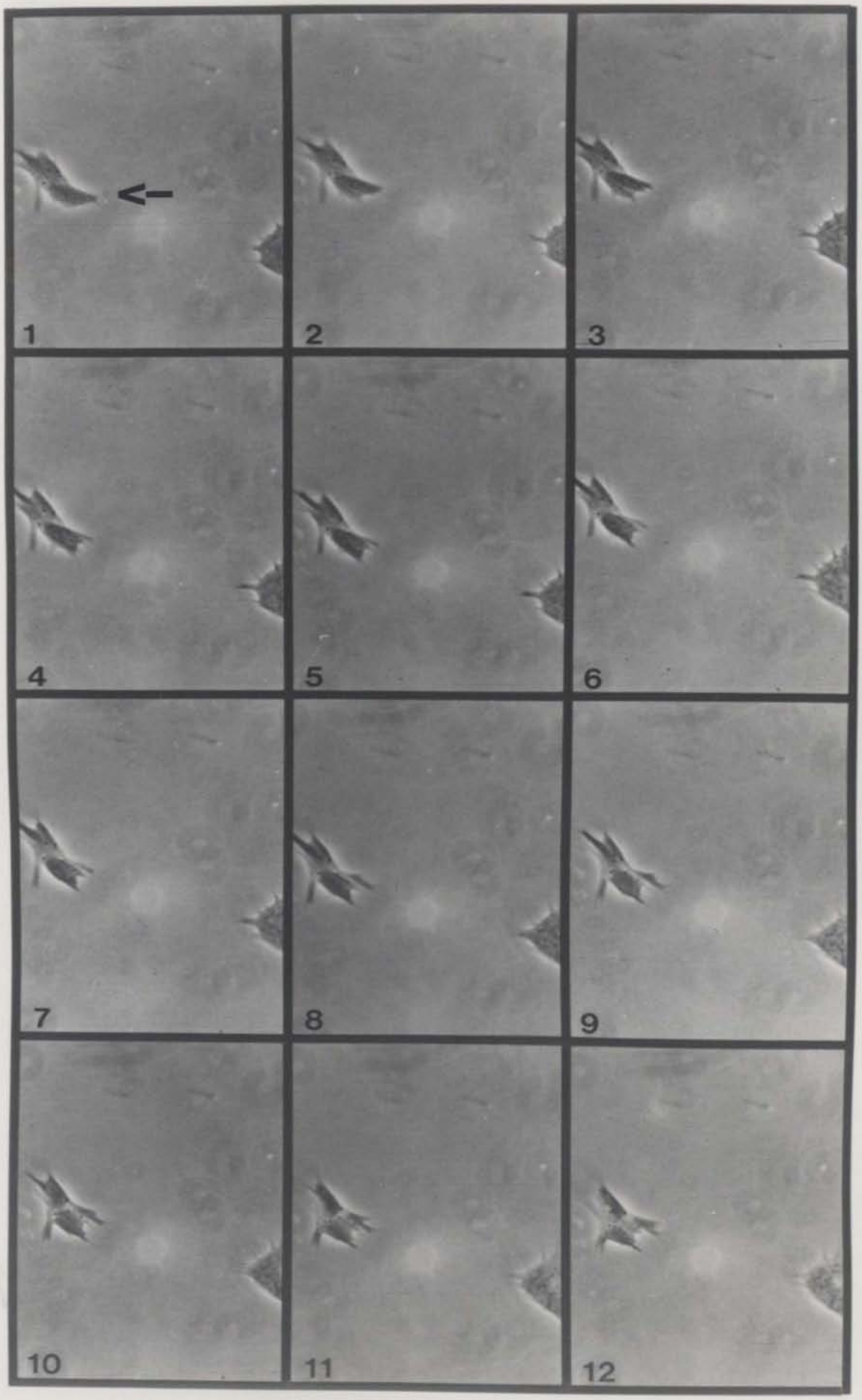
As is often the case, a certain degree of overlapping occurs, so, for clarity, it should be stated that a transition period exists whereby certain pseudopodia at the hemocyte's anterior discontinue their "anchorage" state and begin probing the surrounding area. Due to filming inadequacies, however, this remaining portion of the movement sequence is best illustrated by observing another hemocyte involved in the same process of exploration.

Figure 11-2 verifies how the advancing edge sends out thin, pointed, lightly colored, short pseudopodia. These extensions elongate; becoming wider and darker as more protoplasm flows into them. This lobate form may persist or, as seen more frequently, will subdivide into as many as three minor (smaller) pseudopodia. When no longer required, the projection is seen reverting to its former or original configuration; a process governed by the retreating flow of protoplasm. Furthermore, individual pseudopodia appear which usually maintain their singularity throughout the course of filming. However, several instances have been recorded in which two of the former united to produce one large pseudopodium. The movement sequence comes full circle (Figure 11-22) as the exploratory projections begin adhering to the slide while detachment occurs by a quick jerk of a posterior pseudopodium, thus affirming that the blood cells of Illex illecebrosus not only have an anatomical front and back which execute specific behaviour during the process of locomotion but also are capable of attaching themselves to and moving along a substrate surface.

FIGURE 11. A time-lapse progression illustrating the amoeboid movement of cellular elements from the blood of Illex illecebrosus: - movement across a substrate and detachment. (238x)

An arrow denotes the hemocyte selected for consideration.

A broken arrow (frame 21) denotes the posterior pseudopodium immediately prior to its detachment.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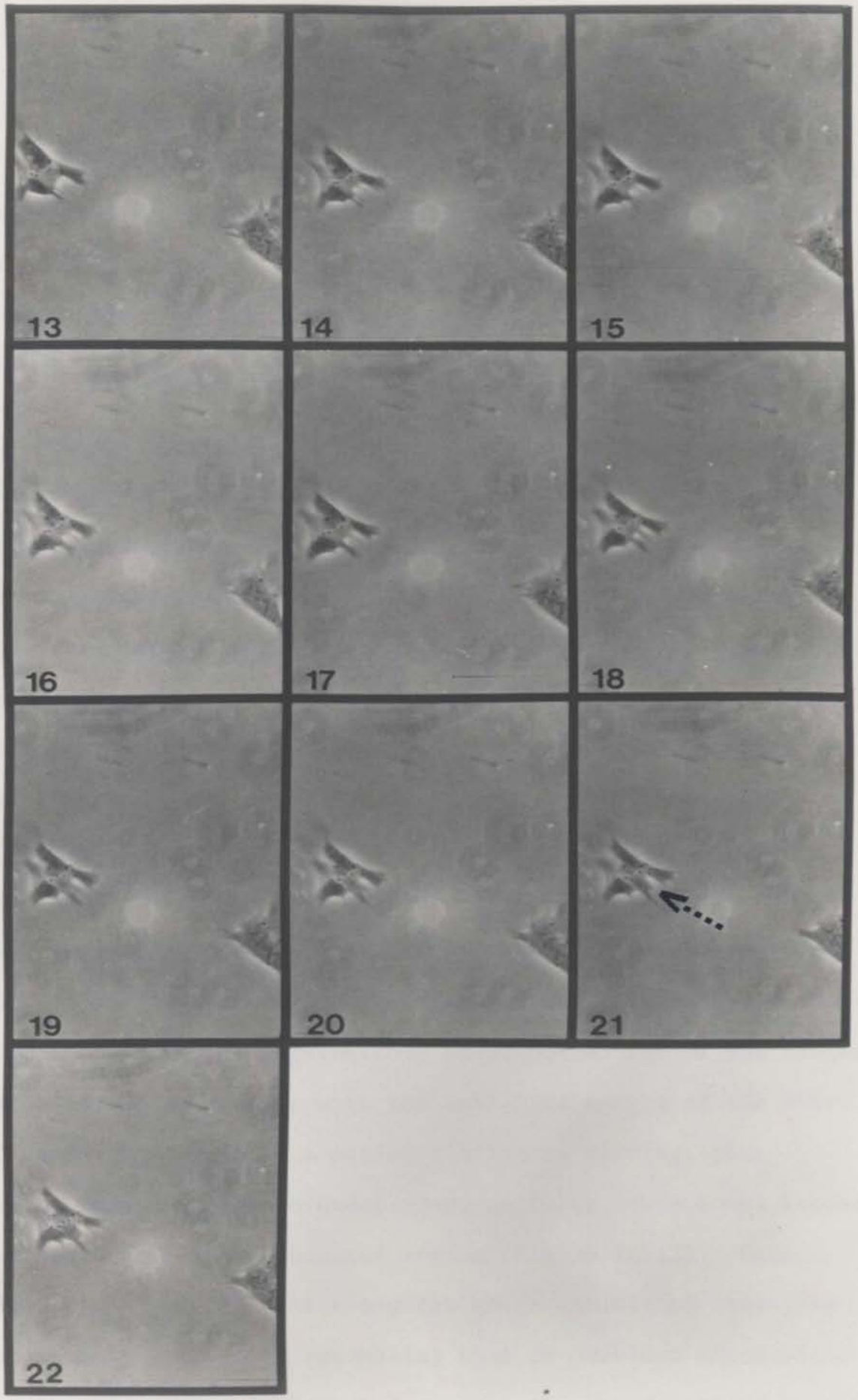
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Internally, 8 dark granules were observed within the central region of this particular cell. These tended to "bob" up and down although rapid, directional movement occurred when protoplasm flowed quickly into a forming pseudopodium. The granules were never seen inside the projection, itself, however.

A second property attributed to the hemocytes of I. illecebrosus is that of agglutination. Fortunately, a sequence which portrays the events surrounding this phenomenon was recorded by means of time-lapse photography (Figure 12). That film shows the approach and subsequent adherence of two blood cells to a third, previously settled corpuscle; the last cell having been described on page 65. Those two have agglutinated or, at least, are being held together by surface tension. The larger one extends typically blade-like exploratory pseudopodia as it "crawls" around the smaller cell (Figure 12-1). Although this smaller hemocyte was seen to change in shape (rounded to pear-shaped, alternately) no projections were extended. Gradually, as these two approach the attached cell, their velocity increases; the outstretched pseudopodial fan of the larger corpuscle coming in contact with the veil-like margin of the third cell (Figure 12-9). With a motion similar to gliding, this projection moves across the underlying, cellular surface and becomes affixed near the latter's central region (Figure 12-12). Once attached, the advancing cell elongates while exhibiting "settling out" or spreading behavior resembling that of previous discussions (Figure 12-18). Throughout such activity, the smaller blood corpuscle

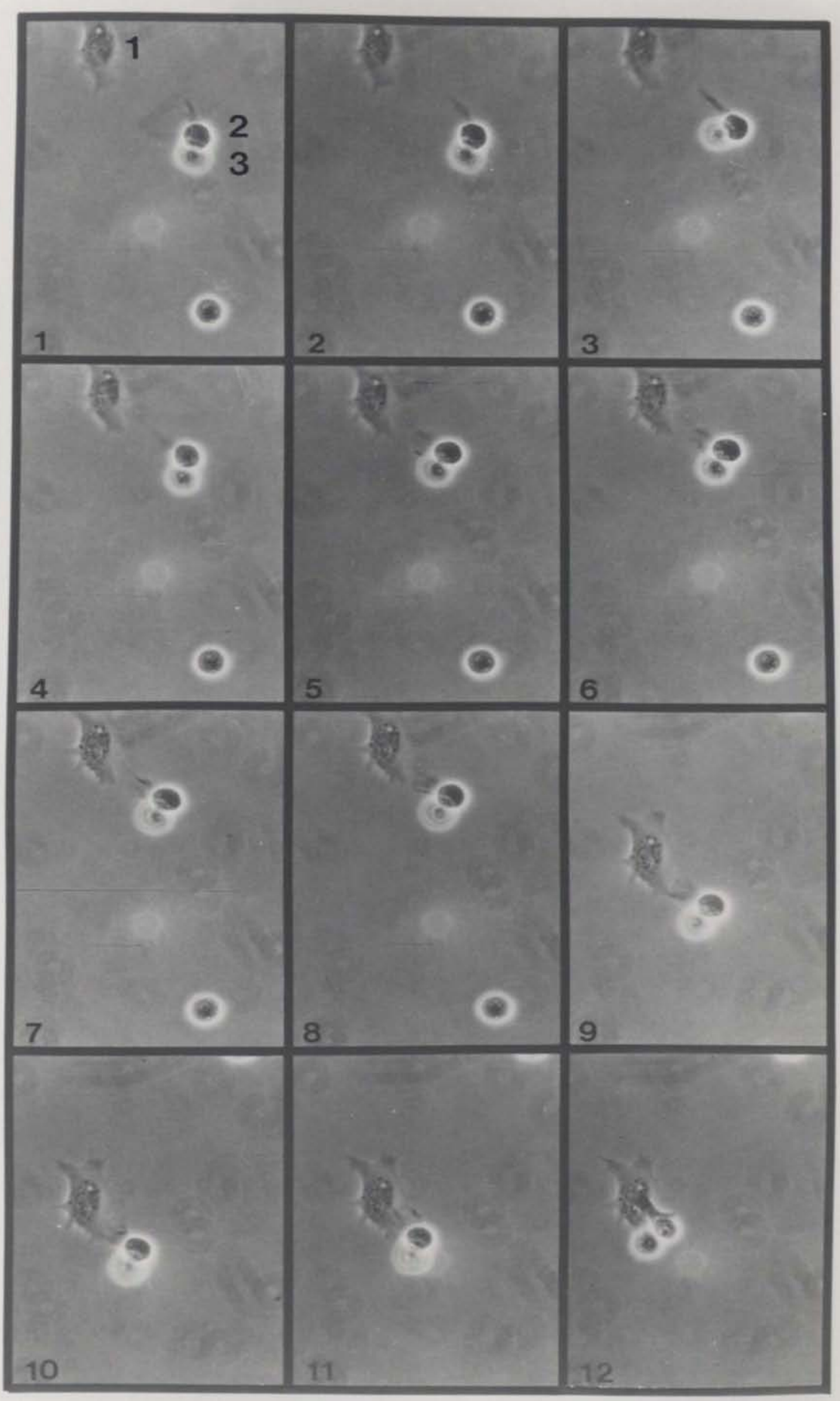
FIGURE 12. A time-lapse progression illustrating the properties of agglutination exhibited by cellular elements from the blood of Illex illecebrosus. (238x)

As described on the previous page of the text;

the "attached cell" is denoted as (1)

the "larger cell" is denoted as (2)

the "smaller cell" is denoted as (3)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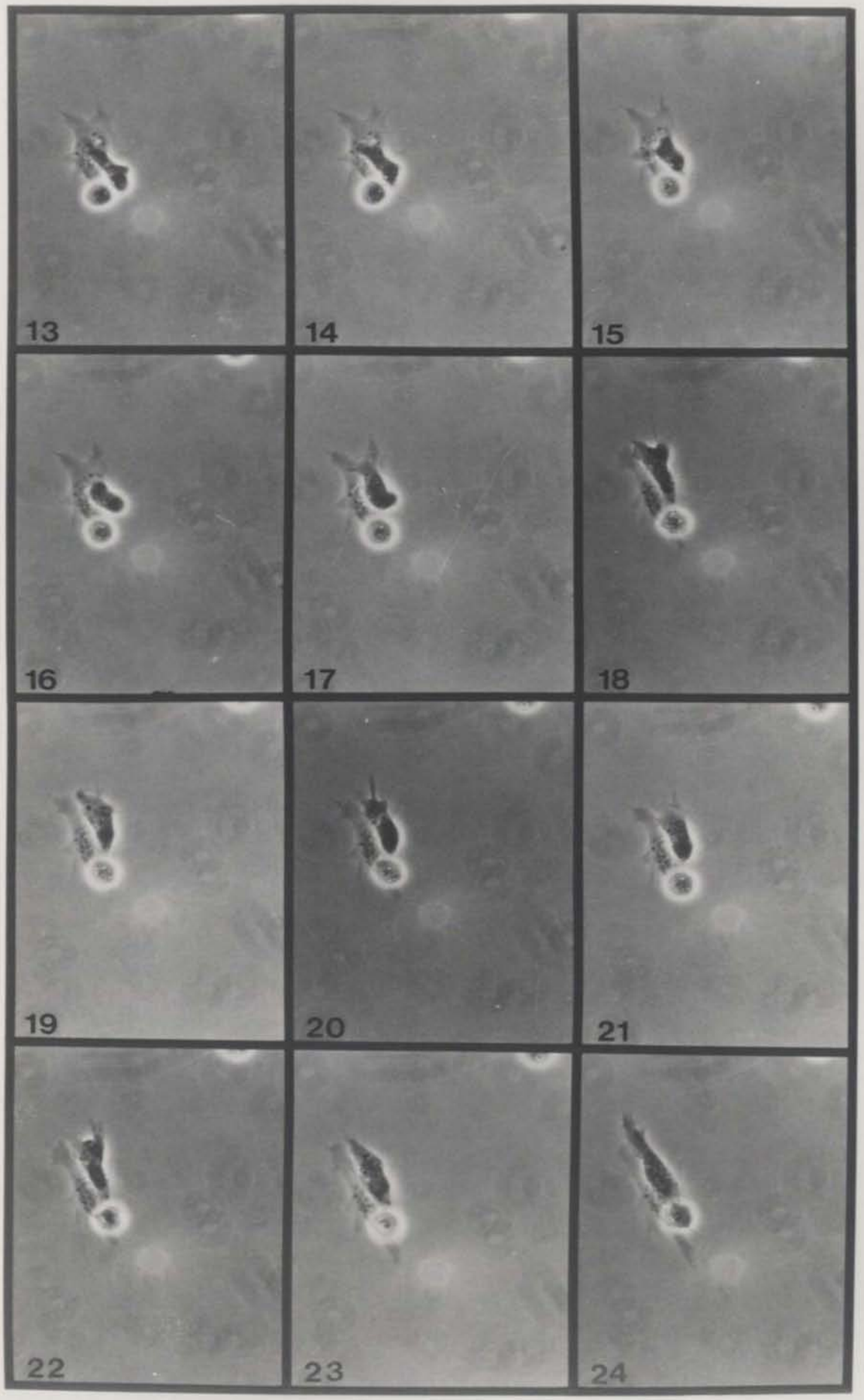
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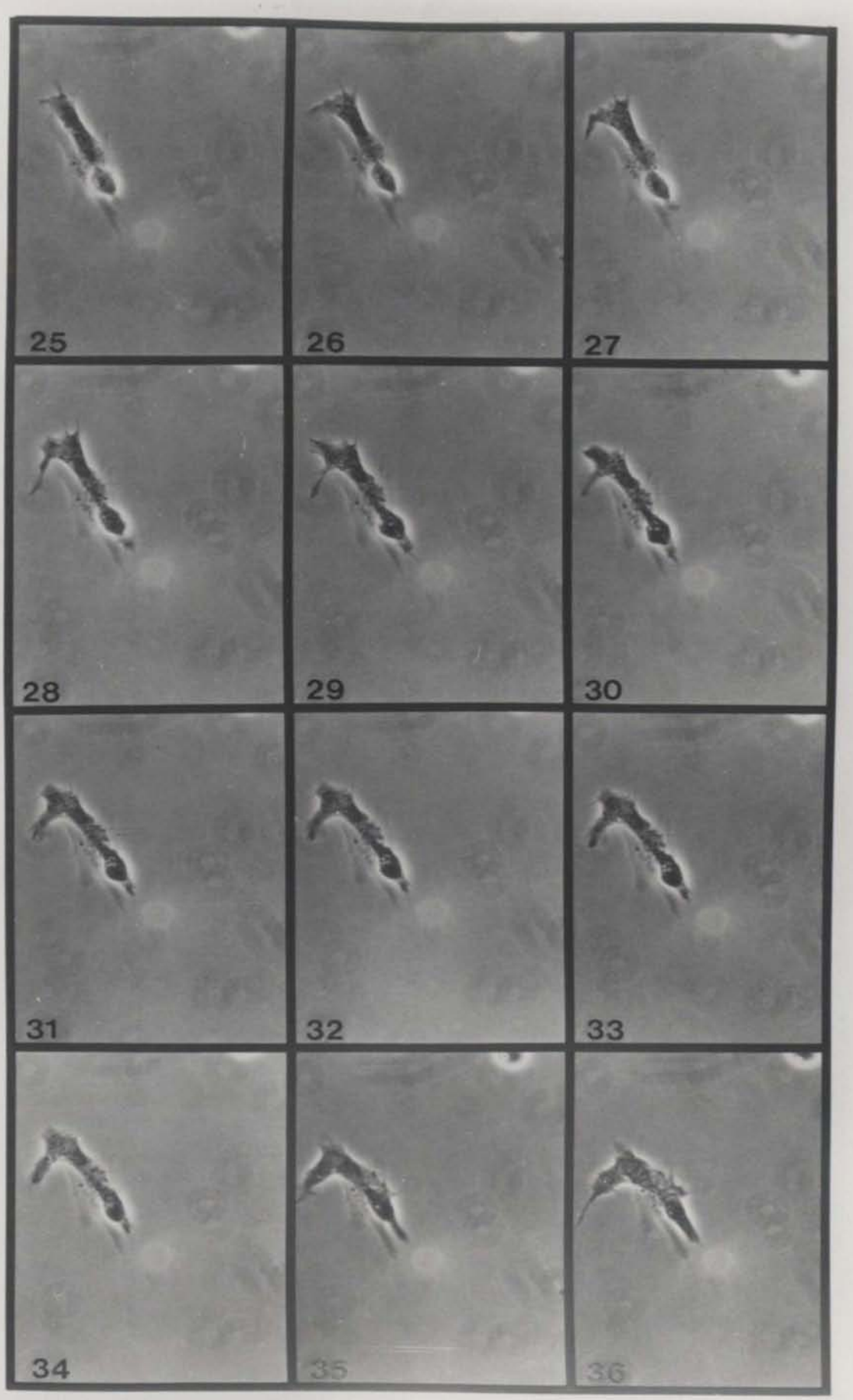
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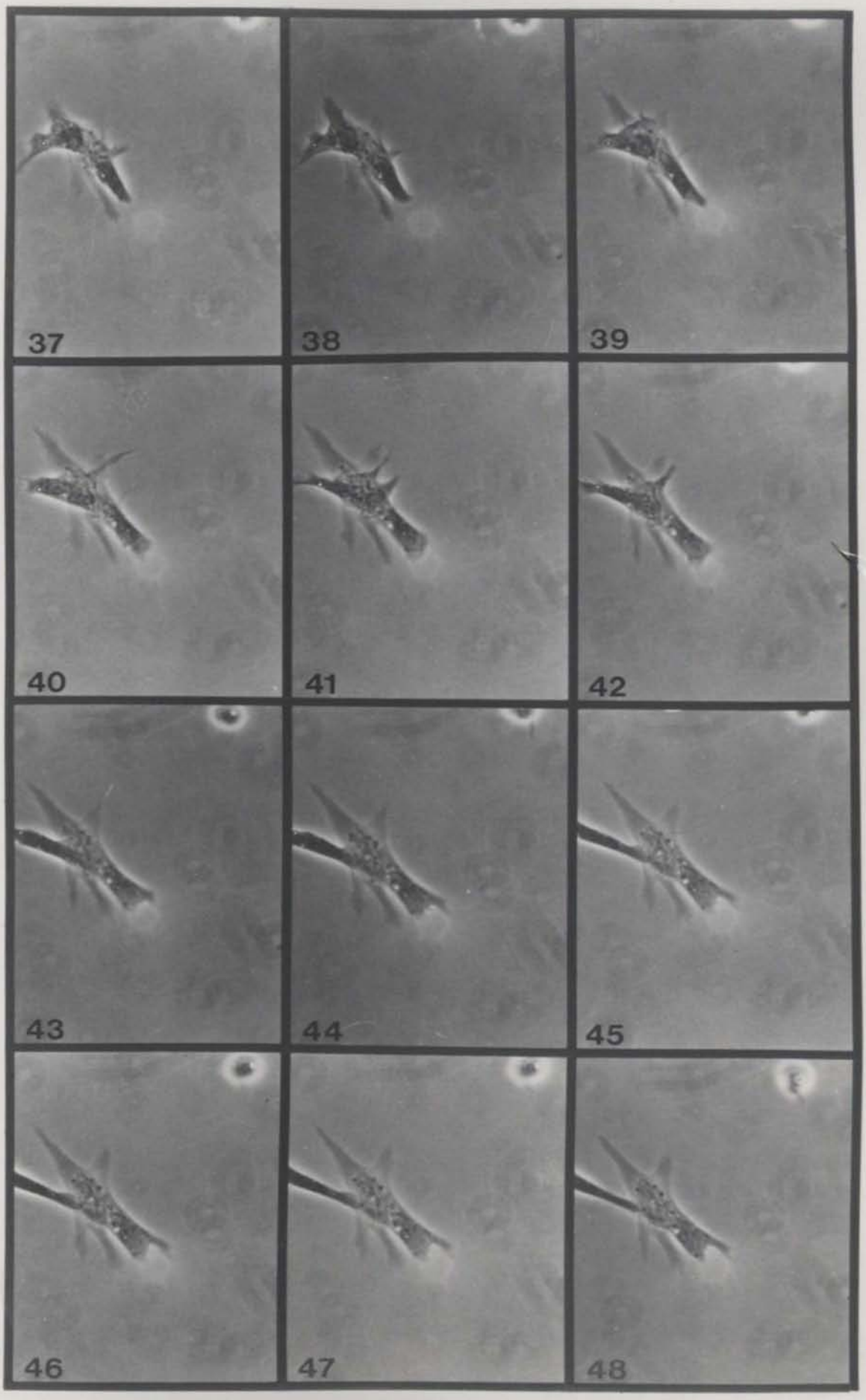
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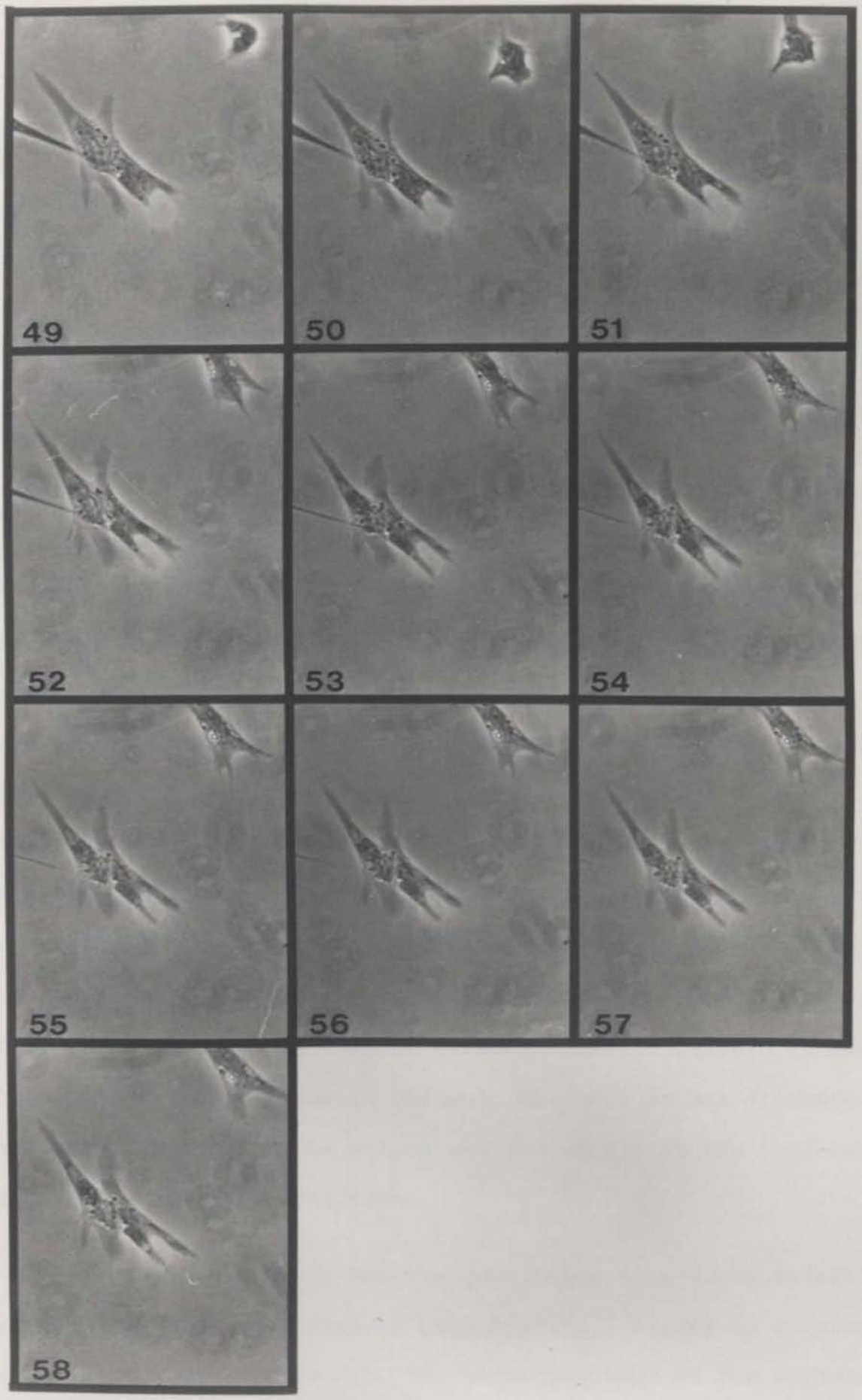
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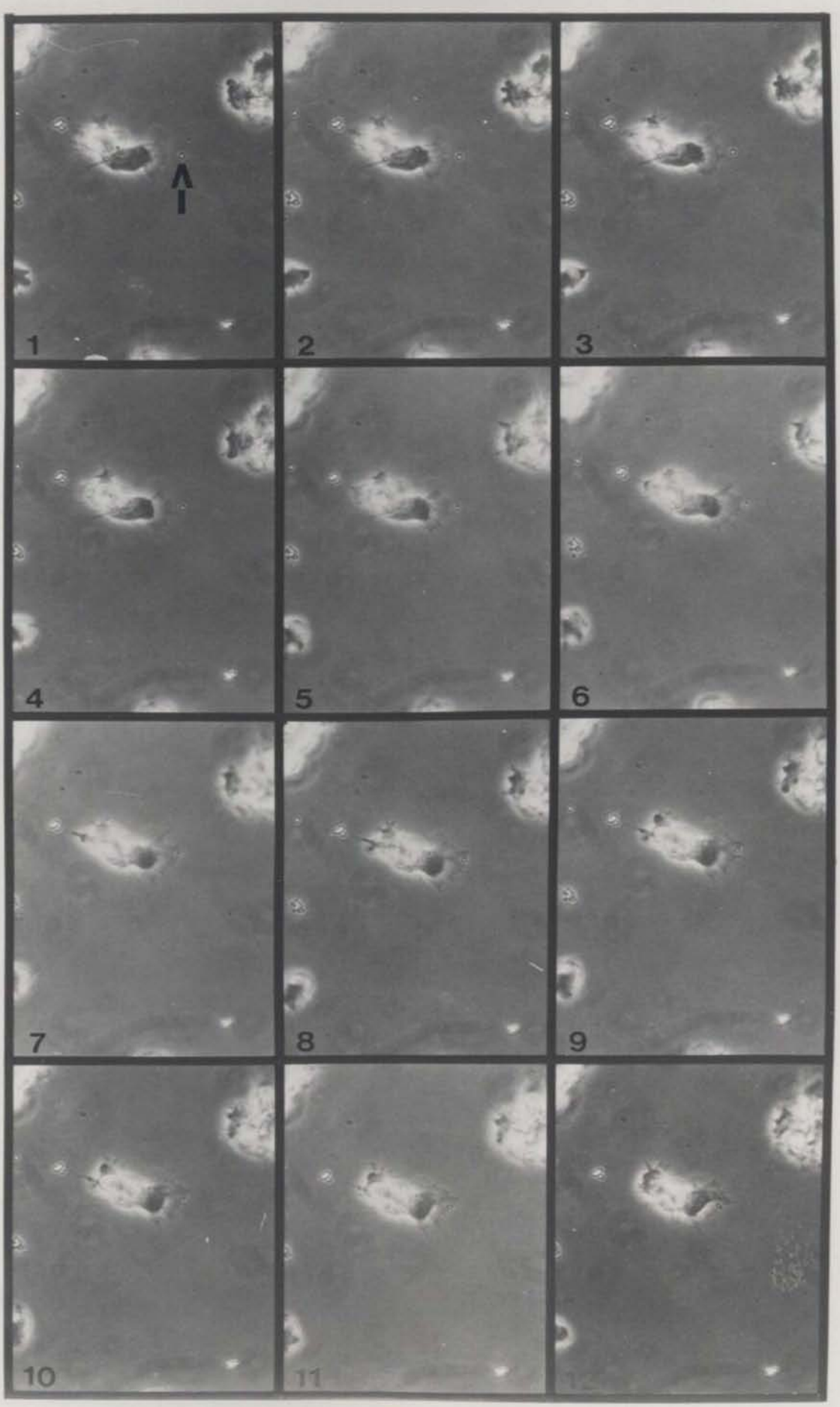
has remained virtually unchanged. It is only upon those final stages of agglutination that it, too, initiates the mechanism for adherence (Figure 12-24).

Although having completed this facet of their behavior the hemocytes still maintain their responsiveness to external stimuli as well as to their agglutinated partners. The study of phagocytosis, which follows, illustrates this point exceptionally well. Apart from the bobbing motion of the dark, intracellular granules, the undermost cell remains relatively inactive as does the smaller, cellular element. One sees a great deal of internal movement and pseudopodial extension associated with the large blood cell, however. In fact, this particular corpuscle becomes highly contorted, being "L-shaped" at one point (Figure 12-32). Later, the same hemocyte was observed moving out of the viewing field (Figure 12-44). A single pseudopodium retaining contact with the originally agglutinated mass now elongates and becomes pale (Figure 12-51). Eventually, it will break free or detach although not so abruptly as the situation recorded earlier (Figure 12-56). One instant the connection is evident; the next it has disappeared completely. Possibly, the motion was too subtle or the projection too pale for accurate detection.

Of special interest was the concluding time-lapse investigation dealing with those processes of phagocytosis. Figure 13 supplements the discussion of this study. The advancing edge of the approaching

FIGURE 13. A time-lapse progression illustrating the phases of phagocytosis exhibited by cellular elements from the blood of Illex illecebrosus. (238x)

An arrow denoted the carmine particle being phagocytized.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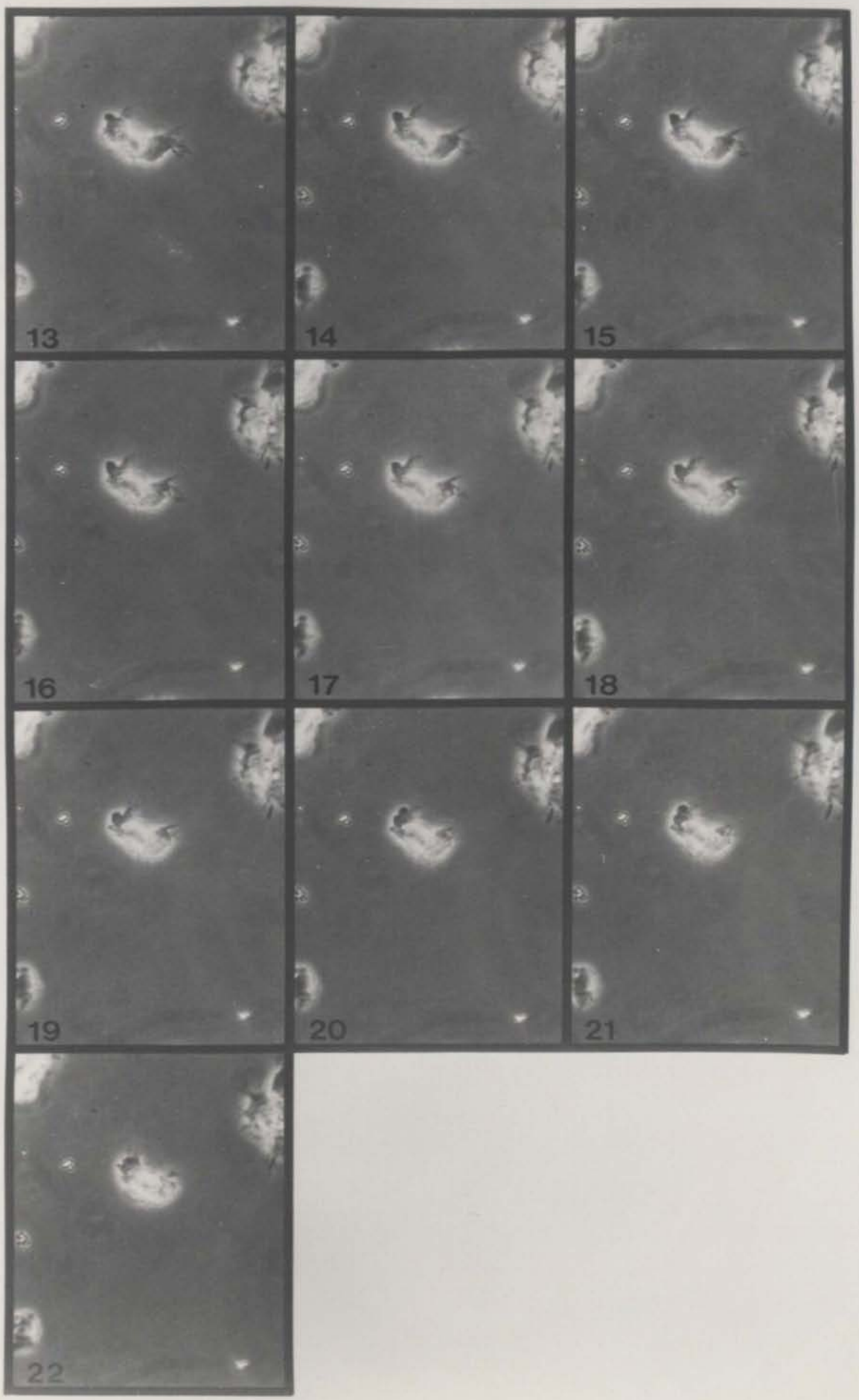
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hemocyte has been extended as a veil-like margin of numerous pseudopodia (Figure 13-1). Upon reaching the carmine particle, the pseudopodium of closest proximity establishes contact with it and then begins invaginating; the lateral extensions progressively surrounding and enclosing the foreign granule (Figure 13-9). As this grain of carmine is drawn toward the cell's endoplasm (the central, innermost region) the adjacent marginal periphery through which it entered ceases to extrude projections (Figure 13-19). However, pseudopodia are present to either side of this area.

Figure 14 serves to augment the information compiled from these previous illustrations.

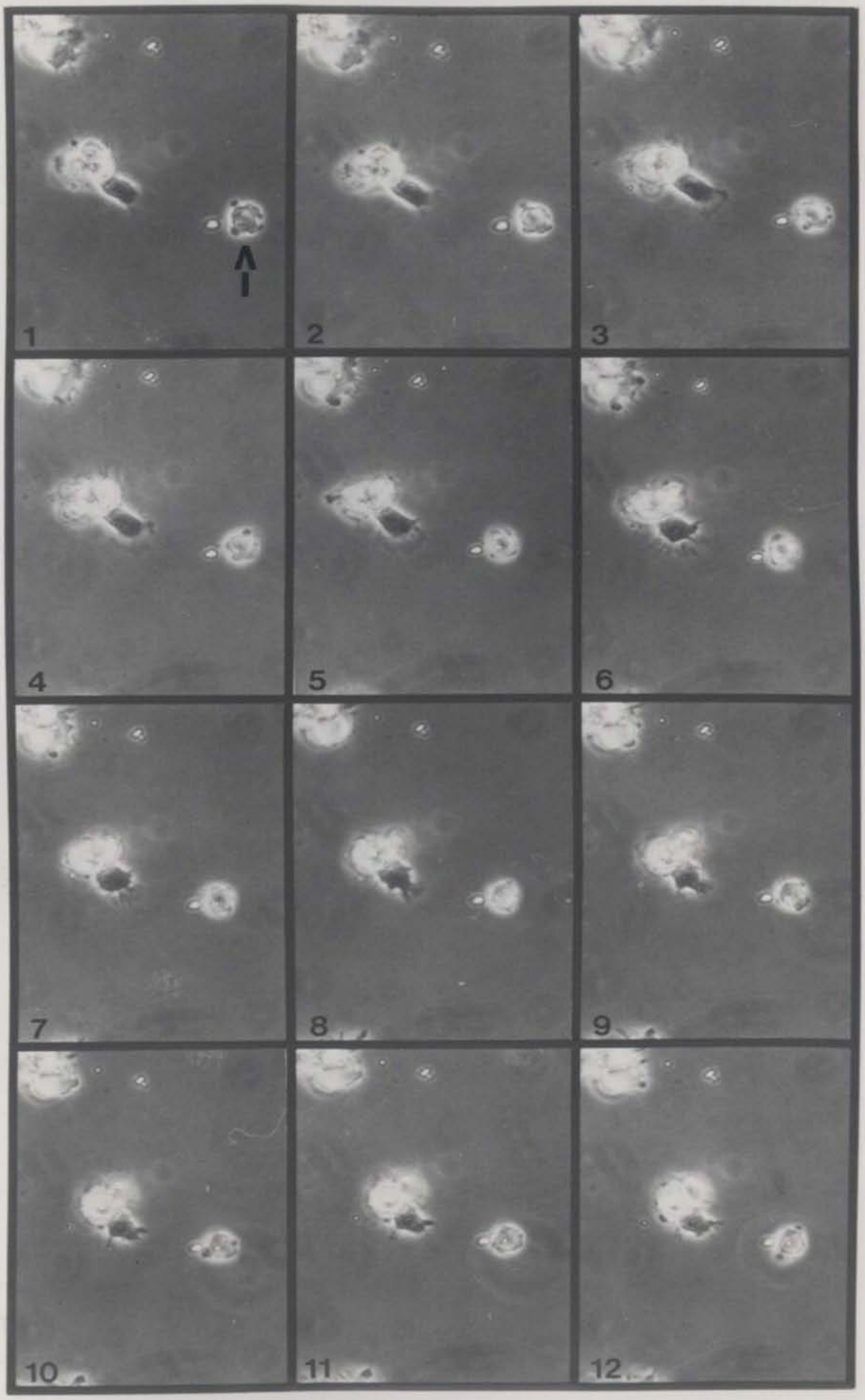
FIGURE 14. A time-lapse progression which substantiates previous information regarding the behavioral responses of cellular elements from the blood of Illex illecebrosus; specifically those responses of movement, agglutination and phagocytosis. (238x)

An arrow denotes the cell selected for consideration.

Phagocytosis: frames 1 through 21, primarily

Movement: frames 6 through 27, primarily

Agglutination: frames 27 through 35, primarily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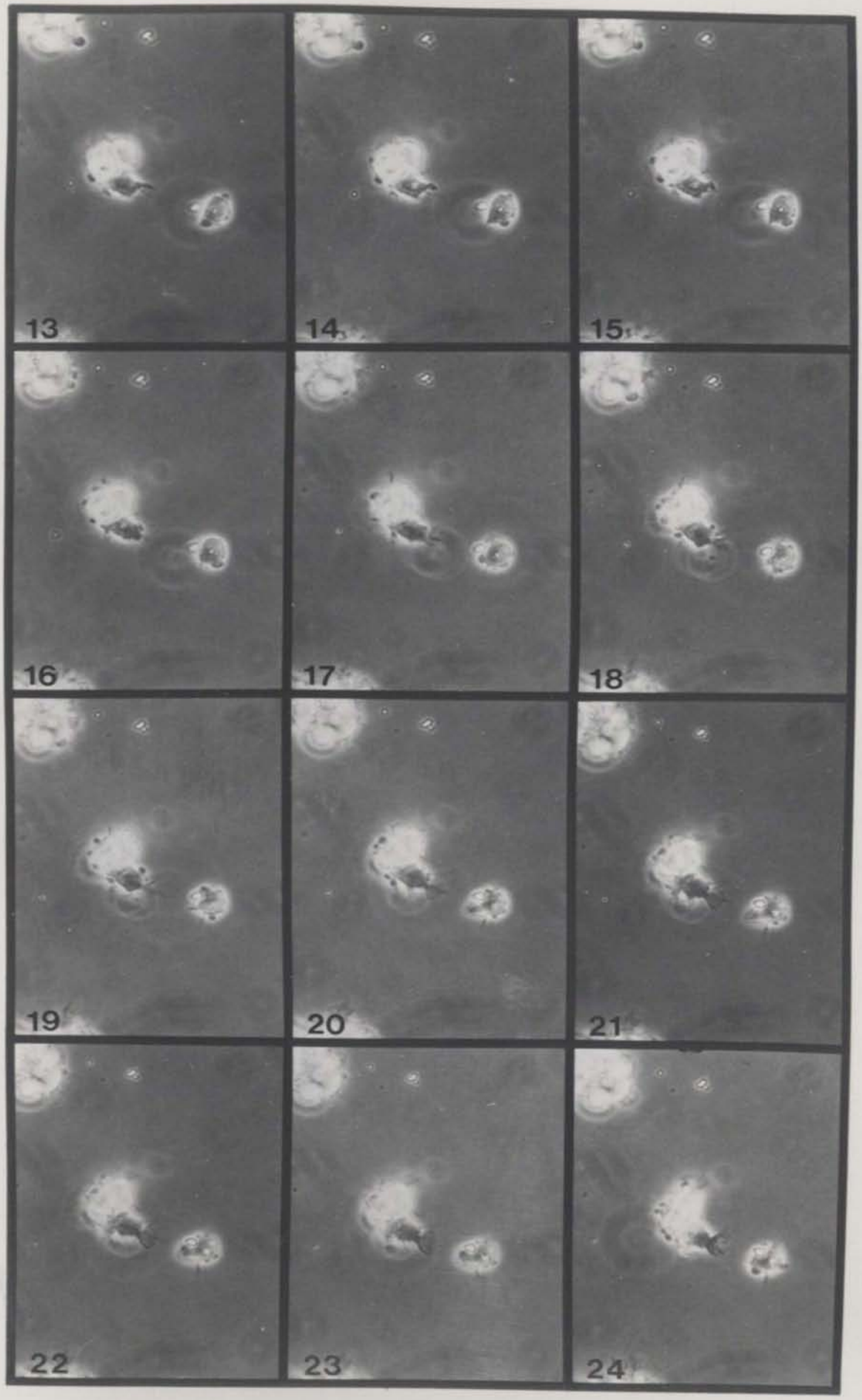
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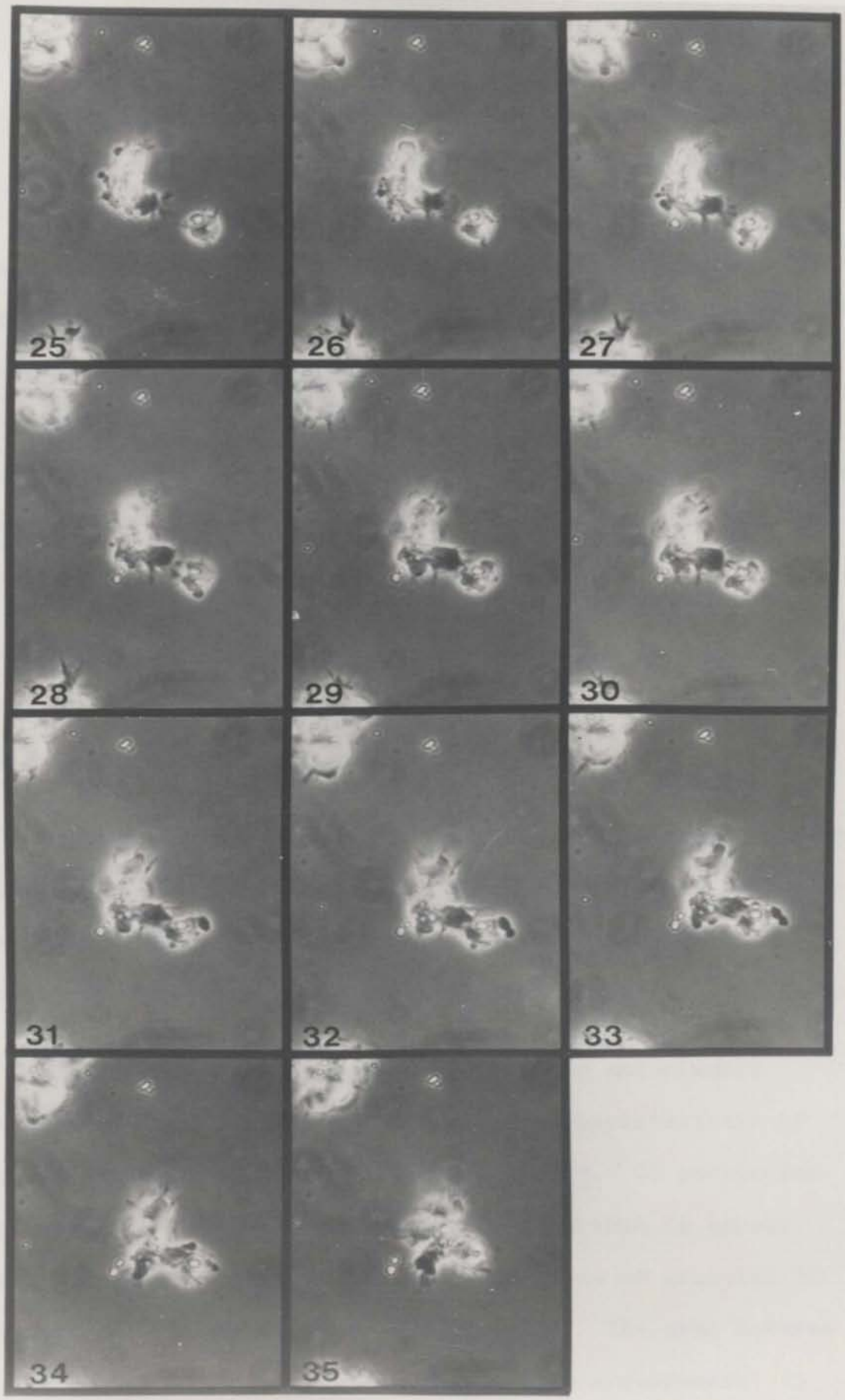
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Electron Microscopy. The eosinophilic granulocytes of I. illecebrosus display levels of cellular organization comparable to those typifying mammals. Figures 15 and 16, representative micrographs illustrating general morphology, show that the nucleus is bound by a double membrane, the outermost portion of which is studded with ribosomes. Nuclear pores have been observed on occasion. Characteristically, the nuclear chromatin is condensed peripherally although aggregations within the central region are not uncommon.

In close proximity of the nucleus are multivesicular bodies. These organelles are membrane-bound and contain smaller vesicles. Infrequently, a darker variation occurs in that the matrix is electron-dense (Figure 17a).

Occupying the surrounding cytoplasm are numerous, individual vesicles. Those found at the cell membrane are indicative of active pinocytosis. Mitochondria whose transversely oriented cristae display typical, chondrisomal organization are encountered as well.

Single and parallel arrays of granular endoplasmic reticulum (ER) constitute another organelle characteristic of leucocytes within the blood of I. illecebrosus. Of particular interest are the unusual configurations which tend to appear randomly throughout these cells. Such portions of granular ER form groups of concentric rings (Figure 17b). The area between these membraneous layers (within a particular arrangement) is often large.

FIGURE 15. A micrograph illustrating the fine structure of leucocytes from the blood of Illex illecebrosus. (4,233x)

1. Mitochondria
2. Golgi complex
3. Multivesicular body
4. Lysosome-like entity
5. Nucleus
6. Cell membrane
7. Linear endoplasmic reticulum

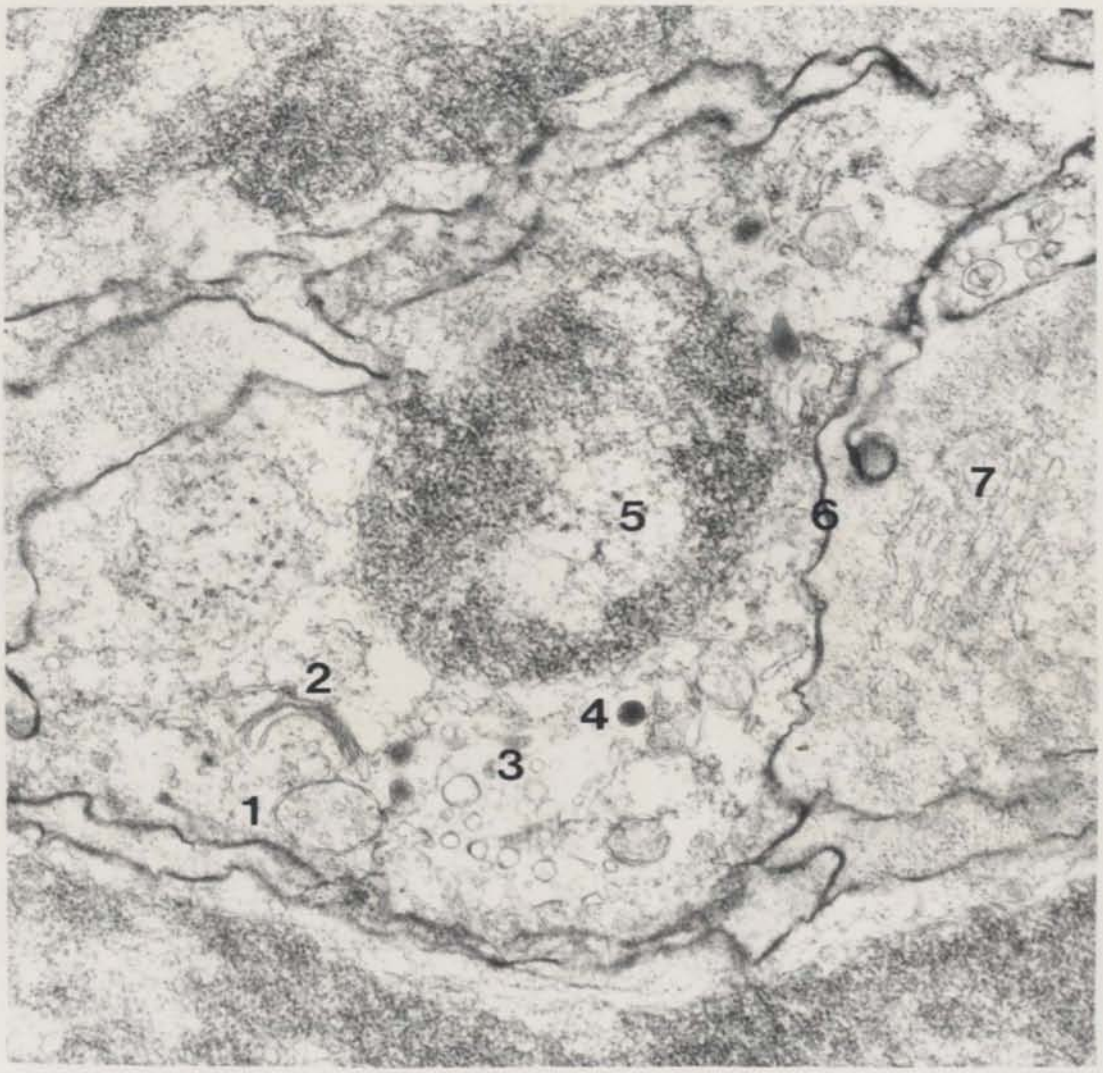
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FIGURE 16. A micrograph illustrating the fine structure of leucocytes from the blood of *Illex illecebrosus*. (4,833x)

1. Granular endoplasmic reticulum
2. Lysosome-like entity
3. Multivesicular body
4. Nucleus
5. Mitochondria
6. Myelin figure
7. Pinocytic vesicle
8. Invagination of cell membrane

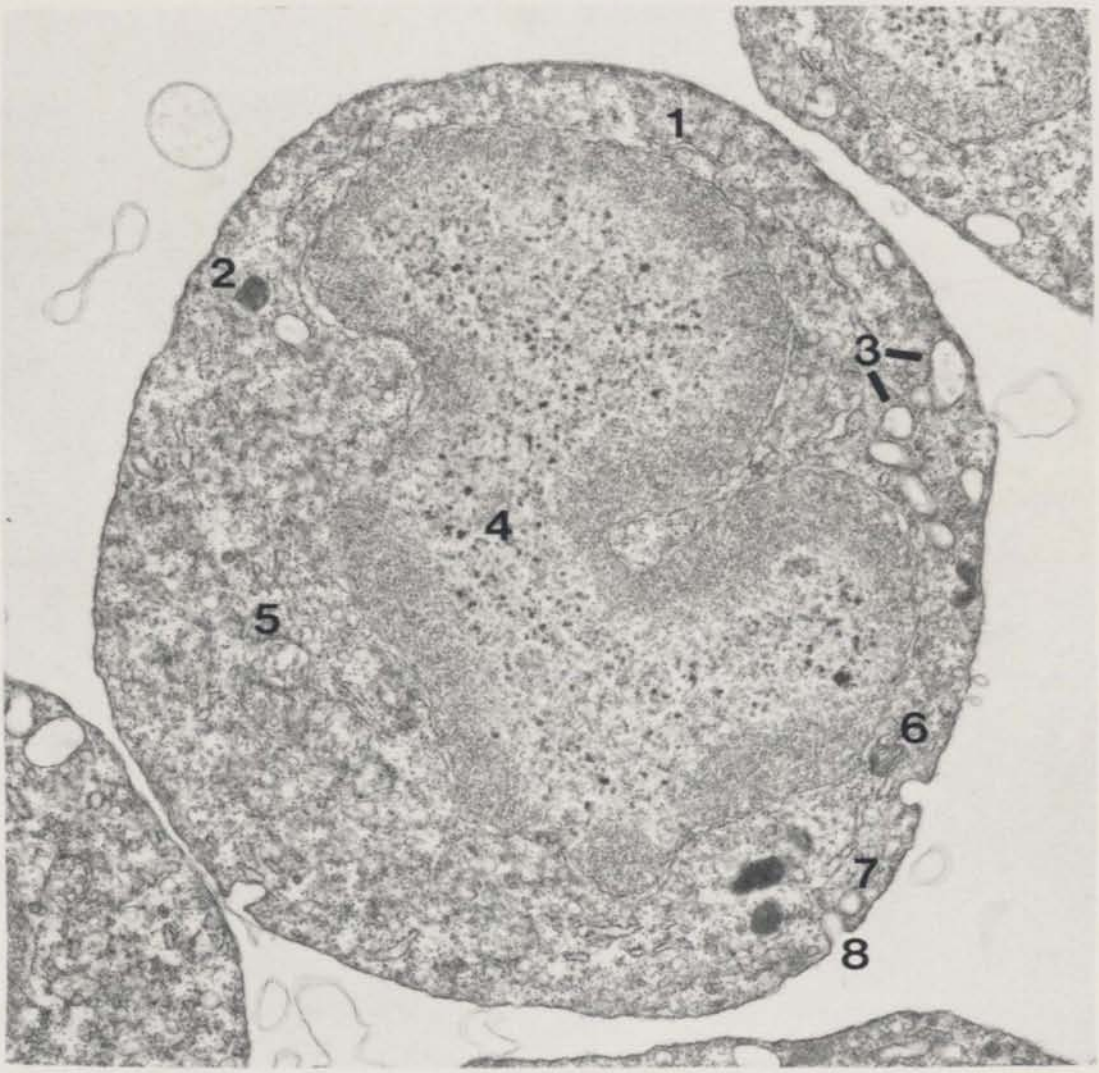
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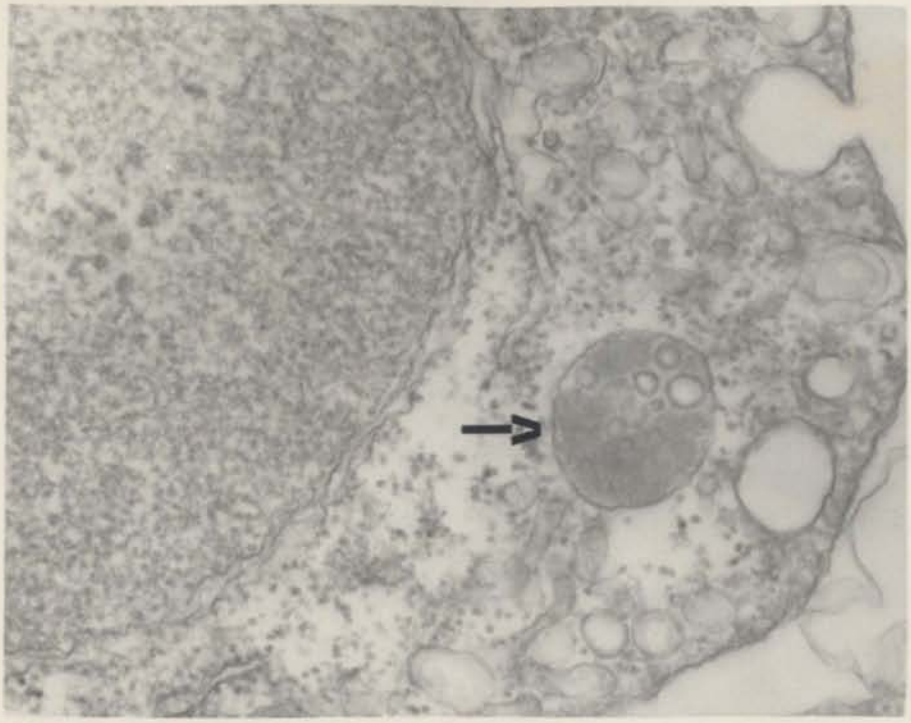
FIGURE 17. Micrographs illustrating the fine structure of leucocytes from the blood of Illex illecebrosus with emphasis on specific organelles.

a. The darker variation of multivesicular body in which the matrix is electron-dense (arrow). (45,000x)

b. Concentric rings of granular endoplasmic reticulum. (50,000x)



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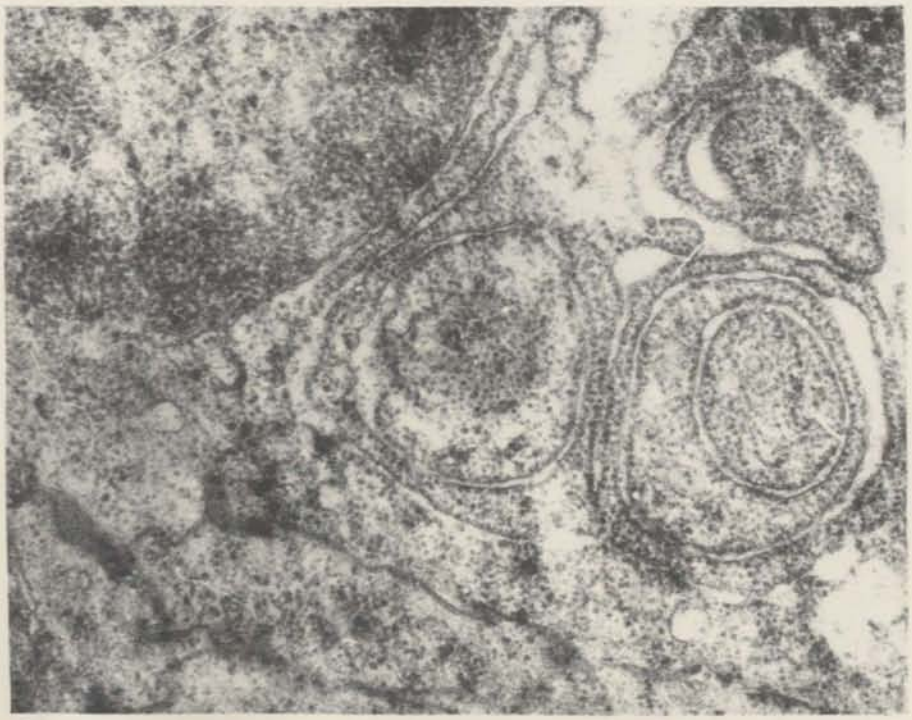

b

The presence of a Golgi complex was verified from electron micrographs as shown by Figure 15. Lastly, lysosome-like entities may be observed. At times, one or two vesicles may be contained within their membranes. Free ribosomes are dispersed in the matrix of the ground substance.

Upon processing, some leucocytes exhibit a high degree of contact with adjacent leucocytes giving the resultant mass a tissue-like appearance (Figure 15). The entire expanse of their cellular membranes is fused and darkly stained.

It should be noted that the above data, while representative, is not comprehensive. Small quantities of fresh squid blood and difficulties in staining procedures during preparation made this aspect of the research a limited one.

DISCUSSION AND CONCLUSIONS

Cellular components of the blood within Illex illecebrosus possess functional and morphological characteristics similar to those described for leucocytes of related cephalopods (Table 1). Indeed, certain traits are reminiscent of mammalian organization. Defined as eosinophilic granulocytes, these cells are round or ovoid in shape and have an average diameter of 20μ-22μ in stained preparations. The nucleus is a most conspicuous feature due to its size and vivid staining properties. High resolution studies reveal such typical structures as a double membrane, interrupted by nuclear pores, with the outermost unit supporting attached ribosomes. Peripheral concentrations of chromatin were visible, as well. A highly polymorphic condition exists whereby this nucleus may assume one of six different configurations. These range from compacted forms through a series of seemingly graded indentations to, eventually, the appearance of three or four lobes. Each configuration originates from the nuclear face, that region which projects into the surrounding cytoplasm. The opposing nuclear periphery is relatively smooth and rounded as a result of its close association with the cell membrane. Heidenhain's rule, accepted by most researchers states that progressive condensation of nuclear chromatin and the degree of

lobulation indicate advancing stages in cellular maturity (Ponder, 1926, p. 221; Ham, 1969, p. 367).

Accordingly, it is quite reasonable to submit that such a morphological series as described above, depicts a maturation sequence within Illex illecebrosus. To ascertain the validity of such a proposal and with the intent of establishing a classification system as a direct consequence, the author collected data through microvideomat analysis. It is interesting to note, having computed the nuclear areas for each of the selected nuclear stages (Figure 7), that values from the Newfoundland bait squid do not adhere to the rule of Heidenhain but, rather, agree consistently with results obtained from normal human beings and normal rabbits as recorded by Ponder (1926, p. 222). The nuclear areas are actually similar throughout these different nuclear categories. One exception was stage six in which only two examples were cited. Their status was inconclusive. The second aspect of Heidenhain's rule that refers to an increase in the number of nuclear lobes with cell maturity appears to be substantiated from data compiled in Figure 6.

The nucleus, in addition to other organelles, is suspended within a homogeneous, cytoplasmic matrix. Electron micrographs reveal the presence of dispersed ribosomes which confirms the basophilic nature of this substance as indicated from stained preparations.

Granular endoplasmic reticulum also contributes to the cytoplasm's affinity for basic dyes, especially those portions of this membrane system that form clusters or groupings of concentric rings.

Large, refractile vacuoles prominent at the level of light microscopy are identified from electron micrographs as multivesicular bodies. Evidence from time-lapse observations (p. 71) and electron microscopy (Figure 16) would suggest that these structures are involved in the process of "heterolysis". Characterized by de Duve and Wattiaux (Friend and Farquhar, 1967, p. 372), this method of intracellular transport commences with the absorption of foreign matter by invaginations of the cell membrane. Vesicles thus formed through pinocytosis convey the matter to lysosomes where digestion occurs. Friend and Farquhar (1967), Straus (1964 a and b) and Ham (1969) support the above outline of events. The relevancy of these statements to processes functioning within the leucocytes of I. illecebrosus can be seen in those time-lapse sequences which recorded undulating movements along the cell membrane. Such behavior is indicative of pinocytosis (Roberts, 1974, personal communication).

Micrographs revealing the presence of invaginations and the subsequent formation of pinocytic vesicles along the cell membrane confirm the existence of this activity upon morphological grounds.

With the occurrence of multivesicular bodies, these blood cells possess all the required machinery necessary for the operation of such an intracellular transport mechanism. Histochemical tests to corroborate this morphological evidence remain to be investigated. However, having established (1) the leucocyte's ability to pinocytize foreign matter and (2) the presence of multivesicular bodies, known to be digestive vacuoles (Friend, 1969, p. 269), the author submits that a similar if not identical mechanism may function within cellular elements from the blood of Illex illecebrosus.

Of final consideration are the cytoplasmic granules. Conventionally stained blood smears reveal two types, eosinophilic granulations and basophilic rods and spheres. The former serve as indicators to the specific nature of this cell type, e.g., eosinophilic granulocytes. While also occurring within supravitally stained preparations and phase contrast observations, these granules were not readily visible during ultrastructural studies. That is to say, those electron-dense, crystaline-like structures which characterize the central portion of eosinophilic granules within man, the rabbit, guinea pig, rat, cat (Bessis, 1973, p. 326) or goldfish (Andrew, 1965, p. 80) seem to be absent from similar granules within the squid I. illecebrosus. Instead, such granules are uniformly electron-dense.

The general structure and arrangement of the basophilic rods and spheres suggest mitochondria, especially when compared to results obtained from leucocytes stained with Janus green. However, this cellular component is said to be invisible when viewed with light microscopy aided by recognized Romanowski stains (Bessis, 1973, p. 35). Electron micrographs do not reveal additional organelles that would correspond to such granules.

Cellular movement, phagocytosis and agglutination, studied by means of time-lapse photography, were shown to be integral parts of the leucocytes' behavioral responses. The spherical shape these blood cells exhibit while circulating throughout the vascular system is lost or deformed upon attachment to a substratum. A property known as "spreading" is responsible for this change in appearance (Bessis, 1973, p. 307). Cytoplasmic veils form and produce a clear margin along the respective portion of cell periphery. No organelles were observed within these structures which could eventually give rise to individual pseudopodia and vice versa. Movement appears to be direct and purposeful as noted by the polarization of anterior and posterior regions. Detachment from a substratum occurs with the quick retraction of a posterior pseudopodium.

An examination of the phagocytic response, as it occurs *in vitro*, disclosed that this phenomenon is initiated when contact has been established between the foreign particle and the cell membrane. Bessis (1973, p. 43) goes one step further by stating that adherence is involved and that "if it (adherence) does not occur, the particle is repulsed by the phagocyte". This is particularly evident from Figure 14. However, once engulfed, the particle is then transported to the cell interior. It was unfortunate that these time-lapse observations did not encompass the process of digestion.

Contact between two or more leucocytes (agglutination) was noted to exist as "free" or "restricted" associations. The former arrangement is temporary, these cells retaining their individuality and the capacity for disassociation. In contrast, those cells joined in a restrictive manner were highly compressed or "clumped" although cell membranes along the periphery of this mass did extend pseudopodia. Disassociation was not observed. Cell aggregations of this type were maintained for long intervals of time.

It is concluded from the foregoing points of discussion that the cellular elements of the blood within *I. illecebrosus* are of one type - eosinophilic granulocytes. Morphological evidence was the criterion for classification. To categorize

a hemocyte solely upon its ability to move or engulf foreign matter would prove both inconsistent and incorrect for the cell, although capable, may not display such behavior at the time of observation. Instead, these characteristics serve to elucidate its role within the animal. However, the presence of specific granules in the cytoplasmic matrix does provide a stable basis for the designation of a cell type. In this case, therefore, the question of function versus form as a means of classification can only be answered through the latter.

It is concluded that the blood cells function as defensive agents for the self-preservation of this teuthoid. Their ability to move by means of pseudopodia and cytoplasmic veils implies a degree of control over cellular activities - these leucocytes are not exclusive subordinates of the bloodstream. The fact that they actively engage in phagocytosis and pinocytosis supports this thesis propounding a protective role against invaders. Organelles necessary for the degradation of ingested material (by pinocytosis) reinforce the premise. Lastly, these cells are capable of agglutinating, a mechanism designed to alleviate the crisis following wound infliction.

The information compiled within this thesis represents only the beginning of a varied and interesting avenue of research. The author recommends the following topics for further consideration:

cell count, the average number of leucocytes in the circulating blood and the extent to which seasonal, sexual or pathological factors may influence this figure; cellular response to injury, a comprehensive look at wound healing; hemopoiesis, developmental stages in the maturation process and the organ site of production; and cell ageing, a comparative study stressing the morphology of cells represented by each category of nuclear configuration.

REFERENCES CITED

ANDREW, W. 1965. Comparative Hematology. Grune and Stratton, New York.

ARVY, Lucie. 1960. Histoenzymological data on the digestive tract of Octopus vulgaris L. (Cephalopoda). Ann. New York Acad. Sci., 90: 929-949.

BAGINSKI, S. 1965a. Studies on the phagocytic system in cephalopods of the Family Eledone. Folia. Morph., 24(4): 351-356.

_____. 1965b. Histophysiology of the parabranchial gland in cephalopods of the Family Eledone. Folia. Morph., 24(3): 215-232.

BARBER, V. and P. GRAZIADEI. 1965. The fine structure of cephalopod blood vessels. 1. Some smaller peripheral vessels. Zeit für Zellforsch., 66: 765-781.

BECK, D. and L. BRAITHWAITE. 1962. Invertebrate Zoology Laboratory Workbook, 2nd ed., Burgess Publishing Company, Minneapolis, Minn., p. 187.

BESSIS, M. 1973. Living blood cells and their ultrastructure (translated by R. Weed). Springer-Verlag, New York.

BOLOGNARI, A. 1950. Sulla Genesi Degli Elementi Figurati del Sangue dei Molluschi Cefalopodi. Boll. Zool., 17, Supple., 369-378.

_____. 1951. Morfologia, Struttura e Funzione del "Corpo Bianco" dei Cefalopodi, Parte II - Struttura e Funzione. Arch. Zool. Ital., 36: 253-287.

_____. 1952. Sulla Localizzazione degli Acidi Nucleinici e Sull Origine delle Granulazioni dei Leucociti nel Corpo Bianco dei Molluschi Cefalopodi. Boll. Zool., 14: 211-216.

BRADBURY, Helen E. 1970. Observations on the functional anatomy of the ommastrephid Illex illecebrosus (Lesueur, 1821) (Coleoidea: Cephalopoda) with emphasis on musculature and the blood vascular system. Doctor of Philosophy thesis, Memorial University of Newfoundland, St. John's, Nfld.

BRADBURY, H. and F. ALDRICH. 1969. Observations on feeding of the squid Illex illecebrosus illecebrosus (Lesueur, 1821) in captivity. Ca. J. Zool., 47(5): 913-915.

CARAZZI, O. 1901. Studi sui Molluschi. Int. Monatsch. f. Anat. u. Physiol., Bd. 18, p. 1.

CARLETON, H. and R. DRURY. 1962. Histological Technique. Oxford University Press, London.

CATTANEO, G. 1889. Sulla Morfologia delle Cellule Ameboidi dei Molluschi e Artropodi. (Translated by Dr. R. Piccinini). Boll. Scient., Vol. XI.

_____. 1891. Gli Amaebociti dei Cefalopodi, e Loro Confronto con Quelli d'altri Invertebrati. Atti. Univ., Genova, XI.

CAZAL, P. et D. BOGORAZE. 1943. Recherches sur les Corps Blans du Poulpe (Octopus vulgaris Lam.). Leur Fonction Globuligéne et Néphrocitoire. Bull. Inst. Océan., Monaco, n. 842.

CINCHONA PRODUCTS INSTITUTE, INC. 1948. The Technique of Blood Examination in Malaria. New York, p. 1-3.

COWDEN, R. 1972. Some cytological and cytochemical observations on the leucopoietic organs, the "white bodies", of Octopus vulgaris. J. Invert. Path., 19: 113-119.

_____ and S. CURTIS. 1973. Observations on living cells dissociated from the leucopoietic organ of Octopus briareus. Exp. and Mole. Path., 19: 178-185.

CUÉNOT, L. 1891. Études sur le Sang et les Glandes Lymphatiques dans la série Animale, 2e partie: Invertébrés. Arch. Zool. Exp. et Gen., 2e série, 9: 13-90.

CUÉNOT, L. 1897. Les Globules Sanguins et les Organes Lymphoides des Invertébrés. Arch. Anat. Micro., 1: 153.

DOAN, C. and P. RALPH. 1964. Supravital Staining. In: McClung's Handbook of Microscopical Technique. R. McClung Jones (ed.), p. 571. Hafner Publishing Co., New York.

FAUSSEK, V. 1893. Über die Sogenannten "Weissen Körper" sowie Über die Embryonale Entwickelung desselben, der Cerebralganglien, und des Knorpels bei Cephalopoden. Mém. Acad. imp. Sc., Saint-Pétersbourg, 7e série, XLI, No. 9.

FAUSSEK, V. 1901. Untersuchungen über die Entwicklung der Cephalopoden. Mitt. Zool. Station, Neaple, Bd., 14:83.

FRIEND, D. 1969. Cytochemical staining of multivesicular body and Golgi vesicles. J. of Cell Biol., 41: 269-279.

_____, and M. FARQUHAR. 1967. Functions of coated vesicles during protein absorption in the rat vas deferens. J. of Cell Biol., 35: 357-376.

HAM, A. 1969. Histology. 6th edition. J. B. Lippincott Company, Philadelphia.

ISGROVE, A. 1909. Eledone, L.M.B.C. Memoirs, No. XVIII, Williams and Norgate, London.

JULLIEN, A. 1928. Sur la Transformation des Cellules Sanguines de la Seiche au cours des Reactions Inflammatoires Aseptiques. C. R. Séanc. Lebd. Acad. Sci., 186: 526-529.

KAWAGUTI, S. 1963. Electron microscopy on the heart muscle of the cuttlefish. Biol. J., Okayama Univ., 9:27-40.

KIYONO. 1929. Quoted from T. Ohuye (1938).

KNOLL, P. 1893. Uber die Blutkorperchen bei wirbellosen Thieren (translated by Norma Joseph). Sitzungsberichte der Mathematisch-Naturwissenschaftlichen Classe der Kaiserlichen Akademie der Wissenschaften, C II Band. Abtheilung III.

KOLLMANN, M. 1908. Recherches sur les Leucocytes et le Tissu Lymphoide des Invertébrés. Ann. Sci. Nat. Zool., 9e série, 8: 1-238.

LANGE, M. 1920. On the regeneration and finer structure of the arms of the cephalopods. J. Exp. Zool., 31(1): 1-40.

LEYDIG, F. 1857. Lehrbuch der Histologie des Menschen und der Thiere (translated by Norma Joseph). Frankfort.

MARTIN, R., V. BARBER and A. BOYDE. 1968. Structural specializations of an endothelium covering neurosecretory axon terminals in a cephalopod vein: scanning and transmission electron microscope studies. Fourth Regional European Conference for Electron Microscopy, Rome, p. 361.

MEYER, W. 1907. Die Anatomie von Opisthoteuthis depressa (Ijima und Ikeda) (translated by Norma Joseph). Zeitscheift für Wissenschaftliche Zoologie, Leipzig, 85: 183-269.

NOEL, R. and A. JULLIEN. 1933. Recherches Histologiques sur le Corps Blanc des Cephalopodes. Arch. Zool. Exp. Gen., 75: 485-499.

OHUYE, T. 1938. On the coelomic corpuscles in the body fluid of some invertebrates. Science Reports of the Tohoku Imperial University, 4th series, Biol., 12:593-622.

PONDER, E. 1926. Studies on the Arneth Count - II. The Relation of Nuclear Configuration to Nuclear Area. Quart. J. Exp. Physiol., No. 221-224.

QUIDLEY, J. 1964. Mechanized Squid Jigger. Trade News, Ottawa, 17(5): 325.

ROWDEN, G. 1973. Rapid Tissue Processing. Philip's Hints, Philips Electron Optics Division, August, p. 1-3.

SERENI, E. and J. YOUNG. 1932. Nervous degeneration and regeneration in cephalopods. Pubblicazioni della Stazione Zoological di Napoli, 12: 173-208.

STRAUS, W. 1964a. Cytochemical observations on the relationship between lysosomes and phagosomes in kidney and liver by combined staining for acid phosphatase and intravenously injected horseradish peroxidase. J. of Cell Biol., 20: 497-507.

_____. 1964b. Occurrence of phagosomes and phago-lysosomes in different segments of the nephron in relation to the reabsorption, transport, digestion and extrusion of intravenously injected horseradish peroxidase. J. of Cell Biol., 21: 295-308.

STUART, A. 1968. The reticulo-endothelial apparatus of the lesser octopus, Eledone cirrosa. J. Path. Bact., 96(2): 401-412.

_____. 1970. Phylogeny of mononuclear phagocytes. In: Mononuclear Phagocytes, R. van Furth (ed.). Blackwell Scientific Publications, Oxford, pp. 316-334.

THOMPSETT, D. 1939. Sepia. L.M.B.C. Memoires, No. XXXII, The University Press of Liverpool.

THORE, S. 1936. Cephalopodstudien. Beiträge zur Kenntniss der Sog. Weissen Korper nebst Mitteilung über ein neues Organ bei Octopus vulgaris. Kungl. fys. Sallsk. Lund. Forh. Bd. 6, n. 16.

VENABLE, J. and R. COGGESHALL. 1965. A simplified lead citrate stain for use in electron microscopy. J. Cell. Biol., 25: 407-408.

VILLANUEVA, A. 1970. A new biological stain for peripheral blood and bone marrow smears. Henry Ford Hospital Medical Journal, 18(1): 11-20.

VOGT, C. and E. YUNG. 1888. Traité d'Anatomie Comparée Pratique. Paris.

WELLS, M. 1962. Brain and Behavior in Cephalopods. Stanford University Press, California.

WITMER, A. and A. MARTIN. 1973. The fine structure of the branchial heart appendage of the cephalopod Octopus dofleini martini. Zeit. Zellforsch., 136: 545-568.

YEAGER, J. and O. TAUBER. 1935. On the hemolymph cell counts of some marine invertebrates. Biol. Bull., 69: 66-70.

YOUNG, J. Z. 1970. Neurovenous tissues in cephalopods. Phil. Trans. Roy. Soc. Lond. (Biol. Sci.), 257: 309-321.