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THE IMMUNOBIOLOGY OF THE ASIAN CLAM, CORBICULA FLUMINEA

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THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

THE IMMUNOBIOLOGY OF THE ASIAN CLAM, *CORBICULA FLUMINEA*

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

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TAI-LAN TUAN

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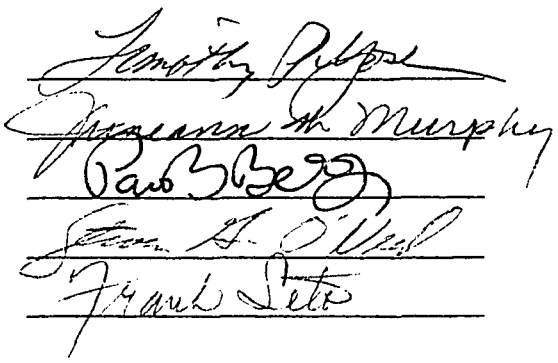
THE IMMUNOBIOLOGY OF THE ASIAN CLAM

CORBICULA FLUMINEA

A DISSERTATION

APPROVED FOR THE DEPARTMENT OF ZOOLOGY

By



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ABSTRACT

The freshwater bivalve, Corbicula fluminea, was selected for the study of the internal defense mechanisms of hemocoelic invertebrates. Corbicula hemocytes, the primary effector cell involved in defense reactions, consisted of a heterogeneous cell population. Based on size, extent of granulation, and granular morphology, these cells were divided into three major cell-types: small hyaline hemocytes, vesicular hemocytes, and large granular hemocytes. The small hyaline cells composed 16% of the hemocyte population, had abundant rough and smooth endoplasmic reticulum, and mitochondria evenly distributed throughout the cytoplasm. Vesicular cells, characterized by the presence of many filopodia and numerous electron-opaque cytoplasmic vesicles, were the most abundant hemocyte-type comprising 60% of total hemocyte population. The rest of the hemocyte population (24%) was composed of large granular cells which possessed large electron-dense or lucid granules in the cytoplasm. All three cell-types varied in their cellular density, and all contained lysosomal acid phosphatase and non-specific esterase activity.

All types of Corbicula hemocytes were capable of adhering and spreading on a glass slide surface. These phenomena were plasma- and divalent cation-dependent. Hemocytes were also capable of phagocytizing formalin-fixed vertebrate RBCs in vitro but only in the presence of a soluble plasma factor(s). The plasma factor(s) had

opsonic properties since RBCs pre-treated with clam plasma enhanced particle uptake by plasma-free hemocytes. The factor was also heat sensitive, being inactivated at 56°C for 30 min and 100°C for 10 min, and its activity was abolished upon lyophilization. The opsonin did not appear to be mediating its activity by interacting with hemocyte/RBC carbohydrate moieties, since thirteen mono- and disaccharides, and the glycoproteins, fetuin and mucin, had no effect on competitively inhibiting hemocyte erythrophagocytosis.

Hemocytes, in the absence of plasma, were shown to exert a hemolytic reaction against 6 species of fresh mammalian RBCs. Using a Hb-release assay, the reaction was shown to be effector cell dose-dependent and also differed between the various RBC species used in this hemolysis assay. Ultrastructural studies and analysis of plaque-forming hemocytes showed that hemocytes lysed RBCs through the active release of their granular contents into the medium. Corbicula plasma also contained a hemolytic factor(s) which also reacted against 5 species of mammalian RBCs tested in a dose- and divalent cation-dependent fashion. The plasma hemolysin was also heat sensitive. The functional similarities between the hemolytic factors from hemocytes and the plasma suggest that these lytic factors may be the same and that hemocytes may represent a major source of soluble plasma hemolysin in the intact clam.

Finally, a naturally occurring hemagglutinin was also found in Corbicula plasma which had the properties of being sensitive to heat and freeze-drying. Plasma treatment with various saccharides and

glycoproteins in competitive inhibition tests, as well as with EDTA at 2 mM, did not reduce its hemagglutinating activity.

Based on these *in vitro* studies, it has been shown that *Corbicula* is capable of recognizing and reacting against foreign particles through both cellular and humoral mechanisms. A functional relationship between cellular and humoral components has been demonstrated in experiments involving hemocyte erythrophagocytosis. Additionally, hemocytes appear to represent a major source of plasma hemolysin.

CHAPTER I

INTRODUCTION

The word "immunity" is derived from the latin immunis, meaning "free from obligation." Therefore, immunity, in a broad sense, denotes the existence in a species of a surveillance mechanism, the immune system, which protects the species against invading harmful or disagreeable agents. From this point of view, a system which participates in internal defense processes for the species can be considered as an immune system (Hildemann and Reddy, 1973; Cooper, 1976; Chorney and Cheng, 1980; Lackie, 1980; Ruben, 1982).

Although the mechanisms of immune function may well vary among different animal groups, the fundamental feature of such a system is to distinguish self from non-self. The recognition of and reaction against foreignness in higher vertebrates are achieved through two different, but complementary systems. One system is responsible for generating cell-mediated immune responses (CMI). This system involves the direct reaction of specialized cells (T-lymphocytes) with foreign materials and results in the direct killing of infectious organisms or transformed cells, or in the regulation of the animal's immune response. The second system involves lymphoid cells derived from the

avian bursa or mammalian bone marrow (B-lymphocytes) which are involved in the production of soluble recognition molecules called immunoglobulins or antibodies. Antibody-mediated reactions constitute what has been termed "humoral immunity" in these animals. Both T and B cells are responsible for specific acquired immune resistance. In addition to B and T cells, which represent the major constituents of this dual system, other accessory cells such as blood monocytes, basophils, eosinophils, polymorphonuclear leucocytes, macrophages, and natural killer cells participate in either regulatory or effector roles in both systems. This latter group of cells largely responsible for the natural (i.e., nonacquired) resistance observed in higher animals. All the above cells interact through specific ligand-receptor bindings. The results of the intricate interactions of these cells and their products in response to foreign materials and the ability of the system to produce an enhanced reaction after second exposure to the same materials (anamnesis or immune memory) form the basis of immune protection in the vertebrates. At the same time, the checks and balances between these immune components results in a fine homeostatic regulation of host responsiveness. Thus, recognition of non-self, specificity, and memory are the characteristics of vertebrate immunity (Fudenberg et al., 1980; Roitt, 1980; Golub, 1981; Hood et al., 1982; Ruben and Gershwin, 1982).

In contrast to the vertebrates, our knowledge concerning invertebrate immunity is still at the descriptive stage. Antibodies and classical complement components have not been reported from invertebrates (Marchalonis, 1977; Warr, 1981). Furthermore, immune

memory and transplantation immunity have only been demonstrated from sparse groups of invertebrates. Nevertheless, invertebrates, composed of a diversity of animals, exhibit a wide variety of immune reactions. These responses can also be divided into the broad catagories denoted as cellular and humoral. Phagocytosis, encapsulation, and natural cytotoxicity are the major cellular events elicited in invertebrates against foreign substances. On the other hand, naturally occurring bactericidal (bacteriolytic), bacteristatic, agglutinating, and opsonic molecules found in the body fluid or tissue extracts are principal mediators of invertebrate humoral reactions against non-self materials. A review of the cellular and humoral responses in the major invertebrate phyla will now be presented in the following sections:

Poriferans

Sponges, considered to have the simplest body organization among metazoan animals, are capable of recognizing foreign materials such as india ink and carmine experimentally introduced into these animals (Cheng et al., 1968). These foreign materials are found to be phagocytized by freely mobile, colorless cells called amoebocytes or archaeocytes. Ink and carmine-laden archeocytes migrate through the excurrent water canal out of the sponge, thus, removing these materials from the sponge body in a "suicidal" fashion (diapedesis). For materials too large to phagocytize, cells form a capsule around the foreign subject (Bang, 1975). The discrimination of non-self in the sponge is further demonstrated between individuals of different species (Spiegel, 1955; Moscona, 1968; McClay, 1971; Maclennan, 1974) or

different strains within the same species (Van de Vyver, 1975; Hildemann et al., 1979a; Van de Vyver, 1980). Moreover, an enhanced second and third allograft rejection accompanied by bilateral cytotoxicity between parabionts of the tropical sponge, Callyspongia diffusa, suggests the existence of polymorphic histocompatibility markers on the sponge cell surface which are responsible for alloimmune memory in this animal (Hildemann et al., 1979a). However, alloimmune memory is not considered to be a common property of all sponges. In Ephydatia fluviatilis, for example, allogeneic incompatibility is marked by an extensive phagocytosis and the formation of a collagen boundary between allogeneic individuals, and this process is not accelerated during second set grafting (Van de Vyver, 1980, 1983; Van de Vyver and Barbieus, 1983).

Coelenterates

Coelenterates have two body tissue layers which are more specialized than that of the sponges. In the body of these animals there exists freely mobile cells called amoebocytes or "lymphocytes", which are morphologically similar to those found in sponges (Hildemann et al., 1977). They are responsible primarily for nutrient delivery, secretion of waste products, and reproduction (Hickman, 1973). In addition, these cells are also the principal mediators for antagonistic reactions like phagocytosis and cytotoxicity against foreign implants in transplantation studies (Hildemann et al., 1977). Similarly to sponges, allogeneic incompatibility is well documented for hydrozoans (hydras and gorgonians) (Theodor, 1970; Ivker, 1972) and anthozoans

(corals and sea anemones) (Bigger, 1976; Hildemann, et al., 1977). However, evidence of immune memory for the members in this phylum is lacking, except the coral, Montipora, in which a short-term memory has been demonstrated (Hildemann et al., 1979b). From an evolutionary standpoint, xenogeneic or allogeneic incompatibility in sponges and coelenterates is thought to be the result of an ecological adaptation aimed at the maintaining of the animal's individual integrity, since most of these organisms lead a colonial style of life (Lackie, 1980).

Nemertines

Nemertines or ribbon worms are acoelomates (animals without a body cavity) with complete digestive and primitive circulatory systems (Hickman, 1973). The tissue mobile cells or "immunocytes", which include agranular leucocytes and granular "macrophage-like" cells, are competent in phagocytic and cytotoxic reactions against foreign materials (Langlet and Bierne, 1977). Similar to sponges and coelenterates, nemertines are also capable of allogeneic discrimination. Langlet and Bierne (1977) found that graft survival was dependent upon species compatibility of grafted cells and immunocytes from the donor and recipient, respectively. Additionally, chimeras from two weakly histoincompatible species generate a stronger acute rejection of xenografts through cytotoxic reactions than those generated in each of the individual species alone, suggesting an analogous stimulatory effect comparable to that observed in vertebrate mixed lymphocyte cultures (Langlet and Bierne, 1983).

In summary, animals (poriferans, coelenterates, and nemertines), representing the lowest level of metazoans in evolution, possess in themselves a highly efficient immunorecognition machinery which is capable of mounting reactions against foreign substances and providing protection for these animals.

Annelids

Annelids are eucoelomates (possessing a true body cavity) with a complete digestive system and a closed circulatory system. Amoebocytes in the body cavity (coelomocytes) are responsible for mediating cellular defense against invading non-self organisms (Metchnikoff, 1968; Cuenot, 1898; Cameron, 1932; Bang, 1966; Cushing and Boraker, 1975). In the earthworm, Lumbricus terrestris, coelomocytes consist of five major cell-types: basophils, neutrophils, acidophils, granulocytes, and chloragogen cells (Hostetter and Cooper, 1974; Stein et al., 1977; Linthincum et al., 1977; Stein and Cooper, 1978). These cells seem to have differential discriminating ability against non-self materials with varying properties (Cameron, 1932; Fitzgerald and Ratcliffe, 1981). For example, coelomocytes phagocytize and clear the human strain of tubercle bacilli from the coelom in 5 days; however, the bovine and avian strain of this bacteria persist in the tissue for as long as 21 days (Cameron, 1932). The phagocytized microorganisms often are incorporated into a dark reddish mass in the body cavity (brown bodies) and become necrotic (Pilgrim, 1965; Fitzgerald and Ratcliffe, 1983). Materials too large to phagocytize, such as metazoan parasites, are encapsulated by coelomocytes and melanized (Poinar and

Hess, 1977).

Transplantation studies have shown that annelids are capable of both allogeneic and xenogenic recognitions (Cooper, 1968) and coelomocytes are responsible for mediating the observed annelid's transplantation immunity (Valembois, 1971). The above conclusion is based on the observations of an accelerated rejection of repeat allo- and xenografts, a heightened coelomocyte numbers in a short period of time during second set transplantation (Cooper, 1968; Hostetter and Cooper, 1973), and by adoptive cell transference of immunity from immunized (i.e., grafted) to naive annelids (Valembois, 1971). An _in vitro_ demonstration of cytotoxicity by _Eisenia fetida_ coelomocytes against allogeneic coelomocytes further strengthens the role of coelomocytes in graft rejection and this reaction has also been suggested as an analogue of the vertebrate mixed lymphocyte reaction (MLR) (Valembois et al., 1980, 1982).

Lysosomal enzymes such as acid phosphatase, β-glucuronidase and lysozyme are found in both coelomocytes and the coelomic fluid of oligochaetes and polychatetes (Valembois, 1971; Stain and Cooper, 1978; Dales and Dixon, 1980; Marks et al., 1981; Stein et al., 1982). The source of the coelomic fluid lysosomal enzymes is still unknown (Stein et al., 1982). In spite of that, the release of an antibacterial chemical, hydrogen peroxide, has been observed to be associated with phagocytic events by _Lumbricus_ coelomocytes (Chateaureynaud et al., 1981). Hydrogen peroxide production in annelid cells is thought to be analogous to the production of reactive oxygen radicals in mammalian

neutrophils and activated macrophages (Nelson, 1976).

The finding of hydrogen peroxide and lysosomal enzymes in annelid coelomic fluid could explain some of the erythrocyte lytic (hemolytic) (Cooper, 1974; Roch, 1979; Anderson, 1980; Chateaureynaud et al., 1981; Chain and Anderson, 1983b) and antibacterial (Roch et al., 1981; Valembois et al., 1982) properties that these animals possess. In the polychaete, Glycera dibranchiater, it is suggested that the coelomic antibacterial factor is also responsible for hemolysis of sheep erythrocytes (RBCs). Preabsorption of coelomic fluid with either bacteria or RBCs can reduce the effect of coelomic fluid on the other (Chain and Anderson, 1983b). A similar finding was also reported by Valembois et al. (1982) from the earthworm, Eisenia fetida andrei, in which the antibacterial and hemolytic activities of the earthworm's coelomic fluid were found to be caused by the same lipoprotein molecules. Furthermore, Roch (1979) and Roch et al. (1981) isolated these molecules and found that they have a molecular weight of about 4 X 10^4 daltons and exist in 4 isoforms. Most antibacterial substances in annelid coelomic fluid are directed against only the highly pathogenic soil bacteria (Valembois et al., 1982). Thus, the presence of humoral antibacterial factors in annelids has a notable value in the species' survival.

Other humoral factors such as agglutinins and opsonins are also found in annelid coelomic fluid. Agglutinins include a bacterial agglutinin (Stein et al., 1981) and a hemagglutinin, so-called, because of its ability to agglutinate various vertebrate RBC species (Cooper et

al., 1974; Gold and Balding, 1975; Garte and Russell, 1976; Anderson, 1980; Stein et al., 1981). Agglutination of foreign particles in the coelomic fluid may increase the efficiency of coelomocyte phagocytosis, and eventually, the clearance of these particles from the body of the animal (Cooper and Lemmi, 1981). In this regard, annelid agglutinin can also be opsonic, although the opsonic effect of agglutinin has only been demonstrated in neutrophil-mediated phagocytosis of foreign particles (Stein and Cooper, 1981). The annelid agglutinins are lectin-like due to the fact that their agglutinative property can be competitively inhibited by some carbohydrates. They are heat-labile and the activity is dependent on divalent cations (Anderson, 1980; Stein and Cooper, 1982).

Although there is much work needed in elucidating the exact roles of cellular and humoral factors, as well as their interaction,in annelid immunity, based on the available information, it is apparent that animals with relatively low position on the evolutionary scale already possess complex recognition mechanisms to protect themselves from infection.

Arthropods

Vast amounts of work concerning self/non-self recognition has been done using arthropods as experimental models. This has been due to the fact that arthropods are not only the most extensive in animal kingdom in terms of abundance and species diversity, but also because of their enormous medical and economic importance to humans. Arthropods are hemocoelic in that their body cavity serves the blood circulatory

function. In the hemocoel flows the hemolymph which contains the cellular elements, called hemocytes, and the soluble plasma compartment. Arthropod hemocytes, as in other invertebrates, are morphologically diverse. Among these cells, plasmatocytes (hemocytes with a homogeneous cytoplasm) and granulocytes (hemocytes with heterogeneous granules in the cytoplasm) are the major cell-types observed to actively participate in cellular defense reactions. Metchnikoff (1884) first observed the phenomenon of phagocytosis in the hemocytes of water fleas, Daphnia, and pointed out a direct correlation between the success of hemocyte phagocytosis on foreign particles and the survival of the animal. Since then, phagocytosis has been observed in many other arthropod species including both insects (Salt, 1970; Anderson et al., 1973; Ratcliffe and Rowley, 1974, 1975) and crustaceans (McKay and Jenkin, 1970a; Patterson et al., 1976). In addition to the free-circulating phagocytic hemocyte, there are also tissues containing fixed phagocytic cells found in the gills, heart, and hepatopancreas which act as blood filtration system. These fixed phagocytic sites are thought to be analogous to tissues comprising the vertebrate reticuloendothelial system and are of primary importance in the clearance of foreign materials from the circulation (Salt, 1970; Smith and Ratcliffe, 1980; Tyson and Jenkin, 1973; White and Ratcliffe, 1980; McCumber and Clem, 1983).

Metabolic pathways of insect phagocytes are similar to those of mammalian polymorphonuclear leucocytes (PMN) and macrophages in that they both utilize the glycolytic pathway as their main energy source. However, the hexose monophosphate pathway which is used by PMN as an

alternative energy source, as well as the potent antimicrobial system, myeloperoxidase-H_2O_2-halide system, are absent in insect hemocytes (Anderson et al., 1973; Anderson, 1975). In addition, hydrolytic enzyme activities are associated with hemocyte phagocytosis (Rowley and Ratcliffe, 1979).

Encapsulation of foreign materials by arthropod hemocytes (mostly granular hemocytes) represents another powerful cellular means of protecting arthropods from microbial pathogens or macroscopic parasite. The formation of multicellular hemocyte layers around foreign targets is accompanied by melanization or the release of lytic or toxic agents around the targets which eventually kills or restricts the growth of the parasite (Salt, 1970; Nappi, 1977; Poinar and Hess, 1977; Schmit and Ratcliffe, 1977; Poinar et al., 1979). Another type of cellular reaction observed against clumps of microorganisms is nodule formation which appears to be a combination of phagocytosis and encapsulation. During nodule formation, hemocytes infiltrate the clump of micororganisms and phagocytize them while others flatten themselves around the combined mass of hemocytes and microbes forming a cellular capsule (Ratcliffe and Gegen, 1976). Nodules formed in the insect body often are melanized, become attached to the wall of the hemocoel and are discharged to the outside during molting. If they occur in adult insects they may persist for the life of the animal.

The recognition and ingestion of foreign materials by arthropod hemocytes can be enhanced by a humoral factor from hemolymph (McKay et al., 1969; Rabinovitch and DeStefano, 1970; McKay and Jenkin, 1970b;

Ratcliffe and Rowley, 1983) and, thus, the humoral factor has been referred to as an opsonin. The receptor-mediated recognition of foreign substances by arthropod hemocytes is further supported by the observation of a differential rate in the phagocytosis of particles which differ in their surface chemical nature, and the discovery of a prophenoloxidase recognition system in crustacean hemocytes (Ratcliffe and Rowley, 1983; Smith and Soderhall, 1983). Moreover, crustacean hemocytes presensitized with endotoxin or killed bacteria show an increase in phagocytosis and RBC rosette formation (McKay and Jenkin, 1970c; Patterson et al., 1976; Goldenberg et al., 1984). Therefore, arthropod hemocytes may also have membrane receptors for opsonins or for directly interacting with foreign determinants. Receptors for binding foreign molecules may be endogenous and/or expressed through activation (Goldenberg et al., 1984).

Cytotoxicity has been demonstrated by hemocytes of the crayfish, Parachaeraps bicarinatus, against various vertebrate tumor cells (Tyson and Jenkin, 1974). In the case of crayfish hemocyte-mediated cytotoxicity, the recognition of the tumor cell appears to be triggered via a trypsin labile receptor(s) on the hemocyte membrane. However, no further characterization of these receptors has been pursued. Arthropods, in general, do not recognize allogeneic transplants and the success of xenogeneic transplant recognition is dependent on the phylogenetic relatedness of the species (Lackie, 1979).

The most fully characterized of invertebrate hemagglutinins is that

of the horseshoe crab, Limulus polyphemus, first described by Cohen et al. (1965). The Limulus hemagglutinins, as well as other naturally occurring hemagglutinins from various species of chelicerates, display a binding specificity for sialic acid (Vasta and Marchalonis, 1983). The molecule is ring shaped with a molecular weight of approximately 400,000 and is composed of subunits of 22,000 daltons each which are non-covalently associated (Marchalonis and Edelman, 1968). Its agglutination activity is Ca^{++} dependent. In addition to the chelicerates, hemolymph agglutination activity against a variety of vertebrate RBCs or bacterial species have been reported from other species of insects and crustaceans (McKay et al., 1969; McKay and Jenkin, 1970b; Scott, 1971; Hall and Rowlands, 1974; Rowley and Ratcliffe, 1980; Lackie, 1981b; and others). In all cases, agglutinins are lectins and demonstrate different sugar binding specificity. Some agglutinins show opsonic properties (McKay and Jenkin, 1970b; Rowley and Ratcliffe, 1980), while others do not contribute to enhancing hemocyte phagocytosis.

In addition to agglutinins, inducible bactericidal substances such as lysozyme are found in the arthropod hemolymph (Stephens and Marshall, 1962; Evans et al., 1968; Anderson and Cook, 1979; Hultmark et al., 1980; Walters and Ratcliffe, 1983). Bactericidin of the waxmoth is a dialysable, non-protein, and heat-stable molecule of small molecular weight (Stephens and Marshall, 1962). In contrast, bactericidins from the moth, Hyalophora cecropia, are composed of three small molecular weight proteins (Hultmark et al., 1980). The largest protein appears to be lysozyme, while the other two smaller molecular

weight proteins are potent, heat-stable, bactericidal substances (Hultmark et al., 1980). There are also inducible humoral factors in the insect hemolymph which appear upon challenge with heterogeneous proteins (McKay and Jenkin, 1969; Karp and Rheins, 1980; Rheins and Karp, 1982). The humoral factors which protect immunized animals from the lethal effects of toxic bee venom proteins are multivalent, because they can interact with venom components in precipitation reactions. Anti-venom factors are also sensitive to the proteolytic enzyme trypsin (Rheins and Karp, 1982). A complex factor, which is thought to be lymphokine-like, also can be induced by injection of latex beads in Galleria mellonella subtoxicus (Mohrig and Schittek, 1979). This factor once transferred to a naive larva of wax moth enables the larva to clear from its hemolymph the normally unphagocytizable bacteria, Bacillus thuringiensis (Mohrig and Schittek, 1979). However, this factor could be a bacterial opsonin which is non-specifically induced through latex bead presensitization.

Molluscs

Like the arthropods, molluscs represent an enormously diverse group of invertebrates in terms of distribution and number of species. Information concerning the internal defense mechanisms of these animals has largely concentrated on the bivalves (clams, mussels, etc.) and gastropods (snails, slugs). Molluscs are hemocoelic and possess open circulatory system. Hemocytes of these animals vary in their morphology and individual chemical composition (e.g. hydrolytic enzyme content or surface membrane structures). Cheng (1981) categorized

bivalve hemocytes into two major cell types: granulocyte and hyalinocyte. Both cells participate actively in various cellular immune functions of bivalves (reviewed by Bayne, 1983). On the other hand, gastropods possess one type of free phagocytic hemocyte, the amoebocytes, and two types of fixed phagocytes, reticulum cells and pore cells (Wolburg-Buchholz, 1973; Sminia, 1981; Sminia et al., 1983). Pore cells are selectively endocytic in that they have a high affinity for proteinaceous materials (Sminia, 1981). Similar to that of the bivalves, gastropod amoebocytes are multi-functional cells. They are capable of both phagocytizing and encapsulating foreign materials (Sminia, 1972; Sminia et al., 1974; Sminia and Barendsen, 1980), synthesizing humoral defense factors (van der Knaap et al., 1981), and the repairing of tissue wounds (Sminia et al., 1973).

Classical experiments by Cuenot (1914), Stauber (1950), Tripp (1958, 1960), and Feng (1965, 1966) have demonstrated that foreign particles experimentally introduced into the bivalve, Crassostrea virginica (Amercian oyster), are phagocytized and degraded intracellularly within phagocytes or eliminated from the animal body by exomigration of the particle-laden phagocytes across epithelial borders through a process called diapedesis. Diapedesis, which also has been observed in other bivalve species, usually takes place at the sites of the foot, alimentary tract, gills and kidney (Stauber, 1950; Cheng et al., 1969; Reade and Reade, 1972; Cheng and Rudo, 1976b; Bayne et al., 1979). Unlike gastropods, bivalves do not appear to have fixed phagocytic cells, and its digestive and excretory system are generally important in the disposal of particle-laden hemocytes from circulation (Cuenot,

1914).

Molluscan hemocytes have a fine capacity for discriminating foreign materials of different size and chemical nature (Tripp, 1961a). Radiolabelled hemocyanins from animal species possessing different phylogenetic relatedness to the chiton, Liolophura gaimardi, were found to be cleared from the chiton's circulation with the farthest related species always being cleared faster than those more closely-ralated species (Crichton and Lafferty, 1975).

Lysosomal enzymes such as lysozyme, non-specific esterase, alkaline phosphatase, acid phosphatase, β-glucuronidase, amylase, and lipase have been found in hemocytes and plasma (cell-free hemolymph) of molluscs (McDade and Tripp, 1967; Rodrick and Cheng, 1974; Cheng and Rodrick, 1975; Cheng, 1976; Cheng et al., 1978; Cheng and Yoshino, 1978). It is evident that plasma lysosomal enzymes have originated from hemocytes (granulocytes). They are released from hemocytes by degranulation during phagocytosis (Cheng et al., 1975; Foley and Cheng, 1977; Cheng et al., 1978; Cheng and Butler, 1979) in a fashion analogous to that observed in phagocytizing mammalian macrophages. In addition, enzymes are thought to be hypersynthesized in hemocytes and subsquently released into plasma upon parasite infection or other forms of non-specific perturbation such as injection of distilled water (Cheng et al., 1977). Other tissues, e.g. the headfoot or visceral mass, of gastropods also may serve as sources of plasma acid hydrolases, since these tissues have also been found to be rich in several lytic enzymes (Yoshino and Cheng, 1977; Cheng et al., 1980).

Lysosomal enzymes are not only important in intracellular degradation of phagocytized foreign materials, but also significant in the extracellular destruction of microorganisms and metazoan parasites (Cheng and Rodrick, 1974; Cheng, 1976; Cheng et a., 1978). Also, enzyme levels can be elevated after parasite stimulation. The aminopeptidase activity was increased in the hemocytes and plasma of Biomphalaria glabrata 20 and 30 day following exposure to irradiated miracidia of the trematode, Echinostoma lindoense (Cheng et al., 1978). This and the enhanced cellular and humoral reactions from other species of molluscs as a result of previous challenge with foreign materials (Cushing et al., 1971; Cheng and Yoshino, 1976; Bayne, 1980; Jeong et al., 1980; Granath and Yoshino, 1983) are considered to be inducible responses which may have functional bearing on the immune systems of molluscs (Cheng, 1983).

Many molluscs serve as intermediate hosts for metazoan parasites such as nematodes and trematodes (review by Bayne, 1983). Some molluscs can mount cellular encapsulation response against parasites. In some cases, a generalized proliferation of hemocytes (leukocytosis) is associated with the encapsulation (Yousif et al., 1979). In addition, hemocytes of a parasite-resistant strain of the gastropod, B. glabrata, are cytotoxic to the trematode, Schistosoma mansoni. It has been further demonstrated that the recognition of the parasite by snail hemocytes is achieved through a cytophilic factor(s) present both in the plasma and on the hemocyte plasma membrane (Bayne et al., 1980a, 1980b). Hemocyte cytolytic properties have also been demonstrated by keyhole limpet hemocytes toward normal and malignant vertebrate cells

(Decker et al., 1981). The recognition of targets by these hemocytes appear to be mediated through a series of sugar-specific lectin-like molecules present on the hemocyte surface, since a variety of defined mono- and disaccharides can block the hemocyte cytolytic effect (Decker et al., 1981).

Rejection of transplants in molluscs has only been demonstrated on the xenogeneic level (reviewed by Bayne, 1983). Transplantation rejection is marked by a differential infiltration of various types of hemocytes around the transplant (encapsulation). These hemocytes eventually invade the implant, and phagocytize dead implanted tissue (Tripp, 1961; Bayne et al., 1979). More convincing evidence of molluscan immune specificity comes from the host-parasite system. When trematode-resistant strains of the snail, B. glabrata, are exposed to normal miracidia of Echinostoma lindoense or if susceptible snails are exposed to irradiated miracidia, the parasites penetrate the snail and migrate to the heart, but are then surrounded by amoebocytes usually resulting in death of the larval parasite (sporocyst stage). If these previously challenged snails are subsquently exposed to normal miracidia, the parasites are quickly destroyed by amoebocytes near the penetrated area. This response is relatively specific, since primary infection with E. lindoense does not "immunize" the snail against a challenge with S. mansoni, although a certain degree of protection is stimulated against E. liei. At the same time, the amoebocyte-producing organ becomes enlarged and its cells show mitosis during primary sensitization (Lie et al., 1976) suggesting a proliferation of effector cells (amoebocytes) in response to parasite infection.

Humoral factors such as agglutinins and opsonins are both present in molluscan plasma (Prowse and Tait, 1969; Hardy et al., 1977; Chorney and Cheng, 1980; van der Knaap, 1980; Renwrantz et al., 1981; and others). Agglutinins are also found as hemocyte surface components (van der Knaap et al., 1981a; Vasta et al., 1982) and associated with cells lining gastropod blood vessels (van der Knaap et al., 1981b), and in the gastropod albumin gland (Hammarstrom, 1972; Anderson and Good, 1976; Renwrantz and Mohr, 1978). Foreign particles in the hemolymph of molluscs have been observed attaching to the surface of blood vessels or in the connective tissue of various organs before they are ingested by fixed phagocytes (van der Knaap et al., 1981; Renwrantz et al., 1981). It has been suggested that such attachment may be mediated through agglutinin molecules present on the surfaces of these cells, although this has yet to be unequivocally demonstrated.

A wide range of hemagglutinating specificities has been noted with molluscan agglutinins. Agglutinins from Helix pomatia (Hammarstrom, 1972) and C. virginica (McDade and Tripp, 1967) agglutinate human A cells and are inhibited by N-acetyl-D-galactosamine and/or N-acetyl-D-glucosamine. Yeast and bacterial agglutinins from Lymnaea stagnalis bind a variety of sugars including D-galactose, L-forms of fucose, galactose, glucose, mannose, rhamnose and the polysaccharides, cellulose, galactogen and glycogen (van der Knaap et al., 1982). Also, the yeast agglutinin from Mytilus edulis expresses a binding specificity for the glycoprotein, mucin (Renwrantz and Stahmer, 1983). Therefore, it is most likely that agglutinins are lectins which agglutinate particles possessing the proper carbohydrate binding site

at their surface membranes.

Renwrantz and Stahmer (1983) isolated an agglutinin from the hemolymph of the mussel, Mytilus edulis, and demonstrated the opsonic property of this purified agglutinin. Others have also recognized the potential opsonic characteristic of molluscan agglutinins (Cheng et al., 1969; Pauley et al., 1971; Anderson and Good, 1976; Renwrantz and Mohr, 1978; Renwrantz et al., 1981), although only a few have used purified preparations to directly demonstrate the connection between agglutinating molecules and their role as opsonins (Renwrantz et al., 1981; Renwrantz and Stahmer, 1983). In addition, hemocytes display a variety of carbohydrates which serve as lectin-binding receptors on their membrane surface (Renwrantz and Cheng, 1977a; Sminia et al., 1981; Yoshino, 1981a, 1981b; Schoenberg and Cheng, 1982). Therefore, it is likely that agglutinin and opsonin may represent the same molecule which could react with foreign particles bearing the proper sugar moieties and subsequently mediate the chemical interaction of particles with phagocytic hemocytes through the binding of similar hemocyte surface carbohydrates.

Other soluble factors including parasite growth or immobolizing substances (Michelson, 1964; Jeong et al., 1980) and an erythrocyte lysin (Anderson, 1981) have also been reported from several molluscan species. However, their functional role in molluscan immunity is still uncertain at this point.

Echinoderms

The phagocytic coelomocytes, which compose 70% of the total echinoid coelomocyte population, constitute the major cellular defense components of these animals (Reinish and Bang, 1971; Bertheussen and Seljelid, 1978; Bertheussen, 1981). Injected bacteria or implanted tissues from other animal species into the coelom are quickly recognized by these cells and are subsequently phagocytized or encapsulated (Johnson, 1969). Bacteria-laden coelomocytes tend to form aggregates in the coelom and these phagocyte aggregates as well as encapsulated foreign materials often become attached to the wall of the coelom and migrate to the tips of papulae (water-vascular system). Following necrosis of these tips, cellular clumps or capsules containing foreign substances fall off and leave the animal (Bang, 1975).

Recognition and internalization of foreign materials by echinoid coelomocytes can take place in the absence of coelomic fluid. An enhancement of coelomocyte phagocytosis of sheep RBCs pre-treated with either vertebrate sera or antibody (IgM) and the loss of this opsonic effect on coelomocytes when RBCs are exposed to vertebrate sera depleted of C 3 or treated with zymosan, strongly suggest that the echinoid phagocytic coelomocytes possess on their plasma membrane, a vertebrate-like C 3 complement receptors (Bertheussen and Seljelid, 1982). Further analogy between the echinoderm and vertebrate immune systems was demonstrated by Brillouet et al. (1981) who showed that two

different cell populations isolated from the starfish axial organ selectively responded to the mitogens concanavalin A (ConA), pokeweed mitogen (PWM), lipopolysaccharide (LPS), or limulin. Also, supernatant from the PWM-stimulated non-adherent cells of starfish axial organ contained vertebrate lymphokine-like substances. The results of this study support the hypothesis that the starfish axial organ represents an ancestral vertebrate lymphoid organ (Leclerc, 1974).

Echinoiderms also exhibit transplantation immunity in which second and third set allografts are rejected more quickly (12 and 8 days, respectively) than the first set grafts (1 month) (Karp and Hildemann, 1976; Coffaro and Hinegardner, 1977). However, memory can only be demonstrated for up to a six month duration.

Hemolysins and hemagglutinins are also present in the coelomic fluid of echinoderms (Brown et al., 1968; Ryoyama, 1973). The functional role of these substances in echinoderm immunity is still unknown. However, the presence of a factor in the hemolymph of Asterias forebesi which interact with purified cobra venom factor and lyses RBCs has prompted the suggestion that echinoderms may possess components which are functionally similar to the terminal components of complement found in vertebrates (Day et al., 1970).

Ascidians

Ascidians, or protochordates, represent an important group of animals in studies concerned with the phylogeny of immune mechanisms, because of their phylogenetic position as the immediate progenitors to

the vertebrates.

Ascidians are hemocoelic and contain several types of hemocytes. Although some of these cells morphologically resemble vertebrate lymphocytes, they do not show any of the functional characteristic of these vertebrate lymphocytes (e.g., blastogenesis or the production of antibodies or lymphokines upon antigen stimulation) (Warr et al., 1977). Instead, phagocytosis and encapsulation represent the primary forms of cellular reactions against foreign materials (Wright, 1980).

Hemagglutinins are found in several species of sea squirts (Parrihello and Patricolo, 1975; Anderson and Good, 1975; Fuke and Sugai, 1972). They display a binding specificity for D-galactose (Parrinello and Canicatti, 1982) or sialic acid (Anderson and Good, 1975). Agglutinins synthesized and secreted by certain hemocytes are considered to be the mediators of hemocyte-related recognition event (Wright and Cooper, 1981).

Ascidians are capable of specific "self" recognition in which separated parts of the squirt's (Amaroecium constellatum) body could re-sort themselves regenerating all missing members (Scott ans Schuh, 1963). Thus, the squirt's cells are capable of recognizing their own specific cell surface molecules with the result that similar cells aggregate together. Also, colony fusion of colonial Botryllus primigenus only takes place between members sharing common alleles for their histocompatibility antigens (Oka and Watanabe, 1960; Freeman, 1970).

Summary

Many invertebrate species have been shown to possess diverse populations of circulating cells which are considered to be very important in mediating their internal defense functions against foreign substances. Many of these cells are morphologically and functionally similar to certain vertebrate immune cells, although, unlike in the vertebrate system, the molecular basis of non-self recognition by invertebrate cells has remained mostly unexplored. For the most part, the immune functions displayed by invertebrates are mostly considered to be forms of natural resistance (e.g., anatomic barriers, cellular phagocytosis, encapsulation, natural cytotoxicity or lytic mechanisms).

Nevertheless, specific cellular reactions elicited in invertebrates against foreign materials have been demonstrated in several host-parasite system (Lie et al., 1975, 1976) and in allograft transplantation studies involving sponges (Hildemann et al., 1977), coelenterates (Hildemann et al., 1979b), annelids (Cooper, 1968; Parry, 1976), and echinoderms (Karp and Hildemann, 1976). In these studies, invertebrates exhibit accelerated cellullar reactions after the second exposure to the same materials. Also, in some incidences, an increase in the number of effector cells as the result of primary sensitization was observed to accompany the accelerated cellular reactions (Lie et al., 1976; Parry, 1976).

Lysosomal enzymes which were found in the body fluid of annelids (Marks et al., 1981), arthropods (Anderson and Cook, 1979), and molluscs (Cheng and Rodrick, 1975), play a major role in hummoral

defense functions of these invertebrates against bacteria and other foreign materials. Other types of bactericidin have also been reported from the plasma of arthropods (Hultmark et al., 1980) and echinoderms (Wardlaw and Unkles, 1978).

Agglutinin and hemagglutinins, another group of molecules found in the body fluid and on the cell surfaces of invertebrates, have been suggested as mediators of invertebrate non-self recognition (reviewed by Stein and Cooper, 1982). These molecules are lectin-like and agglutinate bacteria, yeast, and vertebrate RBCs in vitro. According to Parish's hypothesis (1977), self/non-self discrimination in invertebrates could be based on recognition of carbohydrate determinants by soluble or cell-bound oligomers of glycosyl-transferases. As a result, the individual enzyme specificities could increase the range of invertebrate recognition of foreign materials bearing various carbohydrate molecules on their surfaces. In this regard, the definite functional role of hemagglutinin in invertebrate defense was demonstrated by Renwrantz and Stahmer (1983) in the mussel, M. edulis. They isolated the hemagglutinin molecule from the hemolymph and showed the opsonic effect of these molecules on mussel's hemocytes. Thus, direct evidence for humoral factors interacting with cellular effectors to accomplish a defense-related function in invertebrates is established.

Still, there are more questions than answers concerning the mechanisms of invertebrate internal defense. In order to broaden our knowledge in this area, the study of the invertebrate internal defense

functions is now being extended to a group of freshwater bivalves, Corbicula fluminea. Mature specimens of C. fluminea, distributed widely in most of the North America river systems, is of moderate size (5 cm) and can provide, on average, up to 3 ml of hemolymph. The hemolymph contains numerous circulating hemocytes which are needed for the in vitro manipulations described in this research. Thus, this clam provides a most suitable system for accomplishing the specific aims of this research project, which are as follows: (1) To study the morphological and hematological characteristics of circulating hemocytes from this clam since these cells are considered to be the major effector elements in defense-related reactions, (2) To investigate the cellular mechanisms of Corbicula hemocytes elicited against non-self materials, and (3) To study the humoral mechanisms possessed by Corbicula hemolymph against non-self.

CHAPTER II

MATERIALS AND METHODS

Animals

Clams were hand collected at the outlet of Lake Thunderbird dam, in Cleveland County, Norman, Oklahoma. The size of the clams ranged from 0.5 cm to 5 cm in width, although only the larger size (3 cm to 5 cm) were collected for the study. Species identification was made using the tooth-like structure at the hinge region of the shell. In the laboratory, specimens were placed in a 10 gallon aquarium containing "aged" tap water. Clams were either placed directly on a fine gravel bottom substrate or suspended off from the bottom of the aquarium on a plastic rack covered with a fine screen. Ground commercial fish food bar was provided as food.

Hematological and Morphological Properties of Corbicula Hemocytes

Bleeding

Hemolymph of Corbicula was withdrawn from the clam's posterior adductor muscle sinus using a 1 cc syringe and a 26 G needle or by directly cutting through the muscle using a sharp scalpel after the

shells have been slightly opened and braced with a wooden stick.

Hemocyte Counts

For hemocyte counts, a drop of whole hemolymph (plasma + hemocytes) was placed in an American Optical Bright-Line hemocytometer and the number of hemocytes per milliliter of hemolymph was estimated at 200X using an Olympus phase-contrast microscope.

Light Microscopy

For hemocyte behavioral and morphological studies at the light microscope level, wet mounts were prepared by quickly placing a drop of whole hemolymph onto a clean glass slide and gently overlaying the drop with a glass cover slip ringed with Vaseline to prevent drying. The preparations were then viewed at a magnification of 200X or 400X using an Olympus phase-contrast microscope. Measurements of cell diameters were performed with an ocular micrometer.

Electron Microscopy

For the study of cell structures at the ultrastructural level, hemocytes were fixed in ice-cold 2.5% glutaraldehyde in 0.05 M cacodylate buffer containing 0.05 M sucrose for 30 min. After fixation, cells were collected by centrifuging at 40g and washed 3 times in 0.01 M cacodylate buffer. Glutaraldehyde-fixed hemocytes were then post-fixed in 1% OsO_4 for 1 hr, dehydrated in an alcohol series, and embedded in Poly/Bed 812. Silver to gray sections were obtained on a Porter-Blum ultramicrotome and stained for 8 min with uranyl acetate

and 3 min with lead citrate. The ultrastructure of hemocytes was then studied using a Zeiss 10A electron microscope at an operating voltage of 60 KV.

Enzyme Cytochemistry

The presence of lysosomal acid phosphatase (AP), nonspecific esterase (NE), and peroxidase (PO) activity in Corbicula hemocytes was studied cytochemically using the procedures of Humason (1979) with some modifications. To prepare hemocytes, 4 clams of similar size were randomly selected from the stock aquarium and bled through the posterior adductor muscle using a syringe. Three hemolymph samples (approximately 20 ul per sample) from each clam were placed on glass slides in a humidity chamber. Hemocytes were allowed to adhere to the slides for about 20 min followed by the removal of plasma by washing 10 times with a clam phosphate buffer saline (PBS), adjusted to 100 mOs/kg H_2O. Hemocytes were fixed for about 30 min in 2.5% glutaraldehyde in 0.05 M cacodylate buffered (pH 7.2) containing 0.05 M sucrose, washed 5 times with clam PBS and stored in distilled water (dH_2O) until cytochemical studies were performed.

To demostrate AP activity, hemocytes were incubated at 26 °C for 10 hr in the substrate, β-glycerophosphate in a Tris-maleate buffer (pH 5.0). Then hemocytes were rinsed 10 times in dH_2O, stained with 1% ammonium sulfide for about 5 min, washed 5 times with dH_2O and stored in dH_2O for microscopic examination. Controls consisted of hemocytes incubated in substrate to which 0.01 M NaF had been added. Naphthol

AS-D chloroacetate in a Tris-HCl buffer (pH 7.1) was used as the substrate to demonstrate NE activity. Hemocytes were incubated in the subatrate for about 90 min, after which time they were rinsed and stored in dH_2O for evaluation. Controls consisted of hemocytes incubated in a medium lacking in substrate. To demonstrate PO activity, hydrogen peroxide in the presence of the chromogenic reagent, diaminobenzidine tetrahydrochloride (DAB), in 40% ethanol was used as the substrate. Hemocytes were incubated at 24°C for about 5 min in the substrate, rinsed 5 times with dH_2O and stored in dH_2O. Controls consisted of hemocytes incubated in medium lacking H_2O_2 or DAB, or in medium to which 0.01 M KCN (cytochrome oxidase control) or 0.02 M aminotriazole (catalase control) had been added.

To quantify the distribution and abundance of the enzymes within the hemocyte population, one hundred cells were examined for every clam and treatment. For those samples stained for AP or PO the mean number of granules per cell and percentage of postive cells were calculated for each clam in each treatment group. In those slides stained for NE, each cell was graded as staining heavily (+++), moderately (++), slightly (+), or not at all (-), and the percentage of positive cells was calculated for each clam in this treatment group.

Analysis of variance (ANOVA) was adopted for data analysis. All percentage data were subjected to the arcsine transformation prior to analysis. Duncan's multiple range test was used whenever one-way ANOVAs showed a significant result.

Separation of Morphologically Distinct Hemocyte Subpopulations

A Percoll step gradient centrifugation method was used to separate the morphologically distinct hemocyte subpopulations. A 60% to 90% discontinuous Percoll gradient, in 5% increments, was prepared by mixing Percoll (Sigma, St. Louis, MO.) and 0.1 M cacodylate buffer in appropriate volume to volume ratios and carefully layering the Percoll solutions stepwise in to 1.5 x 10 cm cellulose nitrate centrifuge tubes. Hemocytes were collected from 10 clams by the direct bleeding of hemolymph into a beaker containing ice-cold 2.5% glutaraldehyde in 0.05 M cacodylate buffer containing 0.05 M sucrose. The hemocytes were fixed for 30 min before they were collected by centrifuging at 40g and washed 3 times in 0.1 M cacodylate buffer. In the final wash, hemocytes were adjusted to a concentration of 5×10^8 cells/ml. Aliquots (0.2ml) of hemocyte suspension were layered onto the Percoll gradients and the separation was carried out by centrifuging at 550g at 24°C for 30 min. Bands of hemocytes sedimented at the different gradient interfaces were collected from the top of each tube using a Pasteur pipette. Following microscopical examination and enumeration of the different morphological cell-types, hemocytes from each gradient were washed 3 times with 0.1 M cacodylate buffer and processed for ultrastructural studies.

The Activities of Corbicula Hemocytes Against Foreign Test Particles

Phagocytosis

Test particles. Formalin-fixed vertebrate red blood cells (RBCs) were used as test particles to investigate the recognition and reaction of Corbicula hemocytes against foreignness. RBCs of horses, sheep, rabbits, and humans (types A, B, AB and O) were collected in Alsever's solution and washed 2 times in mammalian PBS before they were fixed in 6% neutral-buffered formalin for 6 hr at 24 °C. After fixation, these RBCs were rinsed 3 times in PBS and one time in 0.02 M glycine to bind the free aldehyde groups. Finally, the RBCs were washed and resuspended in clam PBS at a concentration of 1×10^7 cells/ml.

Hemocyte Preparation and Phagocytosis Assay. Hemolymph of Corbicula was collected through the posterior adductor muscle using a 1cc syringe. Fifty ul aliquots of this hemolymph were dispensed into the wells of tissue culture chamber slide (Lab-Tek, Naperville, IL.) and hemocytes were allowed to adhere for 20 min. The excess hemolymph was centrifuged (300g, 10 min) to obtain a hemocyte-free hemolymph (plasma) fraction. Following hemocyte monolayer formation (20 min), samples of hemolymph in the wells of tissue culture chamber slide were divided into two groups. Hemolymph samples in the first group remained undisturbed. In contrast, the plasma in the second group of samples was carefully drawn from the hemocyte monolayers using a Pasteur pipette and adherent cells of this second group were immediately rinsed

5 times with clam PBS and followed by an addition of 50 ul clam PBS (for no plasma negative controls) or freshly prepared clam plasma (for plasma add-back positive controls). After 5 min of incubation, ten ul of the fixed RBCs were finally added to each hemocyte/plasma or hemocyte/PBS sample and the mixture was incubated for 40 min at 24°C. Following this incubation period, 6% neutral-buffered formalin was added to every sample to stop the reaction. The fixative was then gently aspirated from the cell layers the plastic chambers and rubber gaskets on each slide were removed. Slides were coverslipped and percentage of phagocytizing hemocytes enumerated using phase-contrast microscopy. The experimental protocol is summarized in Figure 1.

The percentage of phagocytizing hemocytes was determined according to the following formula:

$$\% \text{ phagocytizing hemocytes} = \frac{\text{The number of hemocytes containing 3 or more RBCs}}{\text{Total number of hemocytes counted}} \times 100$$

At least 300 to 400 hemocytes were examined for each sample.

The Effect of Plasma on Hemocyte Phagocytosis. Experiments testing for the requirement of plasma on hemocyte phagocytosis were also conducted using methods similar to those summarized in Figure 1. Fixed rabbit RBCs were used as test particles in all experiments. Portions of the clam plasma either heat-treated at 56°C for 30 min or 100°C for 10 min or lyophilized (and subsequently reconstituted in isotonic PBS), were employed in experiments designed to test the effects of plasma on hemocyte phagocytosis. Positive and negative controls consisting of

the adding back of fresh plasma or clam PBS, respectively, were concurrently performed.

In order to study the opsonic effect of plasma, fixed rabbit RBCs were incubated in normal clam plasma for about 2 hr and then washed 5 times in clam PBS before being added to the plasma-free hemocyte preparation (Hemocyte/PBS). This experiment was carried out in tissue culture chamber slides using similar procedures as described in the previous experiment. Hemocyte phagocytosis of fixed, non-opsonized rabbit RBCs in clam PBS (negative control) or normal clam plasma (positive control) was also measured at the same time.

Competitive Inhibition Tests. Because carbohydrates on the cell membrane have been shown to serve as receptors for soluble recognition factors (Harm and Renwrantz, 1980; Stahl and Schlesinger, 1980; Renwrantz and Stahmer, 1983), the possible inhibitory effect of several mono- and dissacharides and two glycoproteins on hemocyte-mediated erythrophagocytosis was tested. Two concentrations (0.03M and 0.1M) of the mono- and dissacharides: L-arabinose, β-D-fructose, D-galactose, α-D-glucose, β-D-glucose, D-mannose, D-cellobiose, α-D-melibiose β-lactose, and N-acetyl-D-glucosamine, N-acetyl-galactosamine, as well as 0.02% or 0.07% solutions of the glycoproteins fetuin and mucin, were prepared in clam PBS. Aliquots of 50 ul of freshly bled Corbicula hemolymph were first dispensed into wells of tissue culture chamber slides. Hemocytes were allowed to adhere for 20 min at 24°C, followed by the addition of 25 ul of each carbohydrate solution into each test hemolymph sample. To provide a normal phagocytosis control,

carbohydrate solutions were substituted with 25 ul clams PBS. The samples were then incubated for 15 min. After which time a volume of 25 ul of fixed rabbit RBCs (1 X 10^7 cells/ml) was added to each preparation. Phagocytosis was allowed to proceed for 40 min, followed by the addition of formalin to stop each reaction. The prevalence of hemocytes participating in erythrophagocytosis was determined for each sample as previously described.

Time Course Study. A test tube method was adopted for measuring the kinetics of erythrophagocytosis and rosette formation, as well as the effect of EDTA on the rate of phagocytic and rosette forming activity. A volume of 0.25 ml of fresh drawn hemolymph was dispensed into each of 6 test tubes. The test tubes were separated into two groups. In the first group, 0.25 ml of clam PBS was added to each hemolymph sample, while in the second group, 0.25 ml of clam PBS containing 4 mM EDTA was added to each sample. The mixtures (total volume of 0.5 ml/tube) were allowed to stand for 10 min before the addition of 0.5 ml of 1.2 X 10^7 fixed rabbit RBCs/ml of clam PBS. At different time intervals (20, 40, and 60 min), the reaction was stopped by adding 0.1 ml of 6% buffered formalin into one test tube from each of the two groups. Attached hemocytes were gently scrapped off from the walls of the test tube using a modified rubber policeman. The contents of each test tube was then mixed well and a 50 ul sample oof this cell suspension was placed on a glass slide. The percentage of phagocytizing and rosette forming hemocytes (i.e. cells with 3 or more attached RBCs) were determined at each of the designated time intervals.

Statistical Analysis. For Corbicula erythrophagocytosis studies, a duplication of each treatment was made in each experiment, and every experiment was performed at least twice. Analysis of variance (ANOVA) were adopted for data analysis. All percentage data were subjected to the arcsine transformation prior to analysis. Duncan's multiple range test was used whenever a one-way ANOVA showed a significiant result.

Hemocytolytic Activity

Modified Plaque Assay. A plaque assay modified from Wittke and Renwrantz (1984) was used to determine the reaction of Corbicula plasma-free hemocytes on fresh RBC targets. Fresh horse, sheep and chicken RBCs collected in Alsever's solution were used as target cells for the assay. RBCs were washed 3 times in Hank's medium (310 mOs/kg H_2O without phenol red indicator, pH 7.4), once in a modified Hank's medium which had been adjusted to 210 mOs/kg H_2O (without phenol red indicator, pH 7.4), and finally adjusted to a 50% cell suspension in the modified Hank's medium. Target cells were stored at 5°C until assays were performed. Hemocytes from 2 clams were collected by the direct bleeding into a beaker containing clam PBS with 2 mM EDTA. The hemocytes were then rinsed twice in clam PBS and finally resuspended in the modified Hank's medium at a concentration of 6×10^4 cells/ml. All of the above procedures were performed in the cold (4°C). The modified Hank's medium (210 mOs/kg H_2O) was chosen as the reaction medium because preliminary experiments had shown that, although hypotonic to RBCs, this medium provided minimum hemolysis of the red cells while viability of hemocytes remained high ($82 \pm 5.6\%$) over a 4 hr period.

Plaque assay slides (Bellco Glass Co.) were used for the assay. Hemocytes prepared as described above were mixed in a 1:1 volume ratio with the prepared fresh RBCs (50% solution), and 15 ul aliquots of this mixture were placed onto each plaque assay slide. Slides were covered with a cover slip and placed undisturbed into a humidity chamber at 24 C for 2 hr. One set of slides, each containing target RBCs of each animal species was placed at 5 C for the same length of time to determine the effect of temperature on the reaction. Two sample replicates from each clam were made for each target RBC species at both temperatures. After incubation, the number of cells capable of forming plaques (i.e. those surrounded by a ring of lysed RBCs) per one thousand hemocytes was enumerated for each target species in both groups. Hemocyte viability was determined by trypan blue dye exclusion. A one-way ANOVA was used in the data analysis.

Hemoglobin (Hb) Release Assay. A measurement of the amount of Hb released from target RBCs in hemocyte-RBC mixtures was used as an indication of the intensity of specific RBC lysis caused by Corbicula hemocytes. This Hb release assay was substituted for the standard ^{51}Cr-release cytotoxicity assay due to its safety, simplicity, and reliability (Anderson, 1980). Fresh RBCs of sheep, cow, goat, horse, guinea pig, and rabbit origin were used as target cells. These RBCs were washed 3 times in Hank's medium and once in modified Hank's medium (210 mOs/kg H_2O) before being resuspended in the latter medium at a concentration of 20 X 10^6 cells/ml and stored at 5 °C until used.

Hemocytes from 8 clams were collected and prepared using the same

method as described in the previous experiment except that, after washing, the hemocytes were adjusted to the following concentrations: 4 X 10^6, 1 X 10^6, 2 X 10^5, and 6 X 10^4 cells/ml of modified Hank's medium. One-half ml aliquots of the fresh, washed RBCs were mixed in 7 X 44 mm test tubes with 0.5 ml aliquots of hemocytes at the different concentrations resulting in hemocyte:RBC ratios of 1:5, 1:20, 1:100, 1:333 for each RBC species tested. Spontaneous and maximum Hb release controls were obtained by incubating the RBCs in the modified Hank's medium or dH_2O, respectively. Hemocyte/RBC mixtures and the controls were incubated for 2 and 4 hr at 24 °C. At the end of each incubation period, cells were gently resuspended and centrifuged at 300g for 5 min at 5 °C. The amount of Hb released in each preparation was then measured spectrophotometrically at 541 nm and used as an indication of the degree of hemolysis caused by Corbicula hemocytes. The percent specific Hb released was calculated according to the following formula:

$$\% \text{ Specific Hb release} = \frac{\text{Test release} - \text{Spontaneous release}}{\text{Maximum release} - \text{Spontaneous release}} \times 100$$

Duplicate samples were performed for each RBC species tested and at each hemocyte: RBC ratio and the experiment was repeated three times. One-way and two-way ANOVAs were used to analyze the arcsin-transformed data. Duncan's multiple range test was invoked whenever one-way ANOVA showed a significant result.

The Activities of Corbicula Plasma Against Foreign Test Particles

Hemolysin

Hemolysis Assay. Five species of fresh RBCs from sheep, cow, goat, guinea pig, and rabbit origin (4% cell suspensions in mammalian PBS) were used as test cells for this assay. Hemolymph was collected by obtaining blood directly from the clam's posterior adductor muscle and centrifuging it at 300g for 10 min to obtain the hemocyte-free hemolymph fraction (plasma). The hemolysis assay was carried out in small test tubes. The plasma was serially diluted (2 fold) with mammalian PBS (0.5 ml/tube). Due to the difference in physiological osmolarity between clams and mammals, in another set of test tubes, the same dilution procedure was carried out using clam PBS instead of plasma. The freshly washed RBCs (0.5 ml) were subsequently added to each test tube of diluted plasma or PBS, and the latter was used as a spontaneous Hb release control. These tubes were then gently agitated and incubated at 24°C for 2 and 4 hr. Maximum Hb release controls were obtained by incubating the RBCs in dH_2O. At the end of each incubation period, samples were centrifuged at 300g for 5 min. The amount of Hb released into the medium from the lysed RBCs was determined spectrophotometrically at 541 nm and used as a quantitative indicator of plasma-mediated hemolysis. The percentage of specific Hb released was calculated by employing the same formula used previously in the hemocyte cytolytic assay. Duplicate samples were made for each

RBC species tested at each plasma dilution. The experiment was repeated twice using freshly collected plasma from two different batches of clams.

Effects of Heat and EDTA on Hemolysin. The effect of heat and EDTA treatment of plasma on hemolysis was also tested. For the heat treatment experiment, the procedures were identical to those described previously (Hemolysis Assay) except that the plasma samples were incubated at 56 °C for 30 min or 100 °C for 10 min before the serial dilutions were made. Fresh cow and sheep RBCs were used as test particles. Duplicate samples were made for each RBC species at each plasma dilution. The experiment was repeated once.

To study the effect of the divalent cation chelator, EDTA, on plasma hemolysis, dilutions of fresh plasma were made in mammalian PBS containing 4 mM EDTA (final concentration of 2 mM). Again the same procedures as outlined in the Hemolysis Assay section were adopted for the experiment and only cow and sheep RBCs were tested. Duplicate samples were made for each RBC species at each dilution level and the test was repeated once.

One-way and two-way ANOVAs were used to analyze the arcsin -transformed data. Ducan's multiple range test was used when a significant one-way ANOVA resulted.

Hemagglutination Activity

Hemagglutination Assay. A hemagglutination assay (Anderson, 1980) was carried out in Cook "V"-shaped well microtiter plates in order to

determine the presence of hemagglutinating factor(s) in Corbicula plasma. A 2-fold serial dilution of fresh clam plasma in mammalian PBS (25 ul per well) was prepared followed by the addition of test RBCs (25 ul at 1 X 10^8 cells/ml mammalian PBS) to each well. Freshly washed RBCs of horses, sheep, chickens, mice, rabbits, and humans (types A and B), as well as trypsinized (0.2% trypsin at 37°C for 30 min) or formalin-fixed rabbit RBCs, were used in this assay. Controls were performed by substituting the plasma with mammalian PBS for the dilutions. In addition, heat-treated (100°C for 10 min), and lyophilized, reconstituted plasma samples were tested to determine the effects of these treatments on plasma hemagglutinin activity. After adding the test RBCs, the microtiter plates were gently tapped against the fingers to mix the samples and the plates were covered and incubated for 12 hr at 24°C. The hemagglutination titers were determined following incubation, and the mean titers from $\log_2$ transformed individual titers were reported for each RBC species and treatment. One-way ANOVA was used to analyze the results.

Competitive Inhibition Assay. Various mono- and dissacharides, identical to the ones used in the phagocytosis experiment (0.1 M final concentration), and the glycoproteins fetuin and mucin (0.01% and 0.035%, respectively) were dissolved in mammalian PBS. Clam plasma dilutions were made in these sugar-containing media. After the dilutions were prepared, the mixtures of clam plasma and sugar-containing mammalian PBS were incubated for 10 min before RBCs were added. Only fixed rabbit RBCs (1 x 10^8 cells/ml mammalian PBS) were used in this assay. Positive and negative controls were prepared

in the same manner as discribed in the previous section (Hemagglutination Assay). Samples were incubated for 12 hr at 24°C and the agglutinin titer for each sample was determined in the same way as described in the previous section (Hemagglutination Assay).

CHAPTER III

RESULTS

Hematological and Morphological Properties of Corbicula Hemocytes

Hemocyte Counts

Hemocytes of Corbicula fluminea were colorless amoeboid cells circulating within the hemolymph. The average concentration of hemocytes in the hemolymph was $7.5 \pm 0.6 \times 10^5$ cells per ml.

Light Microscopy

Morphologically, hemocytes of C. fluminea represented a heterogeneous cell population. Under phase-contrast optics, there were three major cell types which could be distinguished according to their size, degree of granulation, and granule morphology: small hyaline hemocytes, vesicular hemocytes, and large granular hemocytes. Freshly bled hemocytes were round and expressed many short cell processes (Figure 2). Small hyaline hemocytes comprised 16% of the hemocyte population and ranged in size from 5 to 8 microns in diameter. They had a homogeneous cytoplasm and large nucleus-to-cytoplasm ratio.

Small cell processes projected out from the plasma membrane, although, on occasion, long branched cytoplasmic extensions also were observed. Vesicular hemocytes, which varied from round to irregular in shape, were the most numerous cell type comprising 60% of the total circulating hemocyte population. These hemocytes ranged in size from 8 to 15 microns in diameter, possessed a kidney or oval-shaped, eccentric nucleus and a small nucleus-to-cytoplasm ratio. There were small refractile and dark granules present in the cytoplasm. The cells also had short processes extending out from the plasma membrane into its surrounding medium. The large granular hemocytes, comprising 24% of the hemocyte population, had large refractile granules in its cytoplasm. Cells of this type were measured from 12 to 20 microns in diameter. Nuclei of these cells were small with round or oval shapes. Cell processes were also present.

Upon contacting the glass slide, all three types of hemocytes attached to and then spread on the slide surface. After attaching to the substrate, these cells were observed to extend their cytoplasm forming long slender cytoplasmic processes and distinctive ruffled membranes. At this stage, cells were flatten against the substrate and no longer retained their spherical shape. For small hyaline and vesicular hemocytes, the cell membrane could spread out a considerable distance from center of the nucleus (Figure 3). In comparison, the large granular hemocytes usually displayed smaller ruffled membranes with prominent spike-like cell extensions (Figure 4). The cytoplasmic content of the spread small hyaline hemocytes usually had a very fine granular appearance and remained close to the nucleus. The spread

vesicular hemocyte had, in addition to a similar cytoplasmic appearance as that of the small hyaline hemocyte, many refractile or phase-dark vesicles present in the cytoplasm. The presence or absence of these vesicles was used to differentiate between small hyaline and vesicular hemocytes in their spread or flattened state (Figures 3 and 4). For large granular hemocytes, their coarse, refractile, irregular-shaped granules stayed within the perinuclear region and sometimes overlapped with the nucleus (Figure 4).

Hemocytes tended to form clumps soon after bleeding. All three types of cells were found in these clumps. After clumps had settled onto a glass slide, hemocytes tended to move over the glass surface away from the clump, starting on the periphery of the cell aggregates. Thus, crescent-shaped hemocytes with their ruffled membranes positioned away from each cell mass were observed (Figure 5). Hemocyte aggregation could be prevented by collecting the hemolymph in the presence of clam PBS containing 2 mM EDTA. The adherence of hemocytes to the glass substratum could also be prevented by EDTA.

Ultrastructure of Hemocytes

At the ultrastructural level, the small hyaline hemocytes (Figure 6) were round or oval cells with many short cell processes projecting out from the cell membrane. The cytoplasm contained numerous round, oval, or elongate mitochondria, and profiles of rough endoplasmic reticulum (RER) which were located mostly in the perinuclear region. Smooth endoplasmic reticulum (SER) was abundant in some cells. Free ribosomes and glycogen granules were numerous, often giving a localized electron

dense appearance to the cytoplasm of the cell. The nucleus was large (occupying at least 1/2 of the cell volume) and oval, round, or irregular in shape. It contained a moderate amount of heterochromatin.

The shape of the vesicular hemocyte was irregular with many cell processes projecting out from the plasma membrane. The name of the cell was given due to the presence of many vesicles in the cytoplasm of the cell ($\bar{X} = 63.0 \pm 7.2$ vesicles/cell, $n = 10$) (Figure 7). These vesicles, which varied in size and shape, were filled with a homogeneous, moderately electron-dense material. A lucid zone was formed in between this material and vesicular membrane. RER was not as abundant as found in the small hyaline hemocyte and was located primarily around the nuclear membrane. Golgi complexes, mitochondria, and SER were located in the cytoplasm between the vesicles. Ribosomes and glycogen granules were less numerous than in the small hyaline hemocytes. The nucleus was similar in appearance to that of the small hyaline hemocytes except being relatively smaller and eccentric in location.

Large granular hemocytes were mostly round and possessed few cell processes associated with the plasma membrane. The cytoplasm was occupied primarily by large granules ($\bar{X} = 14 \pm 5.7$ vesicles/cell, $n = 5$) which measured 1.8 ± 0.3 microns in diameter (Figure 8). Most of these granules were filled with an electron-dense material which resembled that found in the vesicles of the vesicular hemocyte. However, some granules also were observed to be electron lucid and contained a mucus-like material. Small vesicles, similar to those of

vesicular hemocytes, were also occasionally observed among the large granules in these cells. Cytoplasmic organelles, such as mitochondria, RER, SER, and ribosomes, were scattered through out the cytoplasm. Golgi complexes were scarce in this type of cells. The nucleus was round to oval with several patches of heterochromatin.

In addition to the three major types of hemocytes, a hemocyte which had the combined characteristics of all the above hemocyte types was observed (Figure 9). The size of the hemocyte was 4.3 X 6.1 microns. The shape of the hemocyte was highly irregular due to many short and long cellular projections. This cell was similar to the small hyaline hemocyte in having many elongated or rounded mitochondria which were localized in the endoplasmic region. Many vesicles (about 34) which were characteristic of vesicular hemocytes were present, as well as a large electron-dense granule similar to that of the large granular hemocyte. Sparce amounts of free ribosomes and RER were observed in the perinuclear region and elsewhere the cytoplasm. SER was distributed mostly between vesicles. Golgi complexes were not apparent. The size and structure of the nucleus was similar to that of the vesicular hemocyte.

Enzyme Cytochemistry

Almost all of the hemocytes (97 $\pm$ 1.7%) contained acid phosphatase (AP). AP was localized in the perinuclear region as dark, large (0.5 micron), discrete granules (Figure 10) which were not seen in the control samples. The number of granules present in individual hemocytes ranged from 1 to 32 with an average of 10.4 $\pm$ 1.2 (n = 400).

The average number of granules present in each cell-type was 8.2 ± 1 for small hyaline hemocytes, 11 ± 1.1 for vesicular hemocytes, and 12.1 ± 1.4 for large granular hemocytes. These numbers were not statistically different form each other ($F = 2.9$, $df = 2,9$, $p > 0.05$). Also, hemocytes from different clams possessed similar levels of AP activity ($F = 1.2$, $df = 3,8$, $p > 0.05$).

All of the hemocytes (100%) stained positively for nonspecific esterase (NE), although different degrees and patterns of staining were observed (Figure 11). Moderate NE activity, exhibited by 47 ± 4% of the cells, was characterized by the presence of small granules (0.05 - 0.2 micron) distributed evenly throughout the cytoplasm except the extreme peripheral region of the cell. A heavy NE activity (29 ± 0.3% of circulating cells) was characterized by a dense granular pattern in the perinuclear region in addition to the distribution of numerous small NE granules characteristic of moderately stained cells. Approximately one quarter of the circulating hemocytes (24 ± 3%) stained only sparsely for NE activity throughout the cytoplasm. In addition, most of the small hyaline hemocytes (89.6%) expressed only slight NE activity, and the majority of vesicular hemocytes (52.1%) had a moderate NE activity. In contrast, 64.7% of the large granular hemocytes demonstrated a heavy NE activity. C. fluminea hemocytes did not exhibit peroxidase (PO) activity.

Separation of Hemocytes

By employing a Percoll step gradient centrifugation method (60 to 90% Percoll at 5% intervals), hemocytes could be separated into two

major populations (Figure 12). At the 65/70% Percoll interface, 80% of the cells were vesiculated hemocytes, while 19% were small hyaline cells and only 1% were large granular hemocytes. The 80% vesiculated cells separated in this cell fraction represented 66% of the total vesiculated cell population initially introduced onto the gradient. The 19% small hyaline hemocytes collected at this gradient interface constituted 59% of the total small hyaline cells put on the gradient, while the 1% large granular hemocytes composed of only 5% of the total number of this cell type originally layered on the gradient column. In contrast, at the 75/80% gradient interface, 46% of the cells were large granular hemocytes representing 95% of the total large granular cell population, while 41% of the cells were vesicular hemocytes (34% of the total vesicular cell population), and 13% were small hyaline cells (46% of the initial total small hyaline hemocyte population).

The Activities of Corbicula Hemocytes Against Foreign Test Particles

Hemocyte Phagocytosis

Hemocytes of C. fluminea were capable of phagocytizing formalin-fixed mammalian RBCs from various animal species. Five minutes after the addition of RBCs into the hemolymph, attachments of these target cells to the hemocyte plasma membrane (rosette formation) could be observed (Figure 13). The attached RBCs were eventually internalized by the hemocytes (Figure 14). Individual hemocytes were capable of phagocytizing from 6 to 10 RBCs each and became rounded and detached from the glass slide surface after 40 min (Figure 15).

Results presented in Table 1 indicate that, in the presence of plasma, hemocytes were equally efficient at phagocytizing RBCs from 7 mammalian species with the prevalence of phagocytizing hemocytes varying from 70 to 90% ($F = 0.9$, $df = 6,7$, $p > 0.05$). In the absence of clam plasma, there was a significant reduction in the percentage of hemocytes actively participating in phagocytosis (7.3% to 21.3%) for all the RBC species tested ($F = 12.5$, $df = 1,13$, $p < 0.01$).

The requirement of clam plasma for hemocyte phagocytosis was further tested by adding back plasma to the PBS-washed hemocyte layers before exposure of hemocytes to RBCs. Results, summarized in Figure 16, indicate that the addition of fresh clam plasma to the plasma-free PBS-washed hemocytes (washed hemocytes + plasma + RBCs) restored the ability of these cells in phagocytizing RBC ($80.6 \pm 7.1\%$) to a level comparable to untreated control samples (untreated hemocytes + RBCs) ($83.1 \pm 6.1\%$) ($t = 0.3$, $p > 0.05$).

The effect of heating or lyophilization on the plasma factor(s) mediating hemocyte phagocytosis was studied in experiments in which treated and normal plasmas were added back to washed hemocyte layers followed by exposure to test RBCs. Results presented in Figure 17 indicate that the heating of plasma (56 °C for 30 min or 100 °C for 10 min) or its lyophilization significantly reduced hemocyte phagocytosis ($F = 28.6$, $df = 3,12$, $p < 0.01$). Similar levels of hemocyte phagocytosis between the whole hemolymph (untreated) group and the group of washed hemocytes which had normal plasma added back prior to RBC exposure ($87.3 \pm 1.4\%$ and $82.9 \pm 3.9\%$, respectively, $p > 0.05$)

indicate that the washing and plasma add-back procedures were not a major contributor to the observed reduction in phagocytosis. Overall, the results of this experiment demonstrated that C. fluminea hemocyte phagocytosis of formalin-fixed RBCs was plasma dependent and the plasma factor(s) responsible for mediating erythrophagocytosis was heat-labile and unstable to freeze-drying.

Another experiment was performed to determine if C. fluminea plasma contained factor(s) possessing opsonic properties. Results presented in Figure 18 show that a greater percentage of PBS-washed hemocytes was capable of phagocytizing plasma-pretreated and washed rabbit RBCs in the absence of clam plasma (washed hemocytes + PBS + opsonized RBCs, 85.6 $\pm$ 2%, n =4) than untreated rabbit RBCs (washed hemocytes + PBS + non-opsonized RBCs, 26.2 $\pm$ 4%, n =4) ($t = 12.1$, $p < 0.01$). Thus, it appears that the plasma factor(s) adsorbed onto RBC surfaces was capable of enhancing the direct interaction between hemocyte membrane components and target cells resulting in an enhanced phagocytosis.

Attempts to competitively inhibit hemocyte-mediated phagocytosis using a variety of mono- and dissacharides and glycoproteins were largely unsuccessful. Table 2 shows that the addition of sugars or glycoprotein prior to RBC introduction did not significantly reduce the rate of erythrophagocytosis exhibited by hemocytes ($F = 0.3$, $df = 13,36$, $p > 0.05$).

Finally, Figure 19 summarizes the results of an experiment designed to determine the effect of EDTA on the rate of hemocyte phagocytic and rosette forming activities. In general, there was a trend over time

for a reduction in the percentage of hemocytes forming rosettes for both half-strength hemolymph (1/2 hemolymph + 1/2 PBS) and 1 mM EDTA-containing half-strength hemolymph (1/2 hemolymph + 1/2 EDTA/PBS) samples. However, differences between these two groups over time were not significant at the $p = 0.05$ level. A significant increase in hemocyte phagocytosis was observed by 40 min in the hemocyte group incubated in PBS diluted hemolymph ($F = 7.3$, $df = 2,6$, $p < 0.05$), although this increase was not found in the EDTA containing PBS-diluted hemolymph group ($F = 2.1$, $df = 2,6$, $p > 0.05$). By the end of the experiment (60 minutes), however, both groups reached a comparable value in the percentage of phagocytizing hemocytes. In conclusion, EDTA at a 1 mM final concentration in half-strength hemolymph did not affect hemocyte-RBC rosette formation, but did exert a transient inhibitory effect on the rate of erythrophagocytosis.

Hemolytic Activity

In the modified plaque assay, the viability of hemocytes from the two test clams (clam 1 and clam 2) remained high (89 $\pm$ 2.6% and 86 $\pm$ 3.5%, respectively) at the end of 2 hr assay period. As the results indicate in Table 3, hemocytes in the two clam cell populations were equally capable of causing hemolysis of fresh vertebrate RBCs (plaques), but the amount of hemolysis caused by the hemocytes from clam 2 varied among the different targets ($F = 227.4$, $df = 2,3$, $p < 0.01$). Fresh sheep RBCs were the most susceptible targets for hemocyte-mediated hemolysis with 402.5 $\pm$ 15.5 hemocytes per 10^3 hemocytes capable of forming RBC-lysing plaques. In contrast, chicken

and horse RBCs (60.8 ± 15.8 hemocytes/10^3 hemocytes and 27 ± 9 hemocytes/10^3 hemocytes, respectively) were much less susceptible as targets. Also, there was a reduction in the percentage of plaques formed by hemocytes against sheep RBCs ($F = 245.5$, $df = 1,2$, $p < 0.01$), when temperature was lowered to 5°C.

Results of the Hb-release (2 hr) assay are summarized in Figure 20. It supports the plaque assay data by showing that C. fluminea hemocytes contained hemolytic substances against various mammalian RBCs. In addition, there was a hemocyte (effector) dose-dependency where increasing the ratio of effectors-to-targets produced an increased lytic effect (sheep $F = 35.83$, cow $F = 51.4$, horse $F = 52.7$, rabbit $F = 67.4$, guinea pig $F = 78.6$, and goat $F = 99.1$; $df = 3,18$; $p < 0.01$). In addition, different species exhibited different susceptibility to hemocyte lysis at a 1:5 effector-to-target ratio ($F = 10.55$, $df = 5,30$, $p < 0.01$) (sheep > cow > goat > horse > guinea pig > rabbit). A significant increase in the amount of Hb-release for all target cells was observed when the effector-target incubation was extended to 4 hr (sheep $F = 24.4$, horse $F = 60.1$, guinea pig $F = 55$, cow $F = 10.3$, rabbit $F = 25.2$, and goat $F = 14.3$; $df = 1,35$; $p < 0.01$). However, at this time, most targets reached a comparable amount of Hb-release. At 1:5 effector-to-target ratio, there was no difference found among all target cells, but, at 1:20 and 1:100 ratios, sheep, cow, horse, and guinea pig RBCs had significantly higher amount of Hb-release than those of rabbit and goat RBCs ($F = 8.9$, $df = 5,30$, $p < 0.01$ and $F = 6.6$, $df = 5,30$, $p < 0.01$, respectively).

The nature of the interaction between hemocytes and their RBC targets was further investigated ultrastructurally. The specific types of hemocytes could not be clearly identified due to changes in their cellular morphology (degranulation) during the interaction between effector and target (Figure 21). However, clearly defined subcellular structures and intact plasma membranes indicated that hemocytes were still highly viable. Many hemocytes were observed to possess electron-lucid endocytotic vacuoles instead of their normal granular or vesicular contents (Figure 22). Exocytosis of large granules was also evident (Figure 24). Hemocytes did not seem to take up RBCs after the reaction had proceeded for 10 and 30 min, although many lysed RBCs were seen in the preparation (Figure 21). Some RBC membranes were in close contact with hemocytes and they all exhibited varying degrees of lysis (Figure 23). In conclusion, this study had confirmed that C. fluminea hemocytes in the absence of plasma exerted a cytolytic reactivity toward various species of fresh mammalian RBCs through the release of lytic materials contained in their cytoplasm, presumably within cytoplasmic granules.

The Activities of Corbicula Plasma Aagainst Foreign Particles

Hemolytic Activity

C. fluminea plasma contained a naturally-occurring hemolysin(s) against five species of vertebrate RBCs. The plasma hemolytic activity was dose-dependent as evidenced by a marked decrease in the degree of hemolysis with increasing plasma dilutions after both 2 and 4 hr

reaction times ($F = 100.2$, $df = 4,24$, $p < 0.01$ and $F = 130.2$, $df = 4,24$, $p < 0.01$, respectively) (Figure 25). No apparent increase in the amount of Hb released was found when the plasma-RBC incubation time was extended from 2 to 4 hr ($F = 1.2$, $df = 1,30$, $p > 0.05$). In other words, plasma hemolysin(s) reached its maximum hemolytic capability within 2 hr. At full strength, 1/2 and 1/4 dilutions of plasma, the amount of hemolysis occurring with cow, goat, and sheep RBCs were similar to each other. However, plasma hemolysis of rabbit and guinea pig RBCs, was significantly lower (2 hr: $F = 3.6$, $df = 4,24$, $p < 0.05$, 4 hr: $F = 4.5$, $df = 4,24$, $p < 0.01$) when compared to the above three species, indicating that cow, goat, and sheep RBCs were considerably more susceptible to clam plasma hemolysin(s) than those of rabbit and guinea pig origin.

Corbicula plasma hemolysin(s) was heat-sensitive in that its activity was greatly reduced for both sheep (up to 1/4 plasma dilution) ($F = 4.3$, $df = 3,10$, $p < 0.05$) and cow (up to 1/8 plasma dilution) ($F = 18.2$, $df = 3,10$, $p < 0.01$) RBCs when plasma was pretreated with heat (56 °C for 30 min and 100 °C for 10 min) (Figures 26 and 27). The chelating agent, EDTA, at a 2 mM concentration, significantly reduced plasma hemolytic activity for sheep (up to 1/8 plasma dilution) ($t = 8.5$, $p < 0.01$) and cow (up to 1/16 plasma dilution) ($t = 4.8$, $p < 0.05$) RBCs (Figure 28).

Hemagglutination Activity

Clam plasma also contained a hemagglutination factor(s) active against horse, sheep, chicken, rabbit, mouse, and human A and B type

RBCs (Table 4). Rabbit and mouse RBCs were agglutinated at significantly lower concentrations of plasma (1/194 and 1/256, respectively) than the other RBC species tested. Formalin fixation of rabbit RBCs did not affect plasma agglutination activity on this target species. However, trypsinization increased plasma hemagglutination activity on rabbit RBCs. Also, an increase in the hemagglutination activity against fixed rabbit RBCs was recorded from both heat-treated and lyophilized plasmas. Lyophilized plasma did not dissolve completely into clam PBS. So, the above medium (lyophilized whole plasma) was centrifuged (300g, 10 min) and the hemagglutination activity of the supernatant and pellet were tested separately. It was found that, compared to the lyophilized whole plasma, the hemagglutination activity remained in the pellet, while it was greatly reduced in the supernatant.

Attempts to competitively inhibit plasma hemagglutination activity against rabbit RBCs using mono- and dissacharides and glycoproteins were unsuccessful (Table 5) ($F = 0.4$, $df = 11,24$, $p > 0.05$).

CHAPTER IV

DISCUSSION AND CONCLUSIONS

Cellular reactions involving circulating hemocytes represent one of the principal means by which hemocoelic invertebrates are able to recognize, and therefore, interact with foreign substances introduced to these animals. The clams selected for this study ranged from 3 to 5 cm in shell length and the concentration of hemocytes in the hemolymph varied from 6 to 12 X 10^5 cell/ml. However, no correlation was found between clam size and hemocyte concentration. A similar conclusion also was drawn by Foley and Cheng (1974) when they were studing the hematologic parameters of hemolymph cells of the quahaug clam, Mercenaria mercenaria, from two geographic locations. The concentration of circulating hemocytes in quahaugs varied between 14 to 20 X 10^5 cell/ml. Feng (1965, 1966) reported that variability in cell concentration might be attributed to differences in the physiological state of individual clams, since physiological factors could influence the clam's cardiac action which, in turn, represents a major determinant of circulating hemocyte concentration.

Criteria used for the classification of hemocytes have been quite varied. However, two principal cell-types, the agranular (hyaline) and

granular hemocyte, have been recognized in all bivalve species studied (Cheng, 1981). In this study, the size of hemocytes, the extent of cytoplasmic granulation, and granular morphology were used as criteria for classifying C. fluminea hemocytes. Based on these criteria, the estimated proportion of each hemocyte subpopulation was similar using both light and electron microscope analyses. According to the extent of cellular granulation, C. fluminea hemocytes could be separated into two general categories: hyaline hemocytes (small cells without prominent cytoplasmic granules) and granular hemocytes (including both vesicular and large granular hemocytes). This matches closely the classifications found in studies of other bivalve species, including Crassostrea virginica (Cheng and Rifkin, 1970; Cheng and Foley, 1972; Cheng and Cali, 1976; Cheng, 1975; Foley, 1975; Moore and Eble, 1977), Mytilus edulis (Moore and Lowe, 1977), Mya arenaria (Huffman and Tripp, 1982), and Tapes semidecussata (Cheney, 1971). However, in these studies, reseacher had also used other characteristics such as cytoplasmic staining affinities for certain histochemical stain, presence or absence of selected enzyme activities, and nucleus-to-cytoplasm ratios to subdivide the hemocyte subpopulations. Hemocytes of C. fluminea could also be seperated into three size classes: the small hyaline hemocyte (5 to 8 microns), vesicular hemocyte (8 to 15 microns), and large granular hemocyte (12 to 20 microns). The overlap in size between morphologically distinct hemocyte subpopulations is very common in bivalves (Cheng, 1971; Folry and Cheng, 1974; Moore and Lowe, 1977; Renwrantz et al., 1979). Therefore, classification based on hemocyte morphology may be a more

reliable way to differentiate bivalve hemocytes.

Structurally, small hyaline hemocytes of C. fluminea were most similar to the hyalinocyte of C. virginica (Foley and Cheng, 1972, Renwrantz et al., 1979) and M. mercenaria (Cheng and Foley, 1975; Moore and Eble, 1977), and the leucocyte of T. semidecussata (Cheney ,1971) in lacking prominent cytoplasmic granules. Corbicula hemocytes differed slightly in size, numbers of cell processes, density of cytoplasmic organelles, and shape, however. The high amount of RER, mitochondria, and Golgi complex present in these small hyaline cell also suggest the presence of a high synthetic machinery.

The vesicular hemocyte of C. fluminea with their many small, electron-dense cytoplasmic granules, was similar to the granulocytes of M. mercenria (Cheng and Foley, 1975; Moore and Eble, 1977) and M. edulis (Moore and Lowe, 1977). Also, vesicles of C. fluminea vesicular hemocytes were highly irregular in shape and had a lucid zone between the vesicular content and its delimiting membrane. This lucid zone structure may have been artifactual, being caused by differential tissue shrinkage during fixation, since it was not always observed in hemocytes from other sample preparations. Vesicles appear to correspond to the small refractile granules observed in this hemocyte using phase contrast microscopy based on the close relation between relative cell size and the abundance of vesicular inclusions observed at the light and electron microscopic levels. In electron micrographs, Golgi complexes were often seen in close association with vesicles which suggest that these cellular inclusions may contain Golgi

secretory products. Similar granules in hemocytes of M. mercenaria have been shown to contain acid phosphatase activity (Yoshino and Cheng, 1976), indentifying them as lysosomes. A similar role may be played by granules of C. fluminea hemocytes, since, at the light microscope level, AP and NE activity were found localized in these granules.

Cells similar to C. fluminea large granular hemocyte have not been reported from other species of bivalves. The electron-dense granules of C. fluminea large granular hemocytes were bound by a single membrane and were, structurally, quite distinct from the double unit membrane surrounding the large granules observed in the hemocytes of C. virginica and M. mercenaria (Cheng and Cali, 1974; Cheng and Foley, 1975). Rather, C. fluminea large granular hemocytes were most similar to those found in the granulocytes of crustaceans, both in relative size and fine structural appearance (Sternshein and Burton, 1980). Moreover, a developmental relationship between large granular and vesicular hemocytes of C. fluminea has been suggested based on the following observations: (1) the electron-dense granular content of large granular cells was very similar to that of the vesicles from vesicular hemocytes, (2) cellular inclusions similar to vesicles of vesicular hemocytes were often seen in the large granular cells, (3) the observation of a cell-type which possessed the characteristics of a vesicular cell, but which contained a typical granular hemocyte granule, and (4) the finding that both cell-types contained discretely localized AP and NE activity at the light microscope level. Therefore, it is concluded that the vesicular and granular hemocytes represent

cells of a similar ontological lineage. In this respect, granular cells from other bivalve species are known to be composed of cells which differ in size and granular staining affinity and such differences are thought to depend on the developmental stage of the individual cells (Dundee, 1953; Galtsoff, 1964; Feng et al., 1971; Foley and Cheng, 1972, 1974). Additional cytochemical, histological, and physiological studies of hemocyte are needed to further clarify the ontogeny of hemocytes in C. fluminea.

Studies of the cytochemistry of hemocytes provide important information which may be used, not only in the classification of the various cell types, but also in unraveling questions concerned with the development and function of these cells. Similar to other species of bivalves (Eble, 1966; Feng et al., 1971; Cheng and Rodrick, 1974, 1975, 1976a, 1976b; Moore and Eble, 1977; Huffman and Tripp, 1982), the lysosomal enzymes AP and NE were found in the hemocytes of C. fluminea. The electron-opaque vesicles or granules present in C. fluminea hemocytes are presumed to be the storage organelles (lysosomes) for these cells, since enzyme activity was restricted to discrete granules at the light microscope level. Similar electron-dense granules present in the quahaug clam, M. mercenaria, have been shown to contain AP and are, thus, considered to be true lysosomes (Yoshino and Cheng, 1976). Nevertheless, additional cytochemical studies are needed to clarify the exact nature of these cytoplasmic granules of C. fluminea hemocytes.

Lysosomal enzymes are important in the intracellular degradation of phagocytized material as well as in the extracellular destruction of

infectious agents (Cheng and Rodrick, 1974; Rodrick and Cheng, 1974; Cheng, 1976; Foley and Cheng, 1977; Cheng, 1983). The enzyme content of hemocytes may be quite variable, and appears to be dependent on the age and the physiological state of an organism (Eble, 1966; Feng et al., 1971; Cheng and Rodrick, 1974; Moore and Eble, 1977). The amount of AP and NE activity present in C. fluminea hemocytes was similar among individual clams studied. This result was not surprising, since clams of similar size were selected for the study and were acclimated under similar laboratory conditions and temperature for 3 to 7 days before being tested. The observations of fusion of the vesicles or granules with phagocytized RBCs as well as exocytosis of the granular material by large granular cells suggest that these lysosome-like cellular inclusions are active participants in endo- and exocytotic functions.

Peroxidase (PO), an important intracellular bactericidal enzyme, is found in membrane-bound organelles of vertebrate leukocytes (Master and Holme, 1977). The occurrence of PO in invertebrate hemocytes is not uniform. Cell from the fresh water snails, B. glabrata (Granath and Yoshino, 1983), Lymnaea stagnalis and Bulinus truncatus (Sminia and Burendsen, 1980), have been shown to possess PO activity, while, hemocytes (coelomocytes) of the earthworm, L. terrestris (Stein and Cooper, 1978), insects, Blaberus craniifer and Galleria mellonella (Anderson et al., 1973; Chain and Anderson, 1983) and bivalves, C. virginica and M. mercenaria (Cheng, 1975) apparently lack this enzyme. Like other bivalves, C. fluminea hemocytes did not contain PO. Therefore, intracellular bactericidal activity in these PO-deficient

invertebrates may be dependent on other enzyme systems such as lysozyme (Cheng and Rodrick, 1975; Anderson and Cook, 1979) or other non-lysozyme-like bactericidal chemicals such as the cecropins found in the insect, Hyalophora cecropia (Hultmark et al., 1980).

It is known that morphologically similar hemocytes from other molluscan species can differ in their size, staining affinities, as well as cellular density (Feng et al., 1971; Foley and Cheng, 1972, 1974; Bayne et al., 1979; Renwrantz et al., 1979; Cheng et al., 1980). The results of the Percoll cell separation study have confirmed the above findings by showing that C. fluminea hemocytes with similar morphological features could differ in their cellular density since cells from each of the morphologically distinct hemocyte subpopulations were collected at the same Percoll concentrations (65% and 75%). As it turns out, the density of C. fluminea hemocytes varied tremendously. Preliminary studies, using continuous Percoll or sucrose density gredients, cells could be separated into from 3 to 6 major cell fractions, each containing a mixture of the different cell types. Therefore, it is likely that a much greater range of cellular densities occurs among the hemocyte population than is indicated by the results of the step gradient technique. Nevertheless, the step gredient method was effective in greatly enriching one of the cell-types, the large granular hemocyte population.

Hemocytes of C. fluminea were capable of phagocytizing formalin-fixed mammalian RBCs in an in vitro system. As has been observed in other molluscan species (Tripp and Kent, 1967; Stuart,

1968; Prowse and Tait, 1969; Anderson and Good, 1976; Hardy et al., 1977), C. fluminea hemocytes required a soluble plasma factor(s) before phagocytosis could take place. When erythrocytes were pre-treated with plasma, washed, and then presented to plasma-free hemocytes, phagocytic activity was restored to near normal levels, indicating that the plasma factor(s) may be functioning as an opsonin, probably through the enhancement of surface interaction between the hemocytes and the RBCs. The heat-labile property of this factor(s) suggests that it may be protein in nature. Also, a reduction in the plasma opsonic effect after freeze-drying indicates that the opsonic factor(s) is sensitive to freeze-drying. However, the lyophilized plasma was reconstituted in clam PBS, thus, a possible increase in the overall ionic concentration in the system to hypertonic levels may have significantly contributed to the reduction of hemocyte erythrophagocytosis in test group to below plasma control levels.

Opsonin-like substances have also been indentified in a number of molluscan species including Helix pomatia (Renwrantz et al., 1981), H. aspersa (Prowse and Tait, 1969), Lymnaea stagnalis (Sminia et al., 1979; van der Knaap et al., 1981; van der Knaap, 1983), and M. edulis (Renwrantz and Stahmer, 1983). In some species, opsonins were found to be lectin-like molecules which possess agglutinin activity (Hall and Rowlands, 1974; van der Knaap et al., 1981; Renwrantz et al., 1981; Renwrantz and Stahmer, 1983). The plasma opsonin-like substance and the hemagglutinin from C. fluminea showed similar physical properties such as sensitivity to heat and freeze-dry treatments. In addition, several species of mono- and dissacharides and glycoproteins had no

inhibitory effect on the activities of either material. However, until the opsonin and/or agglutinin in C. fluminea plasma is isolated and purified for further testing, the relationship between these two substances remains an open question.

Like vertebrate neutrophils and monocytes (Rabinowitz, 1964), hemocytes of C. fluminea also required plasma to adhere to and spread on glass surfaces. This was evident when hemocyte monolayers were washed free of plasma components and then incubated in PBS. Hemocytes treated in this manner were not only poorly phagocytic but also retracted their cell processes, becoming rounded. Upon re-introduction of plasma to such preparations, cells would again re-spread on the glass slide surface. Phagocytic activity was also restored. One possible explanation for this phenomenon is that a plasma ligand, specific to cell surface adhesive receptors, was absorbed onto the substratum permitting cellular adhesion and spreading to take place (Grinnell, 1978). Such a ligand, fibronectin, has been shown to mediate substrate attachment and spreading in mammalian fibroblasts (Grinnell and Hays, 1978; Grinnell et al., 1980), and, in fact, both spreading and phagocytosis by vertebrate fibroblasts against various sized latex beads have been shown to be mediated through similar lectin and fibronectin receptors on fibroblast plasma membrane (Grinnell, 1984). Serum-mediated macrophage spreading onto its substratum has been regarded as a cell trying to take up (i.e., phagocytize) a particle of infinite diameter (North, 1968, 1970). Fibronectin (or fibronectin-like) substances are widely distributed in the animal kingdom having been identified in the simplest metazoan, sponges

(Labat-Robert et al., 1981), to more advanced invertebrate forms (Spiegel, 1980; Yoshino, 1983; Akiyama and Johnson, 1983). It is possible that similar molecules in Corbicula plasma are mediating attachment and spreading of hemocytes to the glass surface although other molecules (e.g., agglutinins) may also be involved. Lectins and immunoglobulins specific for cell surface receptor also have been shown to promote cell adhesion and spreading (Grinnell, 1976) as well as cellular activities such as mitogenesis (Nicolson, 1974, 1976), endocytosis (Storrie, 1979) and locomotion (de Petris, 1977). Invertebrate hemocytes have shown to possess cell surface receptors for various lectins (Roch and Valembois, 1978; Cheng et al., 1979; Yoshino et al., 1979; Schoenberg and Cheng, 1980b; Yoshino, 1981 and 1983), and cellular events such as receptor redistribution and endocytosis have been stimulated by binding of lectins to their surface receptors (Schoenberg and Cheng, 1980a, 1982; Yoshino, 1981, 1982). Therefore, it is again possible that C. fluminea hemocytes possess membrane receptors for similar endogenous lectin-like plasma ligands mediating various cellular activities including substrate attachment and spreading, cell-cell aggregation and endocytosis.

Non-specific factors such as charge effects (e.g., electrostatic or hydrophobic interactions) and humidity also may influence phagocytosis and spreading (Wilkinson, 1976). In this regard, a preliminary study showed the addition of Ca^{++} ions to C. fluminea hemocytes, freed of plasma and incubated in PBS, also enhanced the uptake of RBCs by hemocytes. It is likely that Ca^{++} molecules may be acting as non-specific ligands in bridging phagocytes with their targets through

electrostatic interactions (since most cellular membranes carry negative charges) (Wilkinson, 1976; Grinnell, 1978). Another possible explanation for the Ca^{++}-mediated increase in Corbicula hemocyte phagocytosis is that Ca^{++} is able to activate hemocyte membrane bound opsonin(s) and induce hemocyte phagocytosis, since divalent cations are essential for some ligand-mediated phagocytic events of vertebrate phagocytes (Rabinovitch and DeStefano, 1973). The chelator, EDTA, has a higher affinity for Mg^{++}, and thus, the above statement does not contradict the previous finding that EDTA did not inhibit Corbicula hemocyte phagocytosis.

The results of rosette/phagocytosis experiments demonstrated that the attachment of RBCs onto the hemocyte surface was a prerequisite for phagocytosis. This was based on the observation that the percentage of rosette forming hemocytes decreased as the prevalence of phagocytizing hemocytes increased over time. The attachment of target particles on the surface of phagocytes in other vertebrate and invertebrate systems is considered to be the first stage of phagocytosis (Rabinovitch, 1967) and, thus, these observations are consistent with the conventional process of phagocytosis.

Divalent cations have been shown to be required for a variety of cellular functions including cell-substrate adhesion, cell spreading, and phagocytosis of foreign particles by phagocytes (Rabinovitch and DeStefano, 1973, 1974, and 1976). The Mg^{++}/Ca^{++} chelator, EDTA, at a concentration of 1 mM, did not affect C. fluminea hemocyte rosette formation, although, it did delay the process of RBCs uptake by

hemocytes. Two possible explanations for the observed different effects of EDTA on rosetting and RBC internalization include: (1) that there exits a difference in the divalent cation concentration required for each process (particle attachment vs particle internalization) or (2) that binding of targets to the phagocyte membrane induces a divalent cation efflux (Stossel, 1981), which, in turn, could replenish the local concentration of divalent cation in the medium needed for target internalization. Thus, the time lag in phagocytosis observed in the EDTA-treated samples may have been caused by the time required by hemocyte to regulate their local cation concentrations. Similar to phagocytosis, it has been shown that cellular energy and divalent cations, are also necessary for cell-to-cell adhesion (Grinnell, 1978). In this study, an addition of EDTA (2 to 4 mM) into the cell's medium in the cold (0 to 5°C) has been effective in reducing in vitro hemocyte clumping because EDTA reduces divalent cation concentrations in the medium while the low temperature slows down cellular metabolism, reducing the energy necessary for this process.

Like many other molluscan species (Tripp, 1966; Acton et al., 1969; Hardy et al., 1978; Renwrantz and Mohr, 1978; Sminia et al., 1979; Renwrantz and Berliner, 1978; van der Knaap et al., 1983; Renwrantz and Stahmer, 1983), the plasma of C. fluminea also contains a naturally occurring hemagglutinin(s) which reacts with several species of vetebrate RBCs. The sugar binding specificity of the hemagglutinin(s) has not been determined, and, whether differences observed in the titers against the different RBC targets is due to the presence on RBCs of different agglutinin receptors or differences in the density of

receptors on individual RBCs for the various RBC species still remains an open question. Trypsinization of rabbit RBCs increases the hemagglutination titer against this target species. Trypsin-induced proteolysis is probably exposing more hemagglutinin receptors on the RBC surfaces, since this has been shown to be the case in other agglutinin studies (e.g., Renwrantz and Cheng, 1977). Heat-treatment or freeze-drying of clam plasma actually enhanced hemagglutinin titers against rabbit RBCs, although, this activity was found only in the insoluble fraction following heating at 100°C for 10 min (coagulated fraction) or freeze-drying. One explanation for this observation is that protein(s) possessing agglutinating activity under these conditions are being denatured, but, the reaction sites are remaining unchanged. The active agglutinin(s) in an insoluble aggregated form would possess multiple binding sites, thus providing an extremely efficient "nucleus" for RBC agglutination to occur.

The activity of hemagglutinins in vertebrates and some invertebrates have been shown to be dependent on divalent cations (Marchalonis and Edelman, 1968; Acton et al., 1969; Uhlembruck et al., 1975; Renwrantz and Stahmer, 1983). In contrast to these findings, EDTA at a 2 mM concentration did not affect C. fluminea hemagglutinin activity. It is possible that, like some invertebrate agglutinins (Anderson et al., 1972; McDade and Tripp, 1977; Anderson, 1980), the activity of C. fluminea hemagglutinin is not dependent on divalent cations, although, alternatively, 2 mM EDTA in the medium may not have been enough to block out completely the cations necessary for the reaction to proceed. From previous studies (Anderson et al., 1972; McDade and Tripp, 1977) ,

however, 2 mM EDTA represents a relative high chelator concentration and should have been able to at least reduce significantly a divalent cation-dependent reaction.

The spontaneous killing (cytolysis) of non-self targets of cellular entity exhibited by hemocytes of C. fluminea was similar to the natural cytotoxicity of mammalian large granular lymphocytes (Herberman and Ortaldo, 1981) and monocytes (Remington et al., 1975; West et al., 1977) in that effector cell activation is independent of prior antigenic exposure. Hemocytes from the mussel, keyhole limpet, starfish, and bloodworm also display a similar type of reaction toward normal and malignant vertebrate target cells (Decker, et al., 1981) and human RBCs (Wittke and Renwrantz, 1984). Moreover, like that of cytotoxic lymphocytes (Brunner, 1968) and natural killer cells (Patek et al., 1983) where target cell-killing is a dose-dependent phenomenon, a similar kind relationship has been demonstrated using clam hemocytes (effectors) and RBCs (targets). Thus, in experiments using these two cells, it was shown that decreasing the proportion of hemocytes in effector-target (E-T) cell mixtures resulted in a concomitant reduction in the amount of Hb released from RBCs. Also, there were consistent differences in the degree of hemolysis at given E-T cell ratios among the different target RBCs. Decker et al. (1981) suggested a target-specific killing mechanism for invertebrate hemocytes, and presented as evidence the inhibition of target cell killing by specific sugar compounds. Although, target specificity was not directly tested for Corbicula hemocytes, the clam cells did show a difference in their cytolytic activity against RBCs of different species at 2 hr of E-T

incubation. However, since all of the target RBC species used in the assay were susceptible to lysis, the observed differences in the amount of Hb released among target cell species at a given E-T ratio are thought to be caused by differences in receptor density on target cell surfaces, rather than differences in the specific nature of receptors on the different RBC species. Also, the phenomenon of E-T recycling may be occurring in hemocyte-mediated RBC lysis, since extension of the reaction time to 4 hr resulted in most of the targets reaching a comparable maxium amount of hemolysis regardless of the E-T ratio. Thus, 4 hr provided enough time for hemocytes to recycle and kill all targets even if there was a lower density of receptors present on a given RBC species. Moreover, electron microscopic observations of hemocyte-RBC pellets from the cytolytic experiments provided direct evidence that there was contact between membranes of RBCs and intact hemocytes (conjugate formation). However, the rapidity of the hemolytic reaction (2 hr) and the fact that hemocytes are able to form plaques (i.e., RBC lysis at considerable distances from an active hemocyte) strongly suggest that cytolytic activity is probably not based on the conventional E-T conjugation, killing, effector recycling scheme. Alternatively, it is suggested that C. fluminea hemocytes lyse RBCs through a degranulation event, as evidenced by electron microscope observations, and due to its temperature sensitivity. However, whether initial contact between effectors and targets is necessary to start the lytic reaction is not known. Also, the nature of the lytic substance secreted by hemocytes is uncertain at this time. Many molluscan hemocytes are known to synthesize hydrolytic enzymes and subsquently

release them into hemolymph (reviewed by Cheng, 1983), but the specific characterization of membrane-lysing soluble mediators has not yet been accomplished.

The plasma of C. fluminea contains a naturally occurring lytic factor(s) which was found to be active against several species of vertebrate RBCs. Based on its loss of activity in the presence of EDTA and after the heat treatment, it would appear that C. fluminea hemolysin has a requirement for Ca^{++} and/or Mg^{++} for maximum acitivity and most probably is a heat-labile protein. These properties are very similar to those described for other hemolytic substances from several other species of invertebrates (Cooper, et al., 1974; Roch, 1979; Anderson, 1980; Parinello and Rindone, 1981). All of the five species of vertebrate RBCs tested were sensitive to the lytic substance(s), although consistent differences in the intensity of hemolysis were noted between some of the target RBC species. Parinello and Rindone (1981) performed a cross-species absorption study of the hemolysin from the annelid worm, Spirographis spallanzanii, and were able to cross-absorb out hemolysin activity using combinations of the various RBC species. Their results suggest that the various mammalian RBCs tested had similar reactive sites for the hemolysin and, therefore, differences in susceptibility among RBC species was due to differences in the distribution and/or abundance of hemolysin receptors on RBC surfaces. In contrast, Anderson (1980) believes that the differences in hemolytic activity observed for another annelid hemolysin (Glyura dibranchiata) against sheep and rabbit RBCs was due to species' specificity, since, some, but not all, cross reactivity between the two

species could be removed by heterologous RBC absorption. In the present study, differences in target cell susceptibility observed among all five RBC species were consistent within and between experiment using 2 and 4 hr incubation periods. Still, the cause of the difference cannot be determined until cross-species absorption studies are performed.

Many of the lytic substances present in the plasma of molluscan species are lysosomal enzymes and it is believed that the granulocyte, through its secretory activity, represents one of the major sources of these enzymes (reviewed by Cheng, 1983). A preliminary comparison of the nature of the hemolytic substances from hemocyte lysates and the plasma of C. fluminea indicates that cellular and plasma hemolytic components are resistant to a protease inhibitor (PMSF) and are heat-labile. Also, a similar lytic reactivity to the various species of RBCs (sheep and cow > goat > rabbit and guinea pig) was found between the hemocyte and plasma hemolysin. These observed similarities suggest that the hemocytes may be at least one of the sources of the plasma hemolysin, although, further biochemical studies are needed to address this question, as well as to characterize the chemical nature of the lytic molecule(s) itself.

This study has demostrated that the Asian clam, C. fluminea, contains a morphologically heterogeneous population of hemocytes which can be classified into three groups; namely small hyaline hemocytes, vesicular hemocytes, and large granular hemocytes. All three cell-types are capable of recognizing and phagocytizing, in vitro,

formalin-fixed vertebrate RBCs. This *in vitro* phagocytic response is dependent upon a heat-labile component(s) present in clam plasma. In addition, hemocytes, as well as clam plasma, contain a cytolytic substance which is reactive against a variety of vertebrate RBC targets. Similarities in the physico-chemical and functional properties of the hemocyte and plasma lysins suggest that these are the same molecule and that hemocytes may represent an important source of circulating plasma lysin. Plasma alone was capable of agglutinating several species of fresh and formalized vertebrate RBCs.

Therefore, it is clear from this study that *Corbicula* possesses, in its hemolymph, a multifunctional system of cells and soluble factors which can recognize and react against foreign particulate substances. The different components of this system may function alone (plasma lysin) or they may need to work together to achieve their defensive functions, as in the case of phagocytosis.

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Table 1. Corbicula hemocyte phagocytosis using a variety of vertebrate RBCs (n=2).

RBC Species	PBS Control[1]	Experimental[2]
Sheep	21.3 (19.5, 23.1)[3]	87.5 (81.7, 93.4)
Horse	16.5 (18, 15)	70.7 (53, 88.5)
Rabbit	11.8 (14.7, 8.8)	89.1 (86.6, 91.5)
Human A	11.6 (15.4, 7.8)	92.1 (90.2, 94.2)
Human B	10.9 (19.2, 2.6)	94.3 (93.1, 95.5)
Human AB	7.3 (9.1,5.5)	90.1 (84.4, 95.7)
Human O	8.9 (7.0, 10.9)	88.0 (82.6, 93.3)

[1] Percentage of phagocytizing hemocytes which had been washed free of residual plasma prior to addition of test RBCs.

[2] Percentage of phagocytizing hemocytes in the presence of plasma.

[3] $\bar{X}$% (range)

Table 2. Results of the competitive inhibition assay on *Corbicula* hemocyte phagocytosis of fixed rabbit RBCs.

Carbohydrate Species	% Phagocytizing Hemocytes[1] (N)
Control	83.5 ± 5.1 (4)
Monosaccharides (0.03M and 0.1M)	
L-arabinose	9].5 ± 3 (2)
β-D-fructose	87.8 ± 2.9 (3)
D-galactose	88.5 ± 11.6 (3)
α-D-glucose	84.3 ± 0.5 (2)
β-D-glucose	83.5 ± 9.1 (2)
D-mannose	83.1 ± 3.9 (4)
Dissacharides (0.03M and 0.1M)	
D-cellobiose	73.8 ± 13.5 (4)
β-lactose	92.4 ± 3.4 (4)
α-D-melibiose	81.6 ± 4.2 (4)
N-acetylated sugars (0.03M and 0.1M)	
N-acetyl-D-galactosamine	77.3 ± 16.7 (2)
N-acetyl-D-glucosamine	86.8 ± 5.3 (4)
Glycoproteins	
Fetuin (0.02%)	72.3 ± 12.3 (4)
Mucin (0.07%)	83.1 ± 4.1 (4)

[1] $\bar{X}$ ± S.E. %

Table 3. Results of RBC[1] cytolysis slide tests enumerating the number of "plaque-forming" Corbicula hemocytes in two clam cell populations.

	Clam 1 (n=2)[2]		Clam 2 (n=2)[3]	
	5°C	24°C	5°C	24°C
Horse RBCs	2.0 ± 0.6[4]	83.8 ± 30.2	9.0 ± 4.0	27.0 ± 9.0
Chicken RBCs	33.5 ± 6.7	127.3 ± 6.7	15.8 ± 11.3	60.8 ± 15.8
Sheep RBCs	70.4 ± 3.3	412.0 ± 19	56.3 ± 15.7	402.5 ± 15.5

[1] Source of fresh RBCs used as target cells

[2] Hemocyte count 2.0 X 10^4 cell/ml; viability = 89%

[3] Hemocyte count 3.0 X 10^4 cell/ml; viability = 86%

[4] $\bar{X}$ ± S.E. plaque-forming hemocytes per 10^3 hemocytes

Table 4. Hemagglutinin activity of *Corbicula* plasma using RBCs from various vertebrate species as agglutinating test particles.

RBC Species	Sample Size (N)	Log_2 Titer ($\bar{X} \pm$ S.E.)[1]	Activity Units(Y)[2]
Chicken	(4)	3 ± 0.9	8
Horse	(4)	3 ± 1.1	8
Sheep	(4)	4 ± 1.5	16
Human A	(4)	3.3 ± 1.3	10
Human B	(4)	5.8 ± 1.5	56
Mouse	(4)	8 ± 1	256
Rabbit	(4)	7.6 ± 1.2	194
Trypsinized Rabbit	(4)	9 ± 1.3	512
Formalin-fixed Rabbit	(11)	7.1 ± 0.6	137
Formalin-fixed Rabbit with plasma in the presence of 2mM EDTA	(6)	7.5 ± 1.8	181
with heat-treated plasma	(4)	8.8 ± 1.2	446
with lyophilized plasma (Supernatent)	(3)	2.7 ± 1.5	7
(Pellet)	(3)	10.3 ± 1.8	1261

[1] $\bar{X} = \frac{\Sigma x}{n}$, $x = \log_2 y$, y = individual titer point

[2] Activity Units (Y) = $2^{\bar{X}}$

Table 5. Results of *Corbicula* hemagglutinin competitive inhibition assay using fixed rabbit RBCs (n=2).

Carbohydrate Species	Final concentration	Log_2 Titer ($\bar{X} \pm$ S.E.)[1]	Activity Units (Y)[2]
Control	-	5.5 ± 1.2	45
Monosaccharides			
L-arabinose	0.1M	3.5 ± 2.5	11
β-D-fructose	0.1M	4.5 ± 2.5	23
D-galatose	0.1M	3.5 ± 2.5	11
β-D-glucose	0.1M	3.5 ± 2.5	11
D-mannose	0.1M	4 ± 2	16
Disaccharides			
D-cellobiose	0.1M	6.5 ± 0.5	91
β-lactose	0.1M	6.5 ± 0.5	91
N-acetylated sugars			
N-acetyl-D-galatosamine	0.1M	4.5 ± 1.5	23
N-acetyl-D-glucosamine	0.1M	4 ± 2	16
Glycoproteins			
Fetuin	0.01%	4 ± 2	16
Mucin	0.035%	4.5 ± 1.5	23

[1] $\bar{X} = \frac{\Sigma x}{n}$, $x = \log_2 y$, y = individual titer point

[2] Activity Units (Y) = $2^{\bar{X}}$

Figure 1. Protocol used to measure phagocytosis of fixed RBCs by Corbicula hemocytes in the presence or absence of plasma.

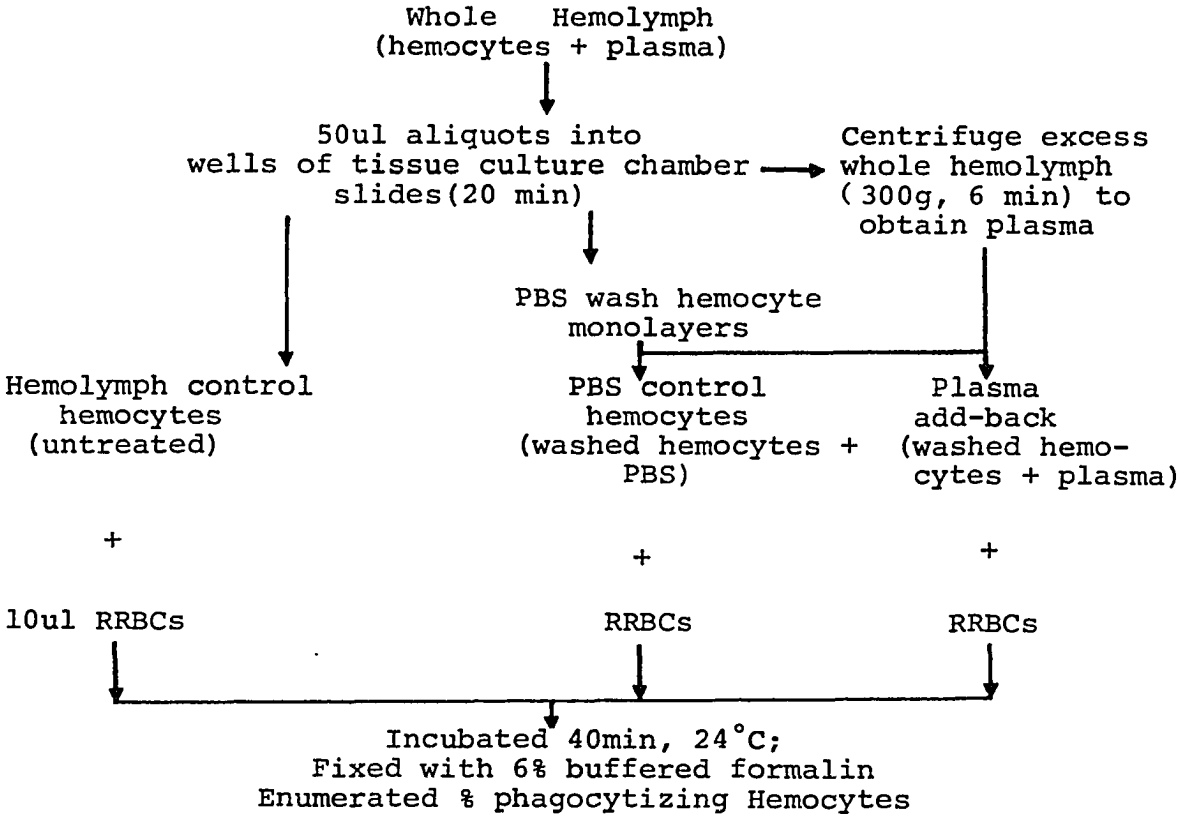
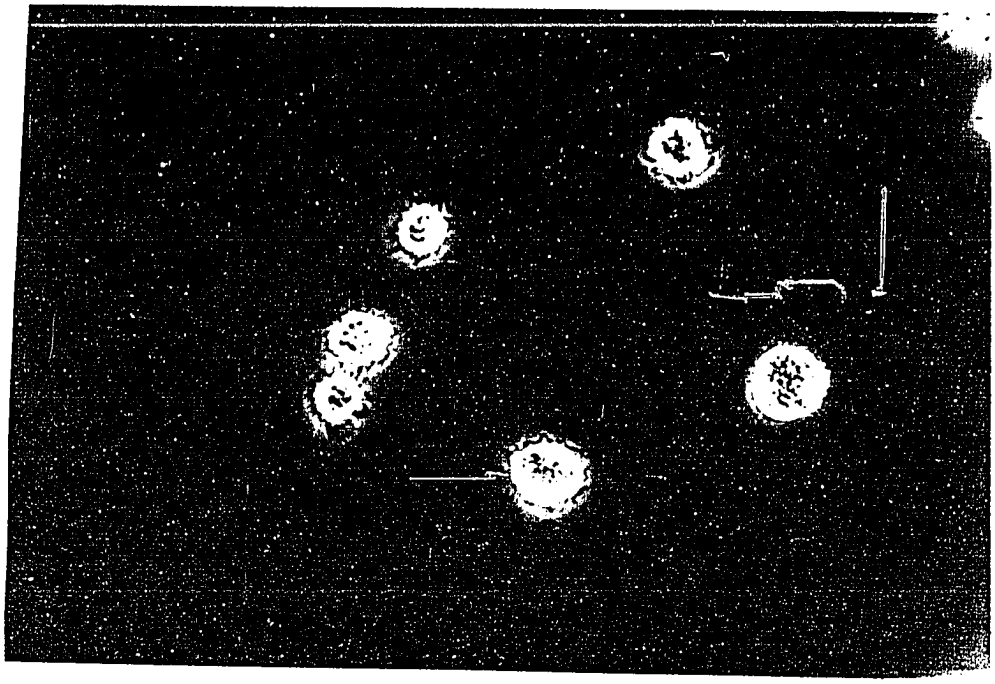


Figure 2. Rounded state of Corbicula fluminea hemocytes seen under phase contrast optics. Bar = 10 um. H - small hyaline hemocyte; V - vesicular hemocyte; G - large granular hemocyte.

Figure 3. Spread hemocytes of Corbicula fluminea. Bar = 8 um. H - small hyaline hemocyte; V - vesicular hemocyte with vesicles (arrow).

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H
H
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Figure 4. Spread hemocytes of Corbicula fluminea. Bar = 8 um. H - small hyaline hemocyte; V - vesicular hemocyte with vesicles (arrow); G - large granular hemocyte with granules (arrow).


4

Figure 5. Corbicula fluminea hemocyte clump with hemocytes migrating away from the clump. Bar = 6 um. C - center of the clump; R - ruffled mambrane.

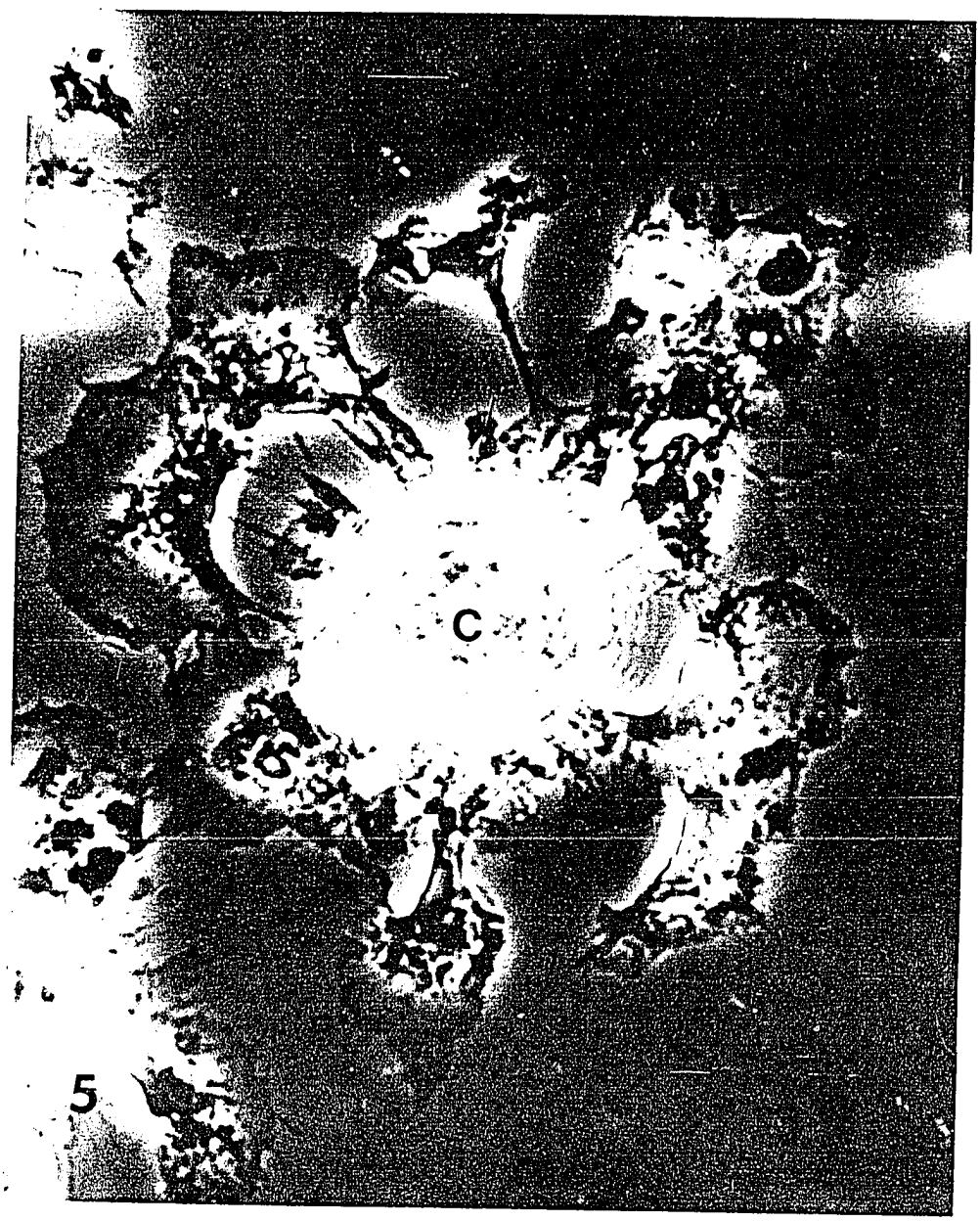
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Figure 6. Electron micrograph of Corbicula fluminea small hyaline hemocyte with prominent rough endoplasmic reticulum and mitochondria. Bar = 3 um. GL - glycogen granules; M - mitochondrion; N - nucleus; RER - rough endoplasmic reticulum; SER - smooth endoplasmic reticulum.

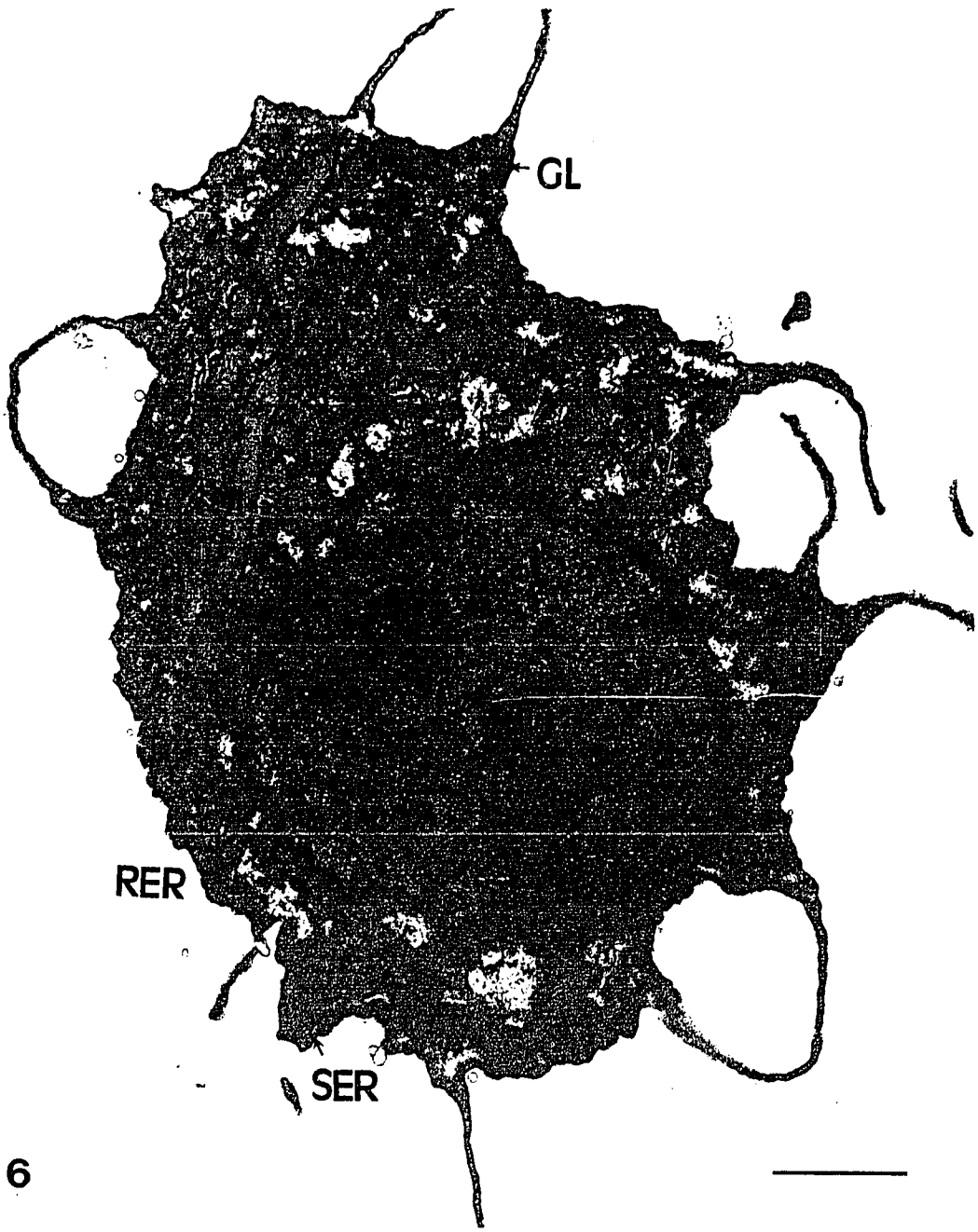

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Figure 7. Electron micrograph of Corbicula fluminea vesicular hemocyte characterized by the presence of many vesicles. Bar = 3 um. GL - glycogen granules; M - mitochondrion; N - nucleus; RER - rough endoplasmic reticulum; SER - smooth endoplasmic reticulum; V - vesicle.

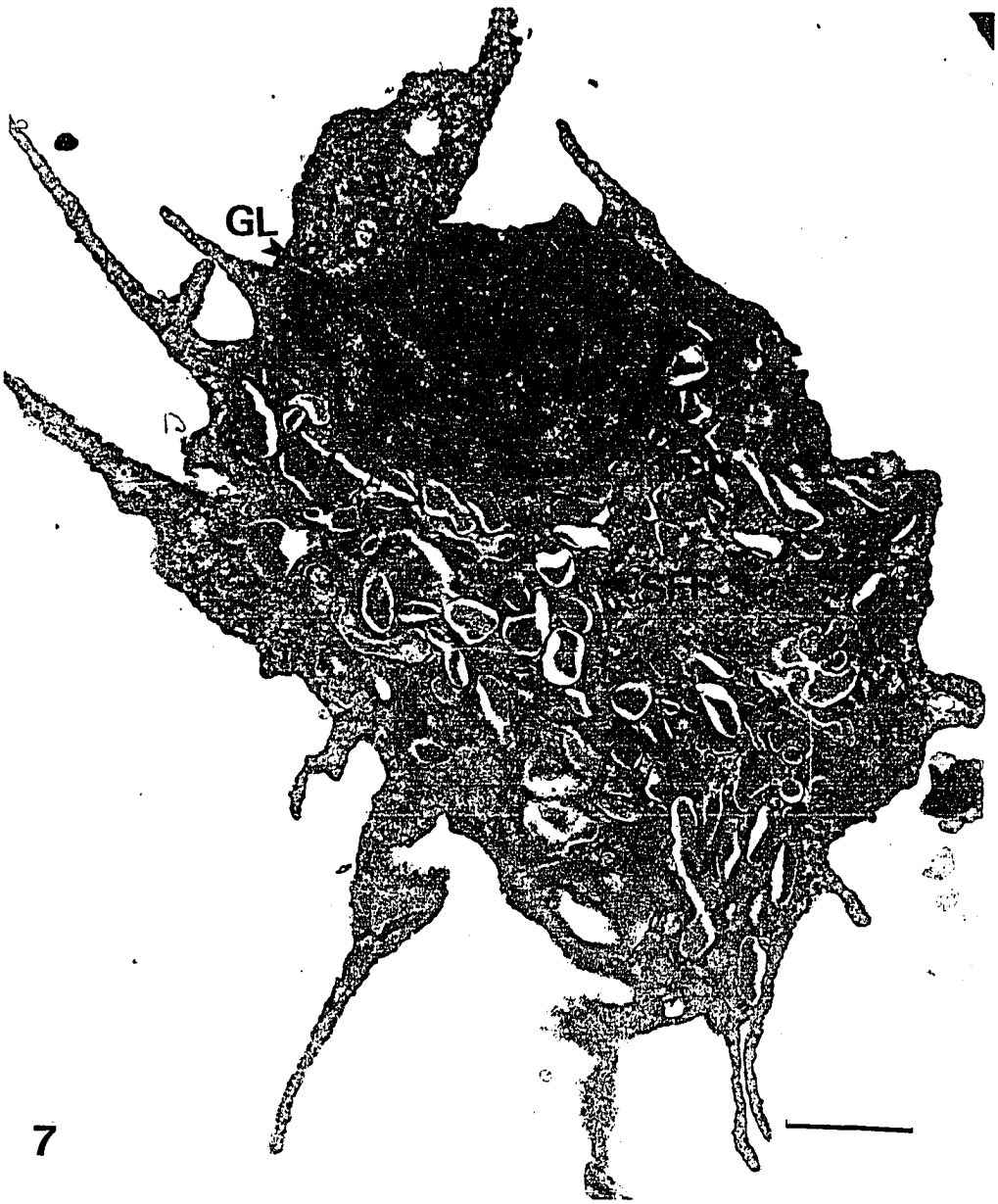

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Figure 8. Electron micrograph of Corbicula fluminea large granular hemocytes with large granules. Bar = 3 um. GL - glycogen granules; LG - large granule with both electron-dense and electron-lucid materials; LDG - large electron-dense granules; M - mitochondrion; N - nucleus; RER - rough endoplasmic reticulum; SER - smooth endoplasmic reticulum; V - vesicle.

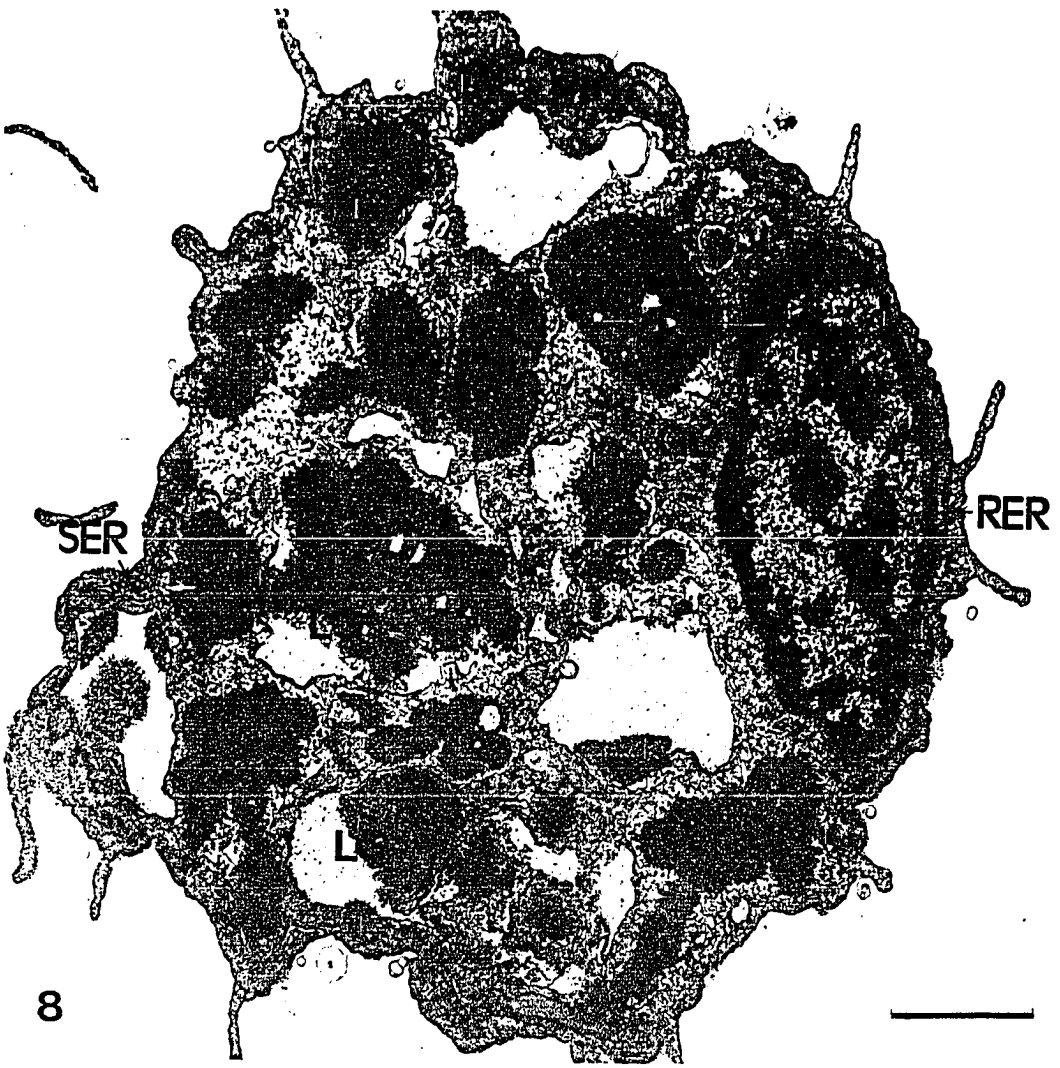

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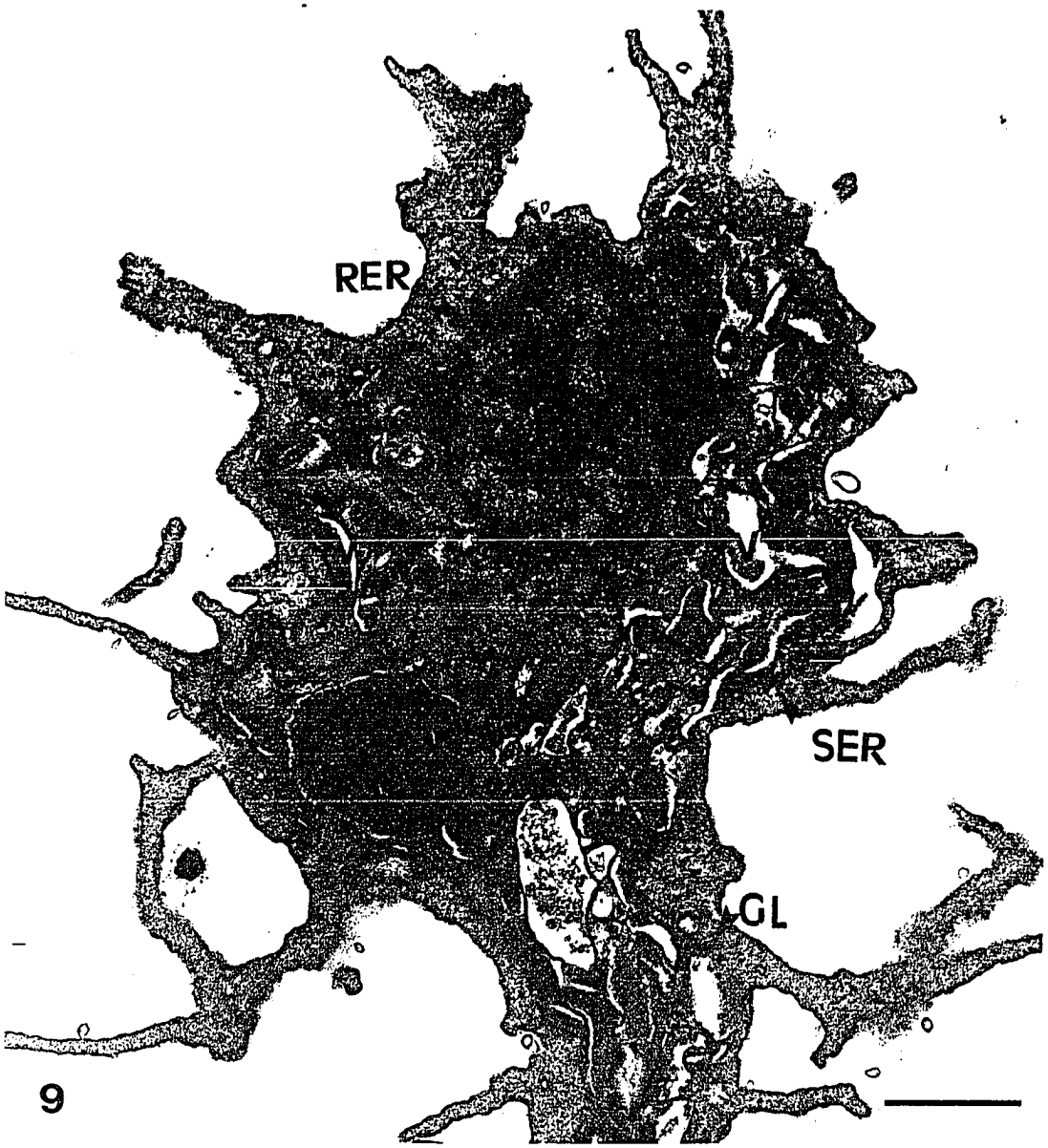
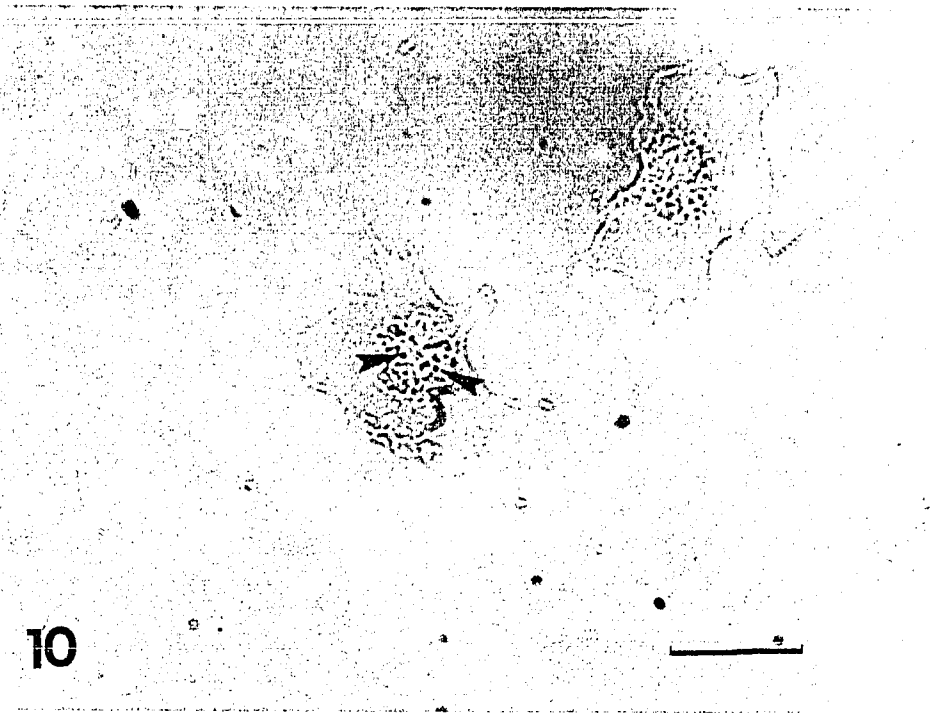
Figure 9. Electron micrograph of Corbicula fluminea intermediate-type hemocyte. Bar = 1 um. GL - glycogen granules; LDG - large electron-dense granule; M - mitochondrion; N - nucleus; RER - rough endoplasmic reticulum; SER - smooth endoplasmic reticulum; V - vesicle.



9

Figure 10. Corbicula fluminea hemocyte with acid phosphatase activity localized in granules in the perinuclear region (arrow). Bar = 8 um.

Figure 11. Corbicula fluminea hemocyte with moderate amount of non-specific esterase activity present in cytoplasmic granules (arrow). Bar = 8 um.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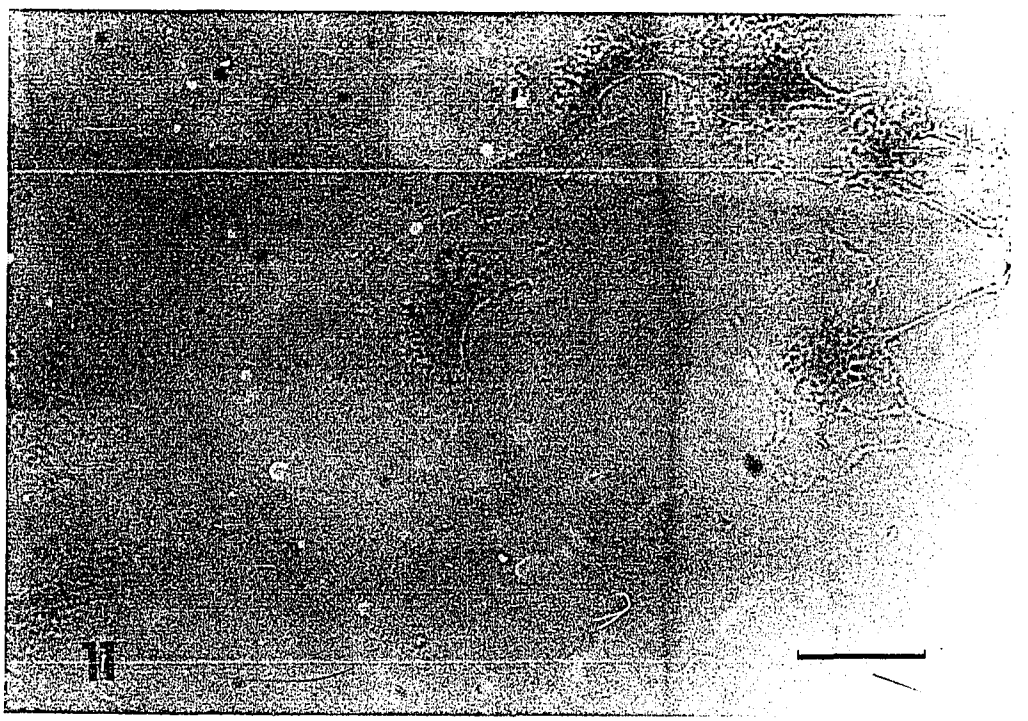
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FIGURE 12. Separation of morphologically distinct Corbicula hemecyte subpopulation using Percoll step gradient.

PERCOLL STEP GRADIENT (PERCOLL:0.1M CACODYLATE BUFFER)

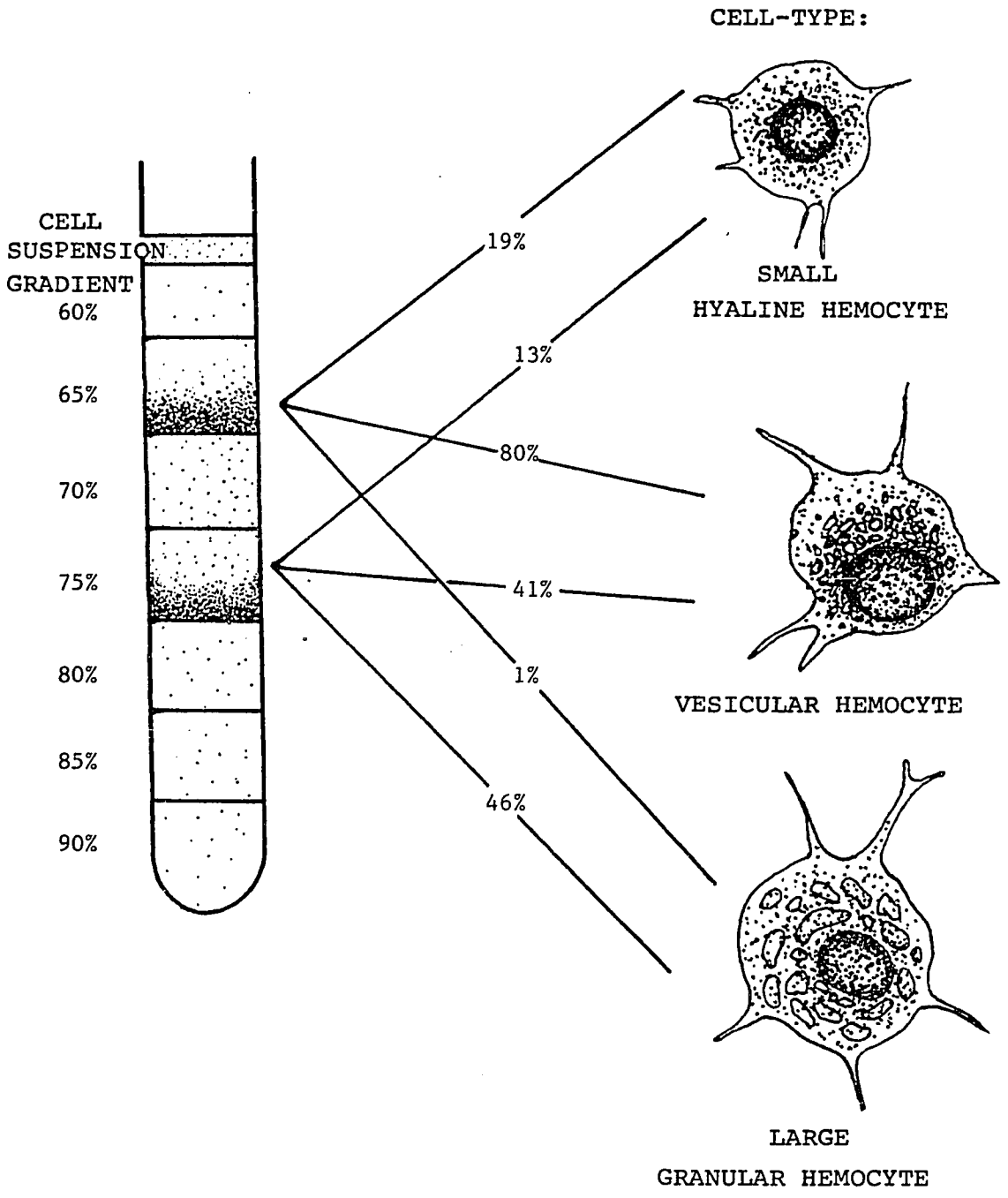
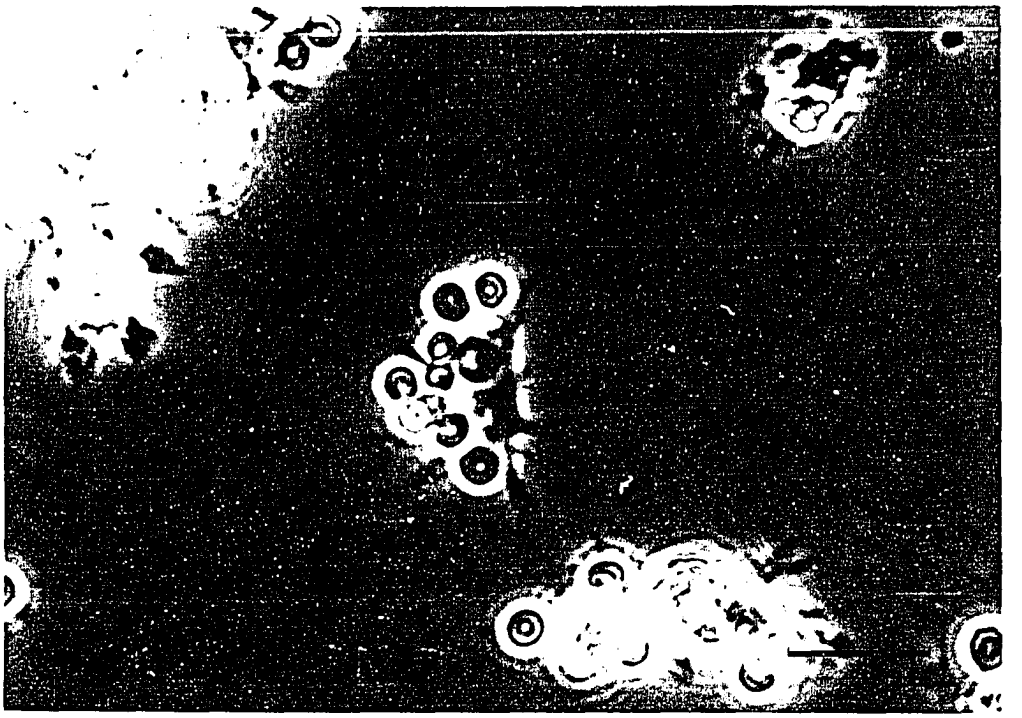


Figure 13. Corbicula fluminea hemocyte with RBC rosette formed around the hemocyte in the presence of clam plasma. Bar = 8 um. H - hemocyte; R - erythrocyte attached on the hemocyte.

Figure 14. Corbicula fluminea hemocyte phagocytosis (10 min). Bar = 8 um. aR - attached erythrocyte; H - hemocyte cytoplasmic extension; iR - internalized erythrocyte.



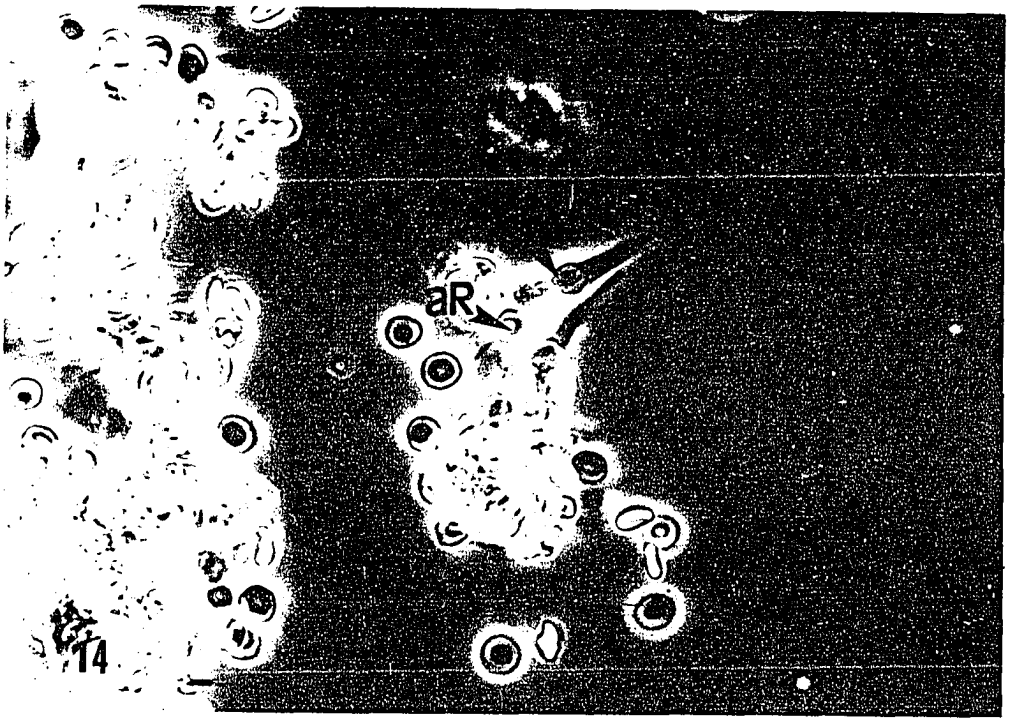

aR
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Figure 15. Corbicula fluminea hemocyte phagocytosis (40 min). Bar = 8 um. H - hemocyte with many internalized RBCs (iRBC).


15

Figure 16. The requirement of plasma for Corbicula hemocyte phagocytosis. Formalin-fixed rabbit RBCs were added to whole clam hemolymph (Untreated Hemocytes +RBCs), or hemocytes washed free of plasma and subsquently incubated in clam PBS (Washed Hemocytes + PBS + RBCs) or plasma (Washed Hemocytes + Plasma +RBCs) (n=10 with two replicates in each sample).

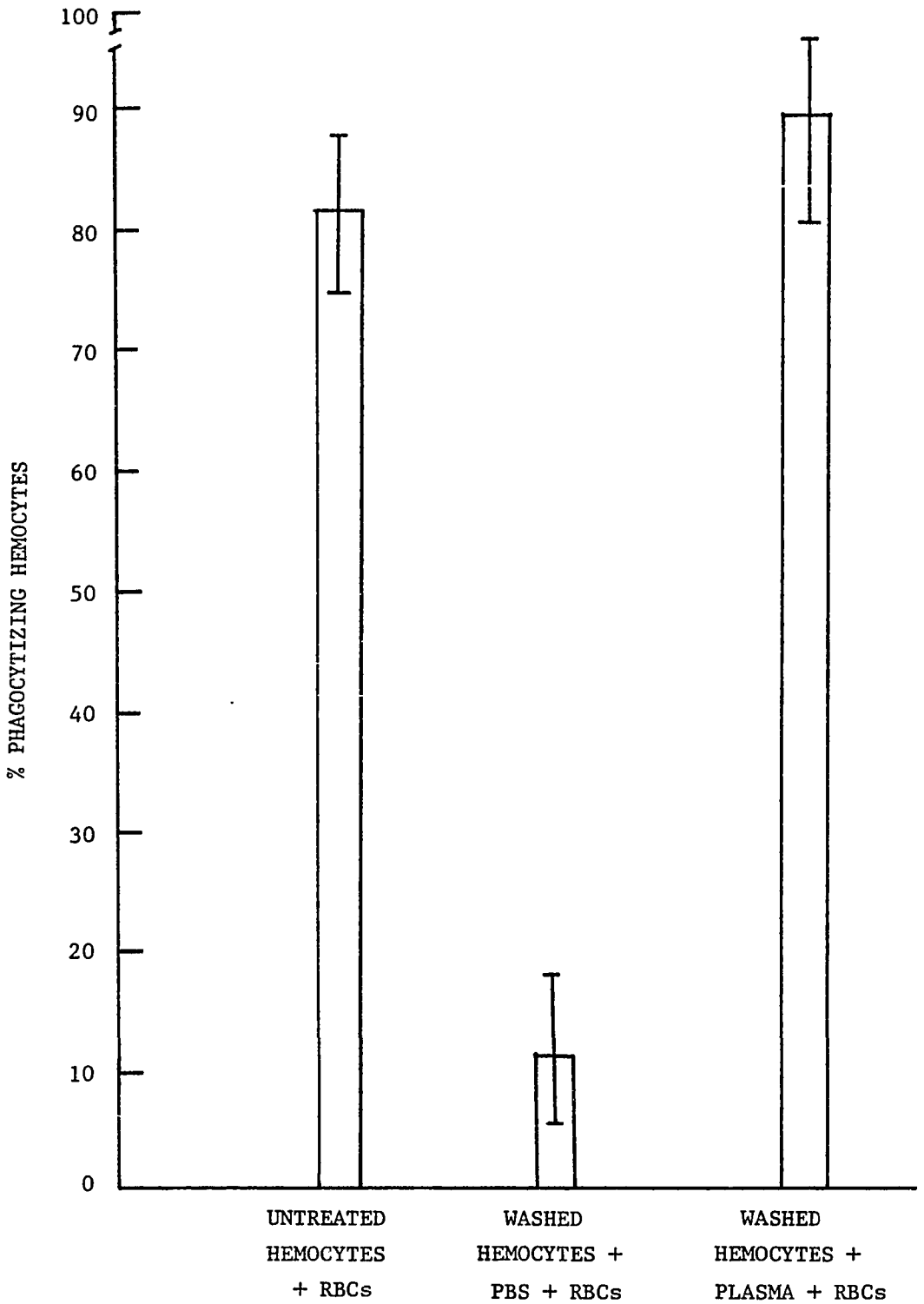

% PHAGOCYTIZING HEMOCYTES
100
90
80
70
60
50
40
30
20
10
0
UNTREATED HEMOCYTES + RBCs
WASHED HEMOCYTES + PBS + RBCs
WASHED HEMOCYTES + PLASMA + RBCs

Figure 17. The effect of some physical treatments on Corbicula plasma factor(s) mediating hemocyte phagocytosis. Portions of clam plasma were heat-treated or lyophilized (subsquently reconstituted in clam PBS) before adding back to the washed hemocytes. Positive and negative controls consisted of adding back of fresh plasma (Washed Hemocytes + Plasma + RBCs) or clam PBS (Washed Hemocytes + PBS + RBCs) (n=4 with two replicates in each sample).

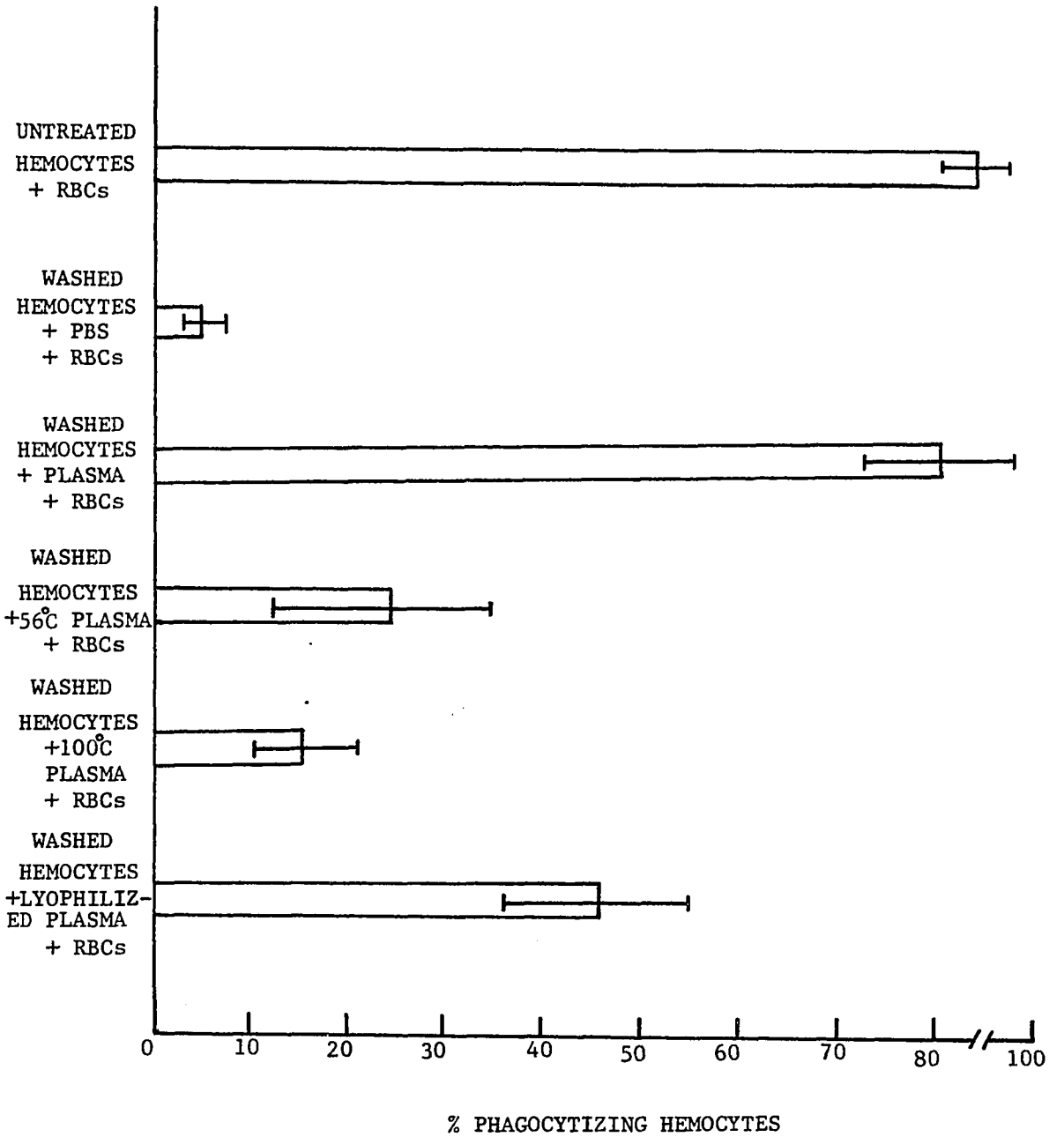

UNTREATED HEMOCYTES + RBCs
WASHED HEMOCYTES + PBS + RBCs
WASHED HEMOCYTES + PLASMA + RBCs
WASHED HEMOCYTES +56°C PLASMA + RBCs
WASHED HEMOCYTES +100°C PLASMA + RBCs
WASHED HEMOCYTES +LYOPHILIZED PLASMA + RBCs
0
10
20
30
40
50
60
70
80
100
% PHAGOCYTIZING HEMOCYTES

Figure 18. The effect of plasma treatment of RBCs (opsonized RBCs) on Corbicula hemocyte phagocytosis. Hemocytes were washed free of plasma and followed by the addition of opsonized (Washed Hemocytes + Opsonized RBCs in PBS) or untreated (Washed Hemocytes + Untreated RBCs in PBS) formalin-fixed rabbit RBCs. These preparations were compared to the controls consisting of addition of untreated RBCs to whole clam hemolymph (Untreated Hemocytes + Untreated RBCs) or hemocytes washed free of plasma and subsquently incubated in fresh plasma (Washed Hemocytes + Plasma + Untreated RBCs) (n=4 with two replicates in each sample).

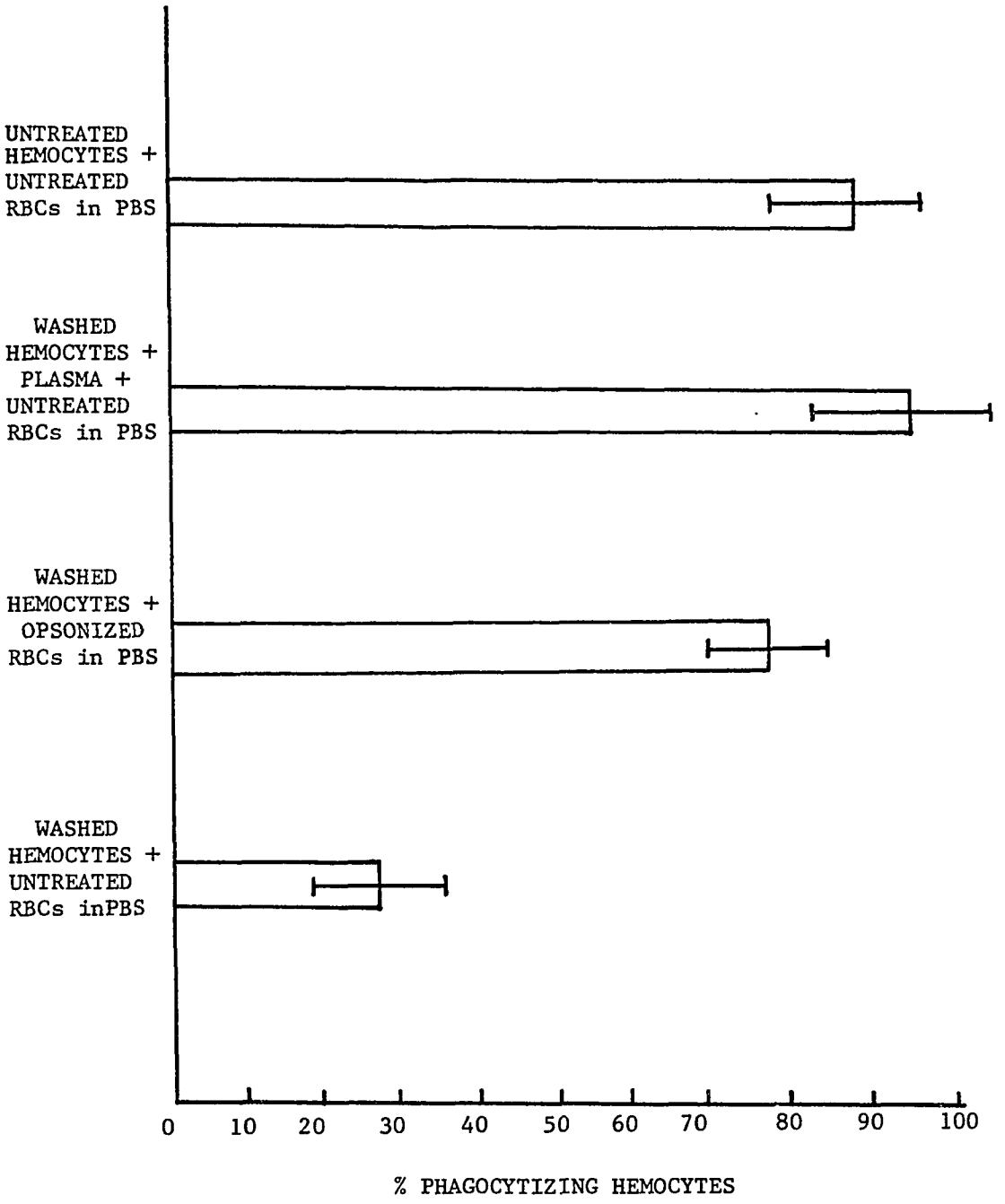

UNTREATED HEMOCYTES + UNTREATED RBCs in PBS
WASHED HEMOCYTES + PLASMA + UNTREATED RBCs in PBS
WASHED HEMOCYTES + OPSONIZED RBCs in PBS
WASHED HEMOCYTES + UNTREATED RBCs inPBS
0
10
20
30
40
50
60
70
80
90
100
% PHAGOCYTIZING HEMOCYTES

Figure 19. Kinetics of Corbicula hemocyte RBC phagocytosis and rosette formation in the presence (EDTA Treated) and absence (Untreated) of 2mM EDTA. Phagocytosis was assessed in a test tube assay as described in the Materials and Methods (n=4 with two replicates in each sample).

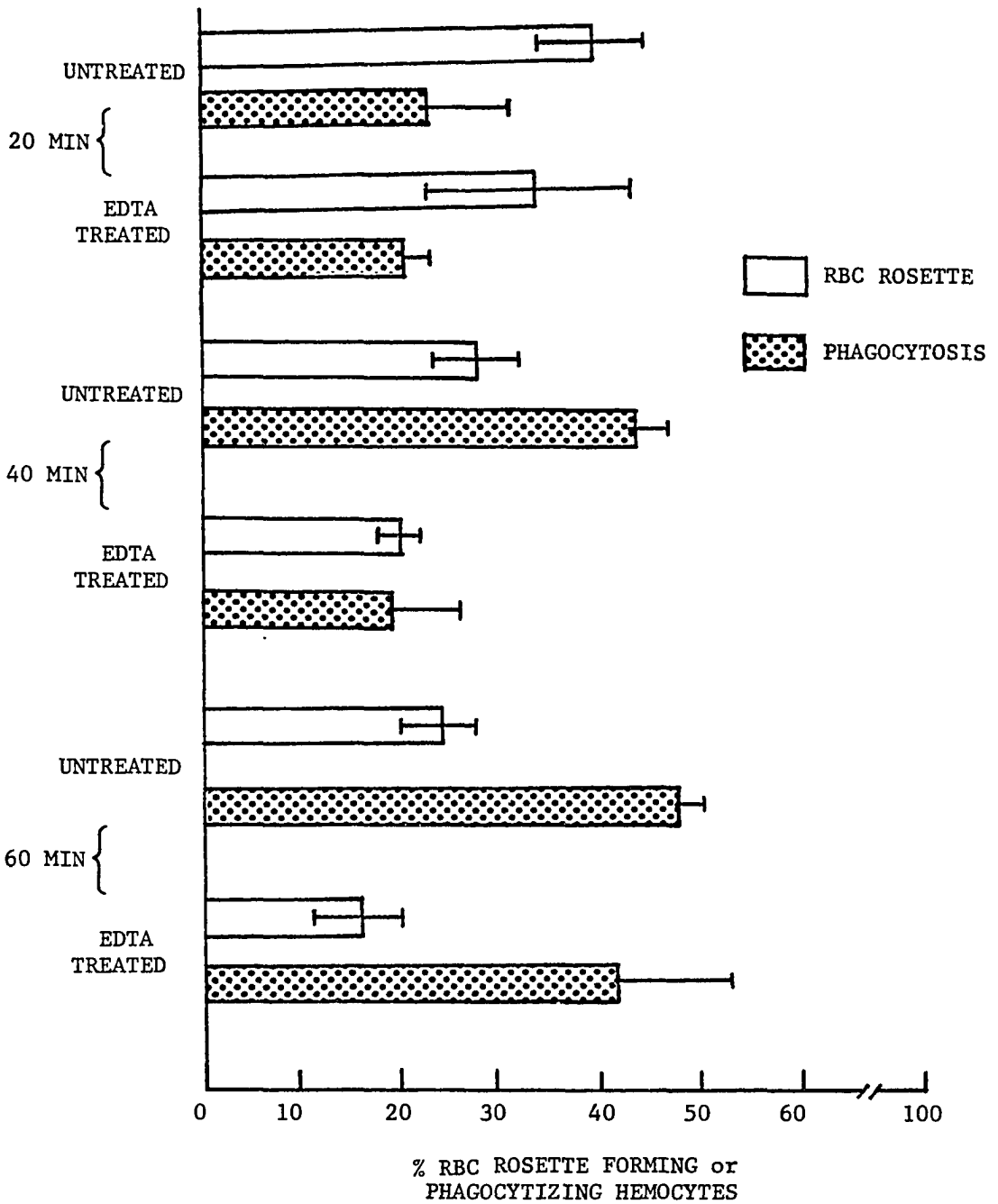

UNTREATED
20 MIN
EDTA TREATED
UNTREATED
40 MIN
EDTA TREATED
UNTREATED
60 MIN
EDTA TREATED
RBC ROSETTE
PHAGOCYTOSIS
0
10
20
30
40
50
60
100
% RBC ROSETTE FORMING or PHAGOCYTIZING HEMOCYTES

Figure 20. Corbicula hemocyte cytolytic reaction against 6 species of mammalian RBCs at various hemocyte:RBC ratios (n=3 with two replicates in each sample). Hemocytes and target RBCs were incubated for 2 hr before measuring hemoglobin levels spectrophotometrically at 541 nm.

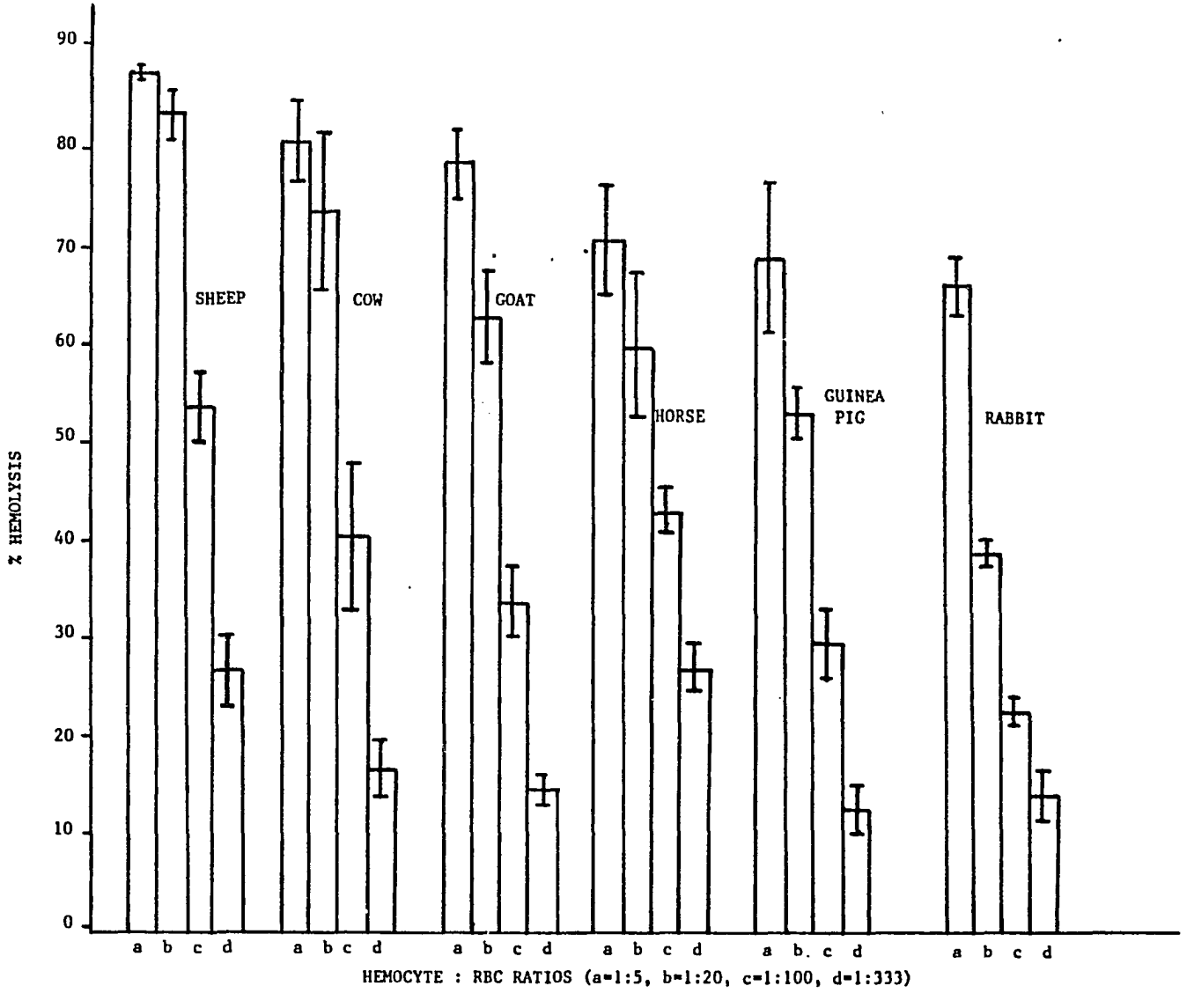
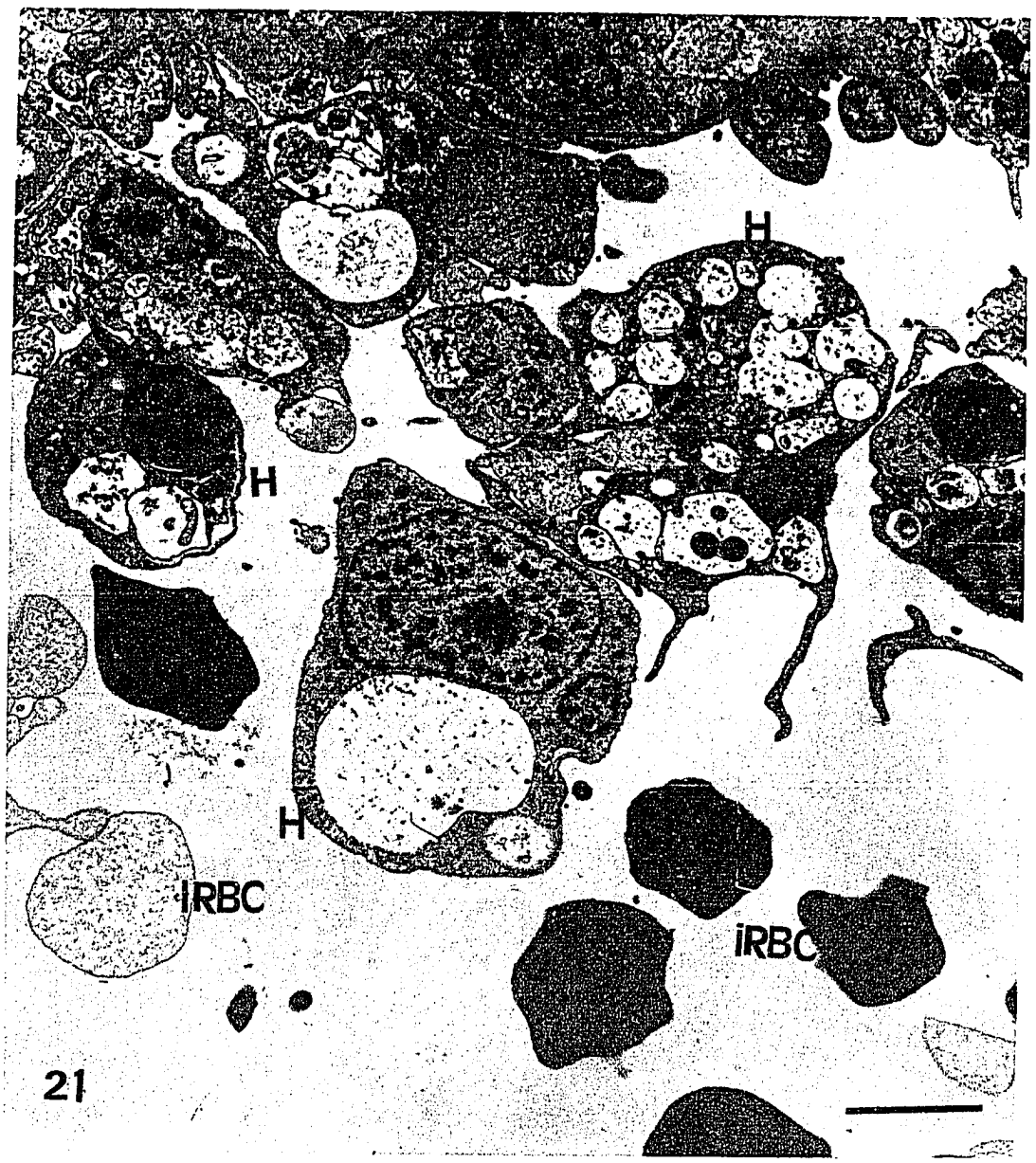

% HEMOLYSIS
90
80
70
60
50
40
30
20
10
0
SHEEP
COW
GOAT
HORSE
GUINEA PIG
RABBIT
a b c d
HEMOCYTE : RBC RATIOS (a=1:5, b=1:20, c=1:100, d=1:333)

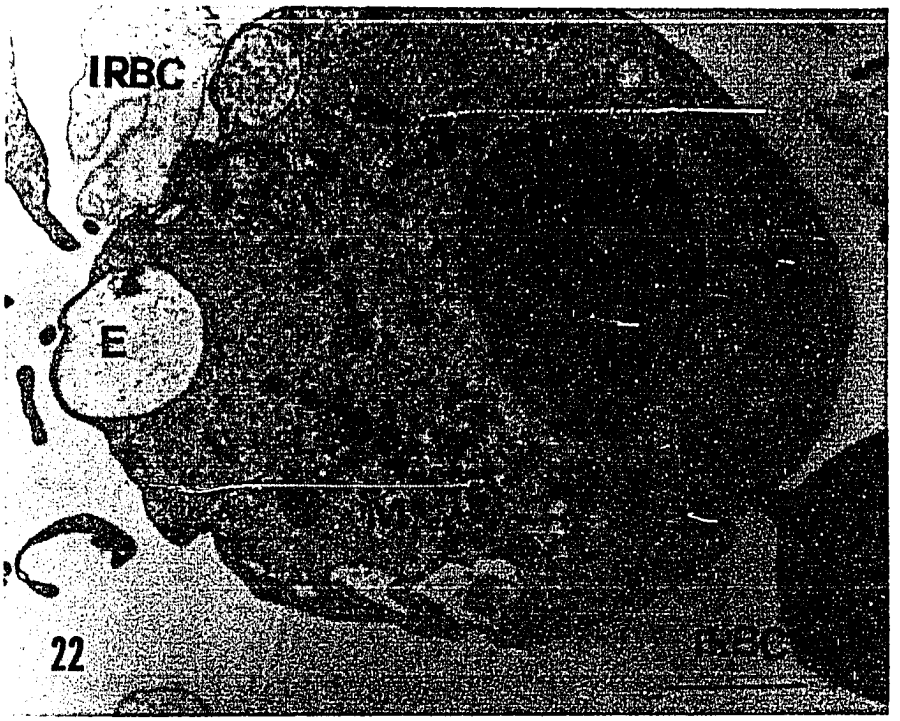
Figure 21. Electron micrograph of Corbicula fluminea hemocyte and rabbit RBC reaction pellet from the hemolytic assay (10 min). Bar = 4 um. H - hemocyte with endocytotic vacuoles; iRBC - intact erythrocyte; lRBC - lysed erythrocyte.



21

Figure 22. Electron micrograph of Corbicula fluminea hemocyte from the hemolytic assay (10min). The hemocyte is devoid of much of its cytoplasmic content. Bar = 2 um. E - endocytotic vacuole; iRBC - intact erythrocyte; lRBC - lysed erythrocyte; L - lysosome-like granule; M - mitochondrion; N - nucleus.

Figure 23. Electron micrograph of Corbicula fluminea hemocyte in close contact with a lysed RBC. Bar = 2 um. E - endocytotic vacuole; F - cell process; L - lysosome-like granule; N - nucleus.


IRBC
E
22

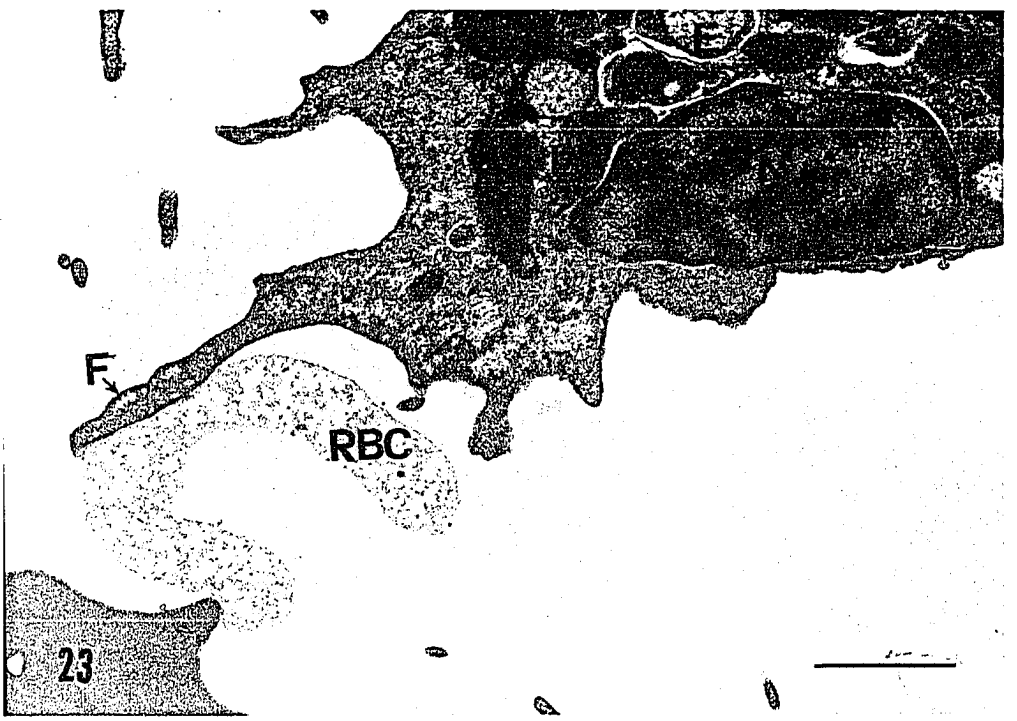

F
RBC
23

Figure 24. Electron micrograph of Corbicula fluminea hemocyte and rabbit RBC reaction pellet from the hemolytic assay (10 min). Bar = 4 um. D - degranulation; H - hemocyte wtih endocytotic vacuoles; iRBC - intact erythrocyte; lRBC - lysed erythrocyte.

IRBC
IRBC
24

Figure 25. *Corbicula* plasma hemolytic reaction against 5 species of mammalian RBCs at various plasma dilutions (n=3 with two replicates in each sample). Plasma and target RBCs were incubated for 2 hr before measuring hemoglobin levels spectrophotometrically at 541 nm.

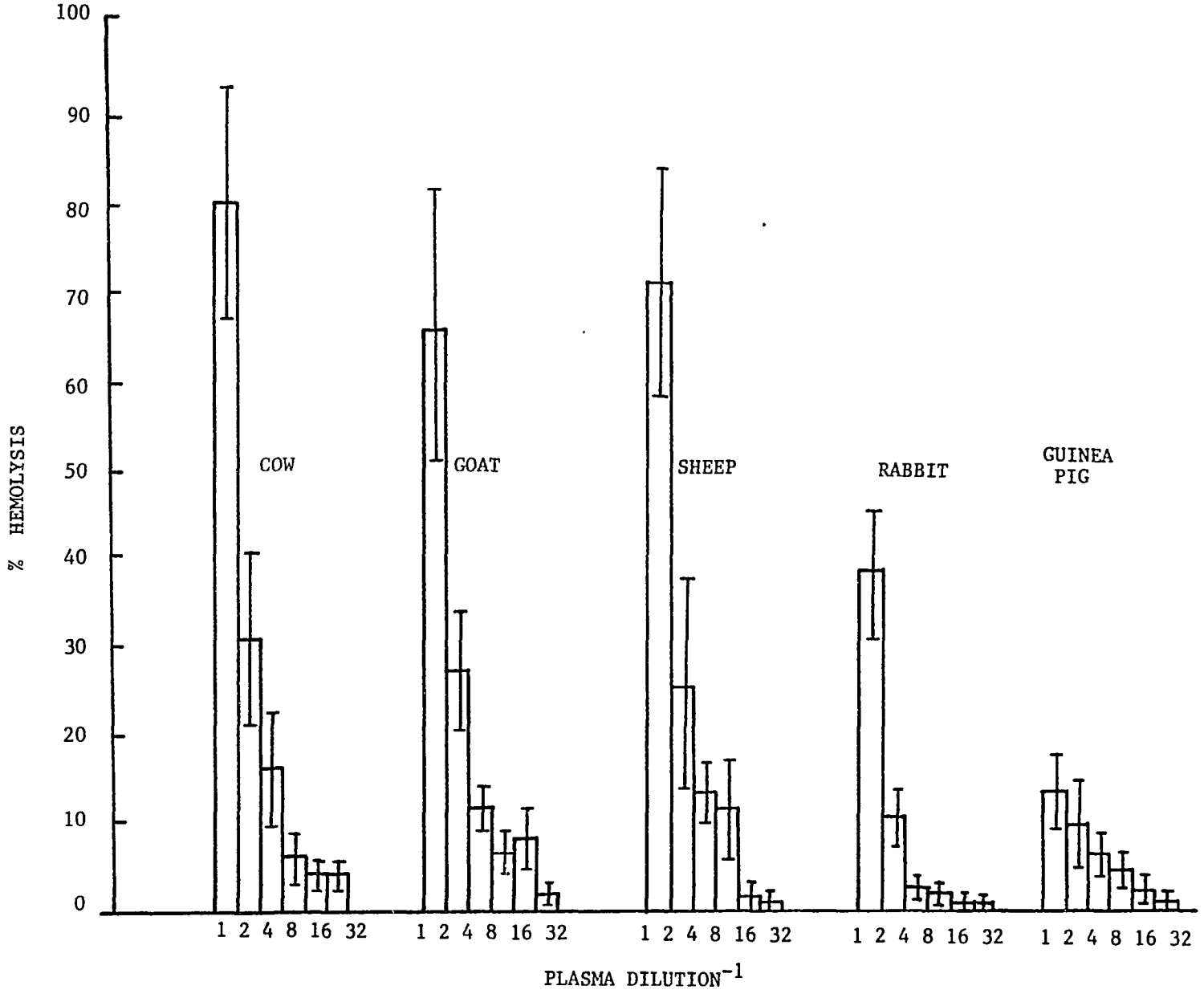

100
90
80
70
60
50
40
30
20
10
0
% HEMOLYSIS
COW
GOAT
SHEEP
RABBIT
GUINEA PIG
1 2 4 8 16 32
PLASMA DILUTION^{-1}

Figure 26. The effect of heat treatment on Corbicula plasma hemolytic activity against sheep RBCs (n=2 with two replicates in each sample).

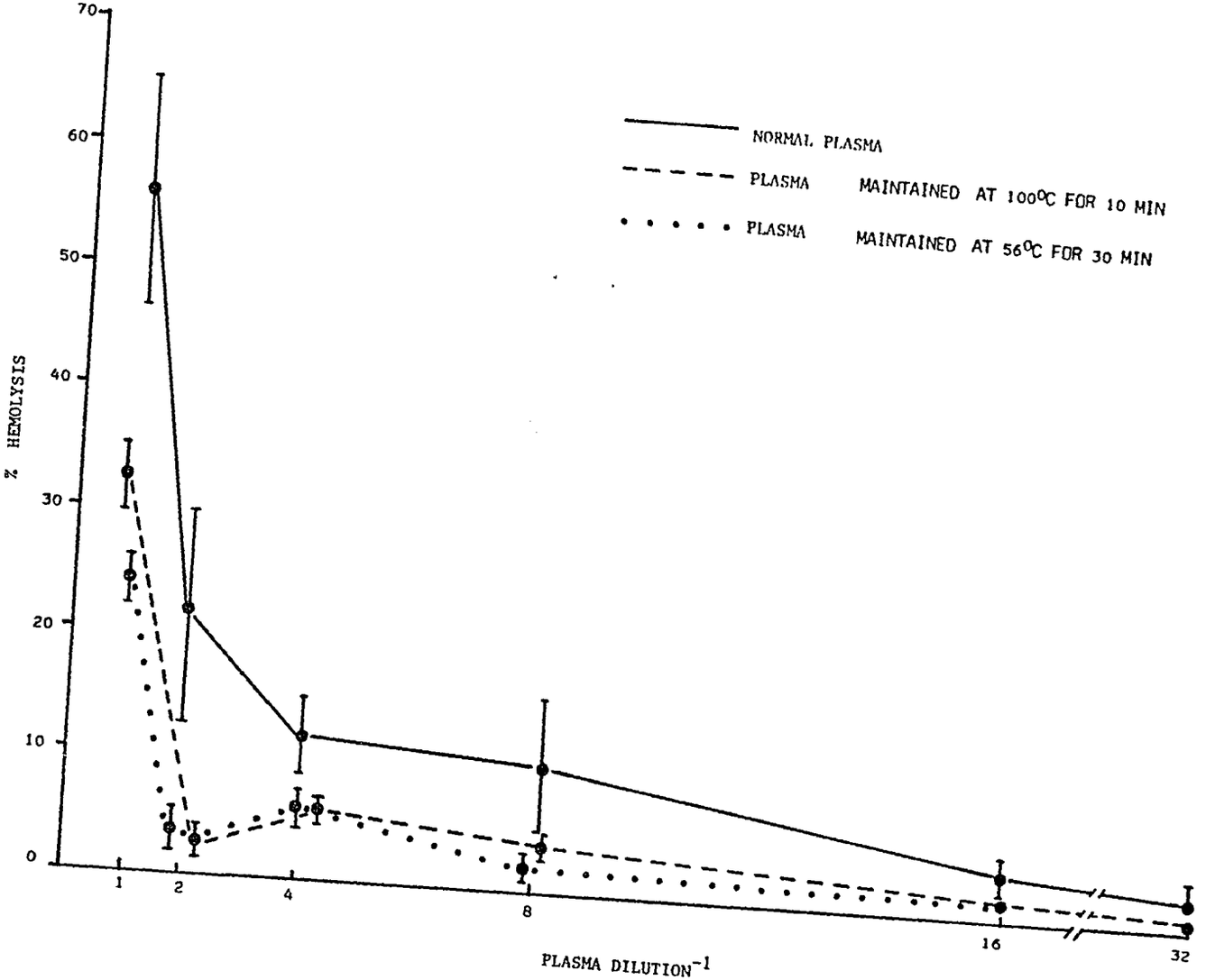

70
60
50
40
30
20
10
0
% HEMOLYSIS
1
2
4
8
16
32
PLASMA DILUTION^{-1}
NORMAL PLASMA
PLASMA MAINTAINED AT 100°C FOR 10 MIN
PLASMA MAINTAINED AT 56°C FOR 30 MIN

Figure 27. The effect of heat treatment on Corbicula plasma hemolytic activity against cow RBCs (n=2 with two replicates in each sample).

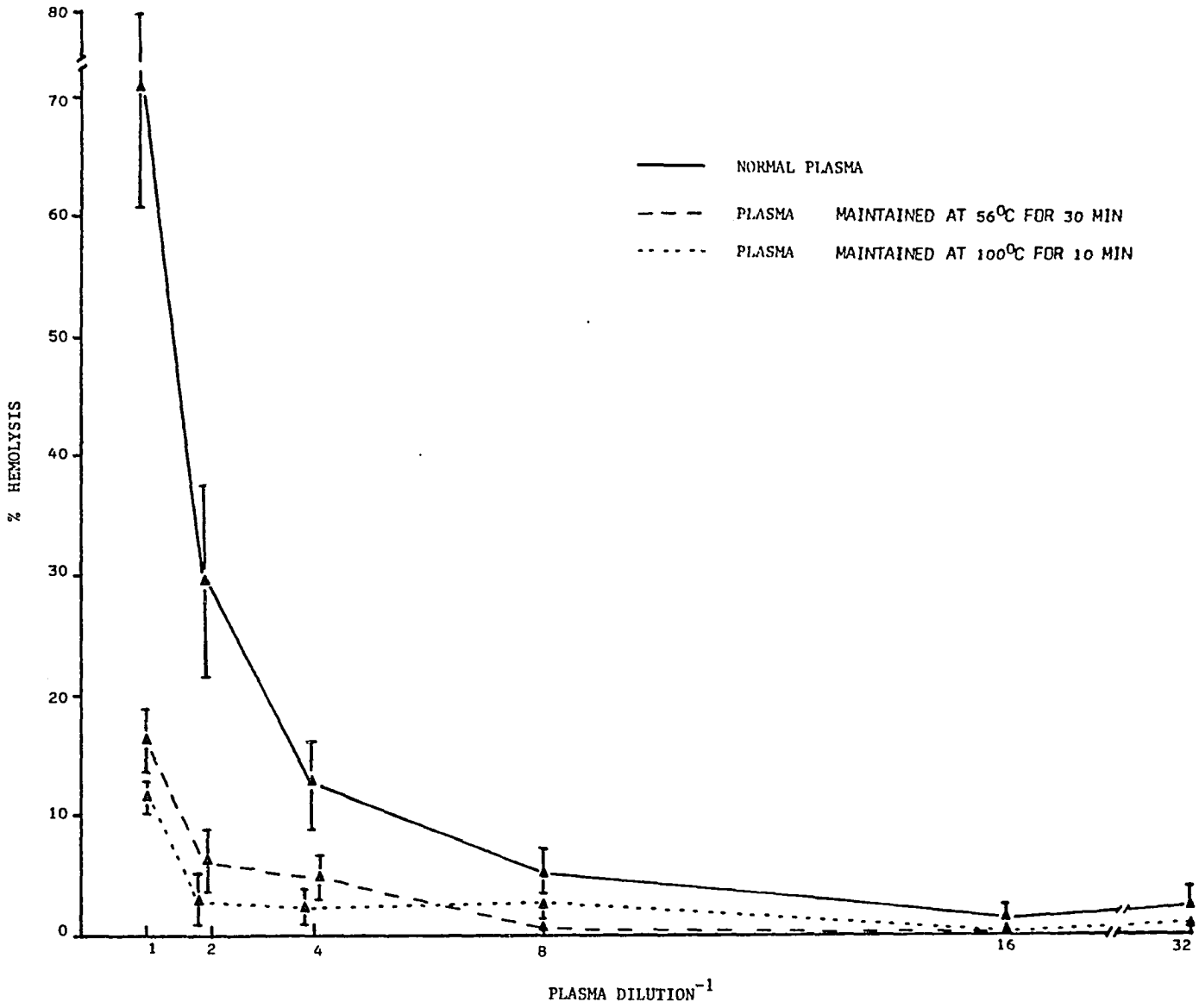
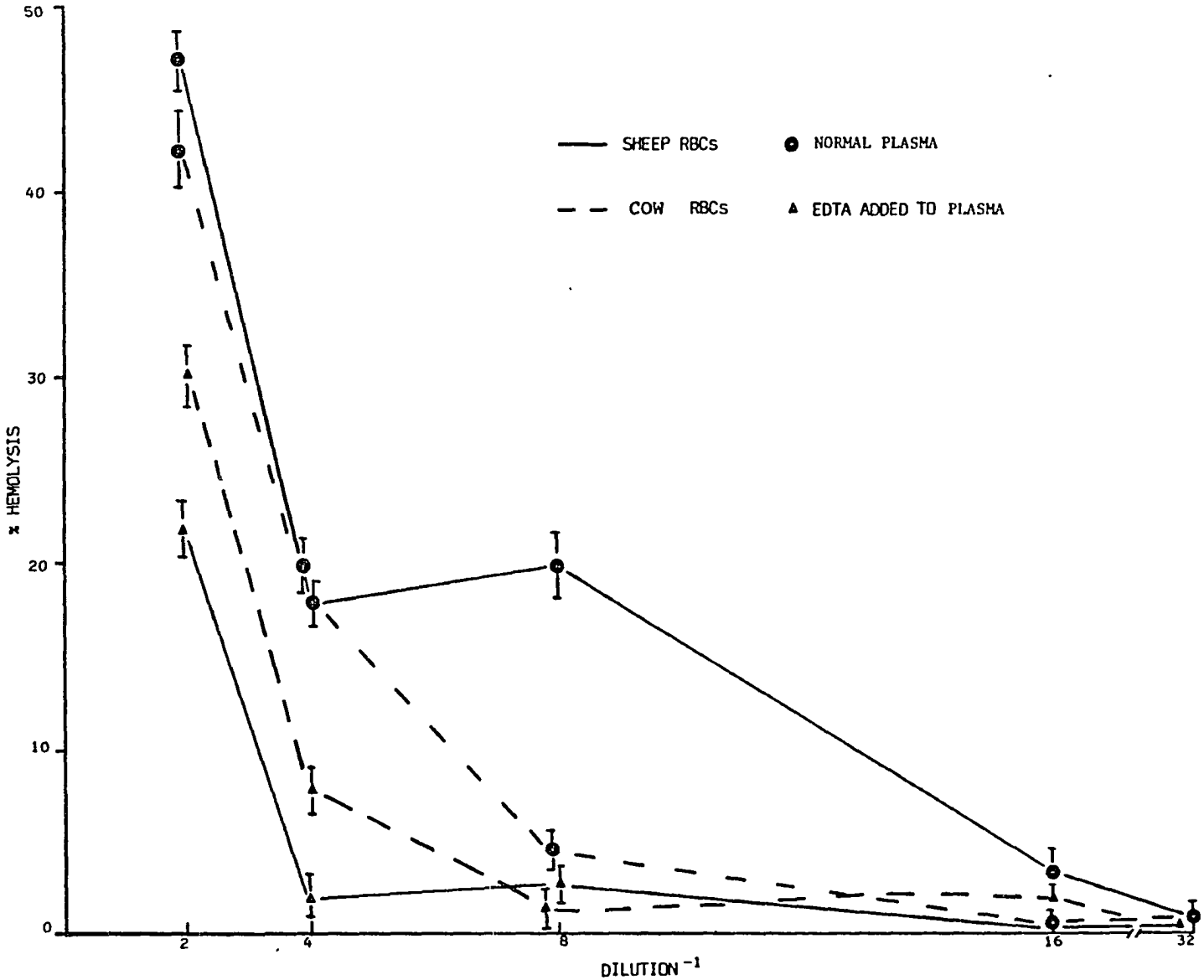

80
70
60
50
40
30
20
10
0
% HEMOLYSIS
NORMAL PLASMA
PLASMA MAINTAINED AT 56°C FOR 30 MIN
PLASMA MAINTAINED AT 100°C FOR 10 MIN
1
2
4
8
16
32
PLASMA DILUTION^{-1}

Figure 28. The effect of 2mM EDTA on Corbicula plasma hemolytic activity against sheep and cow RBCs (n=2 with two replicates in each sample).


50
40
30
20
10
0
% HEMOLYSIS
2
4
8
16
32
DILUTION $^{-1}$
SHEEP RBCs
NORMAL PLASMA
COW RBCs
EDTA ADDED TO PLASMA