STUDIES ON THE PHYSIOLOGY OF HEMOLYMPH COAGULATION IN PERIPLANETA AMERICANA (L.)

by
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ABSTRACT

Title of Thesis: Studies on the Physiology of Hemolymph Coagulation in Periplaneta Americana (L.)

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In the cockroach, Periplaneta americana (L.), hemolymph coagulation (a) is inhibited for as long as 30 minutes at 0° to 4°C, (b) is initiated at 5°C, (c) is permanently inhibited at 60°C, and (d) takes place in 6 distinct stages. Hemocyte agglutination and transformation is inhibited at 0° to 4°C, is permanently prevented at 55°C, and is independent of plasmal factors. Live plasmatocytes, granular hemocytes, and cystocytes are structurally identical, but differ functionally in their capacity to phagocytize and in their fragility. The cystocyte's primary function is the initiation of coagulation and/or precipitation of the plasma by ejecting cytoplasmic material, including mitochondria, into the surrounding plasma. Hemocyte-free plasma will not spontaneously precipitate, but requires either ionic calcium released from transforming hemocytes, and/or material from exploded cystocyte mitochondria. Substances inside mitochondria may well be the source of a coagulation-inducing substance that initiates plasma precipitation and veil formation. Substances involved in P. americana coagulation are present in the plasma of 9 other species of cockroach which react to P. americana cystocytes. Substances in the plasma of Tenebrio molitor, Galleria mellonella, or Rhodnius prolixus do not precipitate in the presence of P. americana cystocytes. The amount and/or effectiveness of a coagulation-inducing

substance released from cystocytes presumably determines the degree of plasma precipitation. Physiologically active substances contained in and/or released from the corpora allata and c. cardiaca, but lacking in the brain, may regulate the percentage of circulating cystocytes, thereby influencing the coagulability of the hemolymph.

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INTRODUCTION

While there is an extensive literature on the process of hemolymph coagulation in insects (Grégoire and Tagnon, 1962; Jones, 1962) most of this knowledge is based on its microscopical aspect (Grégoire, 1951, 1955), and much of it is fragmentary and contradictory.

Coagulation is a most diverse phenomenon and basically concerns the "denaturation" of proteins. The term has been used to refer to the agglutination of hemocytes alone, to gelation of the plasma without microscopically visible precipitation, and to gelation with fine or coarse precipitation of the plasma with or without agglutination of the hemocytes. Each of these may be separate phenomena, but only the last two can involve permanent denaturation. The phenomenon is one of the great elemental reactions of all cells and is intimately associated with the release of calcium (Heilbrunn, 1961). It involves the reaction of the cell to surfaces. Cellular reaction to surfaces can be expressed in a variety of inter-related ways: locomotion, phagocytosis, pinocytosis, agglutination, and coagulation.

In the cockroach, coagulation involves the agglutination and the dissolution of cells and the precipitation of the plasma. To be useful it must be rapid (that is, come to completion quickly). To be effective to the animal it should ideally occur only at alien surfaces (wounds) and at times of stress (that is, normally it would be suppressed).

The purposes of this study on Periplaneta americana (L.) were (1) to develop techniques for controlling coagulation, (2) to analyze the sequence of changes involved in coagulation, (3) to isolate hemocyte-free

plasma and plasma-free hemocytes so that their reactions could be studied separately or after species specific and nonspecific recombination, and (4) to study whether calcium is involved in coagulation. In addition, it seemed desirable to critically reevaluate the function of the cystocyte in coagulation by trying (1) to identify and characterize living, intact cystocytes (="coagulocytes") in fresh hemolymph, and (2) to identify and determine the function of the dark, rounded granules contained in cystocytes and other hemocytes.

LITERATURE REVIEW

There are several comprehensive reviews on insect hemolymph coagulation (Grégoire, 1951 a, 1955; Hinton, 1954; Wigglesworth, 1959; Heilbrunn, 1961; Wyatt, 1961; Grégoire and Tagnon, 1962; Jones, 1962, 1964 in press; and Grégoire, 1964 in press). Information and conclusions from investigations on the hemolymph of other arthropods, especially of crustaceans, have influenced the interpretation of the mechanism of hemolymph coagulation in insects.

In Crustacea and Xiphosura (*Limulus* sp.), Loeb (1903, 1905) noted that blood cells first agglutinated and then disintegrated as they fused into a cell coagulum which was sometimes followed by plasma coagulation. However, Loeb did not recognize any specific cell types that played a major role in the initiation of the coagulation process. The first understanding of the mechanism of blood coagulation in arthropods came when Hardy (1892), Tait (1910, 1911), and Tait and Gunn (1918) discovered in several Crustacea very fragile "explosive corpuscles" which, on contact with foreign surfaces, transformed and disintegrated, and ejected material into the plasma thereby inducing plasma coagulation in surrounding areas. Hemolymph coagulation in insects, as in Crustacea, has been described as consisting of two separate physiological processes which may occur together or independently; (1) hemocyte transformation and/or agglutination, (2) plasma coagulation or precipitation (Muttkowski, 1924; Yeager and Knight, 1933; Ermin, 1939; Beard, 1950; Grégoire and Florkin, 1950 b). In numerous insects, hemocytes analgous to Hardy's explosive corpuscle are termed "coagulocytes" (Grégoire, 1951 a) or "cystocytes" (Jones, 1954, 1962).

Cystocytes are highly unstable hemocytes which during coagulation contract, and then eject cytoplasmic material into the plasma, and cause clouds of plasma precipitate or glassy veils to form around the extruded nucleus.

Grégoire (1951 a, 1953 a, 1955) has classified hemolymph coagulation in more than 400 species of insects into 4 basic patterns: pattern 1 is characterized by a plasmic coagulation or precipitation around cystocytes; in pattern 2, there is formation of hemocyte pseudopodial meshworks and the appearance of glassy veils in the plasma; pattern 3 is a combination of pattern 1 and 2; and in pattern 4, there is an absence of coagulation. Pattern 1 is typically found in most cockroaches and other orthopterans.

Although many recent investigations on the biochemistry of insect hemolymph give information on the various constituents in hemolymph (Wyatt, 1961), little is known about the chemical nature of substances involved in the process of hemolymph coagulation. According to Wyatt (1961), most of these investigations were based on protein components of serum rather than plasma or hemolymph, with no attention given to the nature of coagulable substances. Siakotos (1960 a, b) and Stephen (1961) have biochemically characterized five proteins in Periplaneta americana hemolymph. Siakotos (1960 b) suggested that the major contributing substance to plasma precipitation during coagulation is a large unstable glyco-lipoprotein. Since there is said to be a specific dependence of coagulation on pH and on the ionic ratio in plasma, Franke (1960 a) suggested that enzymes, possibly from hemocyte mitochondria, are critically involved in coagulation of plasma.

Rapp (1947) pointed out that the whole mechanism of clotting in insect hemolymph is very different from that in vertebrate blood. The

microscopically visible crystal-like needles of fibrin which develop during the clotting of mammalian blood have been reported in crustacean blood coagulation (Howell, 1916; Morrison and Morrison, 1952). In insects on the other hand, observations on peripheral parts of islands of coagulation indicated that plasma granules (precipitate) transformed into needle-like structures shortly after hemolymph withdrawal and cystocyte transformation (Grégoire and Jolivet, 1957; Grégoire, 1959 c). Grégoire, Duchateau, and Florkin (1949) noted that the plasma precipitation which develops around transformed cystocytes in Gryllus domesticus appears in the electron microscope either in the form of sponge-like masses (microflocs), or scattered or agglutinated in meshworks against a background of small granules. Identical formations are found in coagulated hemolymph of P. americana (Grégoire, 1959 c).

Differences in techniques of examining the hemolymph are responsible for many of conflicting descriptive accounts of the coagulation process in the same species of insect. When the hemolymph is fixed and stained with Wright or Giemsa, the cells may not be preserved intact or gross distortions in structure may occur (Jones, 1962). In most studies on insect hemolymph coagulation, unfixed, undiluted, or saline-diluted, wet, thin, whole mounts of living hemolymph are examined with bright field, dark field, or phase contrast (Yeager et al., 1932; Rooseboom, 1937; Ermin, 1939; Grégoire and Florkin, 1950 b). A drop of live hemolymph can also be collected in immersion oil (Yeager et al., 1932;Rizki, 1953) or tissue culture medium (Wyatt, 1956). According to Franke (1960 a), the coagulation picture of Blatta orientalis appears different depending on whether it is examined as a thin film or a hanging drop.

Insect hemolymph coagulation has been inhibited by several organic acids: nucleic (Paillot, 1923), citric acid and ascorbic acids (Beard,

1950), vapors of acetic and other fatty acids (Shull, Riley, and Richardson, 1932; Shull, 1936; Fisher, 1935); and by physical treatment, such as (a) heating the insect (Yeager et al., 1932; Babers, 1938; Beard, 1950; Jones, 1962; Wheeler, 1963), freezing or (b) chilling (Beard, 1950; Franke, 1960 a; Siakotos, 1960 a; Rosenberger and Jones, 1960; Jones, 1962), or (c) exposing to ultrasonic waves (Beard, 1950). Franke (1960 a) noted that coagulation in a hemolymph drop of Blatta orientalis is pH dependent, occurs optimally at pH 7-8, is inhibited at pH 2-3 or 11-12, and is incomplete at pH 4-6 and 9-10. Furthermore, in the same insect, Franke (1960 a) reported that coagulation is inhibited by mildly hypertonic saline but is greatly accelerated by hypotonic solutions. Of 33 substances (mostly anticoagulants for vertebrate blood) tested by Grégoire (1953 a), 18 were efficient anticoagulants for insect hemolymph. Among them were four salts (calcium or metallic ion chelating agents), six organic esters of sulfuric acid, two basic dyes, and three reducing agents. Grégoire (1953 a) suggested that these anticoagulants inhibit alterations in coagulocytes or cystocytes, thereby preventing the release of coagulation-inducing substances from the cells. In addition, he reported that coagulation occurs on a variety of surfaces (cellophane, acetophane, plexiglass, glass coated with either silicon or paraffin) and that coagulation occurs independently of pH.

GENERAL METHODS

Rearing cockroaches. Nymphs and adults of the American cockroach Periplaneta americana (L.) were reared separately in Pyrex battery jars (12 x 12 inches) at approximately 27°C and undetermined relative humidity. To increase surface area and provide hiding places for the cockroaches, fourteen, pint-sized, ice-cream cartons (with "V" shaped openings cut in the open end) were inverted and placed in two layers on the bottom of each jar. The cockroaches were fed on Purina Laboratory Chow and provided with water in cotton-plugged 250 ml flasks. The rearing jars were cleaned and new cartons supplied at approximately 3 week intervals. Last stage intermoult nymphs, and moulting nymphs were identified according to the criteria of Flint and Patton (1959).

Hemolymph wet mounts and microscopy. The observations on hemolymph coagulation were made from wet, unstained mounts spread between a slide and a 10 or 12 mm, round coverslips at room temperature or on a special cold stage (see p. 8). For routine work, a dark-medium American optical "Phasestar" microscope was employed. For special work involving the use of a cold stage, light and dark phase were used in conjunction with fluorescent microscopy, and sometimes used in conjunction with a 16 mm time lapse microcinematographic camera attached to a Reichert MeF universal microscope.

Yeager's Cockroach "heart and blood" saline (Buck, 1953) was used unless otherwise stated (saline composed of 10.93g NaCl, 1.57g KCl, 0.85g $CaCl_2$, and 0.17g $MgCl_2$ per liter of distilled water).

Percentages of circulating cystocytes. Small drops of hemolymph from freshly severed antennae were placed in a drop of saline on a glass slide for about 45 seconds, and then fixed with 2% aqueous versene /disodium ethylenediamine tetraacetate (Fisher Scientific Company, No. S-311)/. The percentage of cystocytes was calculated by counting the number of circulating cystocytes among 500 hemocytes in cockroaches using a "Leucodiff Recorder" (Fisher Scientific Company, No. 6-247-10V3).

Photomicrographs. The photomicrographs were taken on Polaroid Land film (type 55 P/N) with a dark-medium American Optical "Phase" microscope or with a Reichert Universal microscope. Time lapse movies were taken on 16 mm Kodak "Linagraph Shellburst" film with a Paillard Bolex movie camera used in conjunction with a Emdeco timer and the Reichert Universal microscope.

Microscope cold stage. In order to inhibit rapid coagulation and still study the hemocytes in as nearly a "natural" state as possible, it was necessary to construct a microscope cold stage. With reference to Plate 1, Figure 1, the following parts were used:

1. Two (75 x 65 x 1 mm) copper plates (A and B).
2. One 1 inch rubber "O Ring" (C).
3. One 2 inch rubber "O Ring" (D).
4. Two number 2, 18 mm round coverslips (E and F).
5. Two elbow, ¼ inch O.D. copper joints (G and H).
6. Twenty 4/40 thread, 3/8 inch, flat or round head brass belts with nuts (I and J).

The dimensions and sizes of the parts listed can be modified to suit optical limitations and stage size of the microscope.

Construction. (1) The periphery of both plates A and B was drilled to accomodate 20 bolts (J). (2) A central 15 mm hole was bored in the

plates (A and B). (3) Two $\frac{1}{4}$ inch holes were bored into plate A in a position between both "O Rings" (C and D) and the elbow joints (G and H) were soldered to these respective holes. (4) A 1/32 inch hole (K) was bored into plate A in a position just inside "O Ring" (C). (5) After placing the "O Rings" (C and D) between the plates A and B, the plates were bolted together. (6) Coverslips (E and F) were glued with "Zut" or another waterproof glue on the outside over the large central holes of both plates and allowed to dry for twenty-four hours. (7) The central chamber enclosed by "O Ring" C and the coverslips was filled by injecting clean distilled water into it through hole K. (8) When filled, the chamber hole K was sealed with a small piece of tackiwax (Cenco-C11444). A completed cold stage is shown in Plate I, Figure 2,A.

Theory of Operation. Cold water is pumped through either elbow joint G or H into the outer chamber (between "O Rings" C and D) and exits from the remaining elbow joint. Cold water circulating through this chamber draws heat from the plates and the inner chamber. "O Ring" C which forms the inner chamber is included in the basic design to help protect the coverslips from the hydraulic pressure of the circulating water as well as to prevent the appearance of air bubbles in the field of view. Specimens are placed directly on the coverslip surface of the cold stage. The cold stage is placed directly on the stage of a microscope in such a position that light from the condensor passes through the central porthole of the central chamber to the objective, (Plate I, Figure 4). When working with phase microscopy, it is necessary to use a long working distance condensor (10 mm) that will allow for the thickness of the cold stage. When using low power objectives up to high dry (470 x), the cold stage can be cooled by pumping ice water directly through it. However, when using high

dry (470 x) or oil immersion (970 x), hydraulic vibration from the pump may interfere with good resolution. With reference to Plate I, Figure 3, this condition can easily be avoided by pumping water through tube D to a small reservoir (H) above the level of the cold stage. Water then flows by gravity from the reservoir through tube B to the cold stage (A), and returns to the pump reservoir (G) through tube C. Tube E is connected to a standpipe in the upper reservoir which drains overflow water back to the pump reservoir. A Bronwill Thermoregulator (F), (A.S. Aloe No. V79900) was used as a combination pump, stirrer, heater, and thermoregulator. Thus, the cold stage could be operated at a wide range of temperatures. The Bronwill Thermoregulator does not provide refrigeration, thus, ice water or a separate refrigeration coil is needed to chill the water. If temperatures below freezing are desired, all water in the system and cold stage can easily be replaced with alcohol or another suitable low freezing point liquid.

PROCEDURE AND RESULTS

Hemolymph coagulation in Periplaneta americana (L.)

Hemolymph coagulation in the genus Periplaneta and in other cockroach genera has been described and classified as pattern I by Grégoire (1955, 1957, 1959 b) and Grégoire and Jolivet (1957)(i.e., a fine cloud of plasma granules appears specifically around cystocytes or coagulocytes). In Blatta orientalis Yeager et al. (1932) and Franke (1960 a) noted the sequence of hemolymph changes occurring during coagulation. According to Franke (1960 a), coagulation in hanging drops of hemolymph occurs in 4 phases: (1) the hemocytes round up or contract, (2) the hemocytes agglutinate and send out pseudopodia, (3) the hemolymph coagulates (i.e., a plasma precipitate forms), and (4) the hemocytes expand and disintegrate to form a thick meshwork.

Preliminary work revealed that in P. americana hemolymph coagulation in thin, coverslip, wet mounts was so rapid that it could not be analyzed without special techniques.

Procedure. The microscope cold stage was used to control the initiation and termination of coagulation as follows. A drop of hemolymph from a severed antenna was immediately placed on a cold stage held at 2°C and coverslipped so that it could be examined with light-phase microscopy at 720 and 1,500 x. When a large number of hemocytes were located in the microscope field, the cold stage was allowed to slowly warm up to room temperature (25-27°C). A 16 mm time lapse movie camera was started to record coagulation at 10 frames/minute until the termination of the process so that the exact sequence of hemolymph changes occurring could be analyzed.

Results. When the hemolymph of Periplaneta americana is collected directly onto a microscope cold stage held at 2°C, the plasma and the hemocytes remain unchanged for as long as 30 minutes before the hemocytes very slowly begin to contract. If the temperature of the cold stage is allowed to go above 5°C, then the following sequence of changes can be readily observed: (1) with the single exception of the granular hemocytes, the hemocytes contract or round up; (2) the contracted cells clump into masses of 5 to 25 cells at random and fine threads of cytoplasm can be seen between the clumps; (3) the cytoplasm of the cystocytes becomes hyaline and the cells suddenly implode to release a single round, barred nucleus and bacilliform mitochondria. The released mitochondria burst and disappear. (4) When the mitochondria burst, a heavy granular cloud appears immediately around the extruded cystocyte nucleus. The granular cloud itself instantly becomes a violently seething mass of tiny vibrating particles (Brownian movement). (5) A finely granular material appears between the clumped hemocytes. (6) Finally, the clumped cells within the granulated plasma flatten out and disintegrate into an amorphous meshwork arrangement of protoplasm and nuclei.

Identification of untransformed cystocytes

Jones (1957, 1962) and Wheeler (1963) reported that cystocytes cannot be specifically identified when they have been fixed by heat, cold, or versene. Plasmatocytes and granular hemocytes are said to be phagocytic in several insects (Yeager 1945; Jones 1956, 1962). Cockroach cystocytes have not been proven to be phagocytic even though the cystocyte is thought to represent a highly specialized granular hemocyte (Jones, 1962).

When Periplaneta americana (L.) injected with chinese ink (Yeager et al,

1942) or with vertebrate erythrocytes (Bettini et al., 1951) there is hemocyte agglutination, reduction of total hemocyte counts and "phagocyte" destruction, but no specific hemocyte types were recognized as being associated with these events.

The present experiments were made to attempt to identify chilled cystocytes before they transformed. It was initially hoped that the cystocytes would not engage in phagocytosis and could therefore be separated from plasmatocytes.

Procedure. Ten last stage nymphs and 5 adults were injected with 10, 20, and 30 µl of a 3% suspension of chinese ink in saline using an Agla syringe and a 30 gauge needle. After 3 and 76 hours, two hemolymph samples were taken from a severed antenna; one sample was fixed in 4% versene on a slide (Wheeler, 1963), and a count made on the number of hemocytes with engulfed ink per 100 hemocytes. The other sample was placed on a cold stage at 2°C. Hemocytes with engulfed ink particles were located and the cold stage then allowed to warm up to room temperature.

Result. There was no significant change in the phagocytic count in adults 3 and 76 hours after 20 µl of 3% chinese ink was injected (i.e.; at 3 hours the phagocytic count was 16.4% ± 3.1; at 76 hours, the count was 21.5% ± 3.4).

When last stage nymphs were injected with 10 µl of 3% chinese ink the phagocytic count was 16.0% ± 2.9 after 3 hours and 14.7% ± 2.7 after 76 hours. Hemocytes with engulfed ink particles were observed at 2°C on a cold stage and then the cells allowed to transform. While nearly all of the hemocytes containing ink were either plasmatocytes or granular hemocytes, nevertheless, in a few cases (about 1 out of 10) hemocytes with ink

inside them transformed into typical cystocytes (Plate 2, Figure 1).

Procedure. Ten milliliters of rabbit blood was collected and preserved in 20 ml of sterile Alsevers solution (Evans, 1957) and stored at 5°C until ready for use. Before use, erythrocytes were washed three times in 0.85% NaCl. An erythrocyte count was made on the erythrocyte suspension before injecting into cockroaches. Percentages of cystocytes from 4 groups of adult female cockroaches (5 per group), were obtained before they were injected with 20 µl suspensions of erythrocytes containing approximately 4,500,000, 3,000,000, 250,000, and 60,000 cells/20 µl. Hemolymph samples were then taken 1 and 20 hours after injection, and the number of hemocytes with engulfed erythrocytes per 500 cells counted on a 2°C cold stage. After this, the stage was allowed to warm to 27°C and the percentage of cystocytes was determined.

Result. When suspended rabbit erythrocytes in 3 concentrations (250,000, 3,000,000, and 4,500,000/20 µl) was injected into 4 groups of adult females (5 per group, injected with 20 µl/cockroach) and these groups were examined 1 and 20 hours thereafter, no erythrocytes were observed in the hemolymph and no phagocytosis of them by hemocytes was seen. When these cockroaches were carefully examined, large agglutinated masses of erythrocytes were seen at the site of injection. These agglutinated masses were partially surrounded by plasmatocytes, transformed cystocytes, and coagulated plasma (Plate 2, Figure 3). Differential counts on these cockroaches showed a normal percentage of cystocytes of about 20% (Table 1). In those cockroaches injected with 60,000 erythrocytes, all of the cold-fixed phagocytes subsequently transformed into plasmatocytes when samples were allowed to warm up (Plate 2, Figure 2). One hour after injecting 60,000 erythrocytes, 5 to 10 free erythrocytes per 500 hemocytes were ob-

served in the plasma and the phagocytic count was 0.5%. No free or phagocytized erythrocytes were observed 19 hours later. These cockroaches showed a marked increase in the percentage of cystocytes 1 hour after injection (of about 18%), but this value returned to normal 19 hours later (Table 1).

Procedure. Various dilutions of rabbit erythrocytes (4,500,000-60,000 cells/ 20 µl) or diluted or whole human blood was added to drops of either fresh unfixed or heat-fixed (55° - 60°C) cockroach hemolymph or cell-free plasma on a slide and coverslipped and examined after 3 minutes.

Result. Strong erythrocyte agglutination occurred when diluted and whole human blood and rabbit erythrocyte suspensions (containing 250,000 to 4,500,000 cells/ 20 µl) were mixed with unfixed or with 55°C - heat-fixed hemolymph or plasma. No agglutination occurred when the 60,000 cell suspension was added to unfixed and 55°C heat-fixed hemolymph and plasma.

Separation of cells and plasma

To study coagulation in the cockroach it is absolutely essential to be able to separate hemocytes and plasma before coagulation begins.

Procedure. Last stage nymph and adult cockroaches confined in petri dishes, were chilled in a refrigerator held at 1 to 4°C for periods of 30, 60, and 90 minutes or heat-fixed by immersion in a water bath at 55° to 60°C for 3 minutes. After this the cockroaches were quickly removed, a hind coxal-sternal joint severed, and hemolymph bled directly into 1 x 75 mm. capillary tubes. These tubes were quickly sealed with plasticine at one end and centrifuged at 15,000 x g for 3 minutes to separate hemocytes from plasma. After centrifugation, a drop of plasma was placed on a slide, coverslipped, and examined (at 970 x) for coagulation-free separation of hemocytes and plasma.

Table 1. The effect of injecting rabbit erythrocytes on the phagocytic index and the number of cystocytes in Periplaneta americana (L.).

Group No.	No. Insects	No. Injected Erythrocytes	Before	1 hr.		20 hrs.	
			% Cysto.	% Phag.	% Cysto.	% Phag.	% Cysto.
1.	5	4,500,000	20	0	18	0	17
2.	5	3,000,000	19	0	20	0	22
3.	5	250,000	21	0	24	0	23
4.	5	60,000	27	0.5	38	0	28
5.	5	Saline injected control	21	-	20	-	19

To collect serum, live hemolymph drawn into 1 x 75 mm capillary tubes was allowed to coagulate for 2 minutes, and the tubes were then centrifuged at 12,000 x g for 10 minutes, after which the supernate (serum) was collected.

Results. Microscopic (970 x) examination of plasma samples from 30 and 60 minute chilled cockroaches revealed that all hemocytes had lysed and that precipitation had occurred before the sample could be centrifuged. Although 30 and 60 minute chilling did not inhibit coagulation, there was a marked qualitative reduction in the number of hemocytes within the hemolymph after 60 minutes of chilling. When hemolymph from 90 minute chilled cockroaches was collected in capillary tubes, it was discovered by microscopic examination that the sample was essentially pure plasma (i.e., free of hemocytes).

When hemolymph from 55°and 60°C heat-fixed cockroaches was centrifuged, the hemocytes did not lyse and the plasma did not coagulate on standing.

Procedure. Doses of 10, 50, 100, and 200 µl of 2% procaine hypochloride plus 4% versene were each injected into separate adult female cockroaches and allowed to circulate for 5 minutes. A drop of hemolymph was placed on a slide, coverslipped and checked for fixation. The remaining hemolymph was bled directly into capillary tubes, sealed at one end with plasticine, and held in a vertical position until about ½ mm of hemocytes settled to the bottom of the tube. The hemocyte sediment in the tubes was recovered by etching the tube with a jewelers file and breaking the tube at the hemolymph/packed hemocyte interface. The packed hemocytes was then expelled from the tube by partially pushing out the plasticine seal from the other end of the tube. These hemocytes were placed

directly in a drop of 4% versene on slide and examined at 970 x to determine if they had maintained their structural identity during sedimentation.

Result. Microscopic (970 x) examination of hemolymph from 2% procaine plus 4% versene injected cockroaches revealed progressively better hemocyte fixation at higher dosage levels. The most lasting hemocyte fixation on the slide and in capillary tubes occurred in cockroaches injected with 200 µl doses of fixative. Therefore, this dosage level was routinely employed for collecting plasma free hemocytes by sedimentation of hemocytes contained in capillary tubes.

Procedure. As previously described, pure plasma samples were obtained by 3 different types of fixation: (a) 90 minutes at 4°C, (b) 1 minute at 55°C or (c) 1 minute at 60°C. Wet mounts of these plasma samples were examined at 970 x during a 30 minute period for any indication of plasma precipitation or other alteration.

Result. Regardless of the method of fixation, pure plasma wet mounts never showed evidence of spontaneous precipitation or other alterations, even after 30 minutes.

Procedure. Plasma-free hemocytes from either 2% procaine or 4% versene-injected cockroaches were combined with a drop of either (1) saline, (2) pure plasma (from 3 different fixations), or (3) serum, using coverslipped preparations.

Result. (1) Both 2% procaine- and 4% versene-fixed, plasma-free hemocytes agglutinated and transformed into plasmatocytes and cystocytes when collected into saline, but at no time did a granular cloud form around the cystocytes or in the saline. (2) When 2% procaine-fixed hemocytes were combined with 90 minute chilled and 55°C heat-fixed plasma, the hemo-

cytes agglutinated and transformed into plasmatocytes and cystocytes and a strong plasma precipitate consistently formed within 2 minutes around cystocytes (Plate 3, Figure 2). Although hemocytes agglutinated and transformed, a plasma precipitate never formed when either 60°C heat-fixed plasma or 4% versene-fixed hemocytes were used. (3) When 2% procaine- and 4% versene-fixed hemocytes were added to serum, they agglutinated and transformed into plasmatocytes and cystocytes. No plasma precipitate formed around cystocytes from 4% versene-fixed hemocyte samples. However, with procaine-fixed cells, in 4 out of 5 cases, a small amount of fine plasma precipitate formed around cystocytes.

For future studies, physiologically active, plasma-free hemocytes were collected from cockroaches injected with 200 µl of 2% procaine in saline, and pure plasma was collected from either (1) cockroaches which were chilled at 1 to 4°C for 90 minutes, or (2) by centrifugation of hemolymph samples taken from cockroaches heat-fixed at 55°C for 1 minute.

The role of mitochondria in hemolymph coagulation

Many investigators have suggested that hemocytes eject some material of unknown composition into the plasma which leads to a granular plasma precipitate or to the formation of glassy veils, or otherwise causes gelation of the plasma. /Yeager et al., 1932; Yeager and Knight, 1933; Grégoire, 1951, a, b, 1953, a, b, c: 1955, 1957, 1959, a, b, c; Grégoire and Florkin, 1950, a, b; Grégoire and Jolivet, 1957; Franke, 1960, a, b; Jones, 1962/. Franke (1956), in his study on the fine structure of hemocyte mitochondria of Blatta orientalis with the electron microscope, noted a very close relationship between mitochondria and coagulation, and stated that mitochondria played an essential role in hemolymph coagulation. Franke (1960 a) noted that during coagulation,

vacuoles appear in the vicinity of the mitochondria and that the mitochondria subsequently undergo Brownian movement, enlarge, and become granular as they disintegrate. In addition, Franke suggested that immediately after hemolymph withdrawal, transforming hemocytes release coagulation-promoting-factors.

Procedure. DuBuy and Showacre (1961) have shown that certain tetracycline drugs, such as aureomycin, specifically combine with mitochondria of living cells and then fluoresce in ultraviolet light. Their technique was used to identify the mitochondria in the hemocytes of P. americana. Cockroaches were injected with 10, 20, and 30 µl doses of 0.02% aureomycin plus 0.88 M sucrose in saline. After the aureomycin had circulated for 3 hours, some cockroaches were bled from a severed antenna into a drop of saline containing 2% versene and 0.02% aureomycin on a slide. Other cockroaches were bled into either (1) a drop of 0.02% aureomycin plus 0.88 M sucrose in saline or (2) a drop of 0.02% aureomycin in saline on a slide. Hemocytes were first observed by phase microscopy and then with ultraviolet light at 3650 Å.

Result. When cockroaches were injected with 10 and 20 µl of 0.02% aureomycin plus 0.88 M sucrose in saline, the numerous, sharply defined, bacilliform inclusions seen in the hemocytes with phase microscopy (Plate 9, Figure 1) fluoresced a pale yellow for 3 to 4 minutes in ultraviolet light, when the hemocytes had been placed in a drop of 0.02% aureomycin and 0.88 M sucrose in saline (Plate 9, Figure 2). When cockroaches were injected with 30 µl of the 0.02% aureomycin solution, an undesirable background fluorescence interferred with observations.

When 10 and 20 µl injected cockroaches were bled into a drop of 0.02% aureomycin plus 0.88 M sucrose in saline, the hemocytes transformed

and cystocytes discharged fluorescing mitochondria into the plasma, but the mitochondria did not explode and no plasma precipitation occurred (Plate 9, Figure 3). But when hemocytes from similarly treated cockroaches were placed in 0.02% aureomycin in saline, without the sucrose, hemocytes transformed, ejected fluorescing cystocyte-mitochondria exploded and a plasma precipitate formed (Plate 9, Figure 4). In this preparation the cystocyte nuclei were outlined by fluorescing mitochondria and the plasma precipitate surrounding cystocytes was faintly fluorescent from disintegrated mitochondria (Plate 9, Figure 4). Presumably, the sucrose inhibits the explosion of mitochondria.

Procedure. Mitochondria were prepared first by grinding thoracic muscles in 0.25 M sucrose, then filtering the brei through surgical gauze, and drawing the filtrate into 1 x 75 mm capillary tubes. The filtrate was then centrifuged at 400 x g for 30 seconds. The mitochondria were decanted, resuspended in sucrose, and centrifuged at 2,000 x g for 10 minutes.

Mitochondria from hemocytes were prepared as follows: (1) hemolymph from a cut antenna was collected in 1 ml.0.25 M sucrose and stirred briefly before drawing the hemolymph-sucrose mixture into capillary tubes and centrifuging at 400 x g for 30 seconds. (2) The mitochondria were then decanted, resuspended in 0.25 M sucrose, and centrifuged at 2,000 x g for 15 minutes.

Mitochondria collected from muscle and hemocytes were each combined with a drop of plasma on a slide, and the preparation coverslipped. These wet mounts were examined for mitochondrial explosion and plasma precipitation.

Result. Muscle and hemocyte mitochondria mixed with hemocyte-free

plasma did not explode but maintained a round shape and no plasma precipitate formed after 20 minutes.

The role of calcium in insect hemolymph coagulation

The calcium content in insect hemolymph is somewhat higher than in vertebrates and lower than in some other invertebrates (Munson, 1953). According to tabulations made by Prosser and Brown (1962) the calcium concentration in Periplaneta spp. is 4.0 mM/L of hemolymph. There are conflicting views concerning calcium and its influence on the coagulation process in insects (Muttkowski, 1924; Yeager et al., 1932; Beard, 1950; Grégoire, 1953 a; and Franke, 1960 a).

According to Franke (1960 a), hemolymph will coagulate normally when placed in 0.4% $CaCl_2$ in Ringer's solution, but will not coagulate when placed in 0.8% $CaCl_2$. Grégoire (1953 a) stated that when $CaCl_2$ was placed in oxalate-treated hemolymph of Gryllotalpus sp., the hemocytes contracted or sent out pseudopodia; but no reaction occurred in the plasma.

Procedures. Kashiwa and Atkinson (1963) used glyoxal bis 3-hydroxy-anil (GBHA) for the specific cytochemical localization of ionic calcium in sectioned mammalian tissue. Their procedures were adapted for identification and localization of calcium in hemolymph and its fractions before and after coagulation.

Hemolymph, plasma, and hemocytes were smeared and dried on a slide and stained according to the technique of Kashiwa and Atkinson (1963). The smears were examined at 430 and 970 x for the presence of calcium as indicated by a red GBHA-Ca precipitate. One part of either fixed or unfixed serum, plasma, or whole hemolymph was mixed with 1/10 part of GBHA

in 1 x 75 mm capillary tubes. All of the reaction tubes, except those containing fresh unfixed hemolymph plus GBHA, were centrifuged at 12,000 x g for 3 minutes. Tubes were then read for the presence of calcium in the hemolymph or its fractions.

Results. Microscopically, no specific localization of calcium ions was observed in dried films of either cell free plasma, plasma of free hemocytes, or whole hemolymph. However, when observed by eye, all of these films appeared very faintly red, indicating the presence of ionic calcium. Heat-fixed (Plate 10, Figure 1) and versene-fixed whole hemolymph, cell-free plasma, and serum in tubes appeared negative for ionic calcium, both microscopically and macroscopically. Unfixed cell-free plasma (Plate 10, Figure 3) and serum (Plate 10, Figure 2) were positive for calcium as indicated by a slight red color. When the GBHA solution was added to unfixed coagulating hemolymph, the resulting coagulum was a deep red color indicating a much higher concentration of free calcium than in serum (Plate 10, Figure 2).

Procedure. Since versene is known to be an effective hemolymph anticoagulant and chelating agent (Hodgman, 1959), it was hypothesized that if just enough versene were added to live cell-free plasma, and then hemocytes added to the preparation, no coagulation would occur. If, indeed, no coagulation occurred, coagulation could then be induced by adding Ca ions back into the system. A mixture of 6 parts plasma and 1 part 4% versene in saline was divided into 2 capillary tubes. Live plasma-free hemocytes were combined on a slide with a drop of versene treated plasma from one tube, coverslipped, and examined for coagulation. One part 0.4% $CaCl_2$ in saline was added to 3 parts of versene-treated plasma from the second tube; and live hemocytes were combined on a slide

with a drop of $CaCl_2$ plus versene-plasma from this second tube, coverslipped, and examined for coagulation.

Result. When the versene-plasma mixture from the first tube was combined with live hemocytes, the hemocytes transformed and agglutinated but no plasma precipitate formed around cystocytes (Plate 11, Figure 1). However, when calcium ($CaCl_2$) was added back into the system (second tube, $CaCl_2$-versene-plasma) and hemocytes added, the hemocytes transformed and agglutinated as they did when mixed with tube 1, but this time a heavy plasma precipitate formed around cystocytes and later throughout the intercellular gaps (Plate 11, Figure 2).

Effects of hormones on coagulation

The relative number of circulating cystocytes or coagulocytes is greatly increased at ecdysis in P. americana, and it seems reasonable to assume that this increase is associated with the marked increase in the coagulability of the hemolymph at this time (Wheeler, 1963). The neurosecretory cells of the brain, corpora allata, corpora cardiaca, and prothoracic glands are all essential for the moulting process in insects (Wigglesworth, 1953). According to Bodenstein (1953), after allatectomizing newly ecdysed P. americana adults, moulting is induced as long as the corpora cardiaca and prothoracic glands are intact. Even in old adults with degenerated prothoracic glands, moulting can be induced by implantation of the corpora cardiaca and prothoracic gland (Bodenstein, 1953).

Since it is conceivable that the percentage of cystocytes is regulated by hormones, it was of interest to briefly examine the hormonal effect of nymphal corpora allata and cardiaca extracts on the percentage of circulating cystocytes in adult cockroaches.

Procedure. As shown in Plate 12, Figures 1 and 2, the corpora allata and c. cardiaca lie on the oesophagus just ventral and slightly posterior to the brain. An extract of the corpora allata and c. cardiaca was made after removing them from last stage moulting nymphs (Plate 12, Figure 3). The fresh glands were homogenized in saline with a Virtis Hi Speed 45 microhomogenizer. Three sets of corpora allata and c. cardiaca were contained in every 20 µl of homogenate. Brains were removed from the same nymphs and similarly homogenized so that 3 brains were contained per 20 µl of homogenate.

The heads of 20 males and 20 females of 24 hour old P. americana adults were ligated at the neck. Control cockroaches were not ligated. The cockroaches were kept at 27°C in petri dishes supplied only with water-dampened filter-paper floors. After 7 days the percentage of circulating cystocytes was determined by a differential count on ligated and control cockroaches. Shortly after making these counts, one group of ligated cockroaches was injected with 20 µl of corpora allata and cardiaca homogenate and another group of ligated cockroaches was injected with 20 µl of brain homogenate. Twenty ligated cockroaches injected with saline were controls. Three days after injection a differential count was made.

Result. As shown in Table 2, 7 days after head ligation, the percentage of circulating cystocytes in adult cockroaches decreased to almost one-half the value in controls. When each of these same head-ligated cockroaches were injected with corpora allata and c. cardiaca from last stage moulting nymphs, the percentage of circulating cystocytes significantly increased (Table 3). There was no change in the percentage of circulating cystocytes when head-ligated cockroaches were injected with brains from last stage moulting nymphs (Table 3).

Table 2

The percentage of circulating cystocytes in 7-day old adult Periplaneta americana whose heads were ligated 24 hours after emergence.

	Males		Females	
	20 ligated cockroaches	10 controls	20 ligated cockroaches	10 controls
Percent cystocytes	13.8 ± 1.8	23.2 ± 2.2	15.4 ± 1.6	29.0 ± 2.0

Table 3

The percentage of circulating cystocytes in head-ligated adult Periplaneta americana (from Table 2), 3 days after being injected with a homogenate of either 3 sets of corpora allata and c. cardiaca or 3 brains from last stage moulting nymphs.

No.	Treatment	Male percent cystocytes	Female percent cystocytes
5	Ca and Cc* injected	17.7 ± 1.3	20.7 ± 1.1
5	Saline injected	13.4 ± 1.7	14.9 ± 1.7
5	Brain injected	8.6 ± 3.3	12.2 ± 1.2
5	Saline injected	13.2 ± 1.9	12.9 ± 1.3

*Corpora allata and Corpora cardiaca

The coagulation process in other cockroaches

It seemed desirable to briefly examine the question of whether the coagulation process was essentially the same in a variety of other cockroaches. Hemolymph removed from unfixed Periplaneta australasiae (Fabricius) (Plate 3, Figure 3), Periplaneta brunnea Burmeister (Plate 4, Figure 1), Blatta orientalis L. (Plate 4, Figure 3), Blaberus craniifer Burmeister (Plate 5, Figure 1), Blaberus giganteus (L.) (Plate 5, Figure 3), and Diploptera dytiscoides (Serville) (Plate 6, Figure 3) coagulated in a manner similar to that of Periplaneta americana (Plate 3, Figure 1), i.e., cystocytes rapidly formed, mitochondria exploded, and a dense granular precipitate formed. When the hemocytes of P. americana were added to the cell-free plasma of the above mentioned cockroaches, coagulation occurred in exactly the same way as with their own hemocytes (Plate 3,4,5, and Plate 6, Figure 3,4).

When hemolymph removed from unfixed Nauphoeta cinerea (Oliver) and Blattella germanica (L.) coagulated, cystocytes rapidly appeared, mitochondria exploded, but only a very fine precipitation appeared in the plasma (Plate 6 and 7, Figure 1). The character of the granular precipitation was thus strikingly different from that in the six previously mentioned cockroaches. Even so, when fresh Periplaneta americana hemocytes were added to the cell-free plasma of either N. cinerea or B. germanica, Periplaneta hemocytes transformed into cystocytes, mitochondria burst, and a very dense precipitate appeared in the plasma (Plate 6 and 7, Figure 2). This suggests that P. americana hemocytes possess more coagulation-inducing factor, or a more potent factor than is found in the hemocytes of N. cinerea or B. germanica.

When unfixed Gromphadorhina portentosa (Schaum) hemolymph is observed in vitro none of the hemocytes transform into cystocytes and the plasma does not precipitate (Plate 7, Figure 3). The hemocytes, all of which looked more or less alike, formed interconnecting pseudopodia which lead to a dense cellular meshwork (Plate 7, Figure 3). When G. portentosa hemocytes were added to P. americana (cell-free) plasma, many of them transformed into cystocytes exactly like the cystocytes of P. americana and furthermore, the plasma of Periplaneta formed a dense granular precipitate (Plate 8, Figure 1). This clearly shows that Gromphadorhina hemocytes possess a factor capable of inducing coagulation. When Periplaneta hemocytes were added to cell-free Gromphadorhina plasma, cystocytes formed, mitochondria exploded, and a dense granular precipitate formed in the Gromphadorhina plasma (Plate 7, Figure 4). Presumably, Gromphadorhina hemolymph does not coagulate like that of other cockroaches because their hemocytes do not lyse and hence the powerful coagulation inducing factors inside the cells is not released and therefore, cannot react with coagulable material in their plasma. Further experiments on Gromphadorhina are clearly desirable in the future.

Coagulation of the hemolymph of the mealworm, Tenebrio molitor (Coleoptera), is of the pattern I type (Grégoire, 1955) (Plate 8, Figure 3), like that of P. americana. The hemolymph of Rhodnius prolixus (Hemiptera) does not coagulate (Wigglesworth, 1959). In the greater wax moth, Galleria mellonella (Lepidoptera), hemocytes agglutinate but the plasma does not precipitate (Beard, 1950). It seemed of particular interest to find if P. americana cystocytes would lead to coagulation or precipitation of cell-free plasma of the mealworm, which possesses coagulable substances, and to determine whether P. americana cystocytes would be capable of induc-

ing coagulation or precipitation of cell-free plasma of Rhodnius prolixus and Galleria mellonella which, theoretically, do not possess coagulable substances. When P. americana hemocytes were combined with a drop of plasma from either Tenebrio molitor (Plate 8, Figure 4), Galleria mellonella (Plate 8, Figure 2), or Rhodnius prolixus, the hemocytes of P. americana transformed into cystocytes, but no plasma precipitate formed.

DISCUSSION

The hemolymph of Periplaneta americana (L.) and its coagulation has already been the subject of numerous studies, mostly with regard to general structure and function of insect blood (Yeager et al., 1932; Ermin, 1939; Grégoire, 1951 a; and Wheeler, 1963). The development of several new techniques for manipulating P. americana hemolymph has greatly facilitated certain lines of investigation that were heretofore not possible.

In vivo chilling of insects to inhibit hemolymph coagulation (Beard, 1950; Siakotos, 1960 a; Franke, 1960 a) is effective for only a very short period, if the hemolymph sample is not kept cold after withdrawal. With the use of a cold stage, the hemolymph can be "cold-fixed" in vitro without treating the whole insect. Since the operator has complete control over the temperature of the cold stage, the rate of in vitro coagulation can be controlled for periods as long as 30 minutes (at 2°C), and this allows careful examination and analysis of the microscopically visible processes which would otherwise be exceedingly difficult or impossible to observe in unfixed preparations.

The process of coagulation in P. americana hemolymph is essentially similar to coagulation in Blatta orientalis (Yeager et al., 1932; Grégoire, 1955; Franke, 1960 a), Periplaneta australasiae and P. brunnea (Grégoire, 1957). Critical analysis of time lapse motion pictures (which were run backward and forward) of the coagulation process clearly shows that both cystocytes and plasmatocytes round up or contract before cystocyte formation occurs, and only after cystocytes appear does the plasma precipitate

form, leading to complete hemolymph coagulation. It was interesting to note that the granular hemocyte maintained its integrity until the very end of the coagulation process, after all of the other hemocytes had disintegrated.

The highly unstable cystocyte is thought to represent a highly specialized granular hemocyte (Jones, 1962). As pointed out by Jones (1962), most workers have agreed that insects have at least 3 fairly well-defined, morphologically distinct types of hemocytes: prohemocytes, plasmatocytes, and granular hemocytes. Numerous physiological and morphological deviations from these types have greatly complicated their nomenclature and classification. A nomenclature and classification based on either morphological or physiological characteristics alone can be misleading. For example, when hemolymph of P. americana is either in 4% versene or on the cold stage, all of the hemocytes appear morphologically identical, and only when coagulation has begun, can plasmatocytes, granular hemocytes, and cystocytes be recognized (Wheeler, 1963). Such a phenomenon shows that the behavior of cells must also be taken into account in their classification. Using a single physiological criterion such as a hemocyte's phagocytic capacity can be misleading; thus, while the cystocyte's primary function is coagulation (Jones, 1962), these cells are also capable of phagocytosis (Grégoire, 1951 a) even though they are less phagocytic than the plasmatocyte and granular hemocyte.

The strong agglutination of human erythrocytes and high concentrations of rabbit erythrocytes by unfixed plasma and whole hemolymph indicates that P. americana hemolymph contains a natural agglutinating factor. This factor is heat-stable up to 55-56°C. Feir and Walz (1964) reported that there is a naturally occurring agglutinating factor in the

hemolymph of the larger milkweed bug, Oncopeltus fasciatus; the smaller milkweed bug, Lygaeus kalmii; and a species of short-horned grasshopper. They stated that this factor was inactivated after 45 minutes at room temperature, after freezing, and after heating to 56°C for 10 minutes.

When high concentrations of rabbit erythrocytes were injected into cockroaches, these erythrocytes agglutinated in vivo and subsequently were encapsulated by both plasmatocytes and cystocytes (Plate 2, Figure 3). While the percentage of circulating cystocytes did not change with these dosage levels (Table 1), when a lower concentration was injected, the percentage of circulating cystocytes increased (Table 1). This increase could have resulted from plasmatocytes selectively falling out of circulation as they encapsulated some of the small agglutinated clumps of erythrocytes, or could have resulted from a destruction of erythrocyte-overloaded plasmatocytes.

In the present investigation, the development of techniques for separating and collecting hemolymph fractions has opened up an entirely new approach to the investigation of the mechanisms of coagulation. In addition, techniques for separating pure plasma and hemocytes from P. americana and other insects, will be of great value in biochemical and seriological studies of hemolymph fractions. These techniques will be essential to an ultimate analysis of the mechanisms involved in coagulation of insect hemolymph.

The collection of pure plasma from chilled cockroaches is related not only to inhibition of hemolymph coagulation, but to the cessation of heart-beat and of hemolymph circulation, as a result of which the intact hemocytes presumably settle by gravity. This hemocyte sedimentation is apparent since progressively fewer hemocytes are contained in hemolymph samples withdrawn from 30 and 60 minute-chilled cockroaches.

Franke (1960 a) has shown that in Blatta orientalis, heating hemolymph from 45° to 55°C decreases the coagulability of the hemolymph and above 55°C, inhibits it. In the present work it has been clearly shown that 55°C heat-fixation of hemolymph is the critical temperature at which only hemocytes are fixed. Pure plasma collected from 55°C heat-fixed hemolymph will precipitate when live hemocytes are added to it, but will not form a precipitate if 60°C heat-fixed plasma is used. Thus, it appears that the hemocyte factor or factors for agglutination and transformation is heat stable up to 55°C and the plasma factor or factors for precipitate formation is heat stable up to 59°C.

Collection of live hemocytes by injecting versene or procaine into cockroaches is effective only when large volumes are injected. This may be related to greater dilution of the hemocytes and to a better distribution of the "fixative" throughout the body. The hemocyte collection technique operates on the same principle as in vivo cold fixation, except that hemocytes remain fixed in capillary tubes as they settle to the bottom of the tube, where they can then be retrieved. The fact that pure plasma from cold- and 55°-59°C heat-fixed cockroaches will not precipitate in vitro supports the findings of Franke (1960 a) and also the generally accepted idea that a coagulation-promoting-substance (Grégoire, 1953 a; Franke, 1960 a) from transforming cystocytes must first be released into the plasma before it will precipitate. On the other hand, the fact that versene- and procaine-fixed hemocytes agglutinate and transform when placed in saline, decisively indicates that plasma factors are not necessarily required for these hemocyte changes. It is interesting to note that versene-fixed hemocytes will agglutinate and transform when placed either in saline or plasma, but will not induce plasma precipitation.

Apparently, versene irreversibly prevents the release of coagulation-promoting-substance from cystocytes, but does not permanently prevent hemocyte agglutination and cell transformation. Procaine-fixation, though effective, is temporary and can quickly be eliminated upon washing the hemocytes in saline or plasma. To be effective, hemocytes must be obtained within 45 minutes after collecting hemolymph from procaine-injected cockroaches. The above findings concerning fixation and separation of hemolymph fractions support the theory of Grégoire (1953 a) that anti-coagulants or fixatives act on the hemocytes or on the plasma.

Variations in the coagulation process of insects can be related to (1) relative numbers of cystocytes or other hemocytes at different stages of an insect's development (Grégoire, 1953 a; Taylor and Millmann, 1938; Wheeler, 1963), (2) to differences in the degree of cystocyte sensitiveness to foreign surfaces (Grégoire, 1953 a), (3) to different amounts of some coagulation-inducing-substance in cystocytes or to the efficiency of this substance, and (4) to different amounts of coagulable material in the plasma.

When live hemocytes are added to serum, only a weak plasma precipitate forms or no precipitation at all occurs. Thus, most or all of the coagulable material in plasma is precipitated during coagulation, and very little, if any, remains in serum. Since P. americana hemocytes combined with plasma of 9 other cockroach species yielded a plasma precipitate like that occurring in P. americana (Plate 3, 4, 5, 6, 7; Figure 2, 4), it is quite clear that their plasma possesses coagulable material. Although the plasma of Blattella germanica, Nauphoeta cineria, and Gromphadorhina portentosa heavily precipitates when combined with P. americana hemocytes, a very finely granular precipitate normally forms

in hemolymph of the first two species (Plate 6, 7; Figure 1) and no precipitate forms in hemolymph of the latter species (Plate 7, Figure 3). It may be that the hemocytes of these latter three species of cockroaches contain lower amounts and/or less efficient coagulation-inducing-substance, as suggested by Grégoire (1953 a). This may well be the case in Blattella germanica and Nauphoeta cineria, since cystocyte formation during coagulation in these species would release some coagulation-inducing-substance, regardless of the degree of activity of this substance.

A cross of Gromphodorhina portentosa hemocytes and P. americana plasma results in hemocyte transformation into cystocytes and this causes a heavy plasma precipitate to form (Plate 8, Figure 1). In their own plasma, Gromphodorhina portentosa hemocytes do not break down or release their powerful coagulation-inducing factor (Plate 7, Figure 3). Presumably, their plasma prevents lysis of the cells.

Beard (1950) suggested the occurrence of a coagulation-inhibiting factor in the hemolyph after freezing and thawing Japanese beetle grubs. As indicated by reactions resulting from combinations of P. americana hemocytes and plasma from insects other than cockroaches, this factor is absent in hemolymph of Galleria mellonella (Plate 8, Figure 2) and Rhodnius prolixus. Because Tenebrio molitor coagulation involves both cystocyte formation and heavy plasma precipitation (Plate 8, Figure 3), it was expected that P. americana hemocytes combined with Tenebrio plasma would result in marked plasma precipitation. However, surprisingly enough, no precipitate formed, even though some of the P. americana hemocytes transformed into cystocytes and were in the presence of coagulable substances in the hemolymph (Plate 8, Figure 4). Since T. molitor, and possibly other insects, possess all the hemocyte and plasma factors for

hemocyte transformation and plasma precipitation, it would seem reasonable to assume that one or more of these factors (1) is chemically different, (2) functions at different concentrations, or (3) requires some other factor or pH, than is present or needed in P. americana hemolymph coagulation.

As suggested by Tait and Gunn (1918), it is possible that hemocytes other than explosive cells could undergo gradual cystolysis and, in later stages of the coagulation process, share to a small extent in completing coagulation. According to Grégoire (1953 a), the process of cellular agglutination does not play any part in the phenomenon of hemolymph coagulation. He also stated that the categories of blood cells other than coagulocytes (cystocytes) are inert, and are scattered or agglutinated at random and become passively embedded in the plasma coagulum. Neither Tait and Gunn (1918) nor Grégoire (1953 a) have decisive evidence to support these views. In the present study, although hemocyte and plasma fractions have been separated, the isolation and collection of specific hemocyte types was not accomplished. Until hemocyte types can be isolated and collected or selectively inhibited, the resolution of their role in coagulation will remain unanswered.

Many investigators have expressed the idea that arthropod blood coagulation is enzymatically controlled as is vertebrate coagulation (Loeb, 1903; Tait, 1910, 1911; Tait and Gunn, 1918; Grégoire and Florkin, 1950 b; Grégoire, 1953 a; Franke, 1960 a). However, biochemical studies of insect hemolymph as yet have only dealt with enzymes of intermediary metabolism (Faulkner, 1956; Dearse and Scarpelli, 1958; Wyatt, 1961). Incidental observations by Grégoire (1953 a) and Franke (1960 a) suggest that enzymatic activity is involved in insect hemolymph coagulation.

Grégoire (1953 a) noted that, of the organic esters of sulfuric acid which were found to be anti-coagulant, the tryanocidal drug "Suramin" is an inhibitor of enzymatic activity. Franke (1956, 1960 a) suggested that since hemolymph coagulation specifically depends on pH and ionic ratio, enzymes are involved in coagulation. If enzymes prove to be involved in insect hemolymph coagulation, investigations would be needed to determine whether these enzymes are located in hemocytes, in plasma, or in both. Franke (1960 a) noted that cystocyte mitochondria are critically involved in coagulation. Among the great variety of enzymes contained in mitochondria, some may be involved in coagulation. The present investigation supports, in part, Franke's view on the critical role of mitochondria in coagulation. Since Periplaneta hemocytes can bring about heavy precipitation of the hemolymph of 9 different species, it is obvious that coagulation cannot be due to a highly specific enzyme or enzymes. It is clear, too, that the mere presence of free mitochondria in the plasma does not induce coagulation. Aureomycin labeling of hemocyte mitochondria (Plate 9; Figure 2, 3, 4) facilitated identification and observations on the fate of mitochondria ejected from cystocytes into the plasma. In normal P. americana hemolymph coagulation, the cystocyte mitochondria explode after they are ejected into the plasma. When the mitochondria are aureomycin-labeled and prevented from exploding, no plasma precipitate forms and the ejected mitochondria remain oriented in circular patterns around the cystocyte nucleus (Plate 9, Figure 3). In unfixed preparations, aureomycin-labeled cystocyte mitochondria explode, and fluorescing mitochondrial material can be seen in the clouds of plasma precipitate around cystocytes and in precipitate generally distributed throughout the plasma (Plate 9, Figure 4). This shows that substances

within mitochondria could well be critically involved not only with the precipitate formation around cystocytes, but also in precipitate formation throughout the plasma. It is also possible that other cytoplasmic substances (e.g. protein and ions such as calcium) are involved. Preliminary experiments involving the addition of intact isolated muscle or hemocyte mitochondria to plasma in an attempt to induce plasma precipitate were inconclusive and require further investigation.

In earlier investigations (Muttkowski, 1924; Yeager, et al., 1932; Beard, 1950; Grégoire, 1953 a; Franke, 1960 a), the determination of the role of calcium in insect hemolymph coagulation has been hampered by technical difficulties. As pointed out by Clark and Craig (1953), most of the calcium present in P. americana hemolymph probably is combined with the proteins, derived proteins, or other organic molecules present in the hemolymph, but only the ionic form is considered physiologically active. As mentioned by Carvalho, Sanui, and Pace (1963), various agents (e.g., electric shock, electromagnetic radiation, acetylcholine, and caffeine) cause a release of bound calcium and the freed calcium then initiates some physiological process. Obviously, contact of hemocytes with certain foreign environments would provide a stimulation for the release of calcium bound to hemocyte cell membranes. However the sensitiveness of certain hemocytes to foreign surfaces could depend on changes in free and bound calcium in the plasma. The poor localization of calcium in hemolymph, hemocytes, and plasma dried films may have resulted from its physical masking by proteins or other hemolymph substances. Since versene is a chelating agent (Hodgman, 1959), no calcium was detected in versene-fixed hemolymph or its fractions. It is not understood why calcium was not detected in heat-fixed hemolymph (Plate 10, Figure 1). It is possible

that heat fixation binds calcium ions to substances in hemolymph so that the ions are not available to react with the GBHA indicator. The pronounced reaction of calcium in coagulated hemolymph (Plate 10, Figure 2) seems to indicate that ionic calcium is released by and/or is closely associated with agglutinating and transforming hemocytes. Serum and plasma both seem to contain qualitatively similar concentrations of calcium (Plate 10, Figure 2, 3), but qualitatively less than is present within a hemocyte coagulum (Plate 10, Figure 2). The experiments involving the removal of ionic calcium from the hemolymph by versene and the subsequent introduction of ionic calcium in the presence of hemocytes indicate that calcium is necessary for plasma precipitation (Plate 11, Figure 2), but is not necessary for hemocyte agglutination or transformation (Plate 11, Figure 2). However, this does not rule out the possible role of bound calcium that may be released by agglutinating and/or transforming hemocytes. Further, it would appear desirable to test the effects of other divalent cations such as lithium or strontium on coagulation. Grégoire (1953 a) using oxalate instead of versene, noted that when ionic calcium was introduced back into the hemolymph system, the hemocytes transformed but no plasma precipitate formed. In his experiment, the failure of the plasma to precipitate could have been due to over-dilution or to only partial elimination of active oxalate from the system. In such indirect tests it is most important to initially remove all the calcium present with the minimum of chelating agent. If the chelating agent is in excess, it will combine with calcium added back into the system. In experiments on mammalian blood, Howell (1916) noted that oxalated plasma readily clotted when a suitable amount (specific quantity not mentioned) of $CaCl_2$ was added to it.

In an earlier study, Wheeler (1962, 1963) reported that the total hemocyte count (= THC) in P. americana significantly increases prior to ecdysis, abruptly falls at ecdysis, and remains about the same for the next 24 hours; and that these changes in THC are related to changes in hemolymph volume. In addition, the percentage of circulating cystocytes is strikingly higher in newly ecdysed adults than immediately before or after ecdysis, and that this increase in percentages of cystocytes at ecdysis is closely associated with the increased coagulability of the hemolymph at that time. Hormones may indirectly or directly regulate the total or differential hemocyte count by influencing blood volume or by acting on the hemocytes or by affecting both. As pointed out by Jones (1962), hormones may promote differentiation of the various types of hemocytes as they do with other tissues or perhaps affect their adhesion to tissue surfaces. The sharp decrease in the percentage of circulating cystocytes 7 days after head ligation of 24 hour old P. americana adults (Table 2) may be due to the exclusion of the corpora allata and c. cardiaca. Hormones and other physiologically active materials associated with moulting and cell differentiation that are released by the corpora allata and cardiaca may also be critically involved in the regulation of the differential hemocyte count. This seems to be supported by the fact that the percentage of circulating cystocytes significantly increases when head ligated cockroaches are injected with corpora allata and cardiaca extracts (Table 3). Extracts of brain injected into head ligated cockroaches had no significant effect on the cystocyte count (Table 3). Further studies are needed to clarify the possible influences of hormones on coagulation.

While the present study offers some useful techniques for an eventual possible solution to the question of plasma coagulation in insects,

we are still far from having even the simplest understanding of the processes involved. The process of coagulation in the cockroach involves first a breakdown of the cell's surface and this is probably related in some unknown way with free calcium ions or possibly other ions in the cells or in the hemolymph. Second, the process involves the release of cytoplasmic components (most conspicuously of mitochondria) and these components react with dissolved substances in the plasma fraction to produce coagulation. While the basic process seems to involve the rupture of the mitochondria, there is no information on whether or not mitochondrial enzymes are involved and if they were, we know nothing about which one or ones are really concerned with coagulation. Since all of the mitochondrial enzymes are concerned with the Krebs cycle (Gilmour, 1961) it would be fascinating to know how various specific inhibitors of the cycle would affect coagulation in the cockroach.

Obviously, the plasma must be capable of coagulation (e.g., Rhodnius plasma cannot coagulate). Apparently the mitochondria must explode (e.g., no plasma precipitate occurs when they are intact, so the surface enzymes on these organelles surely cannot be involved). The enzymes released must react with a plasma component (or components), but the mere dissolution of a cell in an alien environment clearly does not necessarily produce coagulation even when the system is capable of coagulation. It is known that hormones can greatly affect the number of mitochondria in cells and cell permeability (Wigglesworth, 1956), but even so it is very obvious that coagulation does not qualitatively depend upon hormones.

Since the cockroach is a very primitive insect most of their hemocytes are fundamentally structurally very similar, but it is clear that they are functionally very dissimilar, some of them being primarily

phagocytic cells and some of them being primarily coagulocytic.

It is fascinating to conjecture that structural dissimilarities may have evolved long after physiological needs became very obvious. All cells must engage in pinocytosis to live, and phagocytosis is only one gross aspect of a very general phenomenon, but all cells do not have to disintegrate when faced with injury or dissolution.

It is a fascinating phenomenon that certain cells facing dissolution should themselves react to stress by disintegrating and, by so doing, protect the well-being of the whole organism.

CONCLUSIONS

The following statements apply to the physiology of hemolymph coagulation in the cockroach, Periplaneta americana (L.).

1. With the use of a microscope cold stage, hemolymph coagulation (a) is inhibited for as long as 30 minutes at 0° to 4°C, (b) is initiated at 5°C, (c) is permanently inhibited at 60°C, and (d) takes place in 6 distinct stages.

2. Live plasmatocytes, granular hemocytes, and cystocytes are structurally identical, but differ functionally in their capacity to phagocytize chinese ink and erythrocytes, and in their fragility.

3. Although physiologically active substances contained in and/or released from the corpora allata and c. cardiaca (but lacking in the brain) may regulate the percentage of circulating cystocytes and influence the coagulability of the hemolymph, the problem requires additional study.

4. Hemocyte agglutination and transformation is inhibited at 0° to 4°C, is permanently prevented at 55°C, and is independent of plasmal factors.

5. The cystocyte's primary function is the initiation of plasma coagulation. By transforming and ejecting cytoplasmic material, including mitochondria, into the surrounding plasma, it releases a coagulation-promoting substance and leads to plasma precipitation and veil formation.

6. Material from exploded cystocyte mitochondria (a) is mostly concentrated in the plasma precipitate cloud surrounding the cystocyte

nucleus, (b) is present in a small amount in the general plasma precipitate between gaps of agglutinated hemocytes, and (c) is possibly the source of the coagulation-promoting-substance initiating plasma precipitation.

7. Hemocyte-free plasma will not spontaneously precipitate. The release of ionic calcium and of a coagulation-inducing substance from transforming hemocytes, are required for plasma participation. The roles of other ions need further study.

8. The specific plasma component involved in coagulation is present in the plasma of 9 other species of cockroach, but not in the plasma of Tenebrio molitor, Galleria mellonella, or Rhodnius prolixus. The amount and/or effectiveness of a coagulation-promoting-substance released from cystocytes may determine the degree of plasma precipitation.

PLATE 1

The design and construction of a cold stage for a microscope.

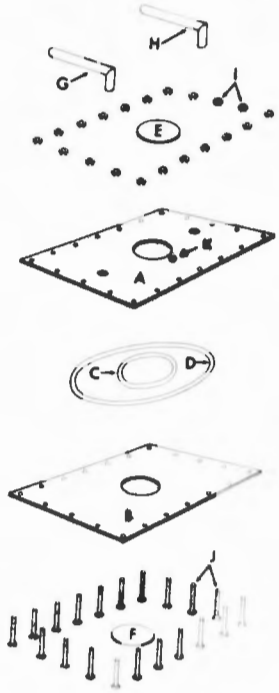
Figure 1. Shows the various parts in an exploded diagram of a cold stage: A and B, copper plates; C and D, "O rings"; E and F, coverslips; G and H, copper elbow joints; I and J, nuts and bolts; and K, 1/32 inch hole.

Figure 2. A fully constructed cold stage. (A) A cold stage constructed from the schematic design shown in Figure 1. (B) A modified cold stage design; note that the nuts are eliminated by tapping the bolt holes in copper plate A (Figure 1), and the copper elbows are brought together (a rubber divider is fitted between the elbow joint internal openings and between the "O rings" C and D to prevent a short water circuit.

Figure 3. Water pump and reservoirs for the cold stage: (A) cold stage; (B) water inlet from reservoir (H); (C) water outlet leading to pump reservoir (G); (F) pump and thermoregulator; (E) overflow from reservoir (H) leading to pump reservoir (G).

Figure 4. The position of the cold stage in operation on the stage of a Reichert MeF, universal, inverted microscope.

PLATE I



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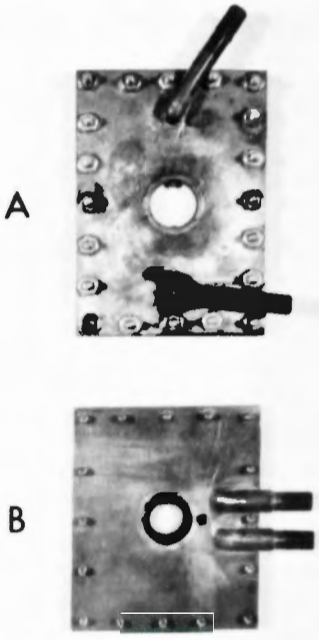

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PLATE 2

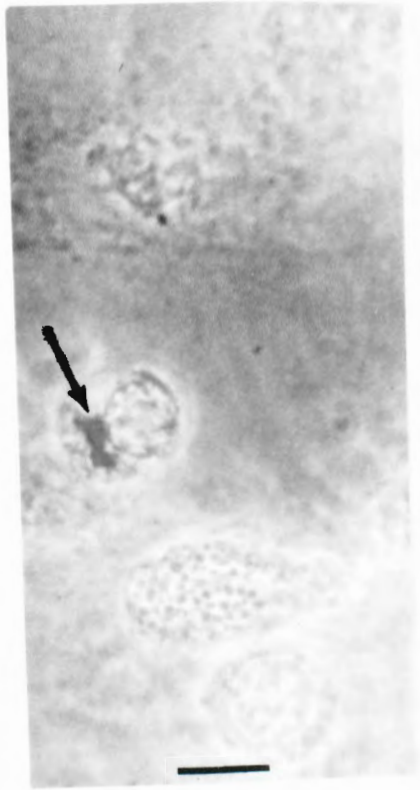
Photomicrographs showing phagocytosis of chinese ink and rabbit erythrocytes by Periplaneta americana hemocytes. (Scale = 10 microns)

Figure 1. Shows a phagocytized particle of chinese ink in the remaining cytoplasm of a transformed cystocyte.

Figure 2. Illustrates rabbit erythrocytes phagocytized by plasmatocytes.

Figure 3. Shows agglutinated rabbit erythrocytes encapsulated by coagulated hemocytes.

PLATE 2



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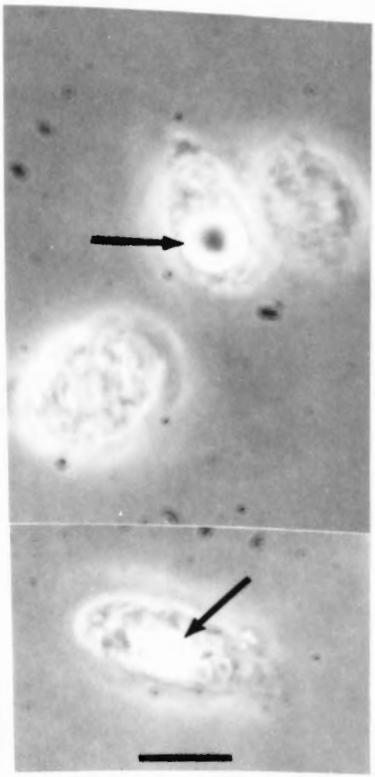

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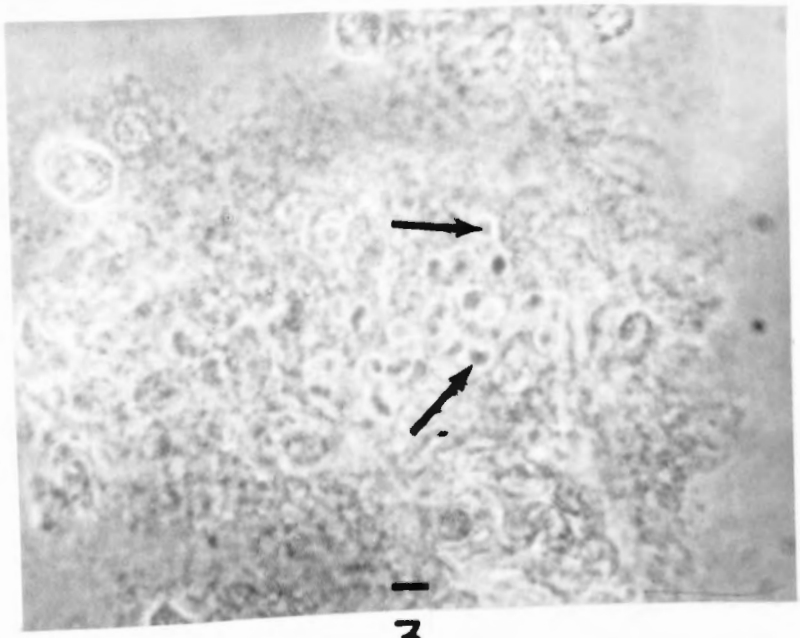

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PLATE 3

Periplaneta americana and *Periplaneta australasiae* hemolymph coagulation patterns compared with patterns after their plasma is combined with *P. americana* hemocytes. (Scale = 10 microns)

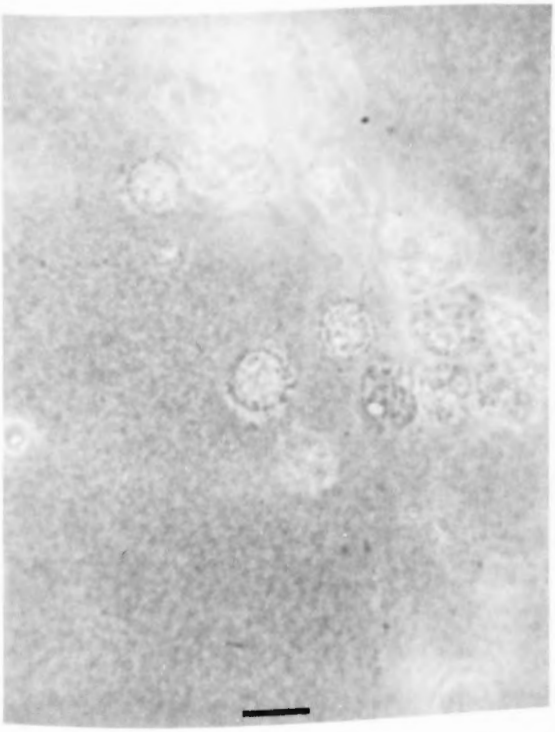
Figure 1. Normal coagulation pattern of *P. americana* hemolymph.

Figure 2. The resulting coagulation pattern when *P. americana* hemocytes are recombined with its own plasma.

Figure 3. The normal coagulation pattern of *P. australasiae* hemolymph.

Figure 4. The resulting coagulation pattern when *P. americana* hemocytes are combined with *P. australasiae* plasma.

PLATE 3



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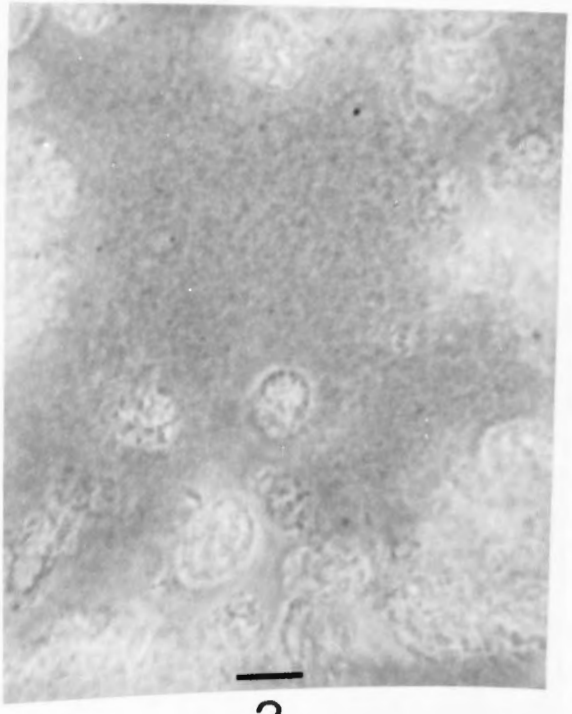

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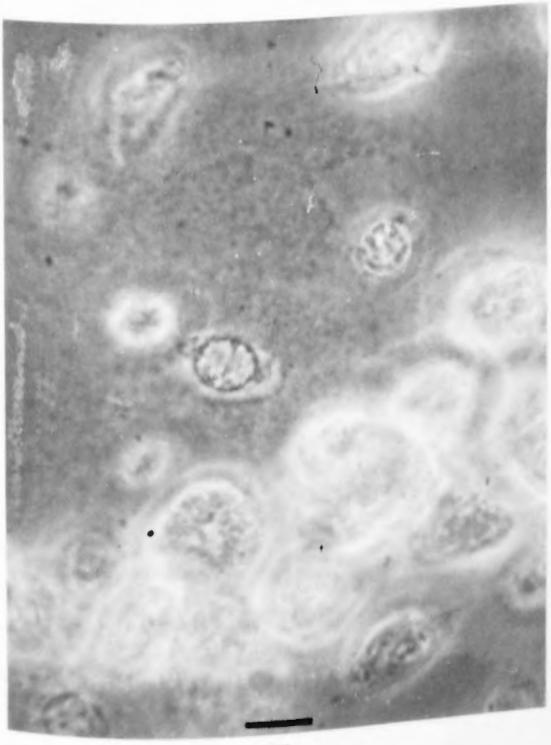

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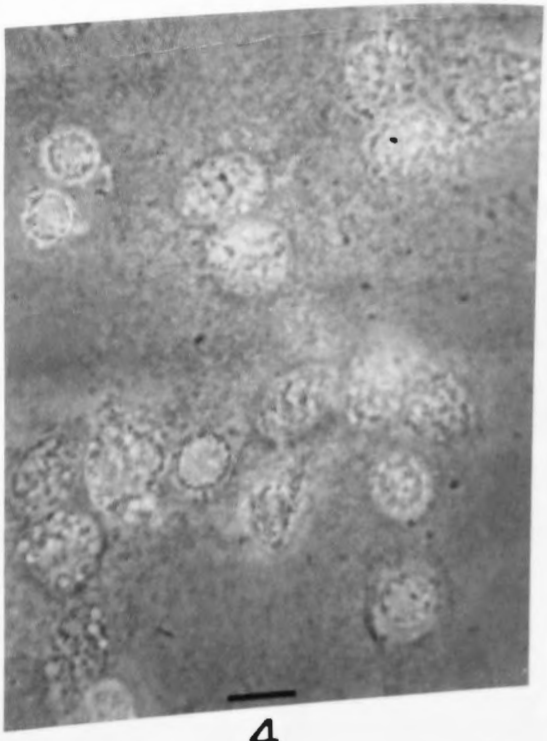

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PLATE 4

Periplaneta brunnea and *Blatta orientalis* hemolymph coagulation patterns compared with patterns after their plasma is combined with *P. americana* hemocytes. Note the similarity of all the coagulation patterns. (Scale = 10 microns).

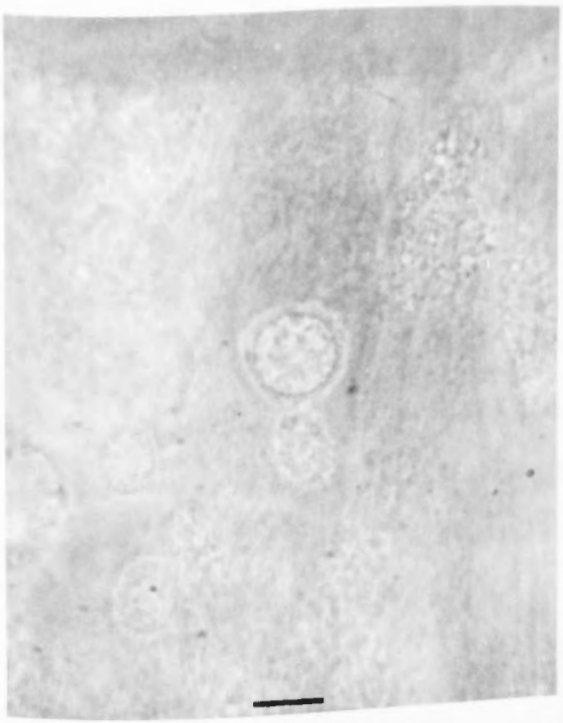
Figure 1. The normal coagulation pattern of *P. brunnea* hemolymph.

Figure 2. The resulting coagulation pattern when *P. americana* hemocytes are combined with *P. brunnea* plasma.

Figure 3. The normal coagulation pattern of *Blatta orientalis* hemolymph.

Figure 4. The resulting coagulation pattern when *P. americana* hemocytes are combined with *Blatta orientalis* plasma.

PLATE 4



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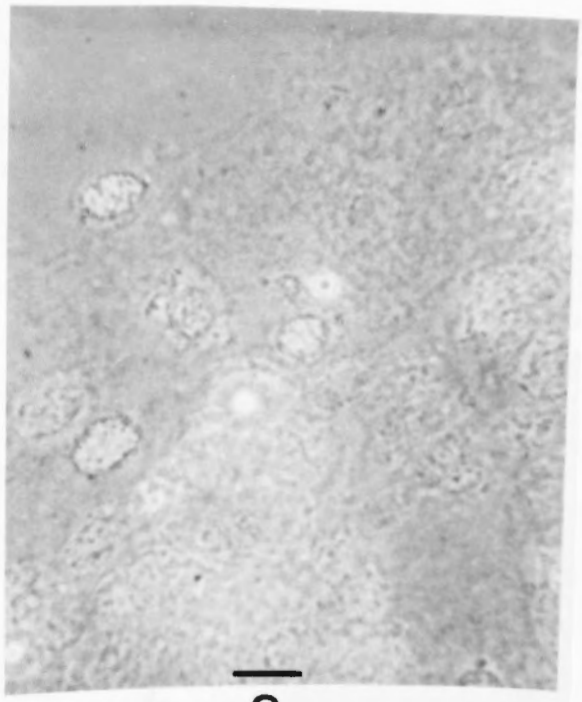

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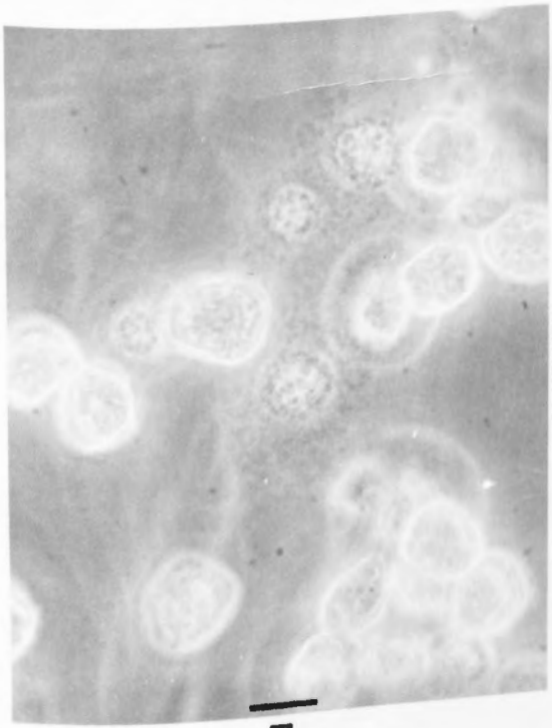

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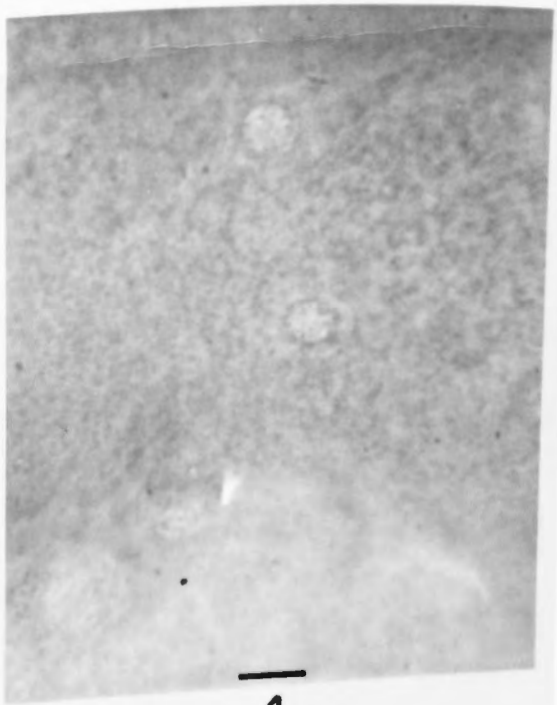

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PLATE 5

Blaberus craniifer and Blaberus giganteus hemolymph coagulation patterns compared with patterns after their plasma is combined with P. americana hemocytes. Note the similarity of these coagulation patterns with those in Plate 3 and 4 . (Scale = 10 microns)

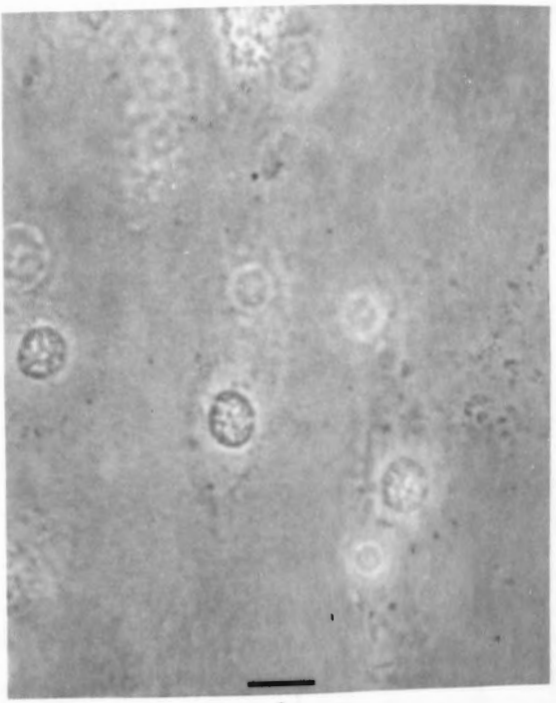
Figure 1. The normal coagulation pattern of Blaberus craniifer hemolymph.

Figure 2. The resulting coagulation pattern when P. americana hemocytes are combined with Blaberus craniifer plasma.

Figure 3. The normal coagulation pattern of Blaberus giganteus hemolymph.

Figure 4. The resulting coagulation pattern when P. americana hemocytes are combined with Blaberus giganteus plasma.

PLATE 5



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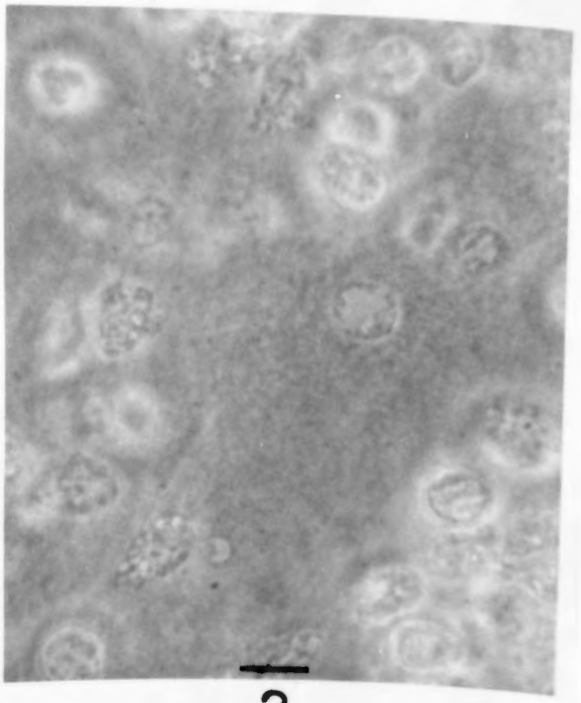

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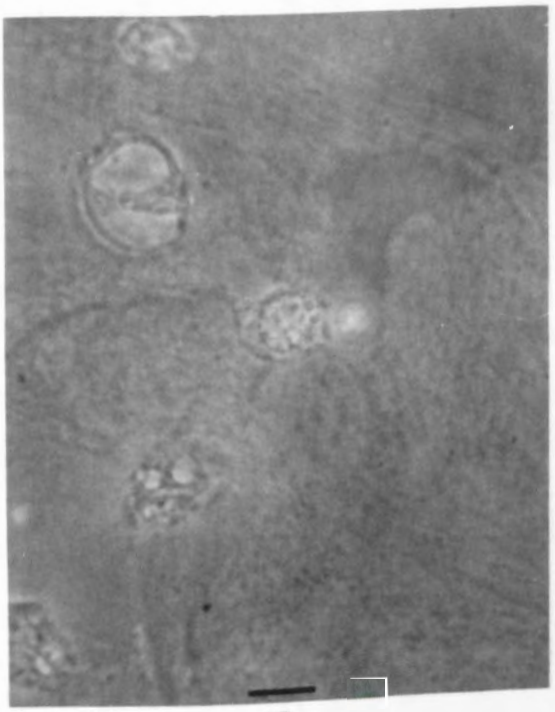

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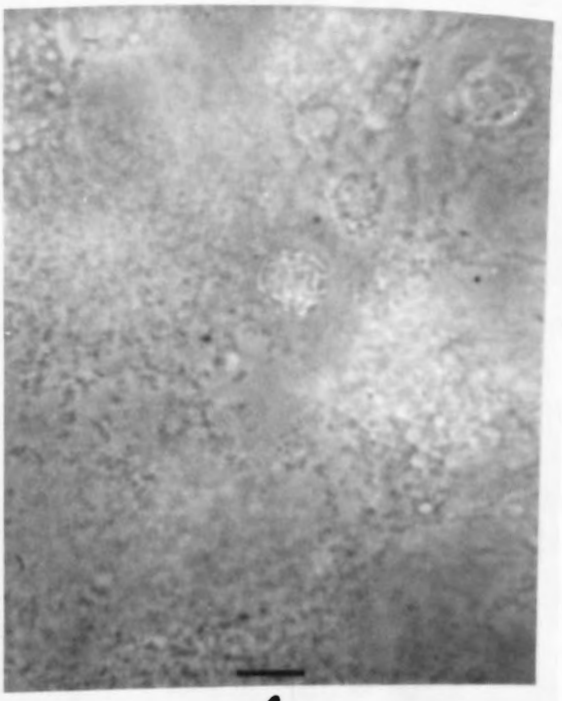

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PLATE 6

Blattella germanica and Diploptera dytiscoides hemolymph coagulation patterns compared with patterns after their plasma is combined with P. americana hemocytes. (Scale = 10 microns)

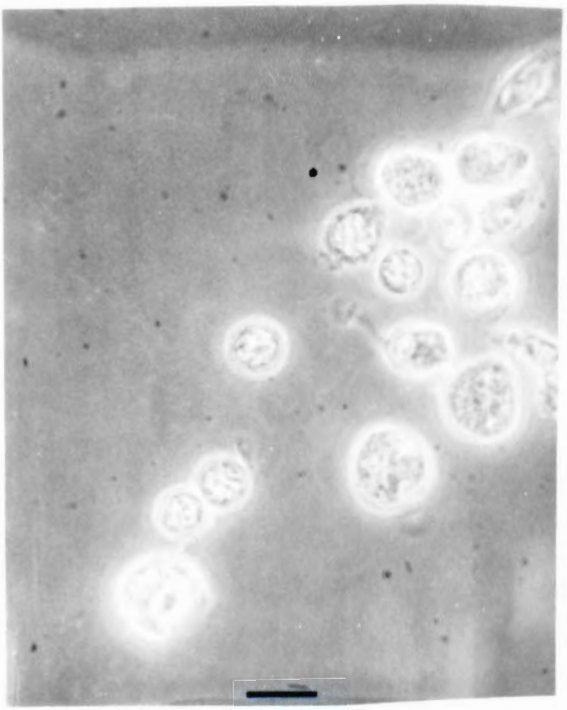
Figure 1. The normal coagulation pattern of Blattella germanica hemolymph. Note that only a slight amount of a very fine plasma precipitate appears.

Figure 2. The resulting coagulation pattern when P. americana hemocytes are combined with Blattella germanica plasma. Note the formation of a heavy plasma precipitate which does not normally occur in B. germanica coagulation.

Figure 3. The normal coagulation pattern of Diploptera dytiscoides hemolymph.

Figure 4. The resulting coagulation pattern when P. americana hemocytes are combined with D. dytiscoides plasma.

PLATE 6



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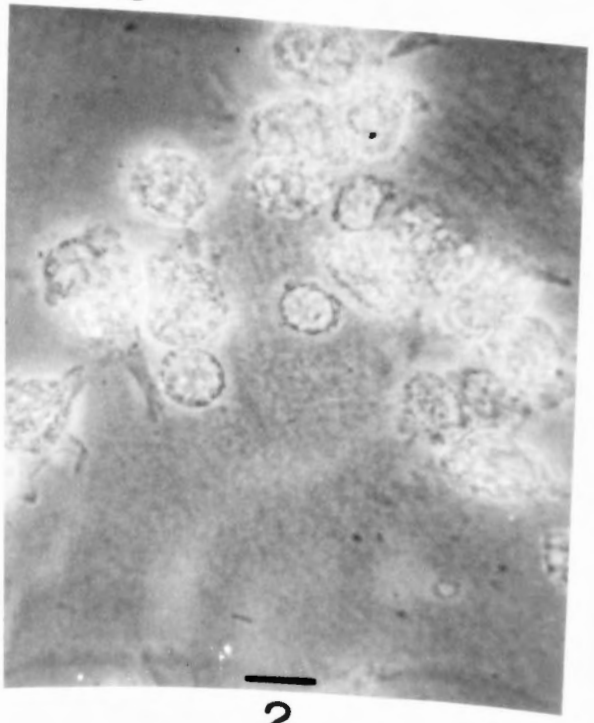

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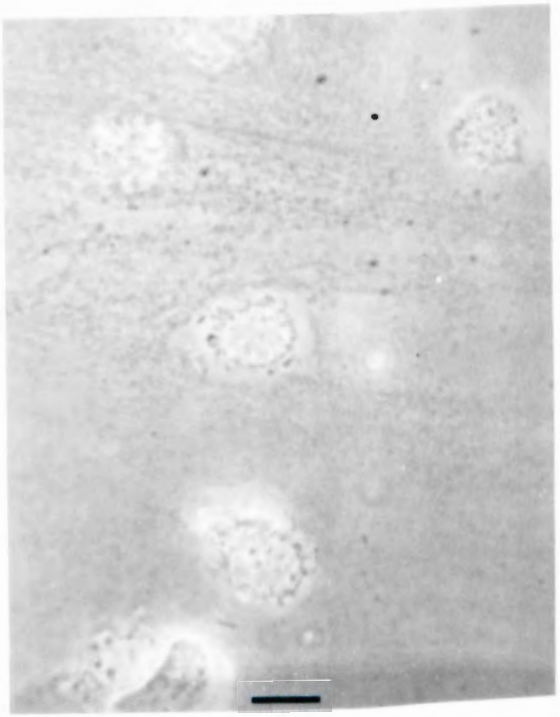

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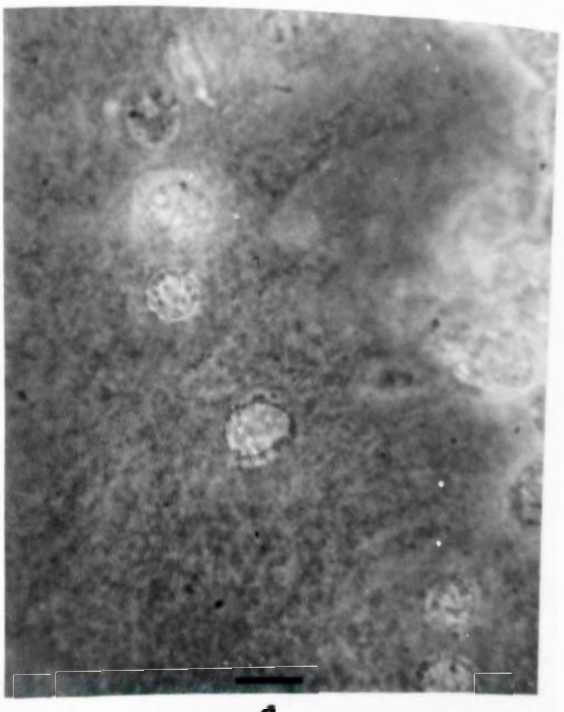

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PLATE 7

Nauphoeta cinerea and *Gromphadorhina portentosa* hemolymph coagulation patterns compared with patterns after their plasma is combined with *P. americana* hemocytes. (Scale = 10 microns)

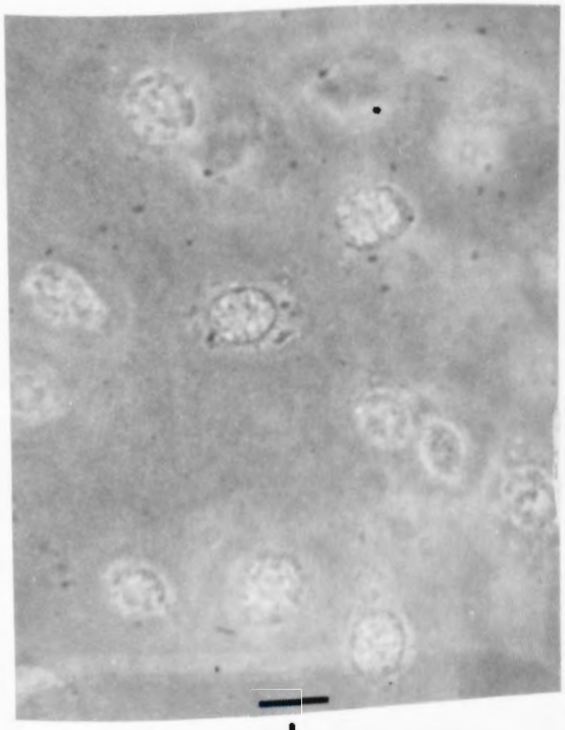
Figure 1. The normal coagulation pattern of *Nauphoeta cinerea* hemolymph. Note that only a slight amount of plasma precipitate appears.

Figure 2. The resulting coagulation pattern when *P. americana* hemocytes are combined with *Nauphoeta cinerea* plasma. Note the formation of a heavy plasma precipitate which does not normally occur in *N. cinerea* coagulation.

Figure 3. The normal coagulation pattern in *Gromphadorhina portentosa* hemolymph. Note that no hemocytes transform into cystocytes and that no plasma precipitate forms.

Figure 4. The resulting coagulation pattern when *P. americana* hemocytes are combined with *G. portentosa* plasma. Note that the hemocytes transform and that a heavy plasma precipitate forms.

PLATE 7



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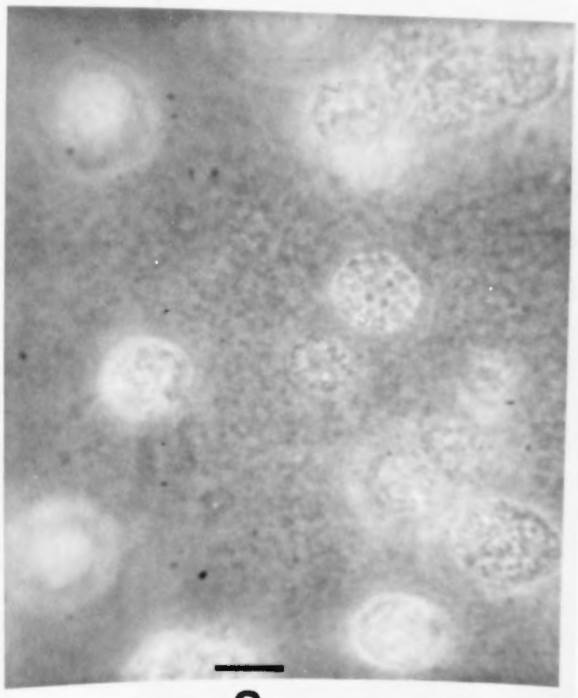

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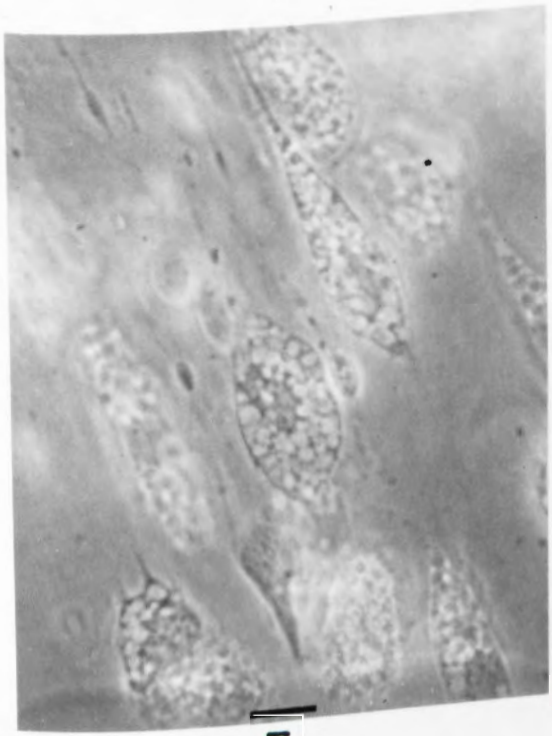

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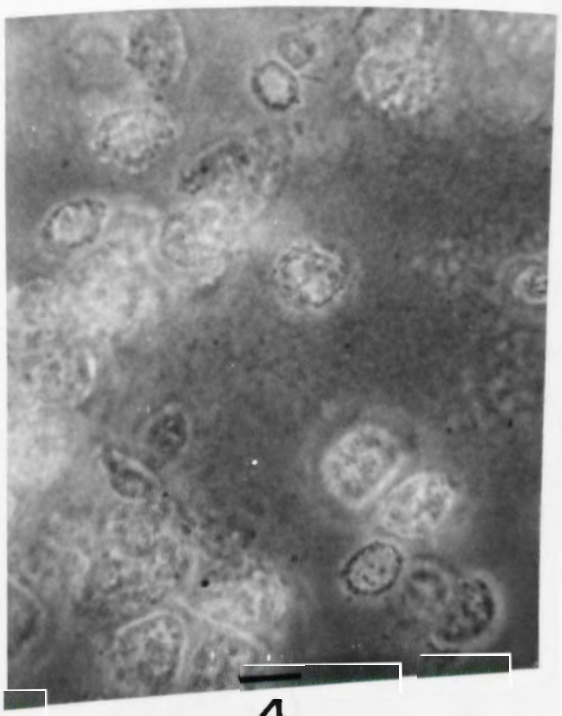

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PLATE 8

Coagulation patterns from inter- and intraspecific crosses of hemocytes and plasma.

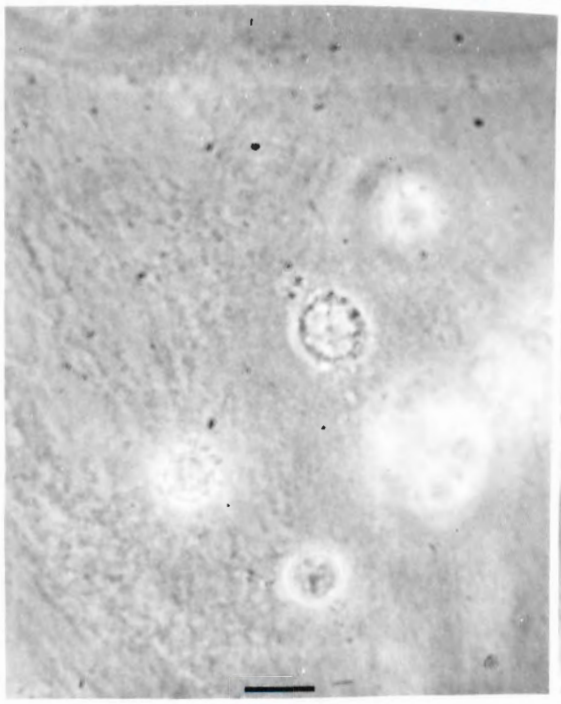
Figure 1. The resulting coagulation pattern when Gromphadorhina portentosa hemocytes are combined with P. americana plasma. Note that cystocytes transform and a plasma precipitate forms even though these events do not normally occur (Plate 7, Figure 3).

Figure 2. The resulting coagulation pattern when P. americana hemocytes are combined with Galleria mellonella plasma. Note that even though cystocytes transformed, no plasma precipitate occurs.

Figure 3. The normal coagulation pattern in Tenebrio molitor hemolymph. Note that cystocyte transformation and heavy plasma precipitate occurs.

Figure 4. The resulting coagulation pattern when P. americana hemocytes are combined with Tenebrio molitor plasma. Note that P. americana cystocytes transform and no plasma precipitate forms even though this plasma normally does precipitate (Figure 3).

PLATE 8



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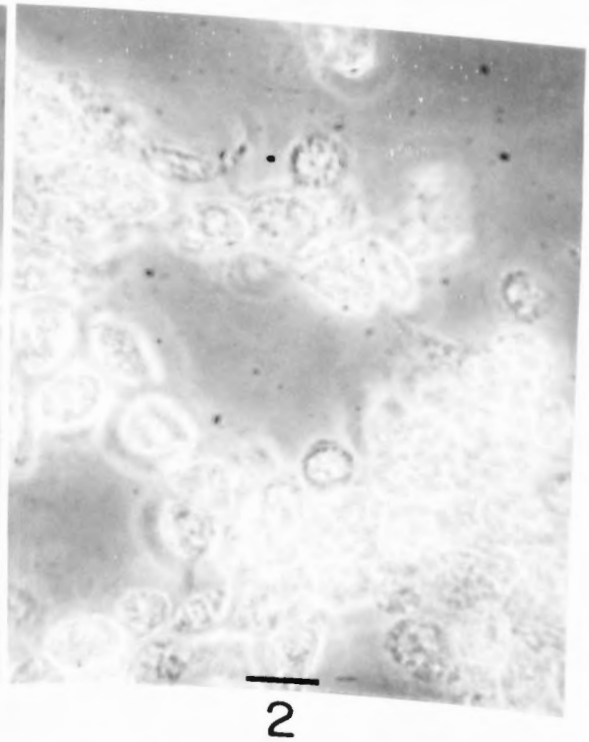

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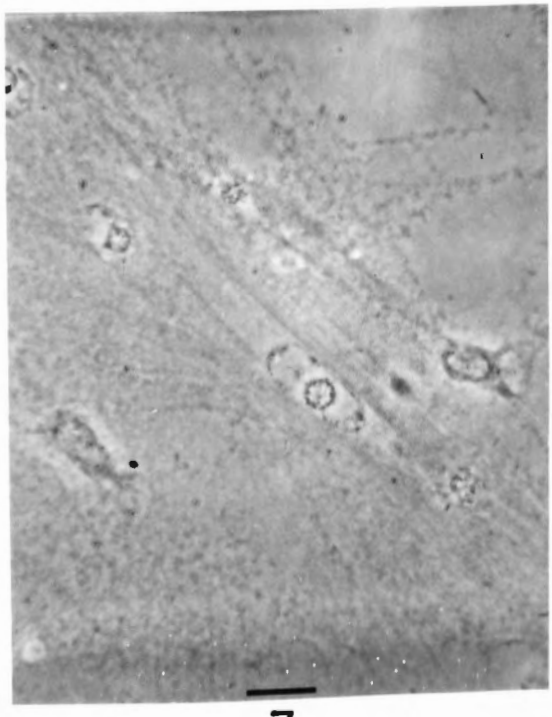

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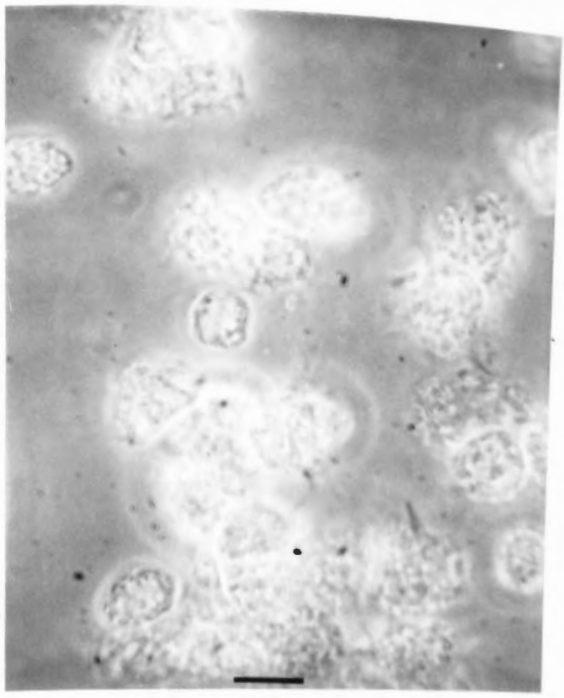

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PLATE 9

The appearance of live hemocytes and coagulated hemolymph from Periplaneta americana injected with aureomycin.

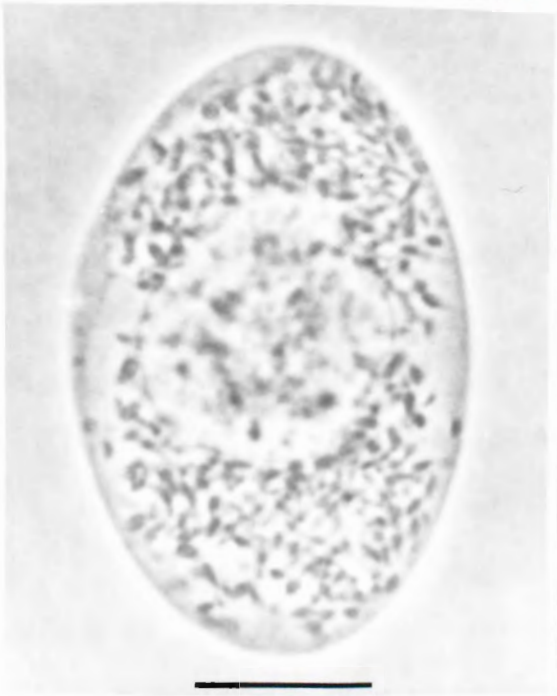
Figure 1. The appearance of a live hemocyte when observed by dark-medium phase contrast. Note the dark granular mitochondria in the cytoplasm (Scale = 10 microns).

Figure 2. The appearance of the same hemocyte in Figure 1 when observed by fluorescent microscopy. Note the specific fluorescence of the mitochondria (Scale = 10 microns).

Figure 3. Incomplete hemolymph coagulation showing the circular patterns of ejected cystocyte mitochondria around cystocyte nuclei (Scale = 10 microns).

Figure 4. Complete hemolymph coagulation showing transformed cystocytes and plasma precipitate. Note the fluorescence of the plasma precipitate cloud around cystocyte nuclei and in the plasma, indicating the presence of material from exploded cystocyte mitochondria (Scale = 10 microns).

PLATE 9



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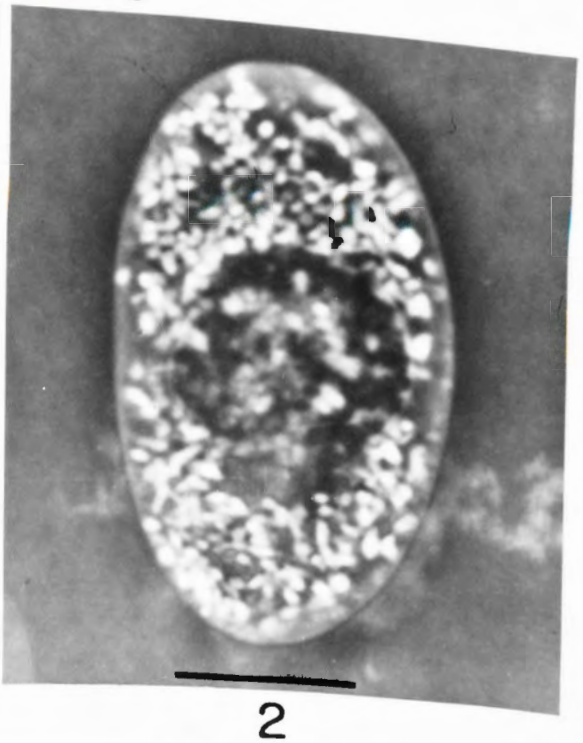

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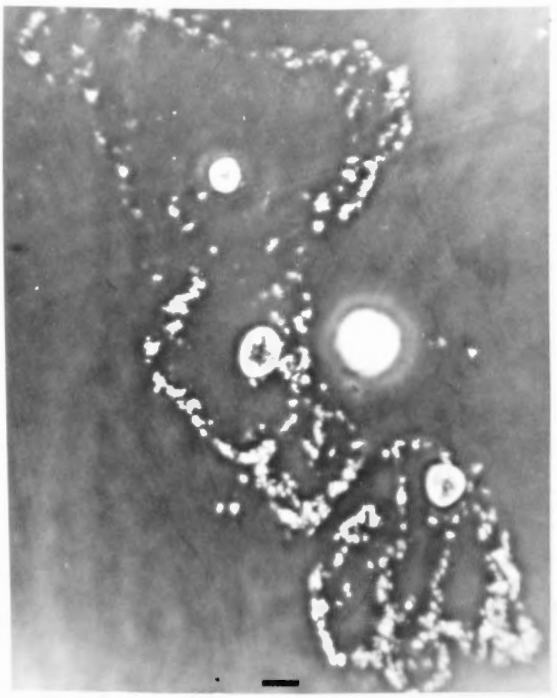

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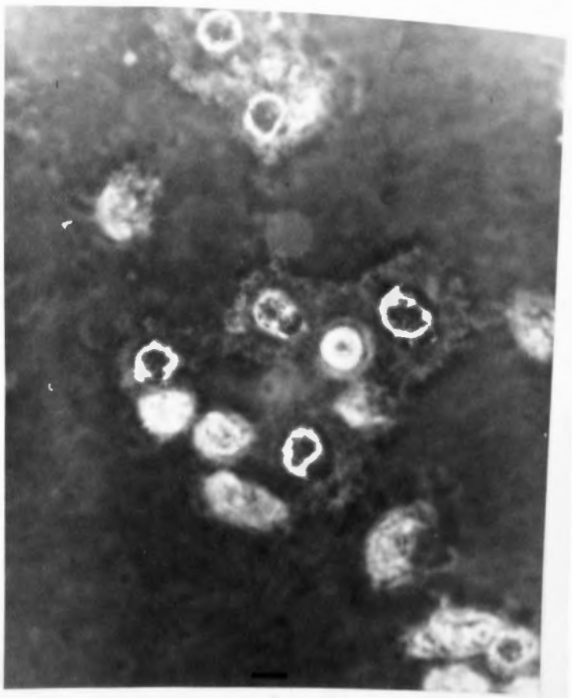

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PLATE 10

Glyoxal bis 3- hydroxy-anal (GBHA) tests for ionic calcium in hemolymph, plasma, and serum of P. americana (L.). A red color is positive for ionic calcium.

Figure 1. A negative GBHA test for ionic calcium in 60°C heat-fixed hemolymph. Note the absence of a red color.

Figure 2. A positive GBHA test for ionic calcium in coagulated hemolymph. Note the sharp red reaction in the cell coagulum and the slight red reaction in the serum at the top of the tube.

Figure 3. A positive GBHA test for ionic calcium in hemocyte-free plasma. Note the slight red color of the reaction.

PLATE 10

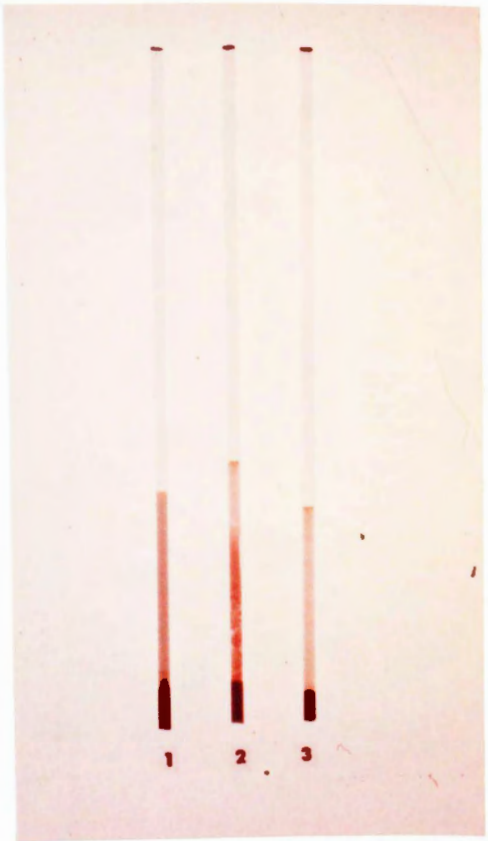


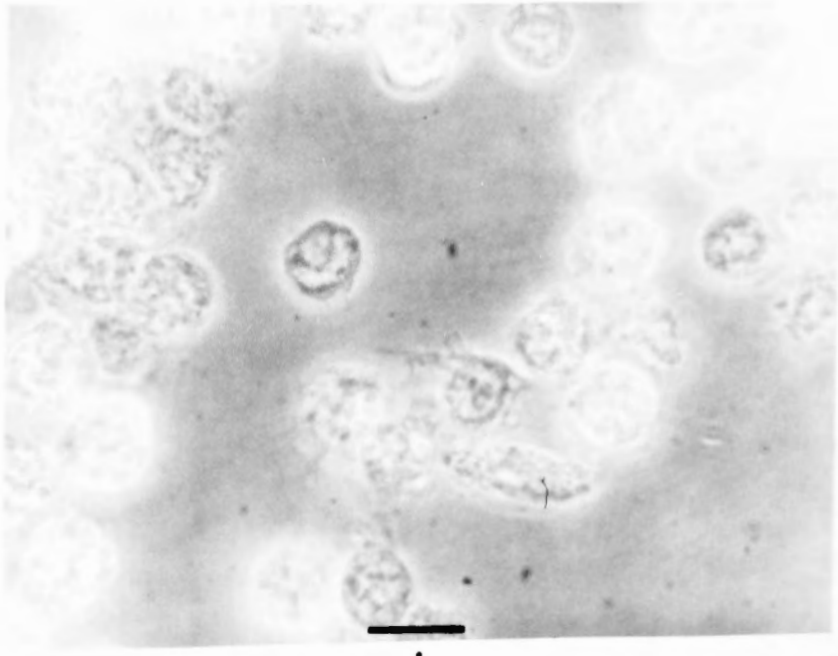
PLATE 11

Coagulation reaction when P. americana hemocytes are recombined with plasma free of ionic calcium and when ionic calcium is introduced back into the plasma. (Scale = 10 microns)

Figure 1. The resulting coagulation reaction when P. americana hemocytes are combined with plasma freed of ionic calcium. Note that only hemocyte transformation occurs and no plasma precipitation occurs.

Figure 2. The resulting coagulation reaction when P. americana hemocytes are combined with calcium free plasma (used in the Figure 1 preparation) resupplied with ionic calcium. Note that hemocyte transformation occurred as it did in Figure 1, but also a plasma precipitate formed.

PLATE II



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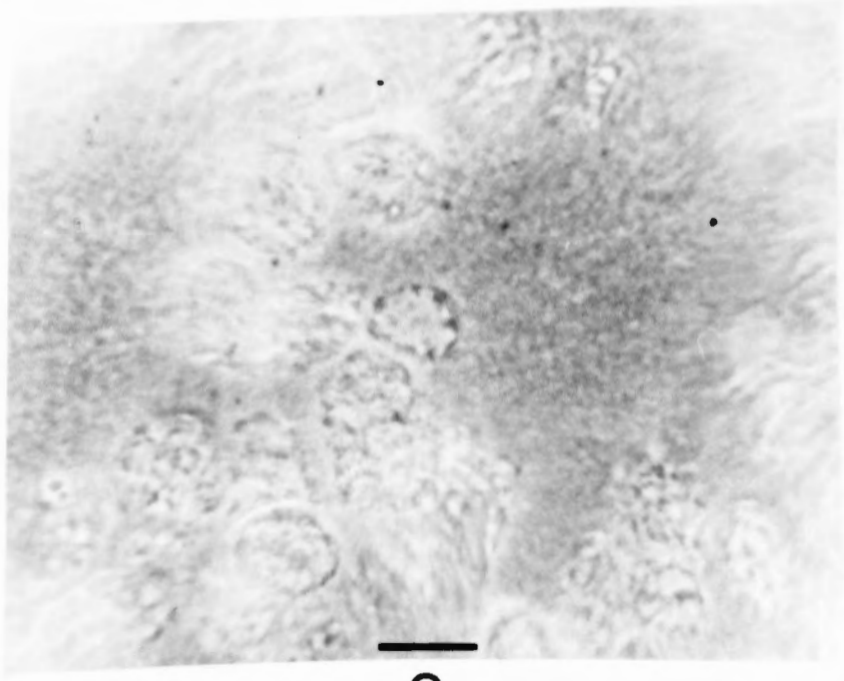


2

PLATE 12

The anatomy of the corpora allata and c. cardiaca in Periplaneta americana (L.).

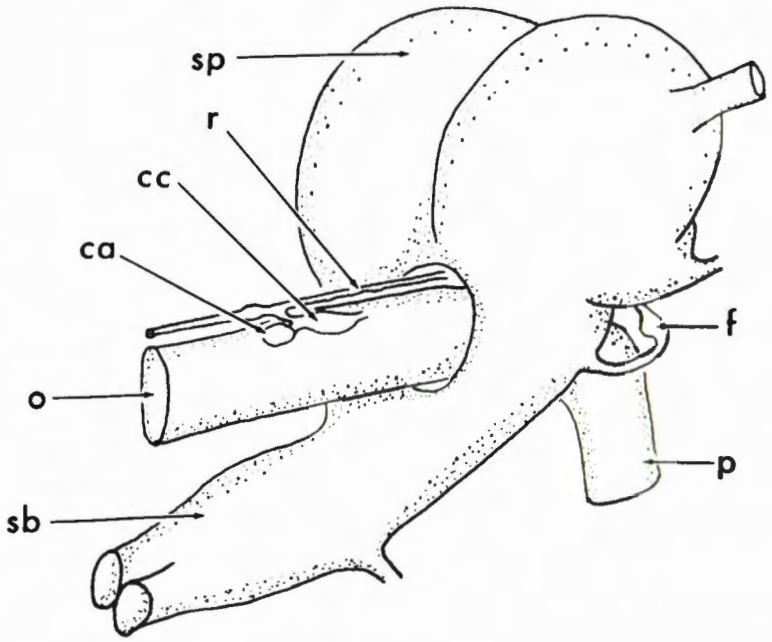
Legend: (ca) corpora allata; (cc) corpora cardiaca; (o) oesophagus; (p) pharynx; (sp) supraesophageal ganglion; (sb) subesophageal ganglion; (r) recurrent nerve; (f) frontal ganglion; and (oa) occipital arch.

Figure 1. A schematic drawing of the retrocerebral gland complex showing the position of the corpora allata and c. cardiaca.

Figure 2. A photomicrograph of a dissection of the corpora allata and c. cardiaca just before extirpation. (Scale = 1 mm)

Figure 3. A photomicrograph of an extirpated corpora allata and c. cardiaca. (Scale = 1 mm)

PLATE 12



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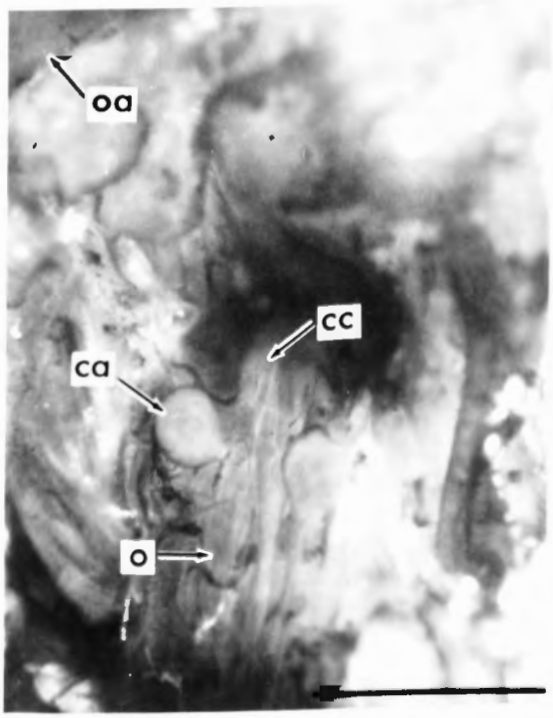

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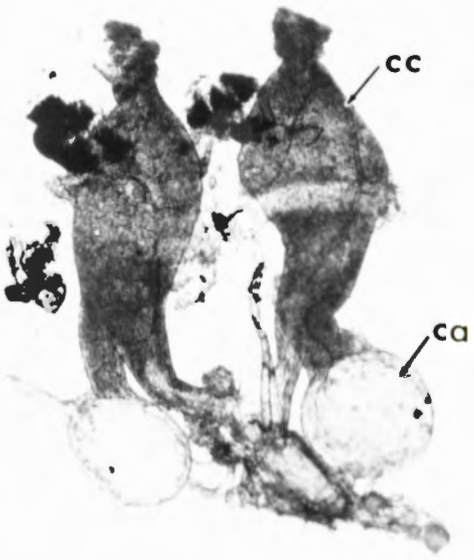

3

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