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The agglutination of formed elements in the blood of invertebrates.

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Thesis

THE AGGLUTINATION OF FORMED ELEMENTS IN THE BLOOD OF INVERTEBRATES

by

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I INTRODUCTION

A Purpose and Scope of Thesis

This thesis, "The Agglutination of Formed Elements in the Blood of Invertebrates", is intended to cover in some detail the role that cellular elements found in the blood and coelomic fluids of invertebrates play in clot formation and in some closely related phenomena. Since the rather broad taxonomic term "invertebrates" includes a great number of extremely diverse groups of animals, it seems most suitable to follow a general phylogenetic plan in arranging the material, especially since most of the investigators in this and related fields have each studied only one or a few closely related species. Thus, most of the experimental work in this branch of physiology of invertebrate blood and body fluids falls into a few large subdivisions.

The animals most carefully studied with respect to hemostasis, blood coagulation, and cell agglutination are some Crustacea and also the Limulus, a species of arthropod now classified with the Merostomata. These animals are the most suitable of all invertebrates for such experimental work, being readily available and comparatively large. They have a relatively well-advanced circulatory system containing copious amounts of blood and cellular elements.

Many investigators have studied blood clotting in the insects, but due, no doubt, to the great difficulty of obtaining more than a drop or two of blood from any but the largest of them, this work consists for the most part of simple descriptions of the clotting process with relatively little study of the chemical and physical factors involved.

There is also a series of observations on the annelids and on the echinoderms, some experimental studies of a few representative mollusks and of the ascidians (lower chordates), and a few isolated notes on scorpions, spiders, brachiopods, and sponges.

The agglutination process is not limited to the animals having a discrete circulatory system, but may occur in the annelids and echinoderms among the cells in the coelomic spaces. Thus, it seems appropriate to include work on agglutination of cells in general, since the coelomic cells of many invertebrates parallel in function those found in vertebrate and invertebrate blood.

The relationship of agglutination to plasma coagulation in the formswhich have both mechanisms is very close, and it appears impossible to give an adequate treatment of one without discussing the other, especially in view of their very close relationship in the vertebrates.

The sources of the material presented here are, for the most part, original papers. Many references were obtained from various reviews of the subject, in particular, those of Silberberg (1938), Rustum Maluf (1939), Mellanby (1939), Rapp (1947), and Glavind (1948). Many of the more recent references were found through a study of Biological Abstracts. The remainder of the material was found through the short reviews or references to previous work which are in most original papers. Several textbooks of physiology were studied to get a general background for this work, in particular those of Dahlgren and Kepner (1908) and of Rogers (1938). The material on agglutination, coagulation, and hemostasis in the vertebrates was obtained from recent reviews, in particular those of Lutz (1951) and Quick (1951), but information was also obtained from the reviews of Silberberg (1938), Quick (1942), Glavind (1948), and Ferguson (1949).

The scientific terms used are, for the most part, those of the original authors although many have been changed to more modern equivalents. Although it is not always possible to make a clear distinction between agglutination and coagulation, I have used the first term to mean the coming together of discrete cells and the second to mean the solidification or jelling of a liquid.

B General Discussion

1. Vertebrates

There are, in general, three types of hemostatic mechanisms in animals, namely, contraction of smooth muscles surrounding a cut blood vessel or wound, agglutination of formed elements in the blood, and coagulation of the blood plasma. All these are found in the vertebrates, but we are particularly interested in the part played by the formed elements. In mammals there are essentially three kinds of blood corpuscles: the red blood corpuscles, the leukocytes, and the blood platelets. The red cells (Ferguson, 1949 a, and Lutz, 1951) usually become entangled within fibrin clots and may be caused to mass together by various serological agents (agglutinins) but are relatively unimportant in hemostasis. The leukocytes have recently been found (Lutz, 1951) to form thrombi and emboli under certain conditions but have not yet been shown to have any important role in blood clotting. The platelets, however, are believed (Quick, 1951, and Lutz, 1951) to be active both as structural components of the clot and as releasers of important substances which act on certain plasma proteins.

Platelet agglutination and fibrin formation are extremely closely integrated, and any scheme to explain blood clotting must deal with both reactions. Until rather recently, Schmidt's classical coagulation theory was considered the best simple explanation, and most of the experimental work done before the last year or two has been interpreted on this basis. It consisted of two steps:

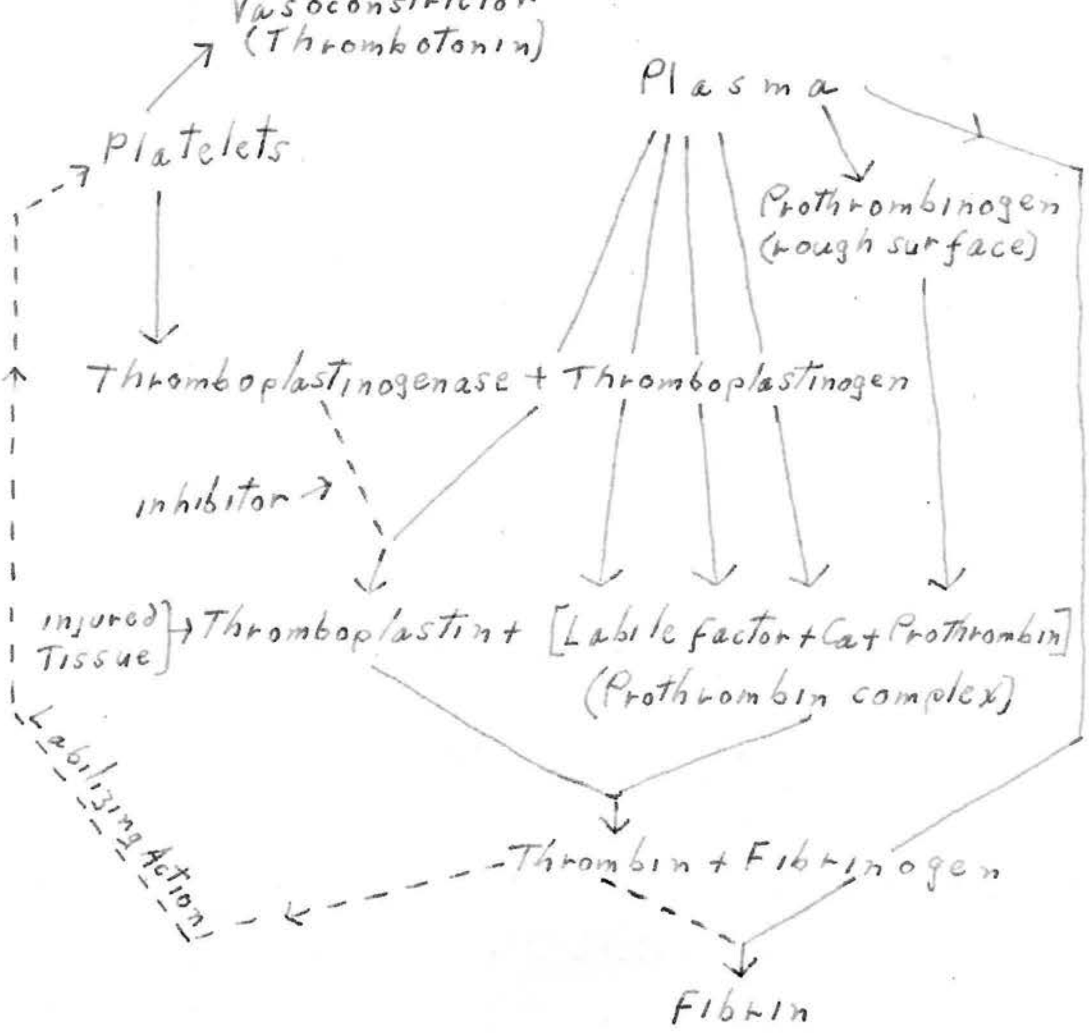
(1) prothrombin + calcium ions + thromboplastin → → thrombin

(2) fibrinogen + thrombin → fibrin

Prothrombin, calcium ions, and fibrinogen were believed to occur free in the blood plasma, while thromboplastin occurred in tissue cells and in the platelets, being released upon their breakdown.

The most recent modification of this scheme is that of Quick (1951). (See chart on page 6.) Several new substances have been postulated, the most important being thromboplastinogenase (in the platelets) and thromboplastinogen (in the plasma). Thromboplastin now is considered a plasma substance rather than a platelet constituent, although it may occur in body tissue cells. Formerly Quick (1942) believed that the platelets agglutinated under the influence of fibrin deposited on their surfaces, but he (1951) now thinks that thrombin has a direct labilizing action on the platelets. He believes that hemostasis depends on a slow, continuous production of thrombin which labilizes the platelets, thus continuing their agglutination and lysis and supplying a vasoconstrictor. Fibrin itself is not essential. Hemostasis is thought to occur in the following steps:

1. The cut vessel undergoes a reflex constriction independent of the platelets.
2. A localized platelet thrombus is formed.
3. Vasoconstrictor substances diffuse out.
4. The endothelial surfaces become sticky and are glued together.
5. The blood flow is slowed and more platelets adhere



Mammalian Hemostasis

adapted from Quick (1951)

> to the injured wall and agglutinate, gradually forming a white thrombus.

A red (fibrin) clot may be formed as thrombin is expressed from the white (platelet) clot during retraction, but the red clot is not essential to hemostasis. Clot retraction depends on the presence of platelets, and it does not occur in a fibrin clot in their absence.

Several substances have been found to have an anticoagulant effect. The decalcifying agents, including the citrates, oxalates, and fluorides, act by removing the calcium necessary for the prothrombin complex. Two other anticoagulants, heparin and hirudin, act by neutralizing prothrombin, while heparin may also deactivate thrombin. (Heparin requires a plasma co-factor, Albumin X, for its action.) Another substance, Dicoumarol, used clinically as an anticoagulant has no direct effect on the clotting process but acts indirectly by interfering with prothrombin synthesis.

Hirudin seems unable to prevent platelets from agglutination, but heparin acts in a variable manner. Quick (1951) believes that heparin preserves the platelets by deactivating the labilizing thrombin. However, according to Lutz (1951) heparin can cause spontaneous platelet or leukocyte agglutination _in vivo_. Dicoumarol completely prevents platelet clot formation _in vivo_, but it can cause intravascular agglutination of the leukocytes. Many other factors are known to increase the stickiness of the platelets. Thrombocytosin (a lipid in subcutaneous fat), a variety of pathological conditions, cellular breakdown anywhere in the body, and certain dietary constituents (milk fat and egg yolk) have this effect.

2. Invertebrates

In the invertebrates, muscular contraction, cell agglutination, and plasma coagulation are all found, plus a fourth hemostatic mechanism, autotomy. However, they usually are not all present and developed in any one animal. It is thus possible to select a suitable form to study a particular process, as did Leo Loeb in his studies of amebocyte agglutination, ameboid movement, and tissue formation, using the merostomatan, Limulus polyphemus.

Autotomy, or the breaking off of an injured part of the body voluntarily at a joint where there is a very narrow and easily closed blood channel, is found in many of the arthropods. Thus, the crab, Maia squinado, (Parsons and Parsons, 1923) commonly prevents loss of blood by this method.

Constriction of the smooth muscle of the body wall is used by certain soft-bodied forms to reduce the size of a wound. The Cephalopoda, for example, can largely prevent bleeding by this method, although their blood cells agglutinate, forming a small clot (Parsons and Parsons, 1923).

The two mechanisms which concern us most are (1) agglutination of the formed elements of the blood or body fluid, and (2) coagulation of the blood plasma. Although autotomy is important among certain crustaceans, and the muscles surrounding a wound contract in many soft-bodied forms, such as the mollusks, the most general method of hemostasis is clot formation. This, also, is the method of primary importance among the vertebrates.

Almost all invertebrates studied seem to have some sort of free-floating cells in their coelomic fluid and in their blood, if present. These cells may be of various sizes, shapes, colors, and functions, but some of them usually can agglutinate to form some sort of cell mass or clot. The cells with this property are usually ameboid, often lack pigments (leukocytes), and have hyaline protoplasm, although granules of various colors may be present. They are often also phagocytic, but not necessarily so. In some animals many cells are normally found reversibly agglutinated inside the body, or readily stick together when stimulated by various factors; in others they do not usually join together or even exhibit ameboid movement _in vivo_, for example the amebocytes of _Limulus_ (Loeb, 1902). However, in most animals the blood cells, upon leaving the body, rapidly develop strong tendencies toward clot formation. They become sticky, send out long processes, and rapidly join on contact to form a solid and often homogeneous mass of cell material. In animals where the plasma gels also, the cells may act by liberating bits of protoplasm which clot in the plasma, or by liberating a substance which effects the coagulation of the plasma itself.

Blood plasma coagulation is found only in relatively few of the higher invertebrates, chiefly among the arthropods, although Ohuye (1937 c) reports the coagulation of cell-free coelomic fluid removed from an annelid. Blood coagulation has been studied in some detail in certain animals, some very careful work having been done with the lobster, the crayfish, and the Japanese shore crab. According to Glavind (1948), the coagulation of lobster blood occurs in a single step. An enzyme, "lobster coagulin", found in blood cells and in body tissue acts on lobster fibrinogen in the presence of calcium ions to convert it to insoluble fibrin. This fibrin is not the same as vertebrate fibrin, but differs from it somewhat in both its chemical and physical properties. For other Crustacea and for insects, the mechanism has been less carefully worked out, with Beard (1950) observing that in the Japanese beetle there is a true coagulation of the blood which is inhibited or activated by various enzymes from body tissues; but the role of blood cells in this process is not yet known, and calcium does not seem to be necessary.

II LOWER INVERTEBRATES

A Protozoa

Certain protozoan cells may be agglutinated by various types of immune substances (Chandler, 1949), move actively about by means of pseudopodia, and ingest solid particles. Such activities of unicellular animals are somewhat similar to those of isolated metazoan cells under certain conditions. However, a detailed study of protozoan physiology is beyond the scope of this paper.

B Porifera

The Porifera have cells which can agglutinate in somewhat the same manner as those in the blood and body fluid of phylogenetically higher animals. Galtsoff (1925) described an investigation using the sponge Microciona prolifera. He separated the cells of the sponge, allowing them to float free in sea water. The archeocytes (the most abundant cells) assumed a globular form. On touching a surface they put out pseudopodia and began to move. Another type of cell, the collencytes, also rounded off, moving when contacting a solid surface. The other cells showed no ameboid movement.

When two archeocytes accidentally touched, the external hyaline cell protoplasm spread out and flowed around the two cells, but the inner granular protoplasm did not coalesce. The aggregate then proceeded to move by means of pseudopodia made of the common hyaline protoplasm. The archeocytes were "sticky", and inert objects would adhere to them and be dragged along; their activity was sensitive to a variety of chemical and physical factors. The cells of <u>Microciona prolifera</u> would not agglutinate with those of <u>Cliona sulphurea</u>.

Within two hours the cells had settled to form a broken membrane which soon broke into many irregular pieces. These gradually contracted so that within five or six hours there were balls of agglutinated cells uniformly distributed over the surface. These continued to move and coalesce for about 24 hours and then began to redevelop into sponges.

C Coelenterata

The Coelenterata are probably the simplest animals to have any free-floating cells in the body cavity (gastrovascular cavity), but according to Rogers (1938) these have no clotting power. However, Wilson (1911) observed that if he broke up the hydroids of either <u>Pennaria tiarella</u> or <u>Eudendrium carneum</u> into cells or small cell aggregates, these would quickly begin to fuse. They formed large solid masses, showing no cell boundaries, and these masses later differentiated ectoderm and entoderm and developed into normal hydranths.

D Some Other Animals

There seems to have been little work done on cell agglutination in many invertebrate phyla, and the few observations recorded show only a lack of such phenomena. Even in some animals which have a coelom or a blood vascular system the contained cells have not been carefully studied. With respect to the nematodes, Chandler (1949) states merely that there are a few large phagocytic cells in the coelom, the "celomocytes". Chitwood and Chitwood (1937-1942) do not mention these. The nemerteans have a few blood cells according to Rogers (1938), but little is known about them.

E Bryozoa

Cuenot (1891) noted that the coelomic cavity of *Bowerbankia imbricata* contained a few small ameboid cells. (These were also found within the various organs.) These cells joined to form small groups which then put forth common pseudopodia. In the body cavity of *Plumatella fruticosa* he noted very large amebocytes on the surfaces of the organs.

F Brachiopoda

About the lowest invertebrates on the phylogenetic tree which have a definite blood cell agglutination are the brachiopods. Ohuye (1936 d) studied the formed elements of the coelomic fluid and blood of Terebratalia coreanica and listed six types of cells, three of which showed a tendency to agglutinate. There were, using his designations, (1) hyaline amebocytes, which were phagocytic and showed a tendency to agglutinate by fusion of their pseudopods, (2) coarsely granular amebocytes, which showed phagocytic activity and would agglutinate less actively, (3) finely granular amebocytes, infrequently found, (4) amebocytes with red granules, rare in the coelom but abundant in the blood vessels, (5) amebocytes with orange granules, also more common in the blood vessels, which showed relatively active ameboid movement and would agglutinate with other kinds of cells, and (6) amebocytes with brown granules, found in the intercellular spaces.

In a later publication Ohuye (1937 b) studied the cellular elements in the coelomic fluid of Coptothyris grayi and of Lingula unguis (L. anatina). He described eight types of cells in Coptothyris; six similar to those of Terebratalia, vesicular cells ("signet ring" shape), and spindle bodies or fusiform corpuscles. In Lingula, Ohuye described five types of corpuscles. They were (1) red blood corpuscles, (2) hyaline amebocytes, rarely found, (3) eosinophilic granulocytes, which were ameboid, phagocytic, and tended to agglutinate, (4) some rare basophilic granulocytes which resembled the eosinophilic ones, and (5) spindle bodies. In both of these publications Ohuye was primarily interested in cytology rather than agglutination.

III MOLLUSCAN-ANNELID STEM

The Mollusks and the annelids both have considerably well-developed blood vascular systems. In both phyla agglutination of the formed elements in blood or body fluid is common, and some annelids seem to have a true coagulation of the coelomic fluid.

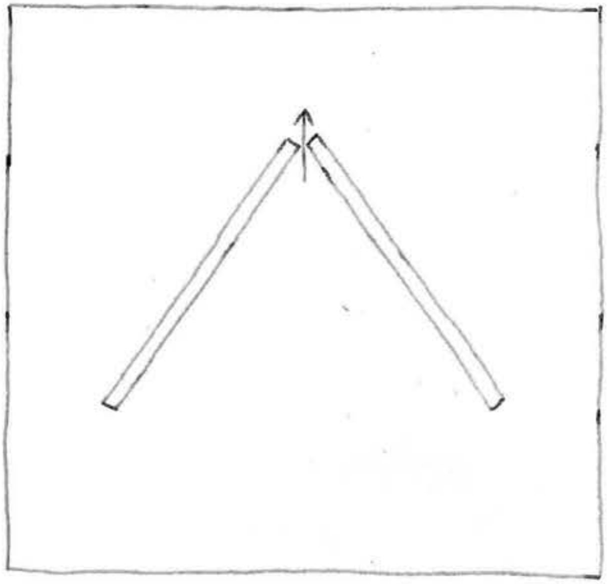
A Mollusca

Jones (1846) was one of the earliest workers to study agglutination in molluscan blood. He observed two species; the whelk, Buccinum magnum and the mussel, Mytilus edulis, and he found granular cells in the blood of both animals. In collected blood these cells shot out processes and agglutinated upon contact. He also described cells with a prominent nucleus which did not take part in this process.

Bottazzi (1902) worked with the blood of *Aplysia depilans*, *Aplysia limacina*, *Eledone moschata*, *Octopus vulgaris* and *Octopus macropus*. He noted agglutination of blood cells and the absence of a true plasma coagulation. (This was later confirmed by Cuénot (1891b), Nolf (1909 b), Parsons and Parsons (1923), and Zunz (1933)). He tried injecting peptone solutions into *Aplysia* and the octopi and found that this did not affect the agglutination of the leukocytes. (He found that peptone would prevent the coagulation of crustacean blood plasma if added *in vitro*.

Nolf (1909 a) tested the blood of various invertebrates for thrombin or its constituents. He added the blood and leukocytes of *Octopus vulgaris* and *Eledone moschata* to fibrinogen from cattle but could not precipitate fibrin. He also tested the blood of the gastropod *Aplysia* and the lamellibranch *Pectunculus violescens* and *Capsa fragilis*, obtaining similar negative results.

Drew (1910) studied the blood of the lamellibranch *Cardium norvegicum* and found that the only cellular elements were amebocytes with short pseudopods. He tried to discover what factors caused the blood cells to agglutinate and found that if he shook the fresh blood in a tube, stirred it with a foreign body, or allowed it to contact a rough surface, the cells would coalesce. He built a plasticene cell with converging walls (see diagram on page 17) and allowed the blood to flow out of it as if it were a wound. He found



Apparatus used by Drew (1910)

(seen from above)

Arrow indicates direction of blood flow.

adapted from Drew (1910)

that if the corpuscles were flowing out slowly and with little force they might touch a rough or foreign surface three or four times before becoming sticky and adhering to the surface, but that if the blood was flowing rapidly, the first or second contact was sufficient to make the cell adhere. He noted that contact with a polished surface was less likely to lead to adherence and, furthermore, that once a cell had adhered to the walls of the chamber it would stick to any other amebocyte that might touch it. He found that if two agglutinated corpuscles were separated, they would remain connected by a strand of protoplasm which would soon contract and thicken. He found that the change in the blood cells leading to agglutination was not due to contact with air, sea water, or hypotonic or hypertonic salt solutions. He also discovered that the amebocytes were phagocytic and would engulf bacteria, but that once agglutinated, they lost this ability.

Drew went further in his observations and studied the role of the amebocytes in wound-healing. Using the technique of cutting the foot of a clam and later fixing and sectioning it, he discovered that within one or two hours there were agglutinated masses of leukocytes with connecting bands on the edges of the wound. They soon completely blocked the wound. As the wound became older the mass of cells became structureless and was invaded by phagocytic and connective tissue corpuscles, muscle fibers grew across the wound, and finally the surface epithelium covered the cut.

Goodrich (1919) showed that the leukocytes of Ostrea and Mytilus, as well as those of other invertebrates, did not have lobular or thread-like pseudopodia as had been commonly supposed. He found (staining the cells with iodine in potassium iodide) that the pseudopods were fine motile membranes which tended to extend over foreign surfaces. Ohuye (1934 b) confirmed his results, but most of the workers describe blood cells as they appear using other techniques.

Orton (1923) studied the blood of oysters and found two cellular elements; large granular cells and smaller hyaline cells. He found that both sorts were actively ameboid and would readily agglutinate to form long chains. He noticed that in sea water the cells would join to form a network. Unlike Drew (1910), he believed that the contact of escaping blood cells with sea water was the stimulus that led to agglutination. He also noticed that the cells functioned in food absorption and in excreting metals.

Ellis, Meirick, and Ellis (1930) observed the presence of a cell clot in blood from 27 species of North American fresh-water mussels but did no experimental work on agglutination.

Takatsuki (1934) studied the oyster Ostrea edulis, describing the same two types of cells as had Orton. He found that the granular leukocytes would readily unite, retaining discrete cell boundaries except in the center of the clot.

He noted that the smaller "lymphocytes" were less active.

He found that the blood cells were sensitive to various reagents and in work similar to that of Loeb (1922 b) on Limulus studied the effects of hypotonic and hypertonic solutions, of acids and alkalis. He noted that fat solvents, such as chloroform, could be used temporarily to stop cell movement. (He confirmed Orton's observation that the blood cells were important in digestion and excretion, noting that they ejected wastes directly from the body).

Ohuye (1937 a) studied the body fluids of the mussels Arca inflata and Glycimeris vestitus, which have red blood cells. In Arca he found two types of blood cells; (1) leukocytes which were actively ameboid and phagocytic, and (2) rare granular amebocytes with similar properties. In Glycimeris he found hyaline amebocytes and two types of granular amebocytes but did not discuss their functions.

The most recent work on molluskan blood is probably that of Stauber (1950), who studied the phagocytic activity of the blood cells of the oyster, Ostrea virginica in eliminating injected India ink.

B Gephyrean Worms

There have been only a few observations on the Gephyrean worms (Echiuroidea, Priapuloidea, Sipunculoidea). Bottazzi (1902) noted that if the body fluid of Sipunculus nudus was removed, the lymphocytes would adhere by slender processes and settle to the bottom but no plasma clot would be formed, and Nolf (1909 a) observed that Sipunculus nudus body fluid would not change mammalian fibrinogen to fibrin. Kyono (1929) studied some Sipunculoidea and observed (1) large colorless leukocytes, (2) small colorless leukocytes, and (3) some smaller leukocytes.

Ohuye (1937 d) studied the cells in the coelomic fluid of two Echiuroids and a Sipunculoid worm. In the euchiuroideans, Urechis unicinctus and Thalassema gogoshimense he noted four kinds of cells. There were (1) hyaline cells, ameboid and phagocytic, (2) finely granular ameboid cells, (3) coarsely granular ameboid cells, and (4) compartmental cells similar to those of some ascidians. In the sipunculoid, Physcolosoma scolops he noted several kinds of cells also: (1) hyaline amebocytes like those in Urechis and Thalassema, (2) small acidophilic granulocytes with blunt pseudopodia, (3) large acidophilic granulocytes similar to earthworm lamprocytes and showing no movement of phagocytic activity, and (4) basiphilic granulocytes similar to the small acidophilic cells. He also noted "urns", made up of a group of cells having an opening with a ciliated border through which waste materials and other substances were ingested. Ohuye did not discuss the agglutination of any of these elements.

C Annelida

Agglutination has been studied to some extent in the blood and body fluids of the various annelids, but little work has been done on the factors involved.

One of the earliest observations on annelid blood was that of Jones (1846), who noticed that the earthworm had several kinds of cells. There were finely granular cells with fine processes and coarsely granular cells as well as some other types. He also observed the presence of small blood cells in the medicinal leech.

Geddes (1880 a) in the course of some investigations on invertebrate clotting noted that the coelomic fluid corpuscles of the earthworm would form clumps (plasmodia) *in vitro*.

Cuénot (1891 a) published a series of observations which covered in a general way a variety of species. In the polychaetes he noted that the cells found in the blood and coelomic fluids differed from species to species, but that most species had non-colored amebocytes and also red cells in the blood, with the amebocytes also being present in the general body cavity. He noted that when the coelomic liquid was moving, the corpuscles were fusiform, but if the liquid was at rest, they emitted pseudopodia. In *Dasybranchus caducus* he observed that the red cells often formed large masses in the living animal. In the blood of the Polychaeta he found that the amebocytes were smaller than those of the coelom. In the species, Amphiglene mediterranea, he found ameboid reddish cells which, if the blood vessel were disturbed, emitted long pseudopodia. Furthermore, he found that in those worms he examined with colorless blood, (Phyllodociens and Syllidiens) the blood had the same cellular constituents as the body fluid. He also found a few species of polychaetes which had no amebocytes in the blood at all.

Cuénot also examined many Oligochaetes. He found that in general, the coelomic fluid was colorless and had many corpuscles. He noted that the blood contained reddish amebocytes, their number varying in the various species observed.

He examined some of the Hirudinea, and found that their blood would not clot on removal from the animal. He found species-to-species variations in the types of blood cells, but concluded that in the leeches there were two general types of blood cells: (1) cells with non-colored granules which he considered food storage cells, and (2) cells with yellow granules.

Griffiths (1891) decided that the blood of annelids did not show a true coagulation but that it did contain some fibrin.

The first investigation which went into some detail concerning clotting in annelids was that of Keng (1895).

He studied the coelomic fluid of the earthworm, Lumbricus terrestris, describing it as being a milky fluid containing crystals, pigments, various living organisms (probably parasites) and several types of corpuscles: (1) small non-granular cells, (2) large hyaline cells which were phagocytic and had short thick pseudopodia, (3) small granular ameboid cells which had fine pseudopodia, and (4) large granular cells which were the most numerous and were ameboid with filiform pseudopodia. These cells could also be found in the intramuscular spaces. Keng noticed that when the fluid was removed from the body, most of the cells showed great activity, and some of the granular cells elongated greatly and fused with extensions of other cells to form a plasmodial network. He found that the different types of cells readily fused, forming a dense plasmodial mass having cells with projecting pseudopodia and vacuoles on its borders. He found that adding water or other fluids or touching the cells would cause them to become vacuolated, emit pseudopodia, and agglutinate to form a clot.

On studying the relation of the coelomic cells to defensive mechanisms, he found that the granular cells would surround bacilli but would not ingest them, while the hyaline cells would ingest the bacilli and digest them. He noticed that if a worm was irritated, it would excrete coelomic fluid from the dorsal pores. This fluid became mixed with sticky threads of mucin, forming a plasmodium.

Benham (1901) investigated the coelomic fluid of two earthworms, Octochaetus multiporus and Acanthodrilus annectens. He found that Octochaetus had opaque, white coelomic fluid which was excreted through the dorsal pores in small quantities if the worm was roughly handled and would also be ejected if the worm was placed in weak alcohol, or acetic acid vapor. If the surface of the worm was cut, the fluid would be discharged from the immediate neighborhood. The fluid was thick and set at once, forming a sticky mass. The Acanthodrilus discharged abundant coelomic fluid on slight handling and this fluid was also thick but hardened to a firm, chalky mass. The cells in it formed a plasmodium and soon died.

In both worms the cells described in the coelomic fluid were as follows: (1) amebocytes with pseudopodia, (2) large and non-motile oleocytes with oily globules, (3) large, non-motile lamprocytes with vacuoles, and (4) small, hyaline linocytes containing a thread which would come out of the cells upon chemical treatment. Octochaetus coelomic fluid contained some long spindle-shaped cells which crept along the body wall.

In Acanthrodrilus, the cells in the coelomic fluid would adhere, forming clumps of from 6 to 12 cells. In Octochaetus the cells remained separate and the amebocytes in the excreted fluid remained alive and motile. Although the fluid rapidly coagulated, "fibrin" threads were only seen after some time and they seemed to connect the cells. In two other species of the genus Octochaetus the coelomic fluid was found to be similar.

Nolf (1909 a) noticed that the cells in the visceral fluid of the Polychaetes Glycera siphonostoma and Dasybranchus caducus would rapidly coalesce in little clumps when the fluid was removed from the worms. He tried to change mammalian fibrinogen to fibrin by adding this fluid to it but was unsuccessful.

Goodrich, (1919) studied the forms of leukocyte pseudopodis, observing cells of the Polychaete, Arenicola, and of the Oligochaete, Lumbricus.

Fauré-Fremiet (1925) observed the amebocytes of the worms, Arenicola marina and Nepthys. He showed that the transference of these cells from a passive to an actively moving state upon removal from the body was not necessarily followed by the death of the cell, but that in vitro the cells would form rather well-organized pseudo-tissues in a suitable environment and gradually approach their original condition. Fauré-Fremiet and Wallich (1925) noted that isolated amebocytes of these worms showed random, disorganized movement. They also studied other factors affecting ameboid movement and tissue culture of these cells.

Kindred (1929) studied the cellular elements in the perivisceral fluid of an Oligochaete, Pheretima indica, finding five types of cells, two of them phagocytic; but he did not discuss agglutination.

Ohuye studied the coelomic corpuscles of several annelids. He examined (1934 b) the earthworm, *Drawida hattamimizu*, finding five general types of cells: (1) lymphocytes which were actively phagocytic and would agglutinate *in vitro*, keeping distinct cell boundaries, (2) monocytes which were phagocytic and ameboid and tended to agglutinate in plasmodia without cell boundaries, (3) granulocytes, (4) lamprocytes, and (5) a few linocytes and occasional detached tissue cells. He confirmed Goodrich's observation (1919) that the pseudopodia were membranous or petaloid rather than lobate.

Ohuye (1937 c) studied the earthworm, *Pheretima sieboldi*, finding that the coelomic fluid could be discharged from both the dorsal pores and the mouth. He classified the cellular elements as (1) lymphocytes, (2) granulocytes, (3) lamprocytes, the most abundant type, (4) linocytes, (5) oleocytes, and (6) a few other cells. He found that the body fluid was opaque when first obtained and that it soon coagulated, deforming the cells in it by the pressure exerted. He found that if he withdrew the body fluid with a syringe (not exposing it to air) it would not clot. He noted that the formed clot would stain intensely, using Weigert's method of fibrin staining. The lymphocytes and granulocytes would agglutinate outside the animal body, but Ohuye believed this phenomenon had no relation to the coagulation of the fluid, as the coelomic fluid would coagulate even after removal of the formed elements. He believed that further study should be undertaken to find out why the body fluid coagulates only in certain annelids.

In a study of cell elements in body fluid, Ohuye (1937 a) found two types of cells in the coelomic fluid of a polychaete, Terebella sp. He noted (1) spindle-shaped amebocytes which exhibited ameboid movement on standing and (2) some scarce granulocytes, but he did not discuss their functions.

IV ARTHROPODA

Introduction: The Arthropoda, as befits the most abundant and successful invertebrate multicellular animals, have been studied in greater detail than have other groups. Most of what we know about invertebrate blood clotting comes from work done with this phylum and, except for a few of the annelids, arthropods are the only invertebrates to exhibit not only cell agglutination but a true coagulation of the blood plasma.

A Limulus

Limulus polyphemus is a rather primitive arthropod of large size commonly found on the eastern shore of North America. The physiology of its blood has been extensively studied especially by Leo Loeb who found this animal admirably suited to studies on blood cell agglutination.

One of the first men to study *Limulus* blood was Howell (1886), who noted that the blood fluid soon clots outside the body and that the clot consists of coalesced blood corpuscles. He also tested the effects of various substances and temperatures on clot formation.

Loeb (1902) noted that *Limulus* had only one type of blood cell; spindle-shaped and granular, showing little ameboid or phagocytic activity within the animal. On collecting some blood, he observed that the blood cells, which he called "amebocytes" underwent a series of changes leading to a variety of forms, but which could be summarized as (1) a partial or complete liquefaction of the cell protoplasm, forming pseudopodia and droplets which broke off, leading to a lysis of the cell (especially marked in those cells which had contacted the cut tissue of the animal or the walls of the container), and (2) a coagulation of the cell protoplasm (both within the cells and floating free in the plasma) producing a mass of fibers. Many of the fibers consisted of strings of agglutinated cells. When collected in sea water (1904 b) the amebocytes were connected by fine strands formed of protoplasm from the cells. In distilled water the cells would swell and agglutinate, and Loeb found it possible to draw out the cell protoplasm into threads, using fine needles. If the blood were collected on a slide (1902) and the cells allowed to coalesce, fibers could be produced by merely lifting or sliding the cover glass.

Loeb considered the Limulus or horseshoe crab to be a crustacean and studied its blood along with that of the Crustacea. He applied the results of work on Limulus blood cells to Crustacean physiology and his ideas about crustean blood cells were formulated largely on the basis of this study. Loeb extended these theories to explain the activity of vertebrate blood cells, embryonic cells, and connective tissue cells.

He observed (1904 b) that lobster blood clotted in two steps; a "first coagulation" made up of agglutinated cells and coagulated cytoplasm and a "second coagulation" which was a true precipitation of cell-free plasma fibrin, and he believed that the clot of *Limulus* blood was homologous to the crustacean "first coagulation". He found that removing calcium with oxalate solution would not prevent the clot formation in the *Limulus* although it would prevent the "second coagulation" in Crustacea. However, much stronger $MgSO_4$ or oxalate solutions would preserve the cells intact.

He found that there were substances (1904 b) in crab *Callinectes*) or lobster (*Homarus*) blood cells and muscle tissue that would cause cell-free plasma of either species to coagulate. However, using these extracts he was unable to demonstrate any plasma clotting in *Limulus*. He found that if he collected *Limulus* blood in saturated $MgSO_4$ to prevent clotting and added just enough distilled water so that the cells would become sticky and could be filtered out, the plasma would never produce a clot. If the blood was diluted instead without filtration a diffuse coagulum of fibers was formed from the lysed cells. *Limulus* amebocytes (1905 c) acted exactly the same in muscle extracts of either *Limulus* or lobster as they did in sea water.

Saturated salt solutions or even simple dilutions would prevent *Limulus* blood from forming a clot. Loeb (1903) found that pyrrhol, resorcin, and hydroquinon had an an inhibitory effect, and that adrenalin chloride, pilocarpine, and atropine were weak inhibitors. Clot formation could also be prevented by heating the blood for 30 to 40 minutes at 50-54°C.

Loeb found (1905 a) that there were several factors that caused the amebocytes to leave their normal inactive state. He found that passage through a narrow wound, breaking through the surface layer of a solution when dropped on a slide, the mechanical shock of the blood falling on a surface, and contacting a solid body on the slide all tended to cause the previously mentioned changes. He found (1905 a) that if he withdrew the blood from the heart with a clean metal cannula and dropped it on a clean slide most of the cells would keep an oval or round shape and would not agglutinate although the cells in contact with the glass would slowly spread out over the surface. He tried withdrawing the blood through an oiled cannula and transferring it into olive oil and found that he could preserve the cells intact for about a half hour. If the blood was removed from the hemocoel rather than from the heart the cells were somewhat sticky and would agglutinate. Collecting the cells into gelatin (1903) would also partially inhibit clot formation. The cells would swell and agglutinate but would remain intact.

Loeb (1902) observed that if he put some foreign body (silk thread, gauze, bits of agar, pieces of coagulated egg white) into the hemocoel of Limulus, the amebocytes would mass about the foreign body but would not penetrate it, unlike certain mammalian blood cells. He also noticed that experimental wounds did not heal. If he injected sea water into a Limulus (1905 c) the blood would clot inside the body, but if distilled water was injected the blood would not clot and only a local coagulum would be formed about the needle hole.

Although Loeb used the term "cell-fibrin" to describe the clot produced by Limulus blood cells, Alsberg and Clark (1908) made a biochemical examination of the cell clot of Limulus and could not find any fibrin.

Loeb (1903) discovered that if Limulus amebocytes were carefully collected they could be grown in Limulus plasma and studied at length. He found (1920) that blood serum differed in various individuals as shown by the growth of amebocytes in their serum and that (1927) the amebocytes from different animals also differed.

Continuing earlier work (1905 a), he studied the effects of osmotic pressure, pH, and various chemicals on the amebocytes.in tissue culture. He noted (1927, 1928) that conditions which tended to harden the cell protoplasm diminished the tendency to agglutination and ameboid movement. Strong isotonic acid solutions, alkali in isotonic NaCl, hypertonic solutions in general, the sulfate sodium ions, and cold temperatures all had this effect. If the cell protoplasm was softened and made more liquid the cells were more likely to agglutinate, and hypotonic solutions in general, potassium ions, ammonium ions, nitrate ions, Limulus blood serum, alkali solutions, and high temperatures caused this reaction. (Loeb's paper of 1928 did not contain any new material but served to illustrate his earlier publication of 1927.)

Loeb (1921 a, b) decided that the changes in the amebocytes which led to agglutination were the same as those leading to ameboid movement. Both phenomena were closely related to colloidal changes in the outer layers of the cell protoplasm (1922 a, 1927). He believed the difference between the two phenomena was merely that (1922 a) if the whole surface of the cell were affected, agglutination would result, while ameboid movement was closely associated with local areas of greater fluidity. He believed that the cells had some independent power of movement, with surface forces controlling them only when in the more liquid state. Loeb (1927) believed that upon stimulation, the amebocytes would (1) take up fluid, (2) soften, (3) extend and harden, and (4) become elastic and contract. The cells thus approached the physical properties of true fibrin. Loeb (1921 b, 1922 b, 1927) explained clot retraction, which occurs in the cell-clot of Limulus, as being caused by gelation of the expanded amebocytes and their pseudopodia into an elastic mass whose tension provided the energy for an elastic contraction of the cytoplasm.

Loeb and Genther (1926 a) showed that this was the only sort of response the amebocytes could show to any stimulating agent although the extent and character of the reaction varied. They showed (1926 b) that the age of the amebocytes could modify the cell response, with the younger cells being more able to alter their fluidity reversibly, while the older cells were more flaccid.

One of the major differences between Limulus amebocytes and other sorts of cells (Loeb and Genther 1926 c) was that the amebocytes were permeable to more substances.

Loeb (1927) never observed any phagocytic activity of the amebocytes. He believed that phagocytosis was not simply a matter of greater surface activity on the part of the cell but that a local softening of the phagocytic cell occurs following contact with the foreign body.

B Other Chelicerata

1. Pyncnogonidia

There has been very little work done on the physiology of blood clotting of Chelicerata other than Limulus. Cuénot (1891 a) studied the blood of several Pyncnogonidia (Ammothea fibulifera, Phoxichilidium exiguum, and Nymphon sp.) and observed that there were amebocytes present in the blood. They were oval and smooth in circulating blood but if stopped by an obstacle put forth pseudopodia.

2. Scorpionidia

Cuénot (1891 a) studied two scorpions living in different habitats, *Buthus occitanus* and *Scorpis europaeus*, and observed slightly ameboid cells and food storage cells in the blood. He observed that the blood when removed from the body rapidly formed a coagulum like that of the Crustacea, containing amebocytes and fibrin.

3. Araneida

Jones (1846) observed the blood of a spider and noted cells which put forth pseudopodia when the blood was shed.

Blackwall (1852) observed blood clotting in several species of spiders. He allowed the spiders to wound each other as part of a study of spider venom and observed that the blood of all species formed a clot. He noted that the clot of *Tegenaria civilis* and *Epeira diadema* were rapidly formed, those of *Ciniflo atrox* and *Lycosa agretyca* more slowly, and that of *Segestria senoculata* blood only after some time.

Cuénot (1891 a) studied the spiders *Tegenaria domestica* and *Epeira diadema* and noted that the blood formed a fibrin clot enclosing most of the amebocytes. He confirmed Jones' observation that the amebocytes would put forth short pseudopodia when in contact with the glass of the collecting slide. He also noted a few large cells which he believed were concerned in food storage.

Yeager and Knight (1933) observed the blood of several unspecified spiders, observing agglutination of the blood cells with subsequent gelling of the cell protoplasm. However, they did not find any true coagulation of the plasma.

In a more recent study, Deevey (1941) observed the blood cells of the Haitian Tarantula, *Phormictopus cancerides*. She found several types of cells: (1) leukocytes, (2) chromophobes, (3) basophils, (4) weak eosinophils, (5) eosinophils, (6) leberidocytes, and (7) cyst cells. There were several types of leukocytes. The larger ones would flatten on contacting a surface, and these cells were active in phagocytosis and in blood coagulation. The hyaline leukocytes were most important in causing the other leukocytes to agglutinate. The eosinophil cells were phagocytic and ameboid, and the chromophobes were slightly ameboid. Deevey observed that blood clotting would not occur if the blood were collected immediately after the death of the spider. She was unable to decide whether Tarantula blood coagulation was entirely a cellular phenomenon or whether the plasma also took part.

4. Phalangida

Cuénot (1891 a) studied some Phalangida, noticing that *Phalangium opilio* blood contained cells similar to those of the spiders, but did not mention clotting.

5. Tardigrada

Shipley (1909) studied the Tardigrada and noted that the blood was a clear fluid which did not coagulate when water was added. He observed numerous corpuscles packed with food reserves.

C Crustacea

The physiology of crustacean blood has been more intensively and carefully studied than that of any other group of invertebrates. Not only have detailed studies been made of the functions of the blood cells in the commoner Crustacea, but the factors producing and modifying plasma clotting have been extensively investigated.

Probably the first study of crustacean blood cells was that of Jones (1846). He observed that there were two kinds of corpuscles in crab and lobster blood. On removing blood from a crab the cells put forth processes and agglutinated, forming an essentially cellular clot. He noticed that lobster blood was about the same, but that the clot was larger and firmer.

Fredericq (1879) proved that the clot of lobster blood starts from agglutination of the blood cells. The plasma released from this "first coagulation" would later coagulate, forming a "second coagulation". The "second coagulation" resembled that of mammalian fibrin, for it could be prevented by adding certain salt solutions, while cell agglutination could not.

Geddes (1880 a) studied the blood of the crabs Carcinus maenas and Cancer pagurus and of the lobster Homarus vulgaris. He noticed two types of crab blood corpuscles; a coarsely granular kind that would not join together and a finely granular kind which would agglutinate in vitro. He thought that invertebrate blood clots were always formed in part by fused ameboid cells and he used the word "plasmodium" to describe such a fused mass.

Pouchet (1882) observed that some crustaceans could remove an injured leg with no loss of blood (autotomy). He also noted that the clot of Palinurus vulgaris blood is a gelatinous mass and that the corpuscles of Palaemon serratus have smooth and regular shapes while within the circulatory system.

In work with hirudin from the medicinal leech, Haycraft (1884) observed that this substance would not inhibit the clotting of crustacean blood.

Halliburton, one of the more important early English investigators, (1885) studied blood clotting in Homarus vulgaris, Carcinus maenus, Astacus fluviatilis, and Nephrops norvegicus. He noted that if the "first clot" is removed a "second clot" will form, but that serum from that clot would not coagulate. However, he believed that both the "first" and "second" clots were identical, being steps in a single process. He thought that the "first clot" was not made up only of agglutinated cells, but included fibrin, for he was able to prevent both clots by adding strong $MgSO_4$ solutions. He observed that the clot finally produced consisted of cells (which had collected together and had shot out long processes) and of fibrin threads.

Halliburton was mainly interested in trying to prepare invertebrate "thrombin". He tested cat, horse, lobster, crab, and crayfish blood and observed that extracts of mammalian or crustacean blood could cause the plasma of either to clot. He believed the coagulating substances were found in the leukocytes.

Howell (1886) was probably the first American to contribute to this field. He observed the blue crab Callinectes hastatus, finding two types of blood corpuscles. He noted that both kinds of blood cell would actively agglutinate in shed blood. He also noted that the plasma gells completely.

Haycraft and Carlier (1888) introduced the technique of collecting blood into oil. They found that coagulation of crab blood depended on its contacting the cut edge of the wound and observed that if the blood was removed with a pipette and put into oil, the corpuscles would not agglutinate and no clot would be formed for about half an hour. However, if the blood was collected on glass, the corpuscles would soon form a branching network.

Cuénot (1891 b) observed great differences in the clotting ability of the blood of various Crustacea. He believed that both clots ("first" and "second") were part of a single process in which fibrin is laid down. He also noted that the blood of some crustaceans kept in captivity gradually lost the ability to coagulate. He thought that the blood proteins, such as fibrinogen, were being used as nutrient.

Heim (1892) studied the blood of some decapods and observed that the "first clot" was made up of cells, while the "second clot" contained fibrin. He noted that the two clots were chemically different, and he found that strong salt solutions which could not prevent the "first clot" could easily prevent the "second". He also showed that calcium ions were necessary for the "second clot" but not for the "first".

Heim prepared "thrombin" and "fibrinogen" from crustacean blood and found, as did Halliburton (1885), that lobster or mammalian "thrombin" could clot the blood of either animal. He also prepared "thrombin ferment" from lobster muscle.

Upon observing the blood of various crustaceans, Heim noted that Platycarcinus (Cancer) pagurus, Maia squinado, Carcinus maenas, Galathea strigosa, and Astacus fluviatilis have only the "first clot", while Homarus vulgaris, Palinurus vulgaris, and Portunus puber have both the cell clot and a plasma fibrin coagulation.

Another of the more extensive early papers was that of Hardy (1892). On studying the blood of the Crayfish, Astacus he found two types of blood cell: (1) explosive corpuscles which would disintegrate upon contact with foreign bodies, and (2) eosinophilic cells which were not as sensitive and were found in only about one-third the abundance of the others. He also noted that some abnormal animals had a few basophilic cells. Hardy observed that the majority of the blood cells, even in apparently healthy animals, were found adhering to the walls of the hemocoel. Upon stimulation by either touching a foreign body or being near other disintegrated explosive cells, the explosive cells would shoot out extremely fine pseudopodia; and blebs of cytoplasm would go along these, expand, and burst. Sometimes there would only be blunt processes or vesicles on the surface of the cells which would swell and burst. The plasma would then clot, enclosing the eosinophiles, which were ameboid but didn't seem to take much part in clot formation. Hardy believed that a "fibrin ferment" was liberated from the explosive cells which acted on fibrinogen in the plasma. He found that iodine, which prevented solution of the granules in these cells, would prevent clotting, but that osmic acid, which preserved the cells intact but allowed their granules to dissolve out, would not. He also noted that the explosive cells were phagocytic although the eosinophiles were not, and that the explosive cells could fuse to form large phagocytic plasmodia.

Hardy also studied the blood of the water flea, Daphnia, and observed that its blood cells were ameboid with basophilic granular protoplasm. He noticed that inside the animals (which are quite transparent) these cells were of very variable adhesiveness, and that sometimes only one part of the cell would be sticky and the cell would detach itself from the hemocoel wall except for a thin protoplasmic strand. At any particular time, most of the cells would not be free-floating. He further discovered that any sort of irritation to the animal or weak electrical stimulation would cause all the corpuscles in the neighborhood to adhere to the body wall. He observed that the increase in adhesiveness of these cells was associated with irregularity of form and increased cell movement. These cells, as Metchnikoff (1892) had observed, were phagocytic and they would normally ingest and digest fat droplets. Hardy noted that if the animal were injured, the blood cells would rapidly disintegrate and disappear. He thought that Daphnia and Astacus showed an evolutionary sequence of blood cell development.

Bottazzi (1902) observed blood clots of several crustaceans, noting that Palinurus and Homarus blood forms a solid clot, but that the clot of Maia blood is semi-liquid. He observed that oxalate would not prevent the "first clot" but only the "second", but like Halliburton (1885) he believed that both clots were formed essentially by the same process. He injected 10% peptone solutions into Maia and Palinurus but found this did not affect blood clotting. Mixing the solution with the blood also had no effect. However, if the blood was collected directly into the peptone solution coagulation was prevented. He also observed that peptone could prevent the formation of blood cell "plasmodia" (clots) in many other marine invertebrates.

Ducceschi (1903) observed that cocain hydrochloride prevented the agglutination of invertebrate blood corpuscles. It could be mixed with the blood in vitro or injected into the animal. Plasma coagulation would also be prevented temporarily by the preserving effect on the corpuscles.

In 1903, Loeb began to study the blood of the Crustacea. He also studied Limulus (which he believed to be a crustacean). Although his German papers are better known, those published concurrently in English seem, judging from reviews of his work, to contain essentially the same material, although perhaps in less detail.

He (1903) compared the clots of the horseshoe crab Limulus, the lobster, Homarus americanus and the spider crab, Platyonchus ocellatus, noting (as previously mentioned) that horseshoe crab blood formed only a large "first coagulum". The lobster and the crab had a similar "first clot", but the lobster also showed a secondary coagulation of precipitated plasma protein. Loeb showed (1904 b) that the first coagulum consisted solely of agglutinated cells or of their solidified protoplasm, while the second coagulum was of plasma fibrin. He also noted that vertebrate blood platelets could agglutinate independently of fibrin coagulation and thought that thrombi within the body might be formed in this manner.

Loeb (1903) discovered that removal of the first clot of lobster blood inhibited formation of the second and that if he diluted the serum left from the "first clot", the fluid would coagulate when shreds of the first coagulum or of lobster muscle were added. He observed that pieces of rabbit, rat, or frog fibrin had no effect on lobster plasma coagulation, and decided that the enzyme that caused the lobster plasma to clot was essentially different from the "coagulins" of vertebrates. He observed that the serum from the "second clot" would not coagulate.

Loeb (1903) also noted that calcium was essential for the second clotting of lobster blood but not for the first.

In a series of investigations Loeb (1904 b) found that he could prevent the second coagulation of lobster blood by diluting it with distilled water and then filtering to remove the agglutinated cells. Adding a piece of "cell-fibrin" (part of "first clot") or of lobster muscle would produce clotting. He treated the blood of <u>Callinectes hastatus</u> similarly but was unable to prevent the plasma from clotting. If he received the blood into water at 70-80°C and then filtered, the plasma would not clot until a piece of Callinectes cell-fibrin or muscle was added. Loeb carried out similar experiments using other crustaceans. However, he could never get Limulus serum to clot by adding any cell or muscle extracts, thus demonstrating a lack of fibrinogen in the plasma.

Loeb (1904 a) examined various invertebrates in an effort to discover the nature of the blood-coagulating enzymes found in body tissues and in the blood cell. Testing the "tissue coagulins" from Homarus and Callinectes muscle, he found a partial species difference, for although either muscle extract could clot either blood plasma, the action was more pronounced in the homospecific preparation. Muscle substances of animals other than arthropods had no effect upon lobster plasma, but extracts of the eggs of some non-arthropod invertebrates had a weak clotting effect on lobster plasma. Loeb found that the specificity of "blood coagulins" from the leukocytes was less distinct than that of the "tissue coagulins". He also noted that clots of vertebrate blood had no effect on lobster plasma.

In a more careful examination of "tissue coagulin" and "blood coagulin", Loeb (1906) came to the conclusion that they were two different substances, for he observed that the tissue coagulins showed greater stability on heating and were more specifically adapted than the "blood coagulins". The "tissue coagulins" required calcium whereas the "blood coagulins" did not.

Loeb (1907) discovered a coagulation-inhibitor in lobster muscle. He discovered (1903) that pieces of lobster hepatopancreas could prevent plasma clotting and that hirudin (1905 b) had no effect. He also (1904 a) noted that chemically inert powdered bodies which accelerated the clotting of vertebrate blood had no effect on that of arthropods.

Loeb (1922 a, 1927) studied cell agglutination most extensively in the Limulus, deciding (as earlier mentioned) that agglutination depended upon changes in the consistency of the outer layers of the cell protoplasm. These changes were related to an uptake of water when the cells were stimulated by various substances or by friction. He explained the behavior of crustacean blood cells largely on the basis of such experiments, for he found it impossible to grow crustacean cells in tissue culture (1921 b).

Nolf (1909 a, b) carried out a series of investigations in which he tested many earlier theories. He first tried (by adding various types of invertebrate whole blood or cell-free plasma to fibrinogen prepared from cattle) to demonstrate thrombin or a prothrombin-thromboplastin complex. Unlike Halliburton (1885) and Heim (1892) he was unable to find such substances in the invertebrates tested, including the crustaceans Palinurus vulgaris and Maia squinado. Like Heim, he noted that the blood of Palinurus clots in two steps like that of the lobster to give a dense clot, but that Maia blood showed only a rapid agglutination of the cellular elements with no subsequent plasma coagulation. On adding Maia blood to mammalian fibrinogen he noted that no fibrin would be precipitated although the Maia blood cells would agglutinate.

He noted autotomy in Palinurus vulguris and observed that if a small wound was made in this animal, the clot of agglutinated cells that formed on the edges of the wound was usually sufficient to stop loss of blood. He confirmed Loeb's observation (1903) on the anticoagulant activity of hepato-pancreatic extracts.

Nolf arrived at a theory of crustacean blood coagulation somewhat like that of Loeb, and the two theories are quite easily compared if one substitutes "coagulins" (Loeb) for "A-fibrinogen" (Nolf) and "fibrinogen" (Loeb) for "B-fibrinogen" (Nolf). Nolf thought that the coagulation of plasma on the Crustacea was caused by the mutual precipitation of two substances, "A-fibrinogen", found in extracts of leukocytes and muscles and to a small degree in the plasma, and of "B -fibrinogen", abundant in normal blood plasma. He believed that their union to form insoluble fibrin was possible only in the presence of calcium ions. Unlike Loeb (1906) he believed that "tissue coagulin" and "blood coagulin" was a single substance.

In comparing various species, he found that "A-fibrinogen (muscle extracts) of all species examined, including not only decapods but also stomatopodes and isopods, would cause the "B-fibrinogen" (cell-free plasma) of _Palinurus vulgaris_ or of _Calappa granulata_ to coagulate. However, he noted that vertebrate thrombin had no effect on crustacean "B-fibrinogen".

Nolf observed that the gelation of the plasma is preceded in whole blood by an agglutination of the blood cells which ultimately destroy themselves in forming the clot. However he believed that agglutination could exist only in those species which had plasma poor in "B-fibrinogen" (so that the plasma gel would not immediately prevent cell movements). He thought that agglutination of the blood cells was always due to a coagulation of "B-fibrinogen" on the surface and in the cortical protoplasm of the cells to give fibrin. He believed that this coating of fibrin was the cause of both agglutination and lysis.

John Tait and his associates studied blood clotting and the functioning of the blood cells in a wide assortment of Crustacea, extending the earlier work of Hardy (1892). Tait (1908) studied the agglutination of blood corpuscles in the amphipod _Gammarus_. He noted that if he cut the antennae of an animal (partly asphyxiated to prevent movement) the cells would adhere to the cut end, forming a clump or mass which stopped blood flow. Later the cells would break down and fuse. The corpuscles would also pile up in the antennae near a wound, and he noted that some animals had their antennae filled with agglutinated blood corpuscles, despite a lack of any visible injury.

In a more extensive series of investigations (1910 a) he studied a variety of isopods and amphipods. On obtaining blood from a cut antenna of the isopod Ligia oceanica he observed three types of corpuscles: (1) eosinophiles, (2) basophiles, and (3) explosive corpuscles, all of which had been observed by Hardy (1892). He noticed that when the blood was collected the explosive cells would rupture (as described by Hardy) and the immediate area about each cell would become filled with a granular coagulum. He noted also that all the explosive cells in any film of blood would rupture simultaneously. Studying the isopods Oniscus and Idotea, Tait observed the same process. About 20 minutes after this first coagulation, all the remaining plasma would gel.

In further work on Gammarus marinus, Tait (1910 a) noticed that in certain animals the cells near a wound (cut antenna) would agglutinate and break down, forming a globular mass of homogeneous material to which other cells would become attached. As noted by Hardy (in the crayfish), the coagulum material seemed to hasten the cytolysis and fusion of the embedded cells. Tait observed that in the Gammarus the leukocytes tended to rupture if near a wound, unlike the blood cells in Astacus (crayfish) and Ligia which did so on contacting a foreign object. In most of the animals only a simple agglutination could be observed and Tait never succeeded in demonstrating explosive cells in *Gammarus marinus* when the blood was collected onto a slide.

Tait (1910 b) compared two species of *Gammarus*, finding that *Gammarus marinus* blood usually formed a clot consisting only of agglutinated cells, while *Gammarus locusta* blood formed a globule of coagulated protein associated with "explosion" of the blood cells as did the blood of *Ligia*.

Soon afterwards Tait (1911) grouped many species of crustaceans on the basis of the type of blood clot they exhibited *in vitro*. He recognized three types: (A) simple agglutination of the corpuscles, (B) agglutination of the corpuscles with subsequent gelling of the plasma, and (C) an insignificant cell agglutination with a plasma gel occurring in two stages: (1) localized clots about specialized cells, and (2) coagulation of all the remaining plasma.

Type *C* clotting required the least blood to detect and was found in isopods and often in amphipods. Types *A* and *B* could not be demonstrated to be either present or lacking using the small amounts of blood found in crustaceans other than the Decapoda. (For a table of his results see pages 53-54).

Tait found no correlation of autotomy with the type of blood coagulation. He found a rather vague correlation between the type of blood clotting and the taxonomic classification, but there were many exceptions.

In a later series of investigations (1918) Tait studied the blood cells of three crustaceans having type *C* clotting:

Types of Blood Clotting in the Crustacea

Type A

Decapoda

Cancer pagurus, Maia squinado, Inachus dorynchus, Macropoda rostratus, Hyas coarctatus

Type B

Decapoda

Carcinus maenus (mostly fluid), Palaemon serratus, Portunus puber, Homarus vulgaris (solid)

(Order indicates increasing amounts and firmness of clots.)

Type C

Isopoda

present: Conilera cylindracea, Idolea baltica, Idolea emarginata, Ligia oceanica, Oniscus, several species of Porcello

absent: Gnathia maxillaris, Dynamene rubra Sphaeroma serratum, Jaera marina

Amphipoda

present: Gammarus locusta (in vitro)

absent: Gammarus marinus, Gammarus polex, Orchestia littorea, Caprella

Mysidacea

absent: a species of Mysis

Decapoda

present: Astacus fluviatilis, Palinurus vulgaris

absent: Pandalus montagui, Pandalus brevirostris, Hippolyte varians, Hippolyte viridis, Palaemon serratus, Crangon vulgaris, Homarus vulgaris, Galathea squamifera, Galathea strigosa, Porcellana longicornis, Porcellana platycheles, Eupagurus bernhardus, Eupagurus prideauxii, Ebalia tuberosa, Corystes cassivelaunus, Carcinus maenas, Portunus puber, Portunus marmoreus, Portunus arcuatus, Portunus depurator, Atelecyclus septemdentatus, Cancer pagurus, Xantho hydrophilus, Xantho incisus, Inachus dorynchus, Macropodia rostratus, Hyas coarctatus, Maia squinado

Brachiura

probably not

adapted from Tait (1911)

Palinurus, Astacus, and Ligia. Studying the cells of Ligia (Tait 1910 a) he divided the eosinophiles into hyaline thigmocytes and granular thigmocytes or amebocytes.

Both types of thigmocytes (or touch-sensitive cells) would put forth pseudopodia and flow out when in contact with a foreign substance such as glass. He noted that only the granular cells could retract their protoplasm after spreading out and that the hyaline ones could not. He believed that this spreading out of the hyaline thigmocytes was due purely to surface tension.

Observing the thigmotactic cells in Ligia and in Astacus, Tait noticed that they were phagocytic, the hyaline thigmocytes being more markedly so. He theorized that cell agglutination and phagocytosis were different aspects of the same basic phenomenon, and believed that the relative surface tensions of the cell and the foreign body (in agglutination the "foreign body" was another cell) caused the cell to flow around the other material. He believed that phagocytosis and agglutination, like the tendency of the cells to spread out on glass, was not dependent on ameboid movement, and he worked out a series of examples which showed that phagocytic activity could be caused by surface tension relationships, but he believed that it might also occur where specific surface activity relationships were lacking. Tait extended his hypothesis to include amphibian spindle cells and mammalian platelets, believing that any cell which would adhere to such materials as glass would also be phagocytic. The differential adhesiveness of the Crustacean blood cells was in very sensitive balance, easily altered by various factors.

Tait and Gunn (1918) carried out a further study of the blood of Astacus fluviatilis. They noted that the hyaline thigmocytes would undergo a cytolysis similar to that of the explosive cells after spreading out and fusing. The granular thigmocytes or amebocytes were actively ameboid within the animal. The amebocytes could also be lysed, but only by relatively severe mechanical or osmotic pressures.

Tait and Gunn believed that the "first clot" formed in Astacus was caused by "thrombin material" being set free from the lysed explosive cells and acting on a small amount of plasma, and that the "second clot" was caused by thigmocytes breaking and releasing "thrombin", which caused the remaining plasma fibrinogen to coagulate (the amebocytes did not produce any "thrombin"). They noted that the cells would not agglutinate and no clot would be formed if the blood were collected in oil and they observed that small non-greasy particles would cause a decrease in the number of circulating blood corpuscles if injected into the hemocoel but that injecting oily substances did not have this effect.

Goodrich (1919) observed the blood cells of Carcinas maenas, Eupagurus prideauxii and Astacus fluviatilis in his studies of pseudopodial form.

Parsons and Parsons (1923) confirmed earlier reports of the lack of plasma clotting in Maia squinado and observed autotomy in this animal. They also observed the blood of Palinurus vulgaris but did not add any new information on blood clotting.

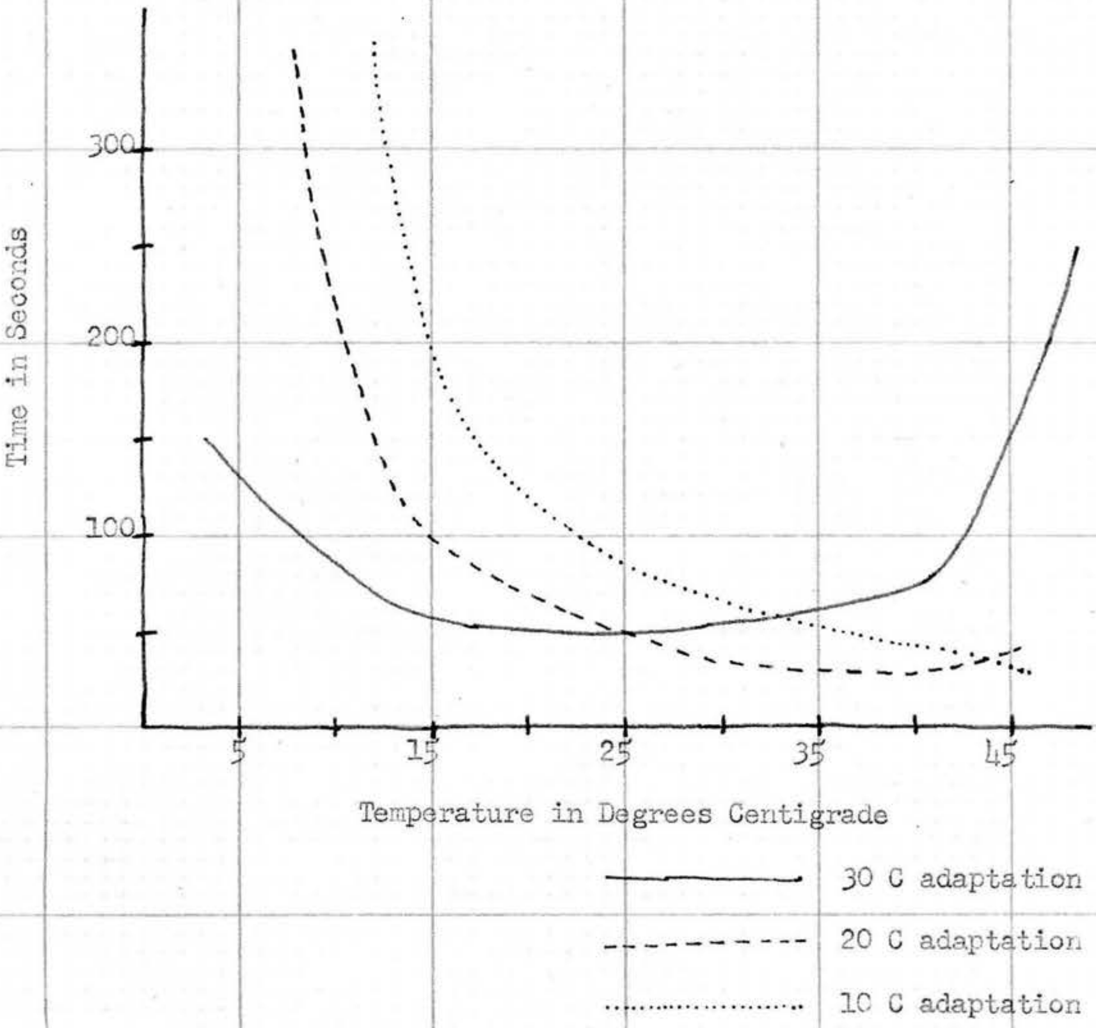
Gruzewska (1932) studied the clotting of cell-free plasma in several crustaceans confirming earlier observations. On studying a group of crabs (Carcinus maenas) he observed as great individual variation in blood clotting as could be found between different species. He also noted marked changes in the blood correlated with the moulting cycle.

Zunz (1932) studied the blood of Maia squinado and Palinurus vulgaris, confirming earlier work on the nature of the clot formed. He collected the blood in mineral oil and centrifuged. The oil retarded cell agglutination, and the cell-free supernatant would not coagulate. He confirmed Heim's observation (1891) that citrate would not prevent cell agglutination and Loeb's observation (1904 b) that muscle extract of these animals added to cell-free plasma would cause a clot to be formed. He also observed that vertebrate thrombin preparations with added calcium had no effect on crustacean plasma, even if the thrombin was from fish blood. Fish muscle extract also had no effect.

Numanoi (1938) studied the blood of the Japanese shore crab, Ligia exotica. He was primarily interested in chemical factors and temperature affecting coagulation of the plasma and did not investigate the role of the cellular elements. In particular, he studied the effects of temperature adaptation. Upon adapting the crabs to temperatures of 10°, 20°, and 30°C, he discovered that for all crabs, the fastest blood coagulation occurred at about 35°C, but that at colder temperatures the blood of cold-adapted animals clotted more slowly than that of warm-adapted ones. At higher temperatures the blood of warm-adapted crabs coagulated more slowly (see graph on page 59). Studying the coagulant enzyme in Ligia exotica, he noticed that the temperature coefficient of enzyme activity was not uniform, being 1.47 in the 10°C-adapted animals, 1.40 in the 20°C-adapted ones, and only 0.42 in the 30°C-adapted crabs. He noticed also that heat would destroy the enzyme, but that freezing stopped its activity reversibly. Numanoi noted that the coagulation rate of Ligia blood varied at different times of the year, being more rapid in the winter, but he thought that the concentration of the blood constituents might play a part in this change for the crabs were marine in the summer and terrestrial in the winter.

He studied the coagulation-inhibiting activity of hepato-pancreatic extract, finding it active in all species studied except one (see table on page 60). He noted that boiling or freezing was without any effect on undiluted hepato-pancreatic fluid (prepared by allowing the organ to

Relation of Blood Clotting Time to Temperature in the Blood of Crabs Adapted to Various Environmental Temperatures



adapted from Numanoi (1938)

Anticoagulant Effect of Crustacean Hepato-pancreatic Extract

species studied	anticoagulant power of Ligia exotica hepato-pancreatic fluid on blood of species studied
Xanthodius distinguendus	+ +
Sesarma dehaani	+ +
Sesarma haematocheir	+ +
Macrocheira kaempferi	—
Panulirus japonicus	+ +

species studied	anticoagulant power of hepato-pancreatic fluid of species studied on Ligia exotica blood
Xanthodius distinguendus	+
Sesarma dehaani	+
Sesarma haematocheir	+
Macrocheira kaempferi	+
Panulirus japonicus	+

adapted from Numanoi (1938)

undergo spontaneous sutolysis) and that addition of calcium to the blood would not prevent the fluid's inhibitory activity. He found that both heparin and hirudin could completely inhibit Ligia exotica blood clotting at dilutions of 1:400, in contrast to the reports of Haycraft (1884) and Loeb (1905 b).

Numanoi mentioned the blood cells only very briefly, noting merely that Tait's explosive cells (1918) were never found in Ligia exotica blood treated with hepato-pancreatic fluid.

Lochhead and Lochhead (1941) studied the blood of Artemia, the brine shrimp. They noticed that Artemia, like Daphnia (observed by Hardy, 1892) had only one type of blood cell. These cells were colorless, nucleated, ameboid, and phagocytic, and were of various sizes . They observed that the cells would undergo various changes when in contact with air, some of them disintegrating. In sea water, a wide variation in the stability of the cells was noted, along with a slight tendency toward agglutination. Within the animal, the blood cells were elongated while in circulating blood, but became ameboid when stationary. If the animal were narcotized, these cells became spherical. If the shrimp were wounded, the blood cells agglutinated to form a plug in which most of the cells retained their individuality, the plasma taking no active part in this process. Wound repair was brought about by a *syncytial* tissue formed of flattened blood cells.

Wolvekamp and Kryt (1947) studied the blood of some crustaceans but added nothing to our knowledge of clotting.

Glavind (1948) carried out a series of investigations on blood coagulation in the European lobster, Homarus vulgaris. He tested the hypotheses of Loeb and Nolf, who believed that lobster blood plasma coagulated in a single stage, unlike the plasma of vertebrates. He confirmed their views and noted that calcium ions were always required. He then investigated whether "tissue coagulin" and "blood coagulin" were different enzymes as Loeb (1906) had suggested, or a single substance (A-fibrinogen) as Nolf (1909 a) believed. He confirmed Nolf's view and in doing so showed that differences between the coagulins of different species were merely quantitative, all animals studied having a common coagulin substance (see tables on page 63).

Glavind compared the properties of lobster coagulin with

Clotting of Plasma from Different Crustadean Species on the Addition of Different Muscle Extracts

(0.2 ml citrated plasma + 0.1 ml non-dialyzed muscle extract
+ 0.1 ml calcium chloride solution)

Plasma of	Muscle extract of				
	Homarus	Nephrops	Astacus	Carcinus	Cancer
Homarus vulgaris	+	+	—	+	+
Nephrops norvegicus	+	+	—		
Astacus fluviatilis	+		—		
Carcinus maenas	+			+	
Cancer pagurus	+				+

adapted from Glavind (1948)

Clotting Time for Plasma of Lobster and Nephrops Norvegicus on the Addition of Muscle Extracts from the Two Animals

(0.2 ml citrated plasma + 0.1 ml non-dialyzed muscle extract
+ 0.1 ml 3 per-cent calcium chloride solution)

Plasma of	Clotting time in seconds by the addition of muscle extract of	
	Lobster	Nephrops
Lobster	55	360
Nephrops norvegicus	30	180

adapted from Glavind (1948)

those of vertebrate thrombin and thromboplastin. He found that lobster coagulin resembled thromboplastin in occurring in muscles and in blood cells and in requiring calcium ions for its activity. On the other hand, lobster coagulin resembled thrombin in acting directly on the corresponding fibrinogen. Glavind repeated the work of Halliburton (1885), who had found that "thrombin" of lobster, crab, and crayfish blood could coagulate cat plasma and that vertebrate thrombin could coagulate crustacean blood. Glavind could not find any evidence of such activity and decided that the earlier conclusions were false.

He studied the properties of lobster fibrinogen in some detail. Blood levels of lobster fibrinogen showed much greater variation than those of human fibrinogen, and Glavind found, like Cuénot (1891 b), that lobsters kept in captivity soon lost much of their fibrinogen, often to such an extent that the blood could not clot. This low level of blood fibrinogen was paralleled by atrophy of the musculature, decrease in blood cell count (as measured by blood cell volume) and a decrease in the hemocyanin level (as measured by color). Glavind noted also that clots from cell-free plasma did not contract, indicating that clot retraction is dependent upon cellular blood constituents in the Crustacea as it is in vertebrates (Quick, 1942). He confirmed Loeb's discovery (1907) of a coagulation-inhibiting substance in lobster muscle.

Glavind studied clotting in several other crustaceans. He noted that <u>Nephrops norvegicus</u> blood rapidly clotted as firmly as that of the lobster, and that <u>Cancer pagurus</u> blood gave a softer and slower-forming clot. Blood of <u>Carcinus maenas</u> slowly produced a fairly firm clot. These clots were produced by mixing muscle extracts and calcium salts with cell-free citrated plasma.

He studied the blood of the crayfish, <u>Astacus fluviatilis</u>, using identical methods, but its muscle extract had no effect. However, using lobster coagulin (muscle extract) a clot was formed although it was somewhat softer than that of the lobster. Glavind noted that the blood cell count of these animals was very low (they were starving) and that the blood of animals kept long in captivity would not clot at all. He believed that the lack of activity of crayfish muscle extract was due to the poor condition of the animals, but thought that possibly some physiological difference between this fresh-water animal and the marine crustaceans might cause the fresh-water extraction method used to be ineffective.

Glavind found that heparin or hirudin could completely prevent the clotting of cell-free oxalated lobster plasma to which calcium ions and lobster muscle extract were added (see tables on pages 66 and 67). He noted that whole crayfish or lobster blood would clot even when the blood was collected into 10% heparin solution, giving a final concentration of 3.3% heparin, although clotting time was delayed (see

Influence of Hirudin on the Clotting Time of Lobster Plasma

(0.2 ml citrated plasma + 0.1 ml non-dialyzed lobster muscle extract + 0.1 ml 0.75 per-cent calcium chloride solution containing hirudin in the amounts indicated in the first column)

Mg. hirudin per ml. $CaCl_2$ solution	Clotting time in seconds
10.0	no coagulation in 24 hours
1.0	370
0.1	180
0.0	180

adapted from Glavind (1948)

Influence of Heparin on the Clotting Time of Lobster Plasma

(0.2 ml oxalated plasma + 0.1 ml non-dialyzed lobster muscle extract + 0.1 ml 0.75 per-cent calcium chloride solution containing heparin in the amounts indicated in first column)

Mg. heparin per ml. $CaCl_2$ solution	Clotting time in seconds
50.0	not coagulated after 12 hours
35.0	"
25.0	2040
15.0	780
10.0	360
7.5	300
5.0	300
3.5	180
2.5	180
0.0	180

adapted from Glavind (1948)

table on page 69). He did not think that this discrepancy proved a difference between tissue and blood coagulins but only indicated that blood cells probably had a far higher concentration of the coagulin than did muscle tissue.

Glavind also studied the effects of heparin on agglutination of the blood corpuscles. Glavind and Plum (unpublished) noticed that in man the concentration of heparin needed to prevent agglutination of the platelets <u>in vitro</u> was about 1000 times as great as that needed to prevent plasma coagulation. The concentration needed to prevent platelet agglutination was similar to that needed to prevent clotting of the lobster plasma. Glavind (1948) noted that lobster blood cells would agglutinate in shed blood even when the blood was collected into 10% heparin solution, as reported above. He did not examine blood cells in the crustaceans carefully, but assumed that the three types of cells described by Tait and Gunn (1918) in the crayfish were also present in the lobster.

Hensill (1948) observed blood clotting in some American Pacific Coast decapods. He noted that the blood of the crab, <u>Hemigrapsus nudus</u> coagulated at once upon withdrawal and that the cells did not agglutinate but became vacuolated and disintegrated. He found that suitable preparations of sodium hydrosulfite would prevent both cellular disintegration and blood coagulation. The blood of <u>Cancer antennarius</u> formed a clot by an agglutination of the blood cells and

Influence of Heparin on the Spontaneous Clotting Time of Crayfish Blood

(2 drops heparin dissolved in distilled water in the concentrations indicated in the first column
+ 4 drops crayfish blood)

Mg. heparin in 0.1 ml. distilled water	Clotting time in seconds for crayfish number 1.	2.	3.
0	210	240	135
5	375	540	405
10	660	670	450

adapted from Glavind (1948)

this also could be prevented with $NaHSO_3$. Hensill studied many other decapods, obtaining comparable results using this salt. He observed that the most effective concentration and pH value varied from species to species.

D Chilopoda and Diplopoda

Cuénot (1891 a) observed the blood of two centipedes, Scutiger coleoptrata and Scolopendra cingulata. He found ovoid and slightly ameboid cells in the blood and some very infrequent storage amebocytes. He found that the blood of these two animals coagulated rapidly to a gelatinous mass (fibrin) enclosing many corpuscles.

Yeager and Knight (1933) studied the blood of the millipede, Spirobolus marginatus. They observed an agglutination and coagulation of the blood cells but no plasma clotting.

Rogers (1938) reported that the myriapods had three general types of blood cells; one, ameboid.

E Insecta

The insects, like the crustaceans, have been carefully investigated with respect to their blood physiology. However, unlike many crustaceans, insects are quite small and work with them is thus comparatively difficult.

One of the first studies of insect blood cells was that of Jones (1846). He found that the blood of various beetles and of both the chrysalis and caterpillar of the cabbage butterfly contained two types of cells similar to those in other invertebrates, as well as some other cells, oil droplets, and granules.

Poulton (1885) studied insect blood clotting and the formation of a dark coagulum. He thought the clotting process was chiefly an oxidation.

Griffiths (1891) carried out a series of biochemical studies on invertebrate blood, during which he observed that insect blood may or may not clot, depending on the species observed.

In the same year, Cuénot (1891 a) published an extensive report on the blood and body fluids of invertebrates. He studied clotting in a wide assortment of insects, finding great variations among the different species (see table on page 70). He observed that the clot was made up of a plasma "fibrin" gel, containing amebocytes and a granular precipitate, which he called "uridine". He observed amebocytes and other types of blood cells in all insects studied except the larvae of <u>Chironomus plumosus</u> and some related Diptera in which cells seemed to be absent.

Barratt and Arnold (1910) studied two beetles, <u>Dytiscus marginalis</u> and <u>Hydrophilus piceus</u>. They observed that the blood of these insects did not coagulate on standing, and they noted fine granules (1-2 micra in diameter) and two

Fibrin Formation in Insect Blood

Life Stage	Insect	Fibrin
larva	Saturnia pyri	2
"	Bombyx rubri	0
"	Bombyx trifoli	0
"	Vanessa antiopa	0
"	Bombyx castrensis	2
"	Chelonia caja	0
adult	Meloe proscarabeus	3
larva	Bombyx quercus	0
"	Pieris brassica	0
"	Pieris rapae	0
"	Libellula depressa	0
adult	Hydrophilus piceus	0
"	Blaps mortisaga	0
larva	Dytiscus marginalis	0
adult	Nepa cinerea	1
"	Notonecta glauca	0
larva	Chelonia pudica	3
"	Liparis dispar	2
"	Harpygia vinula	3
"	Aeschna grandis	0
"	Hylotoma rosae	2
"	Saturnia cynthia	0
adult	Stenobothrus parallelus	0
"	Gryllotalpa vulgaris	3
larva	Acronycta rumicis	0
adult	Pentatoma grisea	0
larva	Chironomus plumosus	0
"	Deilephela elpenor	0
"	Deilephela euphorbiae	(?)

Numerals denote relative amounts of fibrin formed.

adapted from Cuénot (1891a)

types of cells in the blood: (1) phagocytes, and (2) small round cells.

Glaser (1917) tried to grow insect blood cells in vitro, discovering that this method succeeded only with amebocytes, for all other types of cells failed to grow.

Tait (1918) observed cockroach blood cells and studied their movement. He found non-ameboid "hyaline thigmocytes" and ameboid "granular thigmocytes" and noted that the ameboid cells had more power of independent movement than did the corresponding cells of the crayfish. He thought that the cells moved by changing their surface tension in certain areas and allowing the resultant forces to pull a part of the more liquid cytoplasm forward. He noted that these cells could only protrude pseudopodia when in contact with some foreign body.

Glaser (1918) grew blood cells of a grasshopper (Melanopus atlanis), the army worm, and the gypsy moth caterpillar in tissue culture but could not observe any evidence of phagocytic activity. He also observed a lack of phagocytic activity of grasshopper cells in vivo but noted the presence of immune substances in the blood serum.

Bishop (1923) studied the body fluid of the honey bee larva and observed an absence of clotting ability.

At the same time, Paillot (1923) discovered that if nucleic acid was injected into an insect (Agrotis pronubana) the blood would not clot and the cells would remain active and normal in vitro, allowing careful study.

Snodgrass (1924) studied the role of the leukocytes during metamorphosis. He observed that they were not concerned with the breakdown of larval tissue in the apple maggot, Rhagoletis pomonella.

Muttkowski carried out an important series of investigations on the blood of some insects. In general, he found (1924 a) two types of cells: (1) amebocytes with granular endoplasm and thin ectoplasm which were highly thigmotaxic and were often found adhering to surfaces in the body, and (2) chromophil leukocytes which stained deeply with aniline dyes. He separated the leukocytes into several types: (1) secretory, (2) transporting, (3) phagocytic, (4) splanchnocytic, and (5) degenerating. He noted the occasional presence of other types of cells and of non-cellular material in the blood.

Muttkowski (1924 b) studied blood clotting in several species of insects. He found that oxalate would not prevent the clot from forming when added either in vivo or in vitro and that dilute acetic acid would prevent clotting temporarily, but the blood would clot as the acid evaporated. He postulated that the clot was made up largely of gelatin although other constituents of the blood, such as fibrin, were also concerned. He believed that the gelatin was secreted by the amebocytes and that a thin film of gelatin surrounding the leukocytes was necessary for their agglutination.

He observed this gelation in the blood of Dytiscus, Aeshna, Hydrophilus, Leptinotarsa, Pieris rapae, Deilephila, and tent caterpillars.

He observed that in clot formation the amebocytes sent out pseudopods which interlaced to form a network on the surface and bottom of the blood droplet on which other cells and fat droplets were caught. The chromophil leukocytes agglutinated at the same time and then sank down and were caught in the amebocyte network and in a fibrin mesh. The amebocytes and their pseudopodia then contracted and a substance which he thought was gelatin was secreted into vacuoles by the amebocytes. He thought that the amebocytes dried the clot by imbibing the remaining fluid plasma and that the leukocytes secreted a substance into the plasma which did not form the clot but perhaps prepared the plasma for inhibition. The amebocytes gradually shrunk, leaving a surrounding film of gelatin. Muttkowski observed that larger clots would be formed on a warmed or well-lit slide. He noticed that after the death of an insect the blood cells agglutinated in the animal, and the clot persisted until histolysis set in.

Since oxalates would not prevent plasma coagulation, Muttkowski thought that the elements which he believed combined to form the fibrin (thrombin and fibrinogen) were in a very close complex in insect blood.

Muttkowski found no correlation between the type of clot formed and the habitat of the insect but observed that in larvae there were fewer corpuscles and plasma coagulation (gelatin) was relatively more important than in adults. He noted that the insects having longer-lived adults (*Dytiscus*, *Hydrophilus*, *Belostoma*, and the scarabeid beetles) had a greater relative number of corpuscles in the blood and a greater volume of plasma than was found in the shorter-lived forms.

Haber (1926) studied the blood of the cockroach *Blatella germanica*. Like Muttkowski, he believed that all components of the blood were concerned in the clotting process. In some insects he observed granular leukocytes, in others the blood cells were hyaline and granules were found free in the plasma. Haber thought that many of the types of corpuscles observed were merely different stages in the developmental cycle of a single variety of cell. He also observed some cells which he thought might be homologous to vertebrate blood platelets. The quantity of plasma varied with the nutritional condition of the insect and fewer corpuscles were found in starving animals. When the blood was shed, the blood cells sent out pseudopodia which interlaced and fused. Fibrin formed in the interstices of the clot. Later, the elements of the clot contracted, squeezing out a clear serum.

Yeager, Shull, and Farrar (1932) studied blood clotting in another cockroach, *Periplanata orientalis*, also observing

Periplanata fuliginosa. They observed the blood in an oil-water system and noted that certain spindle-shaped cells rounded up and then put forth pseudopodia and agglutinated. The clumps of cells became granular masses, connected by pseudopodia and broader bands of disintegrated cells. A granular precipitate appeared, first in the clumps and later in the whole plasma, and they believed it might be derived from the cells. A second group of blood cells did not round up and their cytoplasm remained fluid. They became attached to the other cells and later disintegrated and their cytoplasm gelled.

They noted that heating the animals at 60°C for ten minutes prevented both coagulation and cell agglutination. At 70°C the blood gelled in the insects. They proved that the formation of the coagulum depended on the presence of unstable blood cells, for in filtered blood they could never observe any fibrous coagulum. They confirmed Muttkowski's (1924 b) observation that oxalate had no effect on blood clotting (as did Loeb (1903) using *Limulus*), and they believed that the cell-clot contained fibrin or had similar properties. They believed that the gelatinous membrane noted by Muttkowski was caused by a drying of the plasma rather than by the plasma being imbibed by blood cells.

Shull, Riley, and Richardson (1932) tested the effects of 34 toxic gases on the blood of *Periplanata orientalis*. Most of the compounds did not produce any visible effect on the blood or on its clotting ability, but a few compounds caused a change in its volume or cell count. Acetic acid-killed insects had blood which would not clot, and the cells appeared to be fixed within the animal.

Yeager and Knight (1933) examined blood coagulation in a wide variety of insects of various classes, collecting the blood into a drop of oil. They classified the insects into three types (see table on pages 79 to 83). *Group 1* had as its type species the honey bee larva. There was no coagulation of the blood but only a few cells having a slight tendency to agglutinate. *Group 2* had as its type species *Periplanata orientalis*. The blood cells would round up and then put forth pseudopodia, agglutinate, and disintegrate, and the cytoplasm would coagulate. There was no coagulation of the plasma. A precipitate might be formed in the blood but it would not form a clot. *Group 3* had as its type species the cricket, *Gryssus assimilis pennsylvanica*. The plasma coagulated to form a fibrous clot, a heavy granular precipitate being observed. The cells were embedded in the plasma but did not seem to take any active part in the clotting.

Not all insect blood clotting exactly followed these three general descriptions. For instance, in *Belostoma fluminea* (Group 3) the blood had relatively few cells and they did not agglutinate, but a granular precipitate was formed coincident with the lysis of certain cells, after

Insect Blood Coagulation

Key

A—Adult
L—Larva
N—Nymph
P—Pupa

Group 1

Order	Species	Developmental stage	Number of Cells	Cell Coagulum	Plasma Coagulum	Plasma Ppt.
Homoptera	Notonecta sp., backswimmer	A	few	none	none	slight?
	Myzus persicae, aphid	A	few	none	none	none
	Aphis maidis, aphid	A	few	none	none	none
	Labidomera clavicollis, beetle	A	few	none	none	none
	Tetraopus tetraopthalmus, longhorn beetle	A	few	none	none	none
	Saperda sp. longhorn beetle	L	moderate	none	none	none
Lepidoptera	Samia cecropia, moth	P	few	none (aggl.)	none	none

Hymenoptera	Camponotus herculeanus subsp. pennsyl., ant	A	few	none	none	none
	Camponotus sp., ant	A	few	none	none	none
	Apis mellifica, honeybee	L	few	none	none	none
	Apis mellifica, honeybee	A	few	none	none	none
	hymenopterous parasite	L	few	none	none	slight?

Group 2

Order	Species	Devel- opmen- tal Stage	Number of Cells	Cell Coagu- lum	Plasma Coagu- lum	Plasma Ppt.
Orthoptera	Anabrus simplex Mormon cricket	N-A	many	occurs	none	none
	Udeopsylla robusta, camel cricket	A	many	occurs	none	occurs
	Melanoplus differentialis, grasshopper	N-A	mod. to many	occurs	none	occurs

Orthoptera	Scudderia sp,. Katydid	N	moderate	occurs	none	occurs
	Diapheromera femorata walking stick	A	many	occurs	none	occurs
Homoptera	Tibicen sp., cicada	A	many	occurs	none	none
	Aphis rumicis, aphid	A?	few	slight?	none	none
Coleoptera	Carabidae; ground beetle	L	moderate	occurs	none	occurs
	Calasoma calidum Fab. ground beetle	A	moderate	occurs	none	occurs
	Elateridae, wireworm	L	many	occurs	none	occurs
	Alobates pennsylvanica Tenebrionidae; beetle	A	many	occurs	none	occurs
	Phyllophaga sp., May beetle	L	moderate	occurs	none	none
	Phyllophaga sp., May beetle	L	many	occurs	none	none
	Geotrupes splendidus, scarabid beetle	A	few	occurs	none	none
	Leptinotarsa decimlineata potato beetle	A	few	slight (aggl.)	none	none

Coleoptera	Anisodactylus verticalis Lec., carabid beetle	A	few	occurs	none	none
Lepidoptera	Lepidoptera sp.	L	moderate	occurs	none	none
	Eucosoma otiosana	L	moderate	slight (aggl.)	none	none
	Geometridae; measuring worm	L	many	occurs	none	none
	Noctuidae; moth	A	few	occurs ?	none	none
	Nephelodes emmendonis cut-worm	L	many	occurs	none	none
	Sphinx moth	A	moderate	occurs	none	occurs
	Samia cecropia, moth	L	many	occurs	none	none
	Bombyx mori, silkworm	L	many	occurs	none	occurs
	Hesperidae; skipper	L	moderate	occurs	none	none
Diptera	Chironimidae; midge	L	many	occurs	none	none
Hymenoptera	Pteronidae ribesii, sawfly	L	many	occurs	none	occurs

Group 3

Order	Species	Developmental Stage	Number of Cells	Cell coagulum	Plasma coagulum	Plasma Ppt.
Hemiptera	Belostoma fluminae, water bug	N	few	occurs (cytolysis ?)	occurs	occurs
	Acanthocephala terminalis, Coreidae	A	moderate	occurs	occurs	occurs
Orthoptera	Gryllus assimilis pennsylvanicus, field cricket	N-A	many	occurs	occurs	occurs
Coleoptera	Cucujus clavipes	L	many	occurs	occurs	occurs
	Phyllophaga futilis, May beetle	A	many	occurs	occurs	occurs
	Phyllophaga fusca, May beetle	A	many	occurs	occurs	occurs
	Phyllophaga rugosa, May beetle	A	many	occurs	occurs	occurs
Lepidoptera	Datana sp., caterpillar	L	many	occurs	occurs	occurs

adapted from Yeager and Knight (1933)

which the plasma clotted. In insects where the blood cells agglutinated there were two different sorts of clots formed. In some insects the cells lost their individuality, forming a granular mass, while in others the cells remained distinct.

Yeager and Knight found that the blood clotting picture of an insect could not be correlated with its taxonomic grouping. They noted a seasonal variation in the field cricket. In the spring and summer the plasma coagulated as previously described but in the fall the cells formed a clot and a precipitate was formed but the plasma did not coagulate. The extent of clot formation in the fall varied greatly among different individuals. They noted that crickets caught in the spring could be kept in captivity, but that those caught in the fall soon died. (All other insects examined were caught in the spring and summer.)

Shull and Rice (1933), using Blatta germanica and Periplanata orientalis, noted that by treating the roaches with acetic acid vapor under controlled conditions any desired degree of clot inhibition might be obtained. They could inhibit clot formation for up to 24 hours without soon killing the insect. They noted that the effects of the acid differed greatly among different individuals.

Tauber and Yeager (1934) performed 221 cell counts on Gryllus assimilis pennsylvanica blood, using acetic acid as an anti-coagulant. They noted a trimodal distribution curve but could not correlate it with any definite known factors, although they believed that a possible variation might be due to the presence of two broods of crickets a season in the locality.

Fisher (1935) studied the cockroach Blatta orientalis, using the clot-inhibiting method of Shull and Rice (1933) and obtaining similar results. He noticed that if blood was completely inhibited from clotting the corpuscles showed no change of shape when removed from the insect. He found that cell appearance was a good indicator of the extent of clot inhibition.

Murray and Tiegs(1935) studied the blood cells of the rice weevil, Calandra oryzae, and noted two types of cells, some phagocytic and some not. They studied the role of blood cells in metamorphosis and like Snodgrass (1924) could not find any clear evidence for phagocytosis of the larval tissues, deciding that this varied in different species.

Shull (1936) studied the effects of fatty acid vapors in inhibiting blood coagulation in Blatta orientalis. He found that vapor of formic, acetic, propionic, butyric, and valeric acid had an inhibitory effect, while that of caprylic and pelargonic acids did not. He observed that the length of treatment needed to prevent clotting varied inversely with the temperature and directly with the physical properties of the acid, complete inhibition occurring only after exposures which killed the insect. Like Shull, Riley, and Richardson (1932) he believed that the carboxyl radical fixed the blood cells inside the body.

In the same year, Fisher (1936) tested the effects of some toxic substances on the blood cell count of Blatta orientalis. He found that HCN and ether had the same effects as acetic acid; while arsenic, mercurous chloride, and carbon disulfide caused agglutination of the cells.

Wigglesworth (1937) studied the response of Rhodnius prolixus to experimentally inflicted wounds. He listed several steps in recovery:

1. Activation of the surrounding cells.
2. Migration of the epidermal cells and their crowding around the site of injury.
3. Accumulation of hematocytes.
4. Spreading of the epidermal cells over the defect.
5. Cell division in the surrounding zone to replace the emigrated cells.
6. Secretion of a new cuticle.
7. Formation of a new basement membrane.
8. Return to the resting state.

He noted that the blood did not clot, but that any which spread to the surface of the wounded area slowly dried and was converted to cuticulin, beneath which chitin was laid down. The blood cells accumulated along the cut margin of the incision within a few hours. In a day or two they formed a solid plug over the perforation, being distributed more sparsely over the surrounding activated area. However, if the cut did not penetrate the basement membrane, blood cells would not accumulate. Both accumulation and migration of the blood cells were found to be due to chemotactic influences of substances from the wounded tissues.

Babers (1938) noted that heating at 60°C for one minute prevent clot formation in the blood of the southern armyworm, Prodenia eridania. He also noted that there was no trypsin in the blood. On adding Prodenia hemolymph to horse serum thrombin or fibrinogen, no clot was formed.

Mellanby (1939) stated in a review that blood clotting was not necessary in insects, for the hemolymph was at less than atmospheric pressure and tended to remain within the body. He noted that some insects used a mechanism of reflex bleeding in which they excreted a small amount of unpalatable blood through the pores or through the mouth to discourage predators. This blood was sucked back into the body when certain muscles were relaxed. Any sort of blood clotting would seriously interfere with this reaction.

Beard (1949) studied the third instar larva of Popillia japonica and observed that although the hemolymph coagulated promptly on leaving a wound, it had no statistically significant effect in reducing hemorrhage.

Beard (1950) carried out an extensive series of investigations on insect hemolymph coagulation, studying the larva of Popillia japonica (Japanese beetle) and the larva of Galleria mellonella (wax-moth). These two insects were found to have fundamentally different types of coagulation, for Popillis blood coagulated on exposure to air, enclosing the hemocytes which did not agglutinate (Group 3 of Yeager and Knight, 1933) while the clotting of hemolymph of Galleria usually involved the cells, which agglutinated to form a flocculent precipitate (Group 1, of Yeager and Knight). However, all three types of clotting could be observed in various wax-moths. If the blood was not disturbed, sometimes there would be no clot formation at all. At other times, the plasma would become somewhat viscous. Differences could not be correlated with any changes, seasonal or otherwise, in these insects.

Beard (1950) confirmed Muttkowski's observation (1924) that calcium was not essential to clotting in insects. He confirmed Babers' discovery (1938) that thrombin was not necessary and found that neither hirudin nor heparin had any inhibitory effects. As result of these observations he decided that clotting in the insects was of no survival value, but was an incidental phenomenon.

Beard (1950) carried out a series of experimental modifications of the clotting process. Using Popillia blood he found that he could prevent coagulation by Yeager and Knight's (1933) heat treatment. Upon adding a drop of untreated hemolymph, the whole mass coagulated. (He noted a slight tendency of the cells to agglutinate if plasma coagulation was prevented). If he mixed untreated Popillia hemolymph with heat-treated Galleria hemolymph, the cells agglutinated and the plasma gelled. However, exposing *Popilla* hemolymph to high temperatures after removal from the body did not prevent coagulation. Beard believed that perhaps the heat-sensitive system was associated with some body tissues other than those cut in collecting the blood. He noted (in contrast to Muttkowski's view, 1924) that the gel of *Popillia* blood did not contain any gelatin.

Beard (1950) studied the effects of cold. After freezing and then thawing a *Popillia* grub, its hemolymph would not coagulate. If hemolymph was allowed to fall from an animal onto a freezing cold surface and later thawed, neither cell agglutination nor plasma coagulation would occur. When a drop of such frozen hemolymph was mixed with untreated hemolymph the response was variable. Beard thought that perhaps this was due to an anticoagulant substance released from the tissues, for the cold-treated hemolymph contained cell fragments. He noted that saline extracts of macerated body-wall tissue reduced the viscosity of the clot when added to hemolymph.

Beard (1950) tested the effects of ultrasonic waves on clotting. On exposing *Popillia* larvae to a frequency of 400 kilocycles per second, the hemolymph was reversibly prevented from clotting, but after the hemolymph was removed from the body, it could never recover its clotting ability. If treated hemolymph was mixed with that of untreated insects, no inhibitory tendency could be demonstrated. *Popillia* hemolymph treated _in vitro_ showed no inhibition of clotting, but if some grub tissue were added before treatment, clotting was inhibited. Beard found that ultrasonic-wave treatment of _Galleria_ caused the hemolymph to lose its tendency to increase in viscosity and prevented cell agglutination. _In vitro_ treatment had no effect.

When Beard added _Popillia_ hemolymph treated _in vivo_ to untreated hemolymph, while treating the mixture _in vitro_, coagulation was prevented. The same technique did not prevent clot formation if wax-moth blood was used.

Beard tested the effects of various substances on the blood. He found that heparin, hirudin, citrates, and oxalates (and Dicoumarol) had no effect on _Popillia_ blood. Chemicals did not necessarily have the same effect on the blood of both species, and he could not find any common factor in those chemicals which prevented clotting. Some chemicals could inhibit plasma gelation without inhibiting cell agglutination.

Beard observed that pseudopod formation by the blood cells might facilitate their adhesion. Inhibitors of cell agglutination did not act by preventing this but, presumably, by altering the whole cell or the cell membrane in other ways.

He thought that the data of Yeager and Knight (1933) suggested that cell agglutination was dependent on the number of circulating hemocytes and that dilution alone could prevent this.

Beard suggested that the plasma coagulation in insects was a polymerization, and he noted the presence of a mucopolysaccharide, using Hale's test (1946). However, he believed that this gelation was probably not simply a polymerization or change-of-state phenomenon but a complex reaction quite dissimilar to that of mammalian blood. He thought that cell agglutination and plasma coagulation were essentially separate phenomena, although the cells might be involved in some unknown manner in the coagulation.

Levenbook (1950) observed the blood of the horse botfly, Gastrophilus intestinalis, noting that it clotted at room temperature. Very few cells were observed and he thought the clot was a coagulation of the plasma elements.

Interestingly enough, Hunzinger, Süllman, and Viollier (1950) noted that supersonic vibrations could cause a lengthening of clotting time of human blood in the presence of thromboplastin and calcium ions, indicating an inactivation of the prothrombin system. A decrease in active fibrinogen was noted, the fibrinogen becoming somehow denatured and inactivated.

V Echinodermata

The echinoderms, having been used extensively in various types of biological research, are a group of invertebrates whose body fluid cells have been studied more intensively than one might expect.

Geddes (1880 a, b) studied the agglutination of blood and coelomic fluid cells in many invertebrates. He observed that if the body fluid was removed from a sea urchin (Echinus sphaera, Toxopneustes lividus, or Spatangus purpureus) the corpuscles separated to form a clot which rapidly contracted. The clot did not contain any fibrin but was made up mostly of white corpuscles which fused to form a homogeneous mass. This mass or "plasmodium" then put forth long anastomosing projections. If the animals were kept a long time, masses of cells might be found in vitro.

Geddes observed several sorts of cells in the sea urchins. There were (1) white ameboid corpuscles which formed most of the clot, (2) cells with a vibratile flagellum which were similar to the ameboid cells and were ameboid within a clot, (3) a very rare type of white corpuscle, and (4) brown-pigmented cells which were actively ameboid. The white ameboid corpuscles were found in all species examined, but the other cells were more variable, and he noted that Spatangus blood contained many green-gray vesicles which easily disintegrated. Epithelial cells, sex cells, and parasites were also found.

In Toxopneustes some types of cells were found in the ambulacral system but not in the blood or perivisceral fluid, while in Spatangus all three fluids contained the same cellular elements.

Schäfer (1882) studied the perivisceral fluid of a sea urchin and found that $MgSO_4$ solutions could prevent clotting. He noted that filtered fluid would not clot, but he believed that the cells acted by exuding a coagulable material which he believed was not fibrin but was similar to mucin.

Howell (1886) studied the holothuroidian Thyonella gemmata. He observed red corpuscles, white corpuscles, and other sorts and, like Geddes (1880 b) believed that the white corpuscles would send out pseudopodia and fuse, entrapping the red corpuscles and other cells.

Haycraft and Carlier (1888) observed that if they pipetted the coelomic fluid of a sea urchin into oil, no clot would be formed for 30 to 40 minutes.

Griffiths (1891) believed the perivisceral fluid of the echinoderms contained fibrin, but Bottazzi (1902), studying clotting in the Holothuroidea, confirmed Howell's (1886) and Geddes' (1880 a, b) theories that the clot was formed of agglutinated cells. He could find neither fibrin nor fibrin threads in the fluid.

Nolf (1909 a) observed that if he added the visceral fluid of the sea urchin, Sphoerechinus granularis, to mammalian fibrinogen, the cells would agglutinate but the fibrinogen would not be affected.

Goodrich (1919) studied the perivisceral fluid cells of Echinus, noting that their pseudopodia were fine membranous structures.

Theél (1921) studied clotting in some Holothurideae, concluding that there was some fibrin material in the coelomic fluid which contributed to the leukocytic cell-clot. He thought that ciliated cells which kept the fluid in motion prevented any fibrous coagulation in vivo, although plasmodia could still be formed. He noticed that the leukocytes were important in excretion and food transport and that they formed a syncytial membrane to repair wounds. He believed they might even be able to secrete spicules to replace damaged areas of the skeleton.

Kindred (1921) studied the perivisceral fluid cells of Arbacia punctulata and found two types of amebocytes: The homogeneous amebocytes, the leukocytes, agglutinated in vitro enclosing the other amebocytes which stuck to them. The leukocytes became very elongated and in an hour and a half their pseudopodia had formed threads which connected the cells, forming a meshwork. In hanging-drop preparations, only those leukocytes in contact with the glass were active in clot formation and they were sticky, tending to adhere to each other. If a piece of the peristomial membrane or the test was removed, the leukocytes formed a clot, closing the wound. They were actively phagocytic <u>in vivo</u>, readily ingesting injected ink particles.

Kindred (1924) observed the perivisceral fluid cells of a wide variety of echinoderms (see table on page 96). All the animals had clear leukocytes which were phagocytic and thromboblastic and, in some species, scleroblastic. Certain highly muscular Holothruoidea had some cells with hemoglobin. Amebocytes with colorless granules were observed in the Ophiuroidea, Echinoidea, and Holothuroidea and amebocytes with red granules were observed in Echinoidea. Vibratile corpuscles (with flagella) were found in the Ophiuroidea and Echinoidea, but in only one holothuroid. Kindred noticed that the amebocytes used flaps of ectoplasm to pull small particles into themselves for digestion. He found some flat cells with filariform processes floating passively in the fluid which were usually joined in a syncytium.

Metalnikov and Rapkine (1925) observed that the larva of <u>Paracentrotus lividus</u> exhibited two growth periods of the mesenchyme, one before and one after the invagination of the entoderm. Using Chinese ink, they found that the cells from the first growth were not phagocytic while those from the second group were.

Fauré-Fremiet (1925)a) grew vesicular amebocytes of a starfish in a tissue culture and noted that they formed a

List of Echinoderms Studied by Kindred (1924)

Asteroidea

Evasterias troschelii
Solaster simpsonii
Dermaster imbricata
Pisaster ochracens
Leptasterias hexactis
Henricia leviuscula
Pycnopodia helianthoides

Ophiuroidea

Ophiopholis aculeata

Echinoidea

Strongylocentrotus drobachiensis
Strongylocentratus franciscanus
Echinarachnius eccentricans

Holothuroidea

Cucumaria japonica
Cucumaria chronjhelmii
Stichopus californicus

adapted from Kindred (1924)

simple network of fine lamellae.

Kawamoto (1927) studied the various body fluids of Caudina chilensis, a holothurid. He found several types of cells: (1) red ameboid corpuscles, (2) white corpuscles containing spherules, (3) brown ameboid corpuscles, (4) crystal-containing corpuscles, (5) minute corpuscles, and (6) fusiform corpuscles. The cells agglutinated but there was never any coagulation of the blood or coelomic fluid.

Ohuye (1934 a) studied the same animal, confirming Kawamoto's description of the cells in the body fluid but divided the white cells into (1) leukocytes and (2) amebocytes with spherules. Using vital dyes, he demonstrated that the cell clot was composed chiefly of leukocytes (white corpuscles). Within the clot these cells usually lost their characteristic form and those in the center of the clot had no pseudopodia. He did not observe any true fusion of these cells but always found a visible boundary between the cells. In vivo they were actively phagocytic. The fusiform cells were observed both with pseudopodia and without them. The amebocytes with spherules contained food particles but were not active in phagocytosis, although those with brown spherules occasionally ingested the dye particles.

Ohuye next studied (1936 b) the holothurid, Molpadia roretzii. The coelomic cells were essentially the same as those described by Kawamoto (1927) in Caudina chilensis, but there were also colorless granular ameboid cells

(Kindred's (1924) amebocytes with colorless granules), spindle cells, and vesicular cells (signet-ring). The hyaline ameboid cells (leukocytes) were active and phagocytic. The colorless granular amebocytes were less active but would agglutinate *in vitro*. The brown cells were motile and phagocytic, and the vesicular cells were motile.

Ohuye (1936 c) studied the coelomic cells of an echinid, *Temnopleurus hardwickii*. He listed (1) hyaline amebocytes, (2) coarsely granular amebocytes, (3) finely granular amebocytes, (4) brown amebocytes which were phagocytic, (5) vesicular amebocytes, and (6) compartmental amebocytes. When the coelomic fluid was removed from the body, a firm coagulum of blood cells was formed. The hyaline amebocytes were especially active, fusing to form a network in which distinct cell boundaries were not observed. The other types of cells kept distinct forms.

Donnellon (1938) studied clot formation in *Arbacia punctulata*, observing that the clot consisted of massed leukocytes. He confirmed Schäfer's observation (1882) that filtered perivisceral fluid would not clot. Donnellon observed that cell extracts caused both cellular agglutination and release of the cell granules when added to perivisceral fluid. On further study he noted that tissue extracts from many invertebrates, potassium, calcium, and strontium salts, fat solvents, rapid temperature changes, any foreign body, mechanical or electrical stimulation, ultra-violet irradiation, and hypertonic sea water all could stimulate clotting. On the other hand, $MgSO_4$, $MgCl_2$, $MgCl_2$ in tissue extracts, 3% formalin, 10% peptone, sea water of pH 4.2-4.6, and oxalate or citrate prevented cell agglutination. Oxalate and citrate prevented agglutination even in the presence of most of the clotting agents but did not prevent agglutination if fat solvents were used.

Donnellon repeated the experiments of Lillie (1909), who found that reagents which caused pigment liberation also caused agglutination of whole Arenicola larvae. Donnellon found that this agglutination could be prevented by first oxalating or citrating the larvae.

As a result of his work, Donnellon concluded that calcium, a tissue factor, and an agglutinin substance were all necessary to clot formation in the sea urchin, and that pigment granule breakdown was related to cell agglutination.

Liebman (1950) classified the leukocytes of Arbacia punctulata into two main categories: (1) trephocytes, which are granular, non-motile or poorly ameboid, and have nitritive functions, and (2) phagocytes. The trephocytes had green, red, or colorless granules and were important in the synthesis and transport of food in the animal. The phagocytes were of several types: flagellated, ameboid, fibroblastic, and petaloid, with the ameboid and petaloid cells having the greatest phagocytic ability.

VI LOWER CHORDATES

Biologists studying morphology, embryology, and evolutionary problems have been especially interested in the invertebrate Chordata. The few non-vertebrate animals of this phylum (Hemichordata, Tunicata, Cephalochordata) exhibit many of the typical chordate characteristics in comparatively simple and unspecialized forms. However, the many special modifications peculiar to these animals have made them quite unlike their hypothetical generalized ancestors.

Cuénot (1891 c) studied the blood cells of a tunicate, Ascidia mentula. He described four types of amebocytes in the blood and noted that the orange-colored cells were not found in the circulating blood but only in limited areas.

Nolf (1909 a) worked with several of these animals, being chiefly interested in trying to demonstrate thrombin in their blood plasma and cells. He was unable to demonstrate any thrombin-like activity of Amphioxus or Cyona intestinalis blood when mixed with mammalian fibrinogen preparations. However, he could not remove blood or lymph from an Amphioxus and had to use a homogenate of the whole animal. Since similar homogenates of fish, whose blood was shown to contain thrombin, had no effect either, Nolf believed that tissue substances inactivated the thrombin. His experiments with Amphioxus were thus inconclusive.

Hecht (1918) observed the blood of the tunicate, Ascidia atra. He found colorless plasma and several types of blood cells. There were two types of unpigmented cells: a homogeneous and readily ameboid sort, and a spherical sort (probably several kinds) lacking any activity. The pigmented cells were all much alike, were slightly ameboid, and contained green, orange, or blue granules. The green corpuscles were the most abundant and were found in all parts of the circulatory system. The blue cells were less abundant and were derived from the green cells. The orange cells were found (like Cuénot, 1891 c) only in certain areas.

Blood clotting was caused by an agglutination of the green and unpigmented corpuscles, plasma coagulation being entirely lacking. The cells agglutinated on contact with sea water, but could also agglutinate inside the animal, for if the animals were wounded, the cells would agglutinate before leaving the blood vessels. If uninjured animals were roughly handled, the blood cells would reversibly agglutinate within the body. Hecht believed that in vivo clotting was due to the release of some chemical into the plasma, for the blood would only clot four or five times in vivo. After that, stimulation of the animal was without effect.

Hecht noticed that in sea water the cells sank to the bottom of the container and agglutinated to give balls which spread out to become discs. The activity of these cells was similar to that observed in sponge and hydroid cells by

Wilson (1911), but no sort of organism was ever regenerated.

Parsons and Parsons (1923) observed the blood of Phallusia mammillata (mammalata) but did not observe clot formation (confirmed by Rogers, 1938).

George (1926) studied the blood of Perophora viridis. He observed (1) green cells which were sometimes ameboid, (2) infrequent orange cells, (3) colorless morula cells, (4) granular ameboid cells which were not phagocytic, (5) compartmental ameboid cells, and (6) signet-ring type cells, sometimes ameboid, which were senescent compartmental cells. The compartmental ameboid cells were thigmotactic and flattened out and became actively ameboid upon contact with glass. They were attracted to other cells. George studied (1930 a, b) the cells of some other ascidians and found them rather similar. Species observed were: Phallusia nigra (Ascidia atra), Ecteinascidia turbinata, Clavelina oblongata, Symplegma viride, Phallusia hygomiana, and Styela plicata.

Ohuye (1936 a) studied the Japanese tunicates, Cynthia roretzi, Styela clava, Chelysoma siboja, and Corella japonica var. asamushi. He found green ameboid cells, orange ameboid cells, brown cells, morula cells, grayish-olive cells, hyaline phagocytic and ameboid cells, compartmental cells, granular phagocytic and ameboid cells, and vesicular cells. The vesicular and compartmental cells were ameboid when young. He noted that the blood plasma of these animals did not coagulate and that the ameboid cells were constantly forming clumps. These cell-clots were not true plasmodia, although the more central cells were somewhat indistinct. The finely granular amebocytes were the most active cells, both in movement and phagocytosis.

VII DISCUSSION

It is apparent that no single scheme or theory can hope at this time to explain all we know about the blood clotting process. The many variations in this mechanism as exhibited by the different invertebrates and by the vertebrates preclude any simple explanation. However, there are a few general points of interest.

Cell clot formation seems to be composed of two related phenomena which may or may not occur together. They are (1) agglutination and (2) lysis. Agglutination of the cells without accompanying lysis is probably the simplest sort of clotting which the blood components can bring about. In such animals as Daphnia and the tunicates this is the only blood clotting mechanism.

In many animals somewhat more advanced in evolution, the blood cells tend to be especially predisposed toward lysis. In the crustaceans this takes the form of a release of small blebs of protoplasm like those released under similar circumstances by the blood platelets (Quick, 1942). Such lysis is often accompanied or preceded by cell agglutination, but it may occur separately. In animals in which the plasma coagulates cell agglutination is not found as often, being impeded by the rapid blood coagulation.

Following lysis of the blood cells there is some sort of gel formation. In some animals, for instance Limulus, only the cell protoplasm solidifies. In others, the plasma proteins also take part in clot formation. Although Loeb seems to feel that coagulation of the cell protoplasm occurs independently of the plasma, other writers, Tait in particular, believe that the so-called "cell coagulation" is really a small-scale plasma gelling caused by release of a substance from the destroyed cells. Two different types of blood cells seem to be necessary for Astacus blood clotting, one causing a local coagulation and the other a more general plasma clot.

In the crustaceans (and in vertebrates) it has been proven that the blood cells release substances which cause the plasma proteins to gel. However, the relationships of the cellular elements to the plasma coagulation found in insect blood are still obscure, although a generalized clot formation following lysing of widely separated blood cells (similar to the phenomenon noticed by Tait in certain crustaceans) has been seen in at least one species of insect by Yeager and Knight (1933).

On the basis of Loeb's work with Limulus amebocytes, Tait's belief that the "first coagulation" in the Crustacea is brought about by certain blood cells releasing substances which cause only a localized plasma gelation appears far-fetched. It seems more likely that the explosive cells of the crayfish release some cytoplasm upon lysis which itself coagulates. However, the formation of the fibrils of coagulum greatly resembles that of the more general "second coagulation" as well as the analogous process of fibrin thread formation upon lysis of mammalian platelets.

A close parallel between the plasma clotting found in invertebrates and that in the vertebrates should not be expected, for the only invertebrate chordates which have been successfully studied (the tunicates) completely lack such a process. The hemostatic mechanism of plasma coagulation probably arose quite separately in the two groups.

Plasma clotting seems to be lacking in most invertebrate animals but is found in some arthropods and a few annelids. The type of coagulation in the crustaceans resembles that of mammalian blood in many respects, but exhibits important differences. For instance, the whole prothrombin-thrombin mechanism seems to be entirely lacking, the coagulins from both blood cells and body tissue cells acting directly on the fibrinogen. Furthermore, the fibrinogen and fibrin found here resemble those of mammals in some chemical and physical aspects but are far from identical. The blood proteins found in these two groups of animals are completely unable to substitute for each other.

In insects, the plasma coagulation process as developed in the Crustacea is lacking. The proteins (and perhaps other substances of the blood plasma) form a firm gel under certain circumstances, but the mechanism is clearly different. A general relationship between the specialized sort of colloidal protein change found in vertebrate blood clotting and the less understood processes in insects is suggested by experiments which show that supersonic vibrations inhibit both insect and human blood clotting.

Most investigators have found the decalcifying agents to be without effect on invertebrate blood cells except when used in such large concentrations that they have a direct osmotic or poisoning effect. However, Donnellon's work (1938) indicates that calcium may be involved in some way in cell agglutination as well as in plasma coagulation. The role of plasma constituents, especially various ionic substances, in cellular agglutination has not yet been sufficiently studied. Furthermore, the work of Lillie (1909) suggests that cell agglutination is the result of surface activity changes not in any way limited to blood cells, although such cells are more sensitive to certain stimuli. In general, cellular agglutination and cell lysis seem to be caused by changes at the cell surface. Such changes in turn depend upon the permeability of the cell membrane, as influenced by certain plasma or environmental factors, and may be related to a release of sticky substances onto the cell. Such sticky material may be derived either from the surrounding plasma or from the cell itself.

A careful evaluation of the data on invertebrate blood cell agglutination and lysis on the basis of what we know of similar phenomena in the vertebrates would be somewhat premature, for our theories of mammalian platelet and leukocyte activity are still in a state of flux (compare Quick 1951 and Lutz, 1951 with Silberberg, 1938 or with Quick, 1942). With respect to the platelets, for instance, Quick's latest theory (1951) that a coating of thrombin causes them to become sticky and agglutinate, and that heparin prevents this agglutination by removing or inactivating the thrombin is in need of considerable modification. Lutz (1951) has shown that heparin actually initiates platelet agglutination *in vivo*. From our knowledge of invertebrate blood cells, it seems more logical to suppose that while thrombin may be concerned at times in the activation of the platelet response, these cells may react in a similar manner to the presence of many other substances (as do the amebocytes of most invertebrates).

Heilbrunn and Wilson (1948) observed that heparin can sometimes prevent protoplasmic clotting, in particular the gelation of the *Chaetopterus* egg preceding formation of the mitotic spindle. Perhaps the present confused picture regarding the effects of heparin on both invertebrate and vertebrate blood systems might be made clearer by further work using materials other than blood components.

The echinoderm theory of chordate development does not seem to be affected by knowledge obtained in blood clotting studies. Neither echinoderms nor lower chordates seem to possess a true plasma coagulation, having only the more primitive blood cell agglutination process. Ascidians and echinoderms resemble each other in having a rather wide variety of blood cell types, but the specific sorts of blood cells found in these two groups also occur in other invertebrate phyla. That no obvious similarities between the blood cells and plasma of echinoderms and of the lower chordates has been found reflects, perhaps, the essentially generalized condition of their blood.

Much of the support for this theory of chordate evolution has been obtained by work in serology. Wilhelmi (1942) prepared tissue extracts from various animals and injected them into rabbits to produce specific antibody preparations. By the use of precipitin tests, he showed that the relationship between echinoderm and prochordate sera was closer than that between annelid or arthropod and prochordate sera. Among the echinoderms, the holothuroids seemed closest to the protochordates, especially to the hemichordates.

Using a direct spermatozoa or erythrocyte agglutination method, Tyler and Metz (1945) demonstrated that <u>Panilurus interruptus</u> serum contains several natural heteroagglutinins. In general, they found that all species of a taxonomic class behaved alike to this serum, any one species absorbing the agglutinins for all animals in its group. Although most of the agglutinins were class-specific, they found that mammals and echinoidea had a single agglutinin substance in common. All vertebrates seemed to possess at least one reactant found in the echinoderms. Tyler and Metz thought that many such reactants may have been lost in evolution, for teleosts and amphibians share a common agglutinin substance with the ascidians which is absent among the amniotes.

In spite of the great diversity found among the invertebrate metazoans, several authors, in particular Liebmann (1946, 1947), have tried to classify their blood and body fluid cells according to a general scheme. Liebmann believes that all these cells are either phagocytic or nutritive in function, and he is able to demonstrate this general division in all animals studied. Unfortunately, he has not been interested in agglutination.

VIII SUMMARY

1. The processes of blood plasma coagulation in the vertebrates and in the invertebrates are two separate phenomena.

2. The plasma coagulation found in crustaceans shows some parallels to that of mammalian blood plasma but seems to have arisen quite independently.

3. Plasma coagulation in other invertebrates is either completely lacking or else is a complex phenomenon occurring in random species. Among the insects, it seems to be a fortuitous process, lacking any true physiological function.

4. Simple cellular agglutination is found in most invertebrates but is not universal.

5. The changes in the blood cells causing them to agglutinate may also lead to their lysis, which is ordinarily followed by a coagulation of the cell protoplasm and of some or all of the surrounding blood plasma.

6. These blood cell phenomena are an expression of certain properties common to all cells but some blood cells are far more sensitive to the precipitating factors.

7. These precipitating factors act primarily at the cell membrane to increase cellular permeability, perhaps by ionic transfer. They may also cause certain materials to be precipitated or released onto the exterior of the cells, thus changing the cell membrane in that way.

8. Cytoplasmic gelling and plasma gelling may be basically similar, although the relationships are as yet obscure.

9. There seems to be no evidence arrived at from blood clotting studies which indicates a particular phylogenetic relationship between the echinoderms and the chordates. However, studies using specific

antisera and agglutinins seem to demonstrate a closer relationship of chordates to echinoderms than to any other invertebrate group.

10. The many sorts of blood cells found among the various animals show marked differences from one another. However, most blood cell types can be collected into a relatively few main groups.

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Abstract

The purpose of this thesis is to discuss the role of invertebrate blood and body fluid cells in clot formation and related phenomena. Invertebrate blood physiology has been most extensively investigated in some crustaceans and in Limulus, a merostomatan. The insects have also been studied a good deal, and there has been some work on the annelids, mollusks, echinoderms, tunicates (ascidians), and others.

The three most important hemostatic devices in vertebrates are (1) smooth muscle contraction, (2) blood cell agglutination, and (3) plasma coagulation. Among the mammalian blood cells only the platelets are important in clot formation, although the leukocytes may sometimes be involved. According to the most recent theory, mammalian blood clots as follows:

1. Thromboplastinogenase (platelet enzyme factor) acts on thromboplastinogen (plasma factor) to give thromboplastin.
2. Thromboplastin + prothrombin complex = thrombin.
3. Thrombin acts on fibrinogen to give fibrin.
4. Thrombin labilizes platelets to give thromboplastinogenase.

In invertebrates the same three basic hemostatic mechanisms are found, although not usually all together. Autotomy is sometimes present also. Various sorts of cells are usual-

ly found in the blood or body fluid. They may not be ameboid <u>in vivo</u>, but usually agglutinate when out of the body, and they may facilitate plasma coagulation.

Upon artificial separation, some of the tissue cells in the Porifera and Coelenterata agglutinate to form small masses. The simplest invertebrates to show an agglutination of naturally free-floating body fluid cells are the Bryozoa, and the Brachiopoda are the simplest to show an agglutination of blood cells.

The Mollusca have abundant blood cells, which readily agglutinate <u>in vitro</u>, forming networks and chains. These cells are important both in hemostasis and in wound healing. Plasma coagulation is completely lacking.

The Gephyrean worms have a variety of body fluid cell types, some of which may coalesce <u>in vitro</u>.

The blood cells of the Annelida vary markedly among the different species, some animals lacking them entirely. When cells are present they usually have some agglutinating ability. Many earthworms eject coelomic fluid through the dorsal pores when irritated. This fluid soon solidifies, usually from the agglutination of its cells, but in at least one earthworm, <u>Pheretima sieboldi</u>, it can coagulate even if the cells are removed.

The arthropod whose blood has been most extensively studied is the <u>Limulus</u>. Although its blood cells are usually non-motile <u>in vivo</u>, they coalesce upon leaving the body to form an elastic clot and will also surround any foreign material placed in the animal's hemocoel. <u>Limulus</u> has only one kind of blood cell, and if the cells are carefully collected they may be grown in tissue culture. Contact of the blood with most foreign materials or any kind of shock causes the cells to (1) become liquefied, leading to lysis, and (2) undergo a protoplasmic coagulation. These cells are unusually permeable to many substances, and their agglutination is closely related to colloidal changes in the outer layers of the cell protoplasm.

The other Chelicerata have been studied only rarely. Cell clotting has been observed in some scorpions and spiders, but the occurrence of plasma coagulation is still in doubt.

Much experimental work has been done on the blood of the Crustaceans. Two main steps have been observed in these animals' blood clotting, although many species exhibit only the "first coagulation", caused by a cell agglutination similar to that found in <u>Limulus</u>. The "second coagulation" is a true plasma coagulation. Various theories have been put forth to explain Crustacean blood clotting. The most recent view is that a coagulin substance is found in blood cells and in some body tissues. This coagulin acts on the plasma fibrinogen in the presence of calcium to precipitate fibrin. These blood proteins seem to be alike in all Crustaceans in which they are found. However, they are unlike the analogous mammalian blood components. The presence of blood cell agglutination, cell lysis, and plasma clotting cannot be correlated with taxonomic relations.

Blood clotting has been extensively studied in insects. In many species the cells will agglutinate to form a clot, and a true plasma coagulation is often observed. However, this plasma coagulation occurs in a random assortment of species and life stages and does not seem to have any physiological significance. It differs from that of the crustaceans in not being inhibited by decalcifying agents.

Many sorts of blood and body fluid cells are found in the echinoderms. Most investigators believe that the clot formed by the perivisceral fluid consists solely of agglutinated cells. Recent work seems to show that calcium, a tissue factor, and an agglutinin substance are all essential.

The only invertebrate Chordata whose blood has been extensively studied are the tunicates. In these animals plasma coagulation is entirely absent, but the blood cells readily agglutinate both <u>in vivo</u> and outside the body.

Although plasma coagulation of crustaceans shows some parallels to that of mammalian blood, the two processes seem to have arisen independently and show essential differences. Plasma coagulation in other invertebrates is lacking or else seems to be an accidental phenomenon.

Simple blood cell agglutination, sometimes followed by lysis, is found in most invertebrate animals. Cell lysis is usually followed by coagulation of the cytoplasm and sometimes by a general plasma coagulation. These blood cell changes are caused primarily by factors which act on the cell membrane, although sticky materials may also be involved. Similar changes may occur in other biological systems. Although their respective blood cells show no special resemblance, serological evidence indicates that the Chordates are more closely allied to the Echinoderms than to any other invertebrates. There are many different sorts of invertebrate blood cells, but most of them can be classified into a few main groups.