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Immunobiology of Eledone cirrhosa (Lamarck).

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Immunobiology of

Eledone cirrhosa (Lamarck).

A thesis submitted to the University of Wales
by

Shelagh Kathryn Malham

in candidature for the degree of

Philosophiae Doctor

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The Lesser Octopus *Eledone cirrhosa.*

This thesis is

dedicated to my

Parents

whose love and support made it possible

and of course to

Cephalopods

the most beautiful invertebrates I know

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Summary

Reliable quantities of blood can be sampled repeatedly from the lesser octopus *Eledone cirrhosa* (Lam.) and the haemocytes cultured for up to 72 h. Sampling causes an increase in the number of haemocytes/ml, in the percentage of haemocytes containing cytoplasmic granules and a change in the staining pattern of the haemocytes. Sampling also causes a decrease in the amount of copper (mg/ml) in the haemolymph and an initial decrease in the amount of protein (mg/ml), which returns to the original values over extended sampling periods.

The haemocytes from *E. cirrhosa* will phagocytose bacteria (*Vibrio anguillarum*) *in vitro* in the absence of haemolymph (dependent on the temperature and duration of incubation) but enhanced phagocytosis will occur in the presence of haemolymph (10-100% concentration). Opsonization is also dependent on the temperature and on the duration of exposure of the bacterium to the haemolymph. Haemocytes migrate towards low concentrations of blood preparations, to lipopolysaccharide (LPS) and to preparations which had contained live bacteria. Haemocytes also have a bacteriostatic effect on the growth of live bacteria with the effect being dependent on the temperature, duration of the assay and the bacterial species used. Haemocytes also produce intracellular reactive oxygen species, detected by nitroblue tetrazolium, after incubation with dead bacteria in particular, but also live bacteria and LPS.

E. cirrhosa haemolymph agglutinates the bacteria *V. anguillarum, V. parahaemolyticus* and *Aeromonas salmonicida* and exerts a bacteriostatic effect on these bacteria which is dependent on the temperature, the amount of haemolymph present and the bacterial species used.

The haemolymph, haemocytes and certain tissues from *E. cirrhosa* exhibit lysozyme and antiprotease activity. The injection of live *V. anguillarum* causes an increase in lysozyme activity in the branchial heart (after 48 h) and a decrease in the haemocytes (after 24 h). Antiprotease activity increased in the haemocytes (4 h) after bacterial injection but decreased in the haemolymph. Live bacteria caused an increase in the number of circulating haemocytes. The bacteria were cleared from the circulation of *E. cirrhosa* in about 4 h by both the haemocytes and tissues (branchial heart, branchial heart appendage and white body) where they were degraded. The large vacuole in branchial heart cells changed in appearance 4 h after bacterial injection and the haemocyte nucleus became pleomorphic. Colloidal graphite was aggregrated in blood vessels only.

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Chapter 1

General Introduction

Invertebrate Immunity

Immune systems in animals involve complex combinations of humoral and cellular components, as well as external barriers, which act to prevent invasion by pathogens. Though invertebrates lack the highly specific immune factors such as immunoglobulins (Igs), and other variable region molecules (Marchalonis & Schluter, 1990; Cooper *et al.*, 1992) which are characteristic of the vertebrate immune response (Manning & Turner, 1976) the invertebrate immune system is adequate for the survival of each species (Klein, 1989). External barriers such as mucus, various arrangements of epithelial cells, and exoskeletons together with antimicrobial and modulating factors serve to prevent external entry by pathogens (Ratcliffe *et al.*, 1985; Millar & Ratcliffe, 1994). If the external barriers are breached the internal cellular and humoral components of invertebrate immune systems act together to destroy the invader. Cellular defence activities performed by blood cells in invertebrates include wound repair, coagulation, phagocytosis, encapsulation, nodule formation and the production of various antimicrobial and cytotoxic substances (Ratcliffe *et al.*, 1985; Pagliara *et al.*, 1993; Millar & Ratcliffe, 1994). Humoral immunity is characterized by factors present in the plasma or serum of invertebrates such as agglutinins and various antimicrobial and immune modulating components, which could have originated from the blood cells or other tissues, and which are involved in various defence activities (Ractliffe *et al.*, 1985; Smith & Chisholm, 1992; Millar & Ratcliffe, 1994). Additional self/nonself recognition factors in ascidians (Jackson *et al.*, 1993) and arthropods in particular, include the prophenoloxidase cascade reaction (Söderhäll, 1982; Söderhäll, & Smith,

1986; Smith & Söderhäll, 1991). In a few invertebrates, some members of the Ig superfamily and the basic components of the vertebrate complement system are present (Ratcliffe *et al.*, 1985; Marchalonis & Schluter, 1990; Millar & Ratcliffe, 1994).

Molluscan Immunobiology: A Brief Survey

Molluscs in general are a diverse group of animals including gastropods, bivalves and cephalopods, however they share with each other and with other invertebrates a number of similar immune functions such as phagocytosis and agglutination. Most of the studies concerned with immunology of molluscs have concentrated on the gastropods and bivalves (Bayne, 1983).

Blood Cells

Most gastropods and bivalves have been reported to have morphologically and functionally heterogeneous populations of blood cells which are used for different functions (Ratcliffe & Rowley, 1979; Sminia & Van der Knaap, 1986; Millar & Ratcliffe, 1994). In some instances, the blood cell type present appears to display different morphological and functional characteristics as it matures (Sminia & Van der Knaap, 1986). Gastropods such as *Lymnaea stagnalis* (Van der Knaap *et al.*, 1983a) apparently have one free circulating blood cell type (haemocyte) which is capable of numerous defence related activities. However, recent work with *L. stagnalis* (Adema *et al.*, 1992) and work with other gastropods such as *Biomphalaria glabrata* (Jeong & Heyneman, 1976; Boyer *et al.*, 1994) indicate the presence of 2 or more haemocyte

types, granulocytes and hyalinocytes (Ratcliffe & Rowley, 1979), probably equivalent to the cells termed spreading and round haemocytes (Ottaviani 1983; Boyer *et al.*, 1994).

Bivalves such as *Mytilus edulis* (Hughes *et al.*, 1991; Noël *et al.*, 1993; Noël *et al.*, 1994), *Mercenaria mercenaria* (Tripp, 1992a) and the clam *Tridacna maxima* (Reade & Reade, 1976) have 2 or more types of haemocyte, the granulocytes and hyalinocytes being the most common.

For bivalves and gastropods the site(s) of blood cell production or leucopoetic organ(s) is thought to vary considerably, thus it may be associated with the mantle epithelium, walls of the blood sinuses, or associated with the kidney and digestive gland (Ratcliffe & Rowley, 1979). In gastropods some of the possible leuopoetic areas have been located between the 'pericardium and the posterior epithelium of the mantle cavity' in *B. glabrata* (Lie *et al.*, 1975; Jeong *et al.*, 1983) and in the heart lung region of *L. stagnalis* (Sminia *et al.*, 1983). However, proliferation of circulating haemocytes has been observed in some gastropod molluscs (Ratcliffe *et al.*, 1985; Jeong *et al.*, 1983; Sminia *et al.*, 1983; Sminia, 1974).

Cellular Defence

The most common cellular defence response in invertebrates, including molluscs, is phagocytosis (Ratcliffe *et al.*, 1985; Bayne, 1983; Millar and Ratcliffe, 1994). Phagocytosis by haemocytes involves the recognition, ingestion, killing and digestion of foreign microorganisms (Ratcliffe *et al.*, 1985; Alberts *et al.*, 1994; Millar &

Ratcliffe, 1994).

Authors who have studied phagocytosis in both gastropods and bivalves have shown for example that the presence of humoral lectins or opsonins in the haemolymph or on the surface of haemocytes can affect the rate of phagocytosis and in some cases lectins are secreted by, or are bound to, the haemocyte itself (Renwrantz, 1983, 1986; Renwrantz *et al.*, 1985). Studies have also revealed that phagocytosis is affected by such factors as the surface nature of the foreign particle (Anderson & Good, 1976; Tripp, 1992a), by the duration of haemocyte incubation with the particle (Abdul-Salam & Michleson, 1980) and by the duration of particle exposure to the opsonin (Fryer & Bayne, 1989). Further factors which affect phagocytosis are the temperature (Tripp, 1992a; Abdul-Salam & Michleson, 1980), pH (Abdul-Salam & Michleson, 1980) and the presence of divalent cations (Tripp, 1992a) in the surrounding medium as well as by the presence of lectins associated with the haemocyte surface (Renwrantz & Cheng, 1977a; Renwrantz & Stahmer, 1983).

The recognition part of phagocytosis includes migration or chemotaxis of the haemocyte to the foreign particle and has been demonstrated *in vitro* in various molluscs (Schmid, 1975; Cheng & Howland, 1979; Schneeweiß & Renwrantz, 1993; Fawcett & Tripp, 1994). Once contact has been established attachment of the foreign particle to the haemocyte surface occurs before internalization of the particle (Alberts *et al.*, 1994). Surface receptors on the haemocyte may aid in this attachment process as well as humoral components (e.g. lectins/opsonins) which may coat the surface of the foreign invader and aid recognition (Prowse & Tait, 1969; Anderson & Good,

1976; Renwrantz & Cheng, 1977a,b; Van der Knaap, 1982; Renwrantz & Stahmer, 1983; Ratcliffe *et al.*, 1985; Fryer & Bayne, 1989; Millar and Ratcliffe, 1994).

Following internalization foreign microorganisms are exposed to various lysosomal enzymes as well as other bactericidal, bacteriostatic and cytotoxic factors (Millar & Ratcliffe, 1994; Ratcliffe *et al.*, 1985). Lysosomal enzymes such as lysozyme (Cheng *et al.*, 1975; Ottaviani, 1991), acid phosphatase (Yoshino & Cheng, 1976) and aminopeptidases (Yoshino & Cheng, 1977) have been detected in various gastropod and bivalve haemocytes and are in some cases inducible by particle challenge (Cheng, 1983). Other cytotoxic (Leippe & Renwrantz, 1988; Bayne *et al.*, 1980a,b) and bactericidal factors include the production during phagocytosis of toxic oxygen metabolites such as singlet oxygen (O_2^-) and hydrogen peroxide (H_2O_2) (Nakamura *et al.*, 1985; Dikkeboom *et al.*, 1987; Dikkeboom *et al.*, 1988; Shozawa *et al.*, 1989; Adema *et al.*, 1991; Adema *et al.*, 1992; Anderson *et al.*, 1992; Pipe, 1992; Noël *et al.*, 1993; Takahashi *et al.*, 1993; Winston *et al.*, 1996).

The presence of overwhelming quantities of small foreign particles induces nodule formation in molluscs where the particles are aggregrated and surrounded by cellular clumps (Ratcliffe *et al.*, 1985; Miller & Ratcliffe, 1994). Where particles are too large to be phagocytosed by single haemocytes e.g. digenetic trematodes (Harris & Cheng, 1975) or large abiotic particles (Sminia *et al.*, 1974) encapsulation occurs by haemocytes surrounding the offending objects (Bayne, 1983; Ratcliffe *et al.*, 1985; Miller & Ratcliffe, 1994).

Haemocytes are also involved in the process of wound healing. Since molluscan blood

does not clot, vasoconstriction and the contraction of muscles around the wound are important in preventing blood loss. Haemocytes migrate to, and aggregrate at, the wound site and initially plug the wound before phagocytosis of necrotic tissue and invading microorganisms and the replacement of damaged tissue (Pauley & Sparks, 1967; DesVoigne & Sparks, 1968; Bubel *et al.*, 1977; Sminia *et al.*, 1983; Sparks & Morado, 1988).

Humoral Defence

Agglutination is one of the most widely studied of the invertebrate humoral defence activities. Molluscan agglutinins are generally glycoproteins and have been shown to cross link and hold in suspension, thereby immobilizing, a variety of particles (Sminia & Van der Knaap, 1986) from bacteria (Fisher & DiNuzzo, 1991; Olafsen *et al.*, 1992; Tripp, 1992b) to erythrocytes (McKay *et al.*, 1969; Stanislowski *et al.*, 1976; Bayne *et al.*, 1979; Stein & Basch, 1979; Boswell & Bayne, 1984; Fisher & DiNuzzo, 1991; Tripp, 1992b). The ability of agglutinins to cross link particles in suspension is however, affected in some molluscs e.g. *M. mercenaria*, by various factors such as the presence of divalent ions, temperature and the nature of the particle surface (Tripp, 1992b), whereas other agglutinins are apparently unaffected by these factors (Michelson & Dubois, 1977). Some agglutinins have binding sites for certain carbohydrate moieties and are therefore often termed lectins. These lectins are possibly involved in self/non-self recognition (Ratcliffe *et al.*, 1985; Renwrantz, 1986; Sminia & Van der Knaap, 1986; Millar & Ratcliffe, 1994). Some agglutinins are

termed opsonins if after incubation with foreign particles they enhance phagocytosis (Ratcliffe *et al.*, 1985; Renwrantz, 1986; Sminia & Van der Knaap, 1986; Millar & Ratcliffe, 1994). Such enhanced phagocytosis has been demonstrated in a number of gastropods (Renwrantz & Mohr, 1978; Bayne, 1983; Fryer & Bayne, 1989) and bivalves (Bayne, 1983; Renwrantz & Stahmer, 1983). In some cases phagocytosis of certain particles will only occur in the presence of the haemolymph containing the opsonin (Prowse & Tait, 1969; Tuan & Yoshino, 1987) and in other cases opsonin independent phagocytosis (Bayne *et al.*, 1979; Abdul-Salam & Michelson, 1980; Tuan & Yoshino, 1987; Fryer & Bayne, 1989) occurs.

Other molluscan humoral defence factors include the lysins, in particular haemolysins (Bayne, 1983; Ratcliffe *et al.*, 1985; Leippe & Renwrantz, 1988; Roch *et al.*, 1996), and other antimicrobial agents (Bayne, 1983; Ratcliffe *et al.*, 1985; Nottage & Birkbeck, 1990; Millar & Ratcliffe, 1994) and modulating factors, such as various protease inhibiting enzymes e.g. the α macroglobulins (Armstrong & Quigley, 1992; Bender *et al.*, 1992; Fryer *et al.*, 1996). The most common antibacterial factor is the bacteriolytic enzyme lysozyme which has been identified in the haemolymph of various molluscs (McDade & Tripp, 1967; Cheng & Rodrick, 1974; Rodrick & Cheng, 1974; Hardy *et al.*, 1976; Cheng *et al.*, 1978; Kassim & Richards, 1978a; Cheng, 1983; Takahashi *et al.*, 1986). Other lysosomal enzymes detected in molluscan haemolymph (Cheng, 1983) include acid phosphatase (Cooper-Willis, 1979) and aminopeptidase (Yoshino & Cheng, 1977). Particle challenge will elevate the amount of these enzymes present in molluscan haemolymph e.g. aminopeptidase (Cheng *et al.*, 1978; Cheng &

Dougherty, 1989), lysozyme (Cheng *et al.*, 1977; Kassim & Richards, 1978b; Cheng & Dougherty, 1989) or acid phosphatase (Cheng & Butler, 1979; Cooper-Willis, 1979; Cheng & Dougherty, 1989).

In Vivo *Clearance of Particles*

Various experiments have demonstrated that injected biotic and abiotic particles are rendered non-viable, or are cleared from the blood, of both gastropods and bivalves (Tripp, 1960; Reade, 1968; Pauley & Krassner, 1972; Bayne, 1973a; Reade & Reade, 1976; Hartland & Timoney, 1979; Bayne 1983; Van der Knaap *et al.*, 1983b; Ratcliffe *et al.*, 1985) The actual process of clearance appears to involve phagocytosis by the circulating blood cells (Prowse & Tait, 1969; Feng, 1966; Bayne & Kime, 1970; Pauley & Krassner, 1972; Crichton *et al.*, 1973; Killby *et al.*, 1975) as well as by fixed phagocytic cells in organs such as the digestive gland, kidney, connective tissues and head-foot muscle (Reade, 1968; Pauley & Krassner, 1972; Bayne, 1973a; Crichton *et al.*, 1973; Killby *et al.*, 1973; Bayne, 1974; Renwrantz *et al.*, 1981; Bayne, 1983). Where injected or introduced particles are too large to be phagocytosed, nodule formation and encapsulation by haemocytes occurs (Tripp, 1961), and lysosomal enzymes are secreted into the capsule by the encapsulating haemocytes (Harris & Cheng, 1975). Clearance times vary between molluscan species and the type of particle injected (Renwrantz *et al.*, 1981), and may also depend on certain factors such as the ambient temperature (Feng, 1966) or the amount and specificity of opsonins present in the haemolymph of the experimental animal (Renwrantz & Mohr, 1978;

Harm & Renwrantz, 1980). Injected pathogens as well as e.g. temperature (Stumpf & Gilbertson, 1978), wounding (Sminia, 1972) and oxygen tension (Wolmaraus & Yessel, 1988) can cause the number of circulating haemocytes in molluscs to either increase (Abdul-Salam & Michelson, 1980; Mounkassa & Jourdane, 1990; Suresh & Mohandas, 1990) or decrease initially before returning to original values (Bayne & Kime, 1970; Renwrantz *et al.*, 1981; Van der Knaap *et al.*, 1983b). Increased haemocyte numbers, or leucocytosis, appears to be related, in some cases, to an increase in the size of the amoebocyte producing organ (Jeong *et al.*, 1983). However, decreased haemocyte counts are apparently related to the attraction of fixed phagocytes containing the injected particles, for the circulating haemocytes, which eventually return to the blood laden with the particles (Renwrantz *et al.*, 1981). Some of the tissues which contain fixed phagocytic cells also display antimicrobial properties such as lysozyme (Kassim & Richards, 1978a; Takahashi *et al.*, 1986) and aminopeptidase (Yoshino & Cheng, 1977) activity. Elimination of unwanted abiotic particles for example carbon apparently occurs by the migration of phagocytes through various epithelial layers such as the digestive tract and mantle epithelium (Stauber, 1950; Tripp, 1961; Reade, 1968). However, biotic particles such as bacteria appear to be degraded (Feng, 1966) and then either voided (Feng, 1966; Ratcliffe *et al.*, 1985) or digested by the cell (Cheng & Cali, 1974; Ratcliffe *et al.*, 1985).

Cephalopod Immunobiology

Cephalopods are soft bodied advanced molluscs which inhabit a variety of marine environments. Though commercially important in a number of countries such as Spain (González *et al.*, 1994; Guerra & Rocha, 1994; Guerra *et al.*, 1994) and Japan (Osako & Murata, 1983; Murata, 1989) very little information exists on the ability of these animals to survive in the microbe-rich marine environment (Ford, 1992).

Various studies using captive cephalopods have demonstrated that once injured cephalopod wounds are invaded by various opportunistic bacteria which can be fatal (Leibovitz *et al.*, 1977; Hanlon *et al.*, 1984; Ford *et al.*, 1986; Bullock *et al.*, 1987; Hanlon & Forsythe, 1990; Ford, 1992). Various factors such as temperature and condition of the seawater have also been shown to have a detrimental effect on the survival of cephalopods in culture conditions (Reimschuessel & Stoskopk, 1990).

Cephalopods have a closed circulatory system encompassing a central systemic and 2 branchial or gill hearts (Wells, 1978; Wells, 1983; Wells & Smith, 1987). The blood is pumped around elastic arteries and veins through 'capillary beds' similar to the vertebrate circulatory system (Browning, 1979; Wells, 1983; Shadwick & Nilsson, 1990) and is also 'stored' in large blood sinuses (O'Dor & Wells, 1984). The blood consists of haemolymph (plasma) containing dissolved protein, of which 98% is the respiratory pigment haemocyanin (Ghiretti, 1966; Wells; 1983). There appears to be one type of haemocyte (blood cell). This apparently originates and matures in the leucopoetic organ, situated in the orbital sockets behind the eyes (Cazal & Bogoraze, 1943; Cowden, 1972; Cowden & Curtis, 1974, 1981; Wells, 1978). As with other

molluscs, cephalopod blood does not clot and blood loss after sustaining a wound is prevented by vasoconstriction of the muscles surrounding the wound. Haemocytes aggregrate at the wound site and form a plug sealing the wound (Polglase *et al.*, 1983; Wells, 1983; Féral, 1988; Ford, 1992).

Stuart (1968) demonstrated that haemocytes from the octopus *Eledone cirrhosa* would phagocytose human macrophages only after their prior exposure to *E. cirrhosa* plasma, indicating the presence of opsonins in the plasma. Cephalopod haemolymph contains agglutinins for various particles (Cushing *et al.*, 1963; Stuart, 1968; Russo & Tringall, 1983; Fisher & DiNuzzo, 1991; Ford, 1992) and agglutin activity has also been found in the skin mucus of cephalopods (Marthy, 1974; Renwrantz & Uhlenbruck, 1974) as well as in other organs (Marthy, 1974). Lectins have been islolated and characterized from *Octopus vulgaris* (Rögener *et al.*, 1985, 1987), and a lactose specific lectin from *O. vulgaris* demonstrated similar properties to 'a sub unit' of haemocyanin, e.g. both contain copper and have comparable molecular weights (Rögener *et al.*, 1986).

O. vulgaris haemolymph also displays antibacterial activity (Russo & Tringall, 1983), and antiprotease activity has been detected in various organs of *Loligo vulgaris* (Tschesche & Von Rücker, 1973) and in the haemolymph of *O. vulgaris* (Thøgersen *et al.*, 1992).

Stuart (1968) observed that carbon injected into *E. cirrhosa* was cleared from the circulation and accumulated in phagocytic cells in organs such as the gill, posterior salivary glands, white bodies and branchial hearts. Bayne (1973b) after injecting

Octopus dofleini with bacteria suggested that fixed phagocytes in the gill of the octopus were primarily responsible for clearance and not the circulating haemocytes. However, Froesch (1979) demonstrated that injection of foreign proteins into *O. vulgaris* caused the mass production and release of particulate material from the optic gland, suggesting that other organs may be involved in the immunocomptence of cephalopods

The work on *Eledone cirrhosa* presented here, though touching briefly on only a few of the well known immune defence responses of invertebrates, aims to increase the knowledge of, and stimulate interest in, this advanced invertebrate.

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Chapter 2

Eledone cirhosa Haemocytes: Isolation and Culture.

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Introduction

Cephalopods are relatively advanced invertebrates, belonging to the phylum Mollusca. They incorporate a high pressure, closed circulatory system where elastic arteries (Shadwick & Nilsson, 1990) are linked, via capillary beds, with the venous system and its associated large sinuses and extracellular spaces (Browning, 1979; Wells, 1978, 1983). It has been proposed that cephalopods have a primitive type of lymphoreticular system which incorporates the branchial gills, the salivary glands and the white body, all containing fixed phagocytic cells, and the blood or haemolymph containing circulating phagocytes called haemocytes (Stuart, 1968; Bayne, 1983). The haemolymph also contains the respiratory pigment haemocyanin, which makes up about 98% of the total blood protein (Ghiretti, 1966; Senozan *et al.*, 1988). The blood is approximately isotonic with seawater, with an average value of 1150 mOsm for haemolymph of the Northern octopus *Eledone cirrhosa*. The inorganic constituents of haemolymph differ from those of seawater, particularly the concentration of sodium (lower) and potassium (higher) (Robertson, 1953; Gnap, 1987). Morphologically, there appears to be only one type of haemocyte present in the blood of species such as *E. cirrhosa* and *Octopus vulgaris* (Ford, 1992) and this is thought to originate and mature in the white body or haematopoietic organ, located in the orbital pits behind the eyes. Once mature, the haemocytes are released into the circulation (Necco & Martin, 1963; Cowden & Curtis, 1974, 1981). In other invertebrates significant advances have been made in our knowledge of the structure and function of blood cells and the presence of different types of cell (Millar & Ratcliffe, 1994). In contrast, very little is

known about octopod haemocytes, their response to infection and therefore what role they play in protecting the animal (Ford, 1992). The study of octopod haemocytes has been hampered by their rapid aggregation into clumps or morulae (Cowden & Curtis, 1974, 1981), a problem frequently encountered with many invertebrates. The methods for collecting octopod haemocytes and obtaining successful short-term cultures for functional assays will be described.

Isolation of Haemocytes

It is possible to sample haemolymph and haemocytes from a number of diverse invertebrates including crustacea (McKenzie & Preston, 1992; Söderhäll & Cerenium, 1992), annelids (Dales & Kalar, 1992), echinoderms (Smith & Söderhäll, 1991), urochordates (Smith & Peddie, 1992; Parrinello *et al.*, 1993) and molluscs. Within the molluscs, gastropod (Adema *et al.*, 1994), bivalve (Noël *et al.*, 1993; Russel-Pinto *et al.*, 1994; Anderson, 1994) and cephalopod (Ford, 1992) haemocytes have been isolated. The haemocytes are usually isolated in an anticoagulant to prevent morula formation. The anticoagulants used for different invertebrates vary but typically include a chelating agent such as ethylenediaminetetraacetic acid (EDTA), or ethylene glycol-bis (ß-aminoethyl ether) tetraacetic acid (EGTA) and exclude calcium and/or magnesium. The effects of EDTA, magnesium (Takahashi *et al.*, 1994) and calcium on morula formation of invertebrate blood cells is well documented (Kenney *et al.*, 1972; Kanungo, 1983); both calcium and magnesium promote morula formation at certain concentrations, and EDTA inhibits aggregration (Shozawa & Suto, 1990). Other anticoagulants used with invertebrate blood cells include heparin or cysteine hydrochloride (Tyson *et al.*, 1974), cysteine (Smith & Ratcliffe, 1978; Smith & Söderhäll, 1983) and caffeine (Bertheussen & Seljelid, 1982; Fryer & Adema, 1993). The maintenance and handling of cephalopods is described by Boyle (1991) in the UFAW Handbook and will not be discussed here. While it is possible to bleed freshly killed cephalopods, it is often difficult to obtain reasonable amounts of blood (Young, 1992). Thus, it is necessary to use live animals. With large octopods (e.g., *O.*

vulgaris) it is possible to anaesthetize the animal and insert a cannula into the dorsal aorta to obtain blood (Wells, 1979) or with *O. dofleini* cannulae can be inserted into the anterior vena cava and the afferent branchial vein (Harrison & Martin, 1965). With smaller octopods such as *E. cirrhosa*, the traditional methods of bleeding involve surgery of anaesthetized animals to gain access to the blood vessels, and as a result the animals do not often recover (Stuart, 1968; Rogener *et al.*, 1985; Senozan *et al.*, 1988; Fisher & DiNuzzo, 1991). A modification of the bleeding technique of Bayne (1973) described below, makes it possible to collect a sample of haemolymph and keep the animal alive and available for the collection of multiple blood samples.

Materials

1. *Eledone cirrhosa* >250g
2. Seawater
3. Absolute ethanol (ultrapure, perfumery grade, 99.9%)
4. 21 gauge 1½" needle
5. 2.5ml syringe (plastic)
6. Marine Anticoagulant (adjusted to pH 7 using sodium hydroxide)-see table 1
7. Haemocytometer
8. Sterile 20ml bijou tubes.

Table 1

Marine Anticoagulant (modified from Söderhäll & Smith, 1983)

Chemicals	g/100 ml Distilled Water
Sodium chloride	2.630
Glucose	1.800
Tri-sodium citrate	0.088
Citric acid	0.055
EGTA	0.030

Methods

1. To ensure maximal survival, all animals to be used in experiments are kept in well aerated seawater at 10°C for 48 h prior to use. Food in the form of shore crabs, *Carcinus maenas*, is provided in excess during this period. Since haemocyte counts start to decrease after 7 days in the laboratory, the animals should be bled between the second and seventh day following capture.

2. Weigh the animal.

3. Immerse the animal in freshly prepared 2.5% ethanol in seawater. Typically 2 L of seawater are sufficient to cover the animal.

4. Observe the animal continuously. As soon as there is a decrease in mantle contractions, highly reduced resistance to insertion of fingers into the mantle cavity, relaxation of chromatophore organs and contraction of the pupils, the bleeding procedure should be initiated.

5. Partially evert the mantle on one side of the ventral septum by reflexing posteriorly the mantle rim. Pressure is required on the rear of the body on the side where the mantle is to be reflexed. Following mantle rim reflection, the gill will partially extrude and the branchial arteries will be exposed (see fig. 2.1).

6. A 21g-1½" needle is then inserted into the blood vessel and the blood collected. A rough guide as to the volume that can be collected is 0.3ml/100g animal, although a maximum volume of 3ml is recommended from animals weighing approximately 1000g or larger. Blood is withdrawn slowly to prevent the vessel from collapsing; should blood movement through the vessel cease the animal is immediately revived. The

Figure 2.2. Haemocytes cultured in octopus Ringer for 72 h, after staining with the supravital dye Evans blue. Attached (AH), spreading (SH) and dead (DH, i.e. stained with Evans blue) haemocytes can be seen.

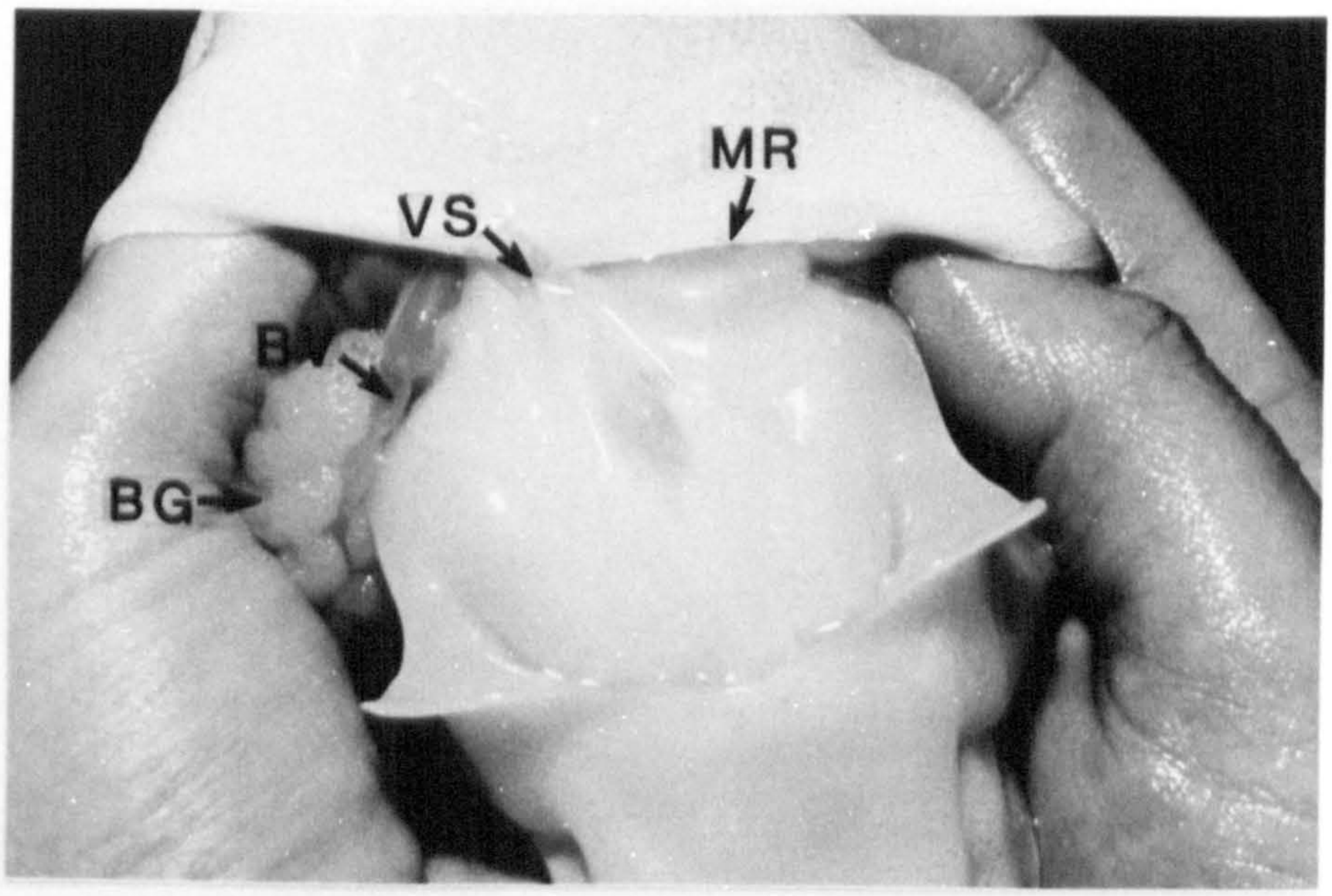


Fig. 2.1

25 mm

volume of blood taken from the animal is small in comparison to the total volume of blood present (Martin *et al.*, 1958) and the amount taken does not harm the animal and allows repeated sampling. Due to the small size of the branchial vessels, bleeding using this technique is not recommended for animals weighing less than 250g.

7. Allow the mantle rim to return to its normal position and return the animal to well aerated seawater and revive by agitating the gills. The animal should recover within 5 min and feed within 6 h. However, if the above procedure (steps 3-6) is allowed to extend beyond 5 min, recovery is less than 100%.

8. Immediately after collection, the blood is diluted 1:10 in ice cold marine anticoagulant (table 1) and a cell count is made using a haemocytometer. The tubes containing the isolated haemocytes should be kept on ice.

Discussion

Large numbers of haemocytes can be isolated using this technique. Care should be taken when inserting the needle to avoid penetrating the kidney sac of the animal as this contains mesozoan parasites (Hochberg, 1982) that would contaminate the isolated haemocytes. The large volume of marine anticoagulant used to rapidly dilute the cells and the haemolymph is to prevent morulae formation. While it is possible to collect haemolymph into syringes containing marine anticoagulant, this is generally not as successful in preventing morulae formation as the described method. Use of other anticoagulants is possible although acidic isolation media, used by workers with some invertebrates (e.g. Söderhäll & Smith, 1983), are not successful for *Eledone* haemocytes which lyse or become highly crenulated at these pH's.

Since the haemolymph isolation procedure is fast, the animal does not need to be under the anaesthetic for long and recovery is good. The amount of stress that the animal suffers is also minimal, an important factor if rebleeding is required. Further, there is little chance of general infections arising which could be detrimental to the animal's health, with implications for altered immunological status of the animal. Thus, blood collection can be repeated, enabling experiments involving haemocyte numbers and activity to be conducted on the same animal. There are, however constraints on the number of times any one animal can be bled. Bleeding should not be performed more than once every 2-3 days and up to a maximum of 5 times. Further, depending on the quality of seawater and the conditions under which the animals are kept, e.g.

temperature, the number of haemocytes obtained progressively decreases over a period of 4 weeks, and the haemocytes themselves may become less amenable to culture.

Haemocyte Culture

Eledone haemocytes cannot be cultured in the isolation media because at the EGTA concentration present, haemocytes will not adhere to the surface of the culture chambers and there is a high haemocyte mortality. Similarly, when EDTA is used at a concentration equivalent to that of EGTA in the isolation medium, there is strong cell to cell adhesion resulting in the formation of morulae within 4 h and high haemocyte mortality. Other workers have cultured invertebrate haemocytes in a Ringer solution post-collection, e.g. as with *Lymnaea stagnalis* (Van der Knapp, 1982) and *Carcinus maenas* (Smith & Ratcliffe, 1978). However, such cultures are relatively short-term. *Eledone* haemocytes and white body cells have been cultured in mammalian tissue culture media (e.g. medium 119 or Minimum Essential Medium) with the addition of sodium chloride and other supplements, i.e. haemolymph, fetal calf serum and vitamins (Necco & Martin, 1963; Stuart, 1968). Even these cultures are rarely successful beyond 12 days, and morula formation is common, especially in the presence of haemolymph. Longer term (1 month) cultures have been described for other tissues such as ovarian and optic gland tissue (Durchon & Richard, 1967).

A Ringer solution adapted from those described for other invertebrates but containing EGTA has been successfully used for culturing *Eledone* haemocytes by the present authors and is described next. It allows haemocytes to survive attached to the surfaces of culture vessels with only temporary morula formation for up to 72 h.

Materials

1. Centrifuge
2. *Eledone* blood in marine anticoagulant
3. Octopus Ringer medium (see table 2)
4. 96-well culture plates
5. 1ml Eppendorf centrifuge tubes
6. Haemocytometer

Table 2

Octopus Ringer for short-term culture of *Eledone cirrhosa* haemocytes.

Chemicals	g/100ml Distilled Water
Sodium chloride	2.433
Glucose	1.400
EGTA	0.015
Potassium chloride	0.082
Potassium di-hydrogen phosphate	0.004

Methods

1. Transfer the *Eledone* blood, in marine anticoagulant to 1ml Eppendorf tubes.

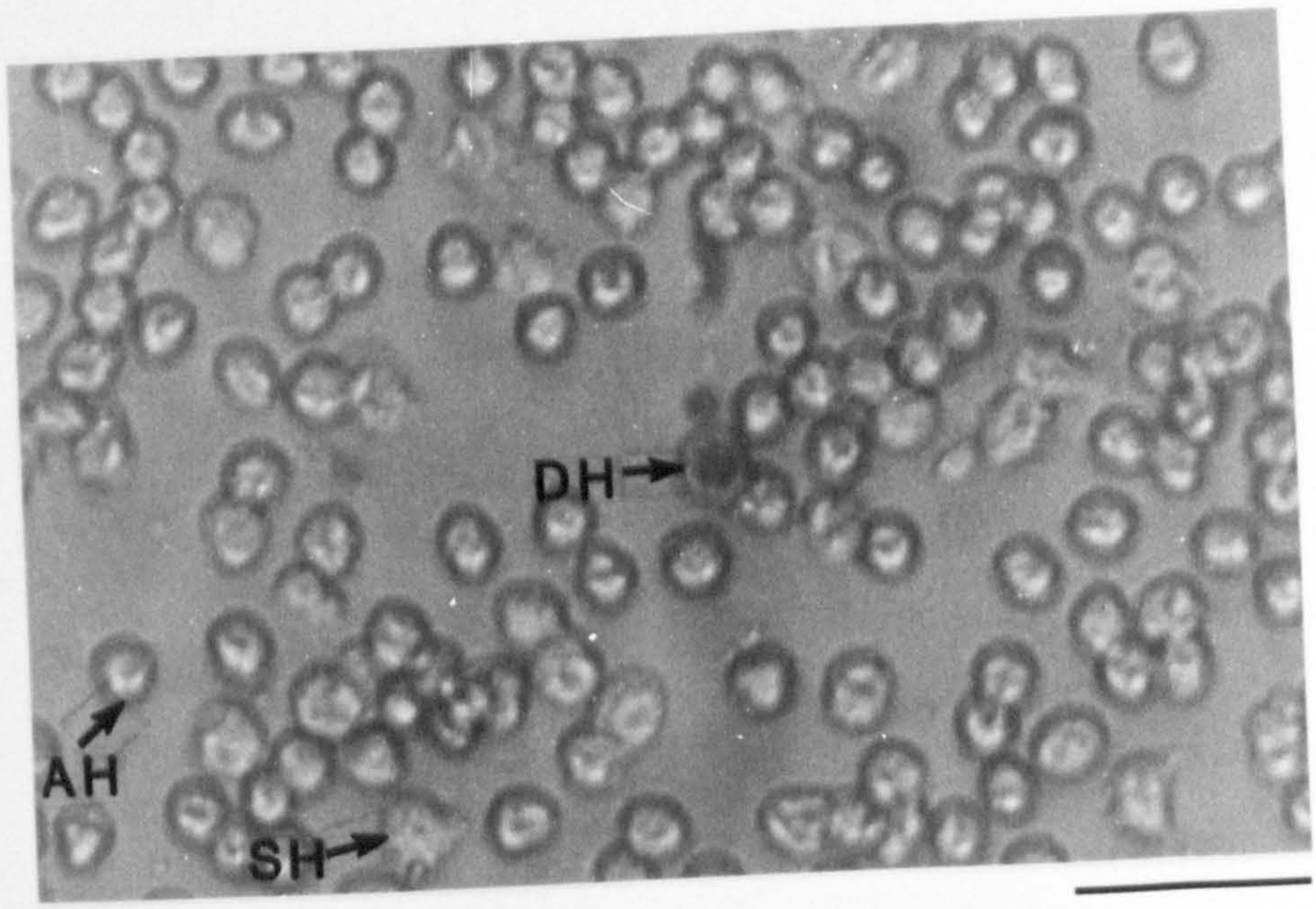
2. Centrifuge the blood at 800g for 5 min at 4°C.

3. Remove the supernatant and resuspend the cells in octopus Ringer medium (table 2). Centrifuge at 800g for 5 min at 4°C.

4. Remove the supernatant and resuspend the cells. Make a haemocyte count and adjust the cells to the required concentration, up to a maximum of 6×10^6 haemocytes/ml.

5. Aliquot 100µl of haemocyte suspension into the culture chambers and incubate at 15°C for a maximum of 72 h.

Figure 2.2. Haemocytes cultured in Octopus Ringer for 72 h, after staining with the supravital dye Evans blue. Attached (AH), spreading (SH) and dead (DH, i.e. stained with Evans blue) haemocytes can be seen.



30 μm

Fig. 2.2

Discussion

High concentrations of haemocytes can be rapidly isolated and cultured for up to 72 h (see fig. 2.2) using the described protocol. Haemocyte viability remains high for this period and the cells can be used in various immunological assays such as phagocytosis. Isolated haemocytes adhere to the surface of the culture chambers within 4 hours, at which time more than 75% of the cells resist dislodgment by vigorous pipetting. After 4 hours, a few morulae may form but these are temporary and have dissociated by 24 hours.

Sterility is one major problem with the isolation and culture of haemocytes. The main source of contaminants entering the culture is from the bleeding procedure. Care should therefore be taken when bleeding the animals to allow no contact of the needle used to withdraw blood with the anticoagulant and to follow sterile procedures thoughout. Even with EGTA in the octopus saline, morula formation can be increased in the presence of as little as 1% haemolymph, and this is a problem for assays looking at the influence of haemolymph on cellular functions. Temperature may also be a problem. *Eledone* haemocytes rapidly lose viability at room temperature and therefore the duration of time "at the bench" must be limited. Temperatures lower than 5°C also have an inhibitory effect on cellular functions such as phagocytosis. Another problem which is frequently encountered is the large variation in haemocyte activity and inter-animal variability. Such variation can be kept to a minimum by using triplicate and quadruplicate assays for each parameter examined.

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Chapter 3

Effects of Sampling on the Haemocytes and Haemolymph of *Eledone cirrhosa* (Lam.).

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Abstract

Sampling blood has an effect on both the number of circulating haemocytes and the concentration of copper present in the haemolymph of *Eledone cirrhosa*. Haemocyte counts increase within 2 h, revert to near starting values after 4/5 days, then increase again to the end of the sampling period. The increase in the number of haemocytes also brings a concomittant increase in the percentage of haemocytes containing visible granules. Acid phosphatase, diaminobenzidine and periodic acid Schiff's reaction gave variable staining results over 10 and 24 days.

The concentration of copper in the haemolymph decreases within 24 h, but further sampling between days 1 - 3 shows no significant change. However continued sampling causes the copper concentration to significantly decrease over the next 7 days. Protein values fluctuate and show no significant change between the first and last samples over 7 days.

Key Words: *Eledone cirrhosa*; Haemocytes; Haemolymph; Copper; Protein.

Introduction

Cephalopods have a closed circulatory system in which the blood is circulated through contractile blood vessels and capillaries by the contraction of 3 hearts (Wells & Smith, 1987; Shadwick & Nilsson, 1990). The blood consists of blood cells, the haemocytes, and plasma, the haemolymph. Unlike a number of other invertebrates (Ratcliffe & Rowley, 1979; Ratcliffe *et al.*, 1986; Millar & Ratcliffe, 1994) *Eledone cirrhosa* appears to have only one haemocyte type. The haemocyte matures in the leucopoetic organ or white body and when released into the circulation, contains a 'U' shaped nucleus with several electron-dense granules present in the cytoplasm (Cowden & Curtis,1974, 1981). Haemocytes from *E. cirrhosa* are involved in cellular defence activities such as phagocytosis (Stuart, 1968; Chapter 4) and demonstrate chemotaxis for certain bacteria and bacterial products (Chapter 5)

The haemolymph of cephalopods, as with other invertebrates, e.g., gastropods and crustacea, contains the respiratory pigment haemocyanin dissolved in the blood. Haemocyanin is a high molecular weight copper protein supposedly synthesized in the branchial glands of cephalopods (Messenger, 1974). Haemocyanin amounts to about 98% of the total protein present in octopus blood (Ghiretti, 1966) and oxygen is transported bound between 2 copper atoms and ligated by protein side chains.

The total blood volume of the octopus *Octopus dofleini* was demonstrated to be 5.8 ± 1% of the body weight (Martin *et al.*, 1958). Using the octopus *Octopus vulgaris*, Rögener *et al.* (1987) demonstrated that the total protein concentration of haemolymph was 108mg/ml, with an estimated copper concentration of 183 ± 5μg/ml

of haemolymph. Wells and Wells (1993) demonstrated that removal of a large amount of blood (up to 40%) from *O. vulgaris* was followed by the rapid replacement of blood fluid over 24 h but no replacement of haemocyanin. The authors implied that no reserve of haemocyanin existed and that haemocyanin could not be synthesized rapidly. However O'Dor and Wells (1984) suggested that in times of stress or blood loss, *O. vulgaris* might utilize blood from the large blood sinuses around the gut.

Elucidation of the immune functions of *E. cirrhosa* necessitated the development of a sampling technique (Chapter 2). Very little information existed on the effects of removing blood on, in particular, the haemocytes of *E. cirrhosa*. This paper investigates some of the effects of taking blood samples on the haemocytes and the haemolymph of the octopod *E. cirrhosa*.

Materials and Methods

Animals

Eledone cirrhosa (Lam.) were caught in crab pots around the North Wales coast. They were brought into the aquarium at the University of Wales at Bangor and assigned to particular tanks. The animals were allowed to settle for 48 h before being weighed, sexed and marked. They were anaesthetised before being marked by use of a syringe and needle (21 gauge 1½"needle) containing a 1% solution of alcian blue in octopus Ringer (Chapter 2). The alcian blue mark was introduced into the tissue of the web as near to the base of the arm(s) as possible, and the arm thus marked used in a numbering system (left or right, 1-4).

Sampling

Several sampling routines were performed on different sets of animals. In each case the weight of the animal and the arm marks were recorded. The animals were then anaesthetised and sampled as described in Chapter 2. The volume of blood obtained was recorded and a 100µl sample were placed in 900µl of marine anticoagulant (MA) (NaCl, 2.63g/100ml; glucose, 1.8g/100ml; tri-sodium citrate, 0.088g/ml; citric acid, 0.055g/100ml) containing ethylene glycol-bis(β-aminoethylether) N, N, N', N', -tetraacetic acid (EGTA) (0.029g/100ml) and duplicate blood counts made using a haemocytometer. In some cases blood smears were made by placing 20µl of blood directly from the syringe onto glass slides and the blood drawn out over the slide. The glass slides were air-dried and stored at 4°C before staining. During some sampling

routines the remaining blood was then centrifuged, at 800g for 5 min at 4°C, and the cell-free haemolymph stored at -70°C for further analysis. This stored sample was used to determine the effect of sampling on protein and copper levels in the haemolymph.

Sampling Routines

Four sampling periods were utilized. One set of five animals was bled at 0 h, (taking between 1-2ml) followed by smaller samples (about 400µl) at 2, and 4 h (fig. 3.1). A second set of 5 animals was bled as the first set but sampling times were; day 0 and then days 1, 2, 3, 4, 7 (fig. 3.2) with 1 - 2ml samples being removed. A third set (of 10 animals) were all bled on day 0, sampling between 1-2ml, and then 2 animals were bled every 24 h, taking about 1ml of blood per animal, allowing all ten animals to be sampled over a 5 day period (A-E) (fig. 3.3). The final set of five animals were all sampled on day 0 taking as much blood as was possible (between 1 - 2 ml) and then less blood (about 400µl) was taken on subsequent samples at days 1, 2, 3, 5, 8, 12, 17, 24 (fig. 3.4).

Staining

Twelve blood smears were obtained per animal per sample for the first and third sampling routines, (i.e., 6 originals and 6 duplicates per sample). The second sampling routine was for blood counts only, but the fourth routine used only the blood stain, Giemsa (Sigma). The staining techniques employed for the first and third sampling

routines were acid phosphatase, alkaline phosphatase, peroxidase, Giemsa, periodic acid Schiffs (PAS) reaction and bromophenol blue. In all cases controls were run to ensure that the techniques were working. Acid phosphatase activity in the blood smears was detected by incubating the slides for 30 min in veronal acetate buffer containing sodium α-naphthyl phosphate (Gurr) and diazotized pararosaniline (Gurr) and adjusted to pH 6.0. Alkaline phosphatase activity was detected by incubating the glass slides for 60 min in a Tris buffer (pH 10.0) medium containing sodium α-naphthyl phosphate and fast red (Gurr). Peroxidase activity was determined by incubating the slides in a solution of diaminobenzidine (DAB) in tris buffer (pH 7.75) and hydrogen peroxide (Sigma). General blood stains using Giemsa in Gurr buffer (pH 6.8) and bromophenol blue were used for detection of major morphological differences in the haemocytes using a light microscope. Carbohydrates were detected using the PAS reaction.

Protein Determination

Ten µl of haemolymph diluted with 500µl distilled water were precipitated in 400µl of 12% trichloroacetic acid (TCA) for 2 h at 4°C. Individual samples were centrifuged at (4000g) for 30 min and the protein pellet resuspended in 200µl of 0.3M sodium hydroxide (NaOH) with the addition of 800µl of Biuret reagent (6g sodium potassium tartrate; 1.5g copper sulphate; 30g sodium hydroxide/1000ml distilled water). Samples and bovine serum albumin (BSA, Sigma) standards (0.125 - 2mg/ml) were incubated at 37°C for 30 min. One ml of heptane was added and the samples were vortexed and

centrifuged. The heptane was removed and 350µl of each sample were added in duplicate to a flat bottom 96 well plate(Dynatech). End point readings were taken at 550nm after a 30 min incubation of the plate at room temperature.

Copper Determination

Standards of 1, 2, and 3µg/ml of copper (B.D.H.) in distilled water were aspirated directly into an air-acetylene flame and a standard curve obtained using a Thermo-Jarrell-Ash atomic absorption spectrophotometer at 324.7nm. Twenty µl haemolymph samples were added to 5ml of distilled water and the mean atomic absorption readings calculated from the standard curve. Distilled water blanks were passed through the spectrophotometer after each haemolymph reading.

Analysis

Duplicate blood cell counts were averaged and converted to counts per ml. The mean and standard error of the replicates were then used to plot haemocyte numbers against the time used of sampling. Student t-tests were used to compare replicated blood counts at different sampling times. The Giemsa stained slides were used to determine differences in the number of granules in the haemocyte cytoplasm. The number of haemocytes with visible granules was expressed as a percentage of the total number of haemocytes recorded for each sample and replicate means and standard errors were recorded. Student t-tests were then used to compare the percentage number of haemocytes containing visible granules with the sampling time.

Observations on acid and alkaline phosphatase, peroxidase, the PAS reaction, and bromophenol blue stain were recorded and used to demonstrate possible haemocyte differences over the sampling periods.

Haemolymph protein and copper concentrations were determined and the mean and standard error of the replicates were plotted. T-tests were used to determine significant differences between sampling times.

Results

Blood Counts

It has been demonstrated that sampling has an effect on the haemocyte population present in the blood of *Eledone cirrhosa*. When a sample(1-2ml) is taken, the haemocyte number per ml increased within the first couple of hours.

Haemocyte counts over 4 h indicate that within 2 h of removing 1 - 2 ml of blood from the animal the count nearly triples, but it has decreased significantly ($p<0.05$) at 4 h (fig. 3.1). Samples taken from a second set of 5 animals sampled over 7 days indicate a significant increase ($p<0.05$) in haemocyte numbers/ml within the first 24 h (fig. 3.2). Over the following 6 days the haemocyte numbers decreased and have decreased significantly by day 7 in comparison with the 24 h sample.

Sampling over 10 days with all 10 animals sampled at day 0 (fig. 3.3), then 2 animals per day resampled over the following 10 days indicates a dramatic increase in haemocyte numbers within the first 48 h (B^1). All subsequent haemocyte counts were significantly less than the 48 h sample but apart from the day 4 sample (D^1) were all significantly higher than the day 0 sample. All 10 animals were sampled 3 times over the 10 day sampling period, on day 0 and then twice in rotation on subsequent days, e.g. animals in set A were bled on day 0, day 1 (A^1) and day 6 (A^2). The haemocyte counts obtained over the 10 days appeared to indicate a possible cycle of increasing counts followed by a decrease, e.g. the values between days 0 & 3, 4 & 7 and 7 & 10. However when results for different individuals are compared there is an indication that the timing of sampling is important.

Figure 3. 1. The number of haemocytes/ml in blood in *Eledone cirrhosa* over a 4 hour sampling period. The bars are the means of 5 animals and the error bars are the standard errors of the mean.

Figure 3. 2. The number of haemocytes/ml in blood in *E. cirrhosa* over a 7 day sampling period. The bars are the means of 5 animals and the error bars are the standard errors of the mean.

Figure 3.1

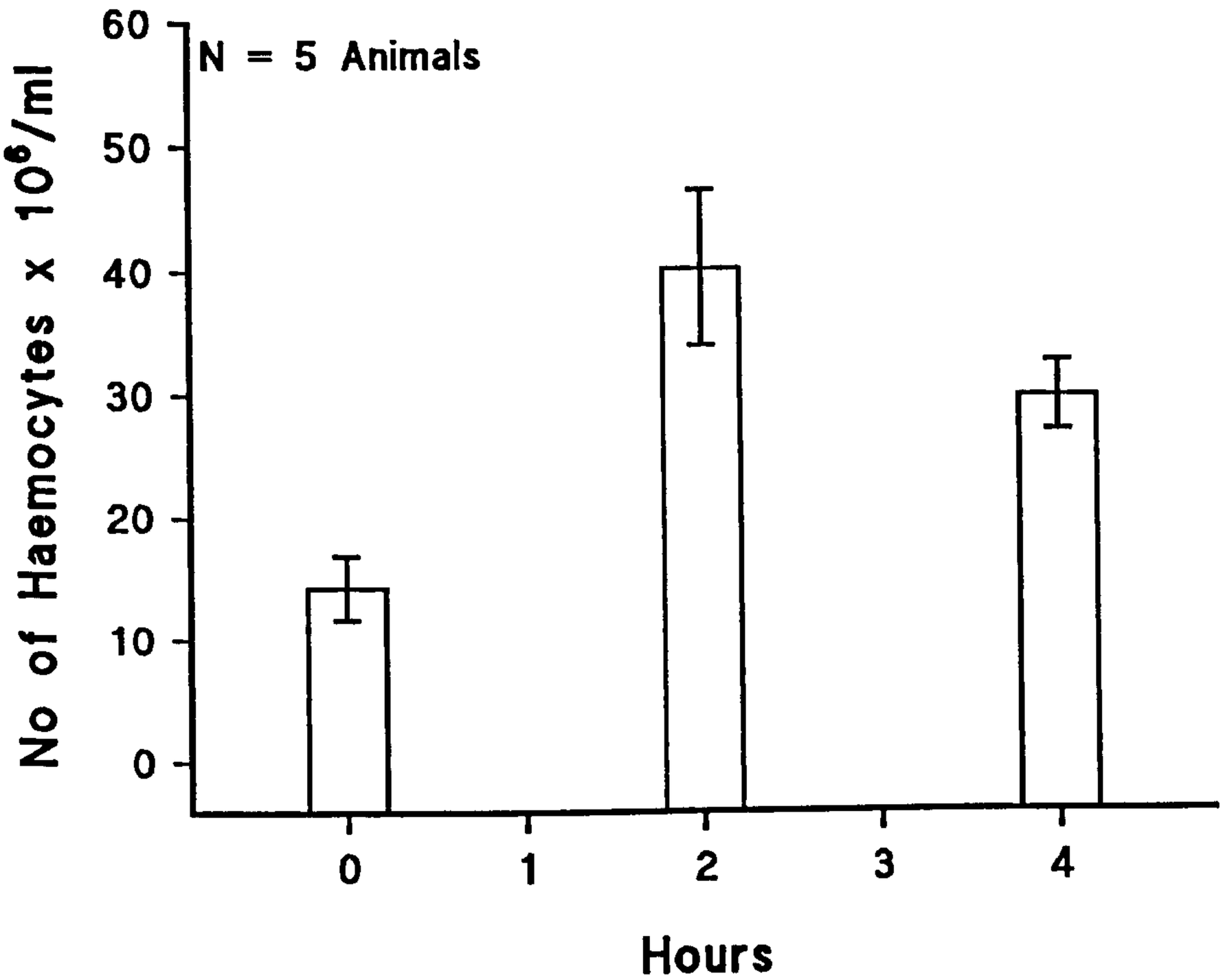


Figure 3.2

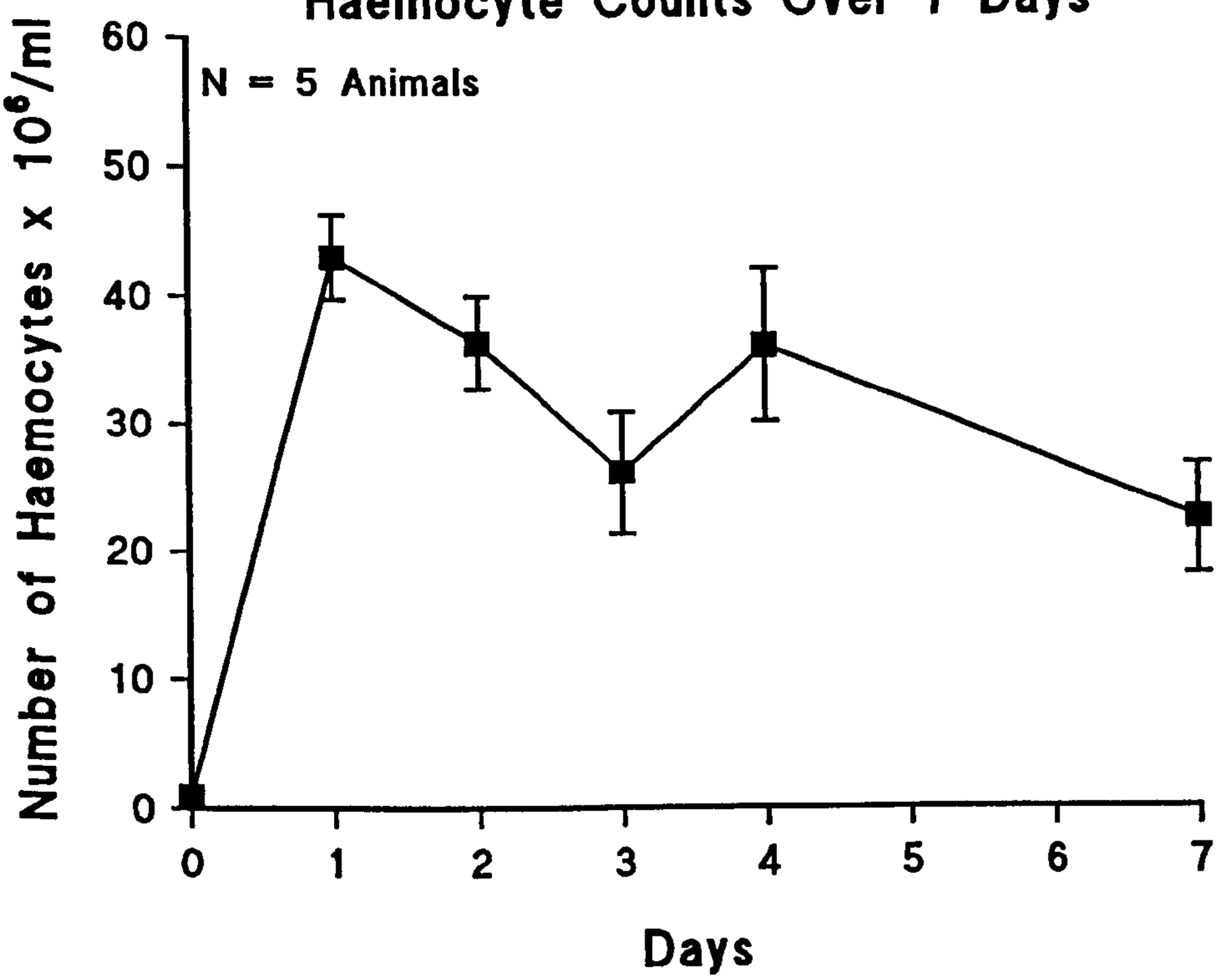


Figure 3. 3. The number of haemocytes/ml in blood in *E. cirrhosa* over a 10 day sampling period. Ten animals were bled on the first day, day 0, and 2 animals/day over the remaining 10 days. The bar on day 0 is the mean of 10 animals whereas the bars for days 1-10 are the mean of 2 animals. The error bars are the standard errors of the mean.

(A1 - E1 = days 1 - 5; A2 - E2 = days 6 - 10).

Figure 3. 4. The number of haemocytes/ml in blood in *E. cirrhosa* over a 24 day sampling period. The bars are the means of 5 animals and the error bars are the standard errors of the mean.

Figure 3.3

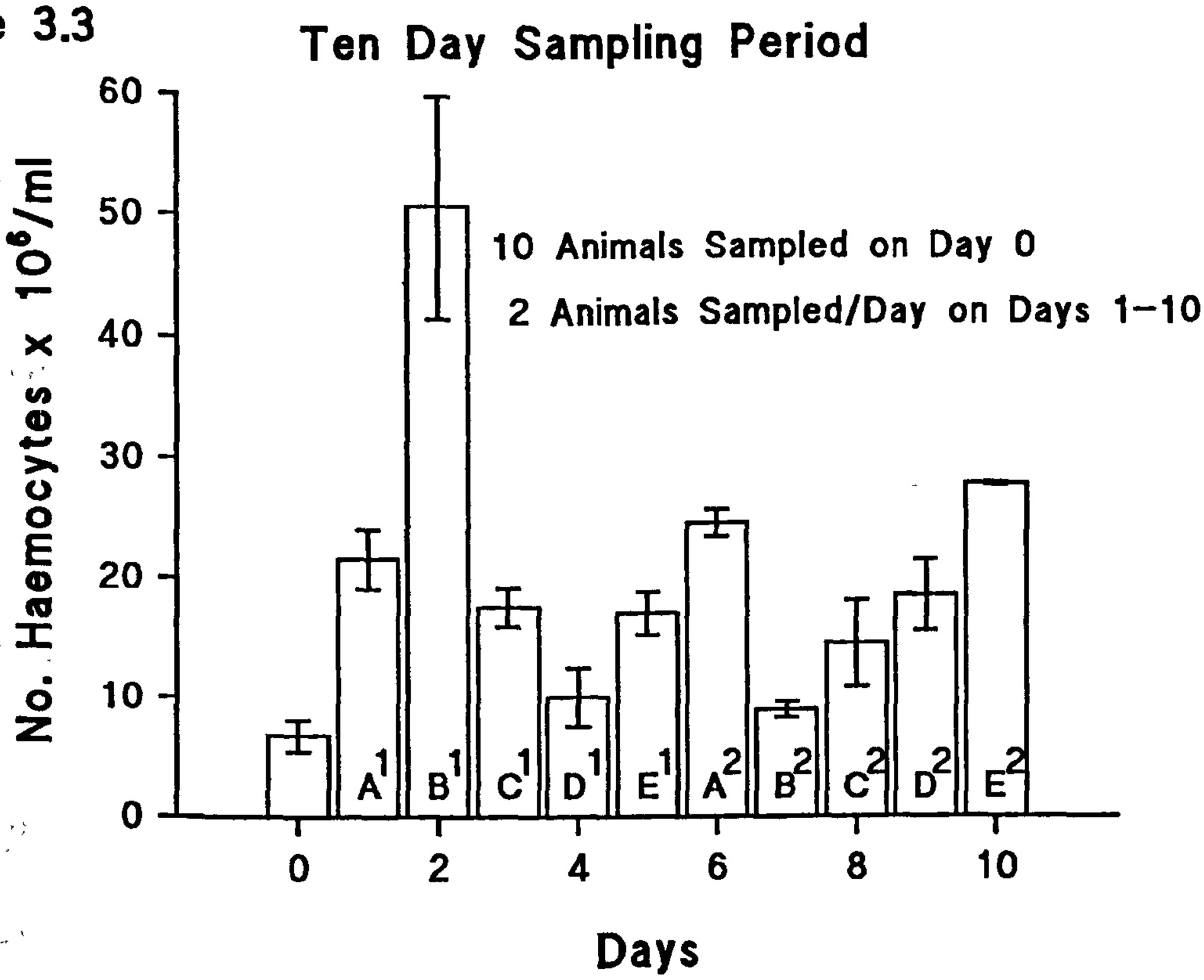
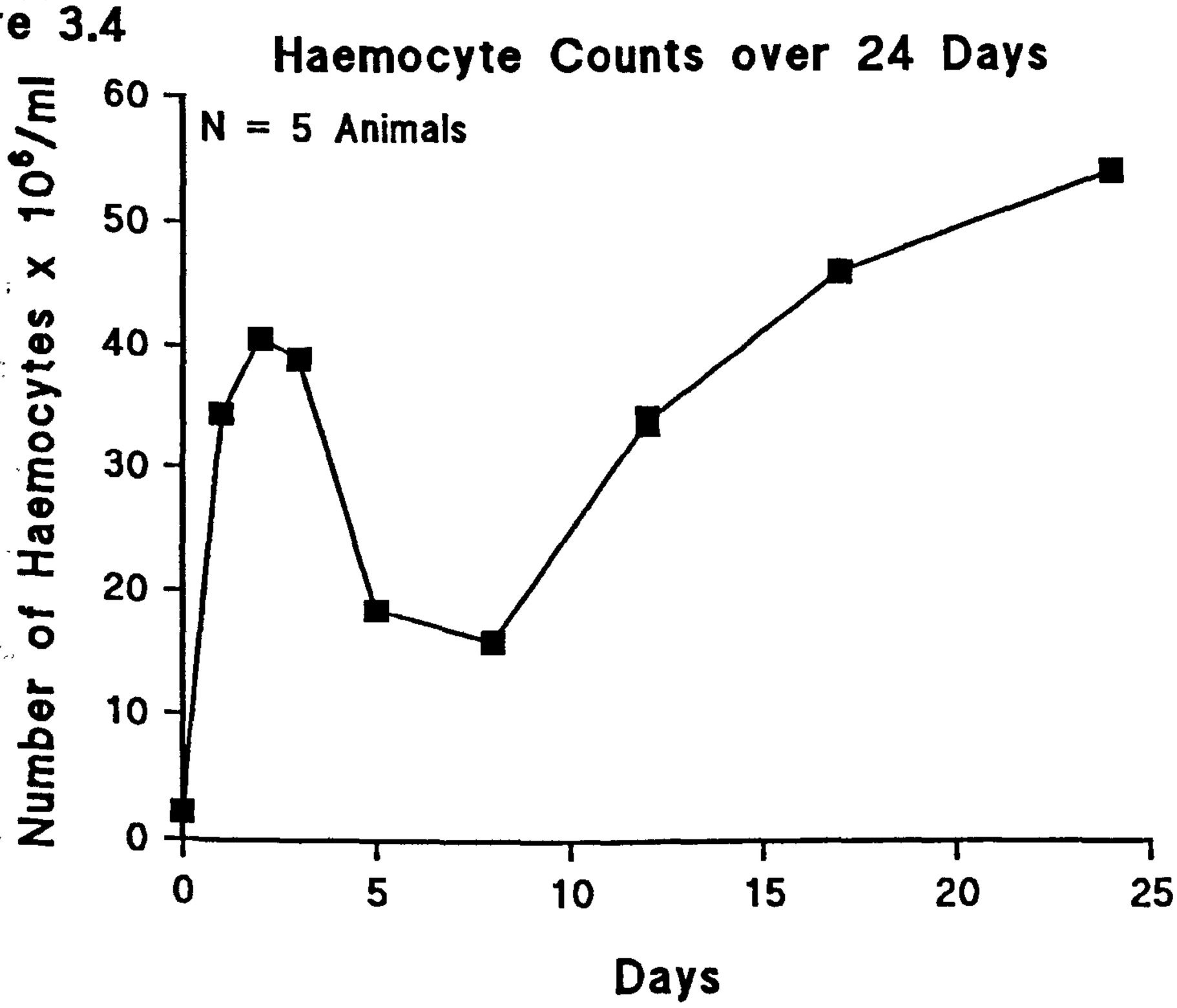


Figure 3.4



When the sampling period was extended to 24 days, sampling animals on days 0, 1, 2, 3, 5, 8, 14, 18 and 25 (fig. 3.4), the haemocyte numbers again increased significantly ($p<0.05$) to day 2. This was followed by a decrease to day 8. Then haemocyte numbers continued to increase again until the end of the experiment.

Blood Smears

The percentage of haemocytes containing Giemsa positive granules increased over the 4 h sampling period (fig. 3.5). Similarly there was an increase in the percentage of haemocytes containing granules up to day 3 of the 10 day sampling period (fig. 3.6) and up to day 5 of the 25 day sampling period (fig. 3.7). The set of animals (A) bled on days 1 and 6, in the 10 day sampling regime, showed large increases in the percentage of haemocytes containing granules visible in the cytoplasm (fig. 3.6), while both sets B and C showed a significant decrease from the first to the second sampling sets. This may again indicate that the time of sampling is important. Animals in the 25 day sampling group showed an increase in the percentage of haemocytes containing granules to day 5 (fig. 3.7) followed by a dramatic decrease to day 8 with a slower rate of decline to a value less than the day 0 value by day 24.

Haemocytes obtained in samples from the 10 day group were negative or only stained weakly with the PAS and DAB reactions for carbohydrate and peroxidase respectively (table 1). Weak staining in comparable smears stained with bromophenol blue for protein indicated a slight increase in staining for 3 animals (L1 @ day 6, R1 @ day 3

Figure 3. 5. The bars represent the mean value for the percentage of haemocytes containing cytoplasmic granules from 5 animals over a 4 hour sampling period. Error bars are standard errors of the mean.

Figure 3. 6. The bars represent the percentage of haemocytes containing cytoplasmic granules over a 10 day sampling period. The mean for 10 animals is presented on day 0 whereas over the remaining sampling period (days 1-10) the mean of 2 animals was taken. Error bars are standard errors of the mean.

(A1 - E1 = days 1 - 5; A2 - E2 = days 6 - 10).

Figure 3.5 Percentage of Haemocytes with Cytoplasmic Granules Sampled Over 4 Hours

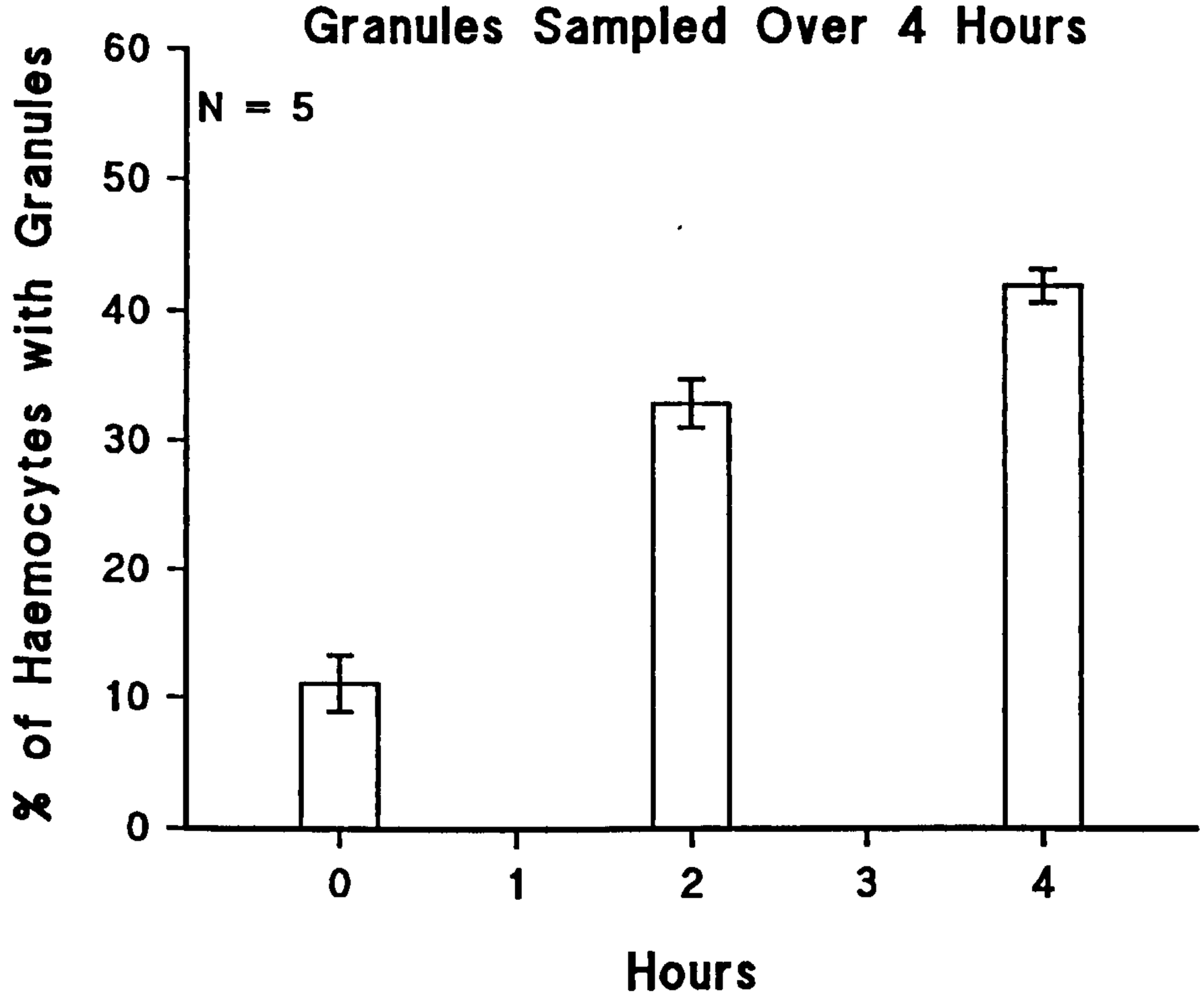


Figure 3.6 Percentage of Haemocytes with Cytoplasmic Granules Sampled Over 10 Days

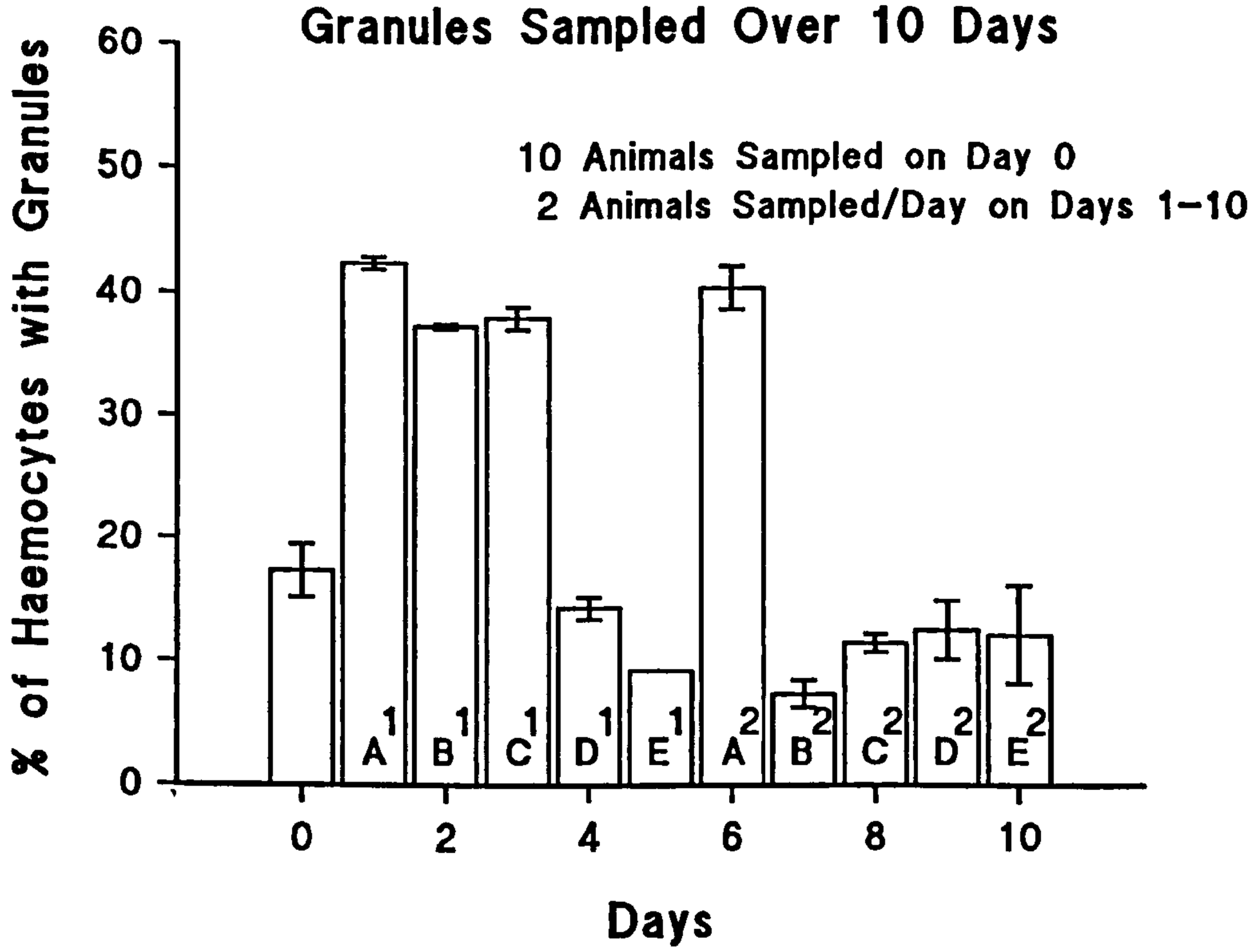


Figure 3. 7. The bars represent the mean value for the percentage of haemocytes containing cytoplasmic granules from 5 animals over a 24 day sampling period. Error bars are standard errors of the mean.

Figure 3.7 Percentage of Haemocytes with Cytoplasmic Granules Sampled Over 24 Days

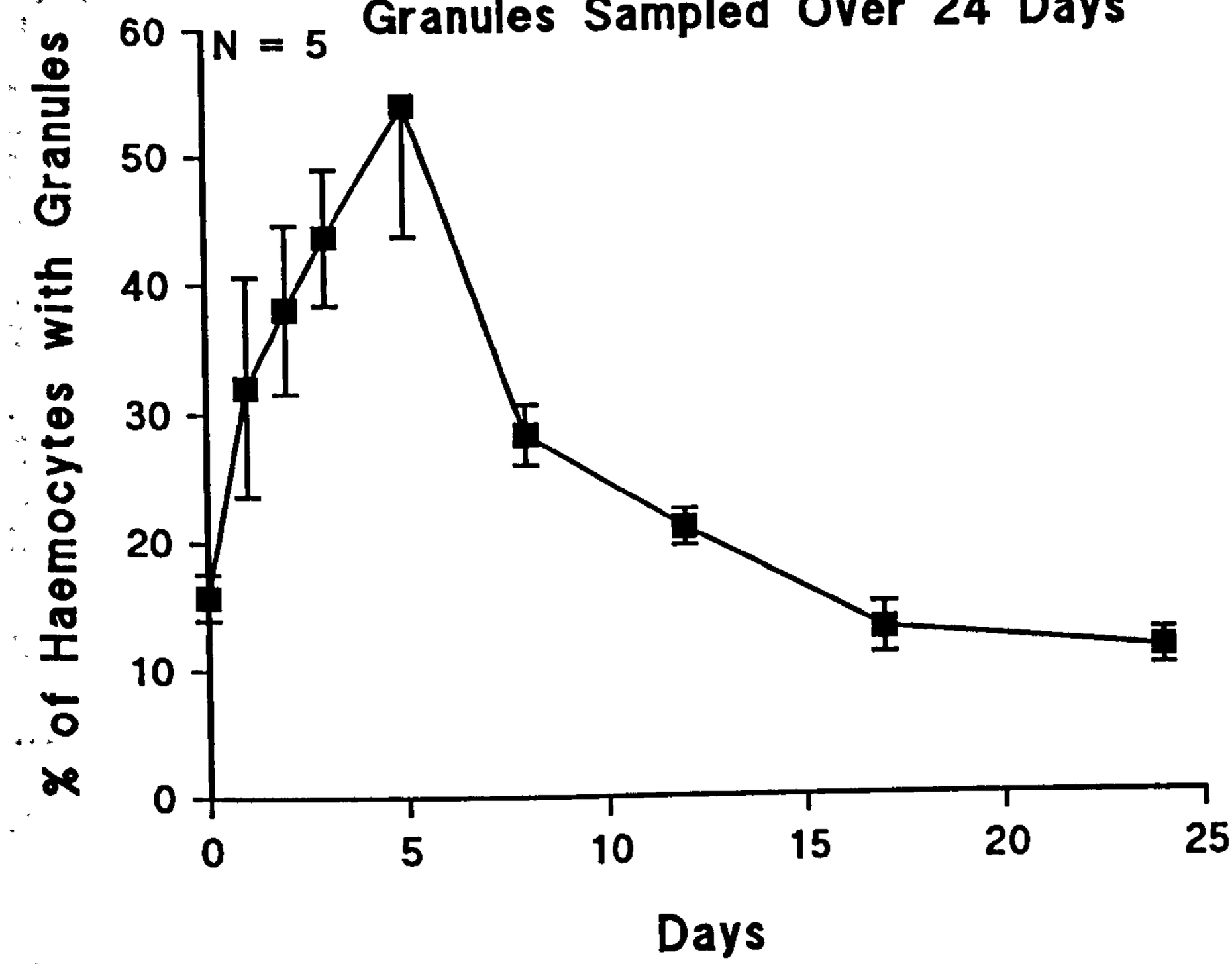


Table 1. Blood smears were taken from 10 animals on day 0 and from 2 animals/day for the next 9 days. The smears were stained with periodic acid Schiffs reagent, bromophenol blue, acid phosphatase and diaminobenzidine. The staining intensity for each of the stains used was recorded.

1.a. Periodic Acid Schiffs

Day	L1	L2	Day	L3	L4	Day	R1	R2	Day	R3	R4	Day	L1 R1	L2 R2
0	-	-	0	-	-	0	-	-	0	+	+	0	+	+
1	+	+	2	-	-	3	+	+	4	-	-	5	-	+
6	-	-	7	-	-	8	-	-	9	+	+	10	-	-

1.b. Bromophenol Blue

Day	L1	L2	Day	L3	L4	Day	R1	R2	Day	R3	R4	Day	L1 R1	L2 R2
0	+	+	0	+	+	0	+	+	0	+	+	0	+	+
1	+	+	2	+	+	3	++	++	4	+	+	5	+	+
6	++	+	7	+	+	8	+	+	9	+	+	10	+	+

1.c. Acid Phosphatase

Day	L1	L2	Day	L3	L4	Day	R1	R2	Day	R3	R4	Day	L1 R1	L2 R2
0	-	+	0	-	-	0	-	-	0	-	-	0	+	+
1	+	++	2	-	-	3	+	+	4	+	+	5	-	-
6	++	++	7	+	-	8	+	+	9	++	+	10	-	-

1.d. D.A.B.

Day	L1	L2	Day	L3	L4	Day	R1	R2	Day	R3	R4	Day	L1 R1	L2 R2
0	-	-	0	+	+	0	+	-	0	+	-	0	+	+
1	+	-	2	-	-	3	-	-	4	-	+	5	-	-
6	-	-	7	-	-	8	-	-	9	-	-	10	-	-

L1-L2R2 = 10 Individual Animals

- = No Reaction
+ = Slight Positive Reaction
++ = Moderate Positive Reaction
+++ = Strong Positive Reaction

and R2 @ day 3). Considerable variation was noted with staining for acid phosphatase but an increase in staining in the second and third samples was noted for 7 of the 10 animals.

All 4 staining techniques indicated increased positive results compared to day 0 for all animals in the 25 day sampling group (table 2). The haemocytes from 3 animals showed increased levels of staining with PAS at day 3 and this was maintained or increased until day 24. Levels of staining increased at day 1 but then decreased to day 0 levels at day 8 in the other 2 animals. Staining for protein in the haemocytes from all animals increased by day 8 or 12 but decreased to day 0 levels at day 24 in 3 of the 5. Staining for acid phosphatase and peroxidase increased within 1 to 3 days but returned to day 0 levels in all but 2 animals. No positive results were obtained for alkaline phosphatase, (results not shown).

Protein Concentration

Over 4 h (fig. 3.8) the protein concentration of the haemolymph decreased significantly ($p<0.05$) from about 116 mg/ml to 64 mg/ml. Large individual variations were observed over the 7 day sampling routine (fig. 3.9). The protein concentration at day 7 was not significantly different ($p=0.4$) from the day 0 value. The possible increase at days 2 and 3, of the 7 day sample, although not significant because of the high level of variation, requires further study.

Table 2. Blood smears were taken from 5 animals over a period of 24 days. The smears were stained with periodic acid Schiffs reagent, bromophenol blue, acid phosphatase and diaminobenzidine. The staining intensity for each of the stains used was recorded.

2.a. Periodic Acid Schiffs

Day	L1	L2	L3	L4	R2
0	+	+	+	+	+
1	+	+	++	++	+
2	+	+	++	+	+
3	++	++	++	++	++
5	++	++	++	+	++
8	++	++	+	+	+
12	+++	++	+	+	+
17	++	++	+	+	++
24	++	++	+	+	++

2.b. Bromophenol Blue

L1	L2	L3	L4	R2
-	-	-	-	-
-	+	-	+	-
+	+	+	+	-
-	+	+	-	+
+	+	-	-	+
+	++	+	+	++
++	+	++	+	++
+++	++	++	++	++
+	-	++	+	++

2.c. Acid Phosphatase

Day	L1	L2	L3	L4	R2
0	-	+	+	+	-
1	-	+	+	++	+
2	-	++	++	++	+
3	+	+++	++	+	+
5	-	++	+	+	+
8	+	++	+	+	+
12	++	++	+	++	++
17	+	+	+	++	+
24	+	+	+	+	+

2.d. D.A.B.

L1	L2	L3	L4	R2
-	-	-	+	-
+	+	+	++	-
++	+	+	++	-
++	++	+	++	+
++	++	+	+	+
++	+	+	+	+
+	+	+	+	+
++	+	-	+	+
++	+	-	+	+

L1-R2 = 5 Individual Animals

- = No Reaction
+ = Slight Positive Reaction
++ = Moderate Positive Reaction
+++ = Strong Positive Reaction

Figure 3. 8. The protein concentration in samples of haemolymph collected from *E. cirrhosa* over 4 h. Each value is the mean of 5 animals. Error bars are standard errors of the mean.

Figure 3. 9. The protein concentration in samples of haemolymph collected from *E. cirrhosa* over 7 days. Each value is the mean of 5 animals. Error bars are standard errors of the mean.

Figure 3.8

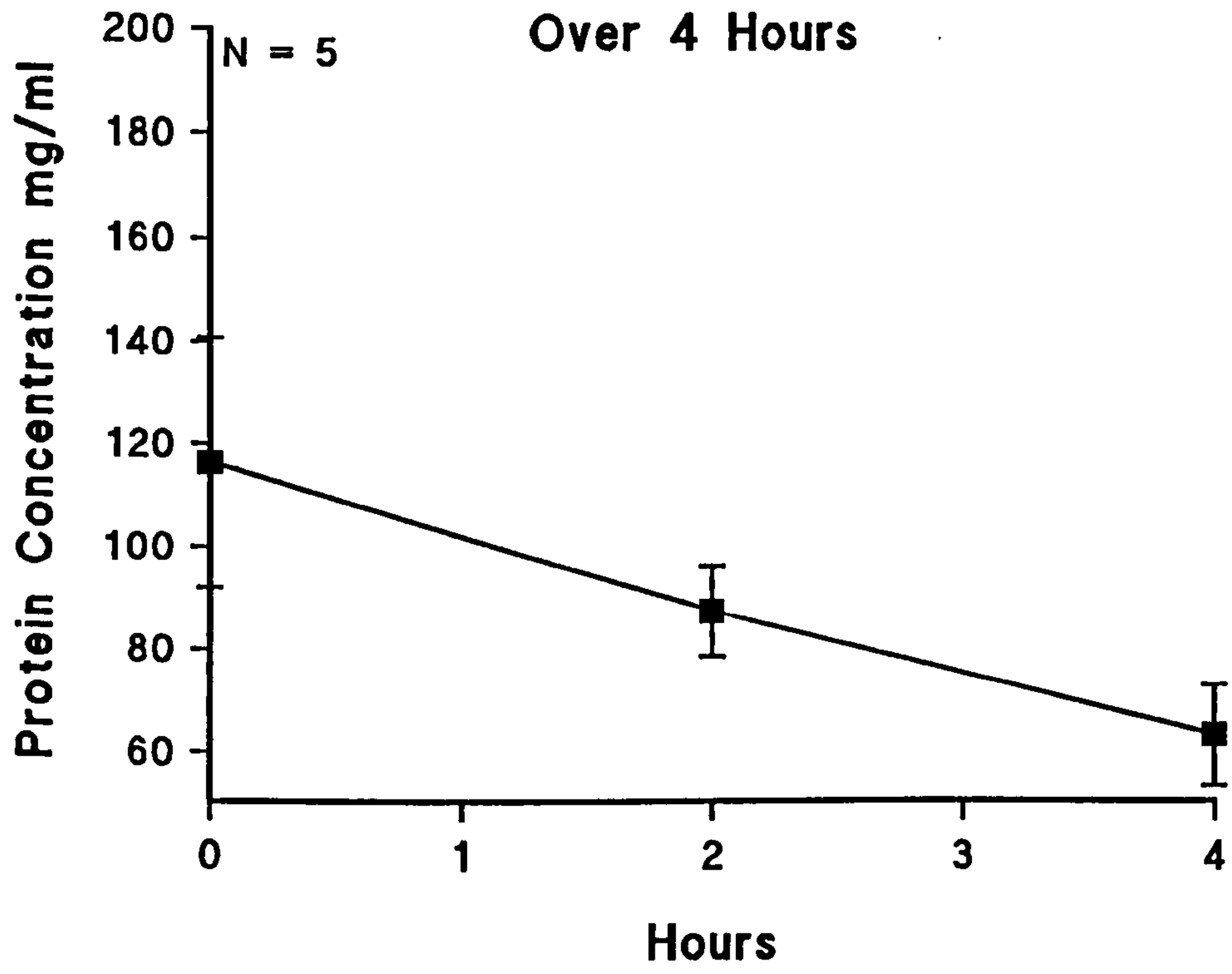
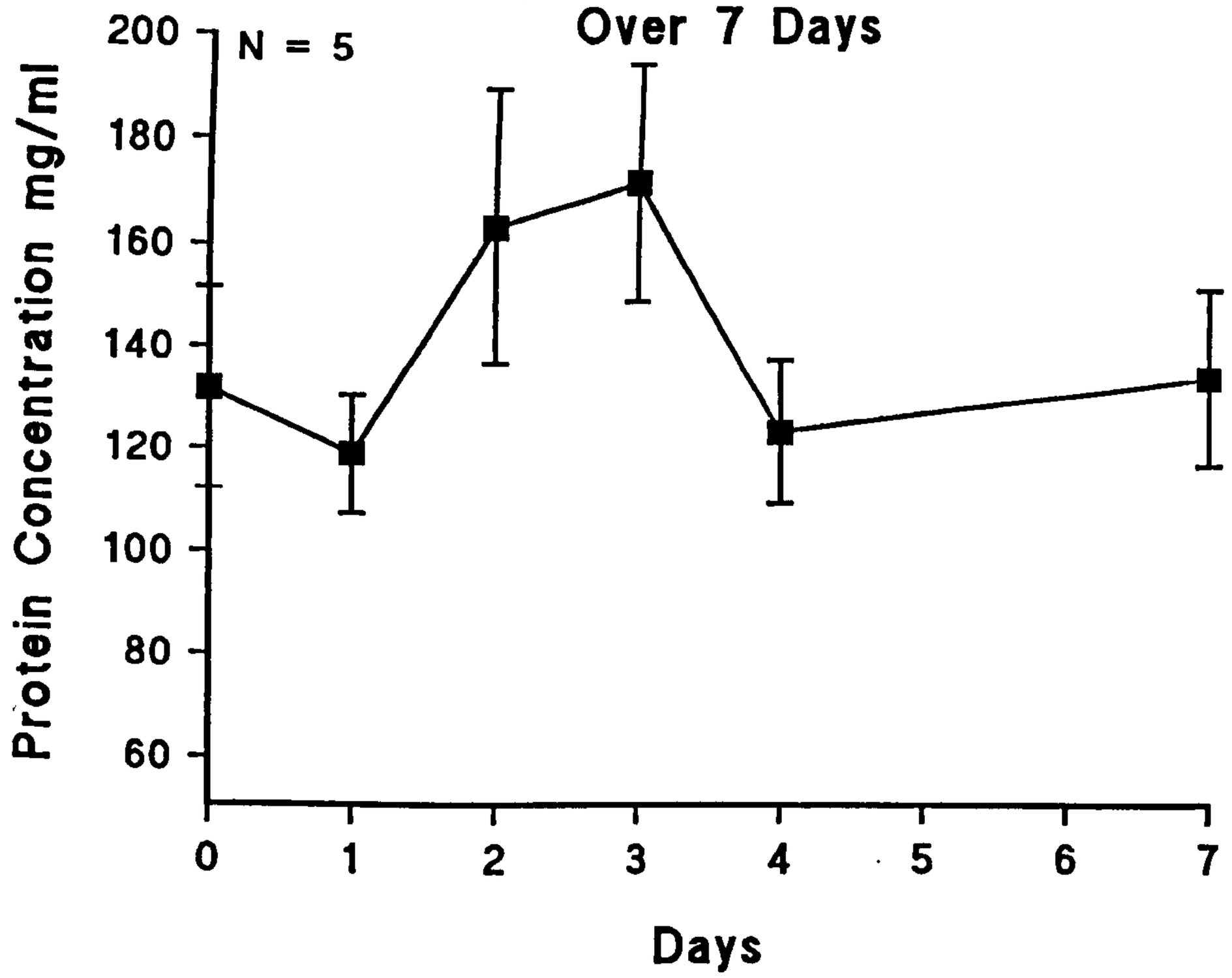


Figure 3.9



Copper Concentration

The concentration of copper in the haemolymph decreases in the 4 h sampling group (fig. 3.10), and the decrease was significant ($p<0.05$) between the initial (0 h) and final (4 h) bleeds. The concentration of copper in the haemolymph decreases in the 11 day sampling group after the initial sample on day 0 (fig. 3.11). Over days 1, 2 and 3, the copper concentration does not change even though on each day between 2 - 4% of the animals blood is being removed. After day 3 however continued sampling at the same rate resulted in the copper concentration continuing to decrease and no recovery was observed over the rest of the sampling period.

Figure 3. 10. The copper concentration in samples of haemolymph collected from *E. cirrhosa* over 4 h. Each value is the mean of 5 animals. Error bars are standard errors of the mean.

Figure 3. 11. The copper concentration in samples of haemolymph collected from *E. cirrhosa* over 11 days. Each value is the mean of 5 animals. Error bars are standard errors of the mean.

Figure 3.10

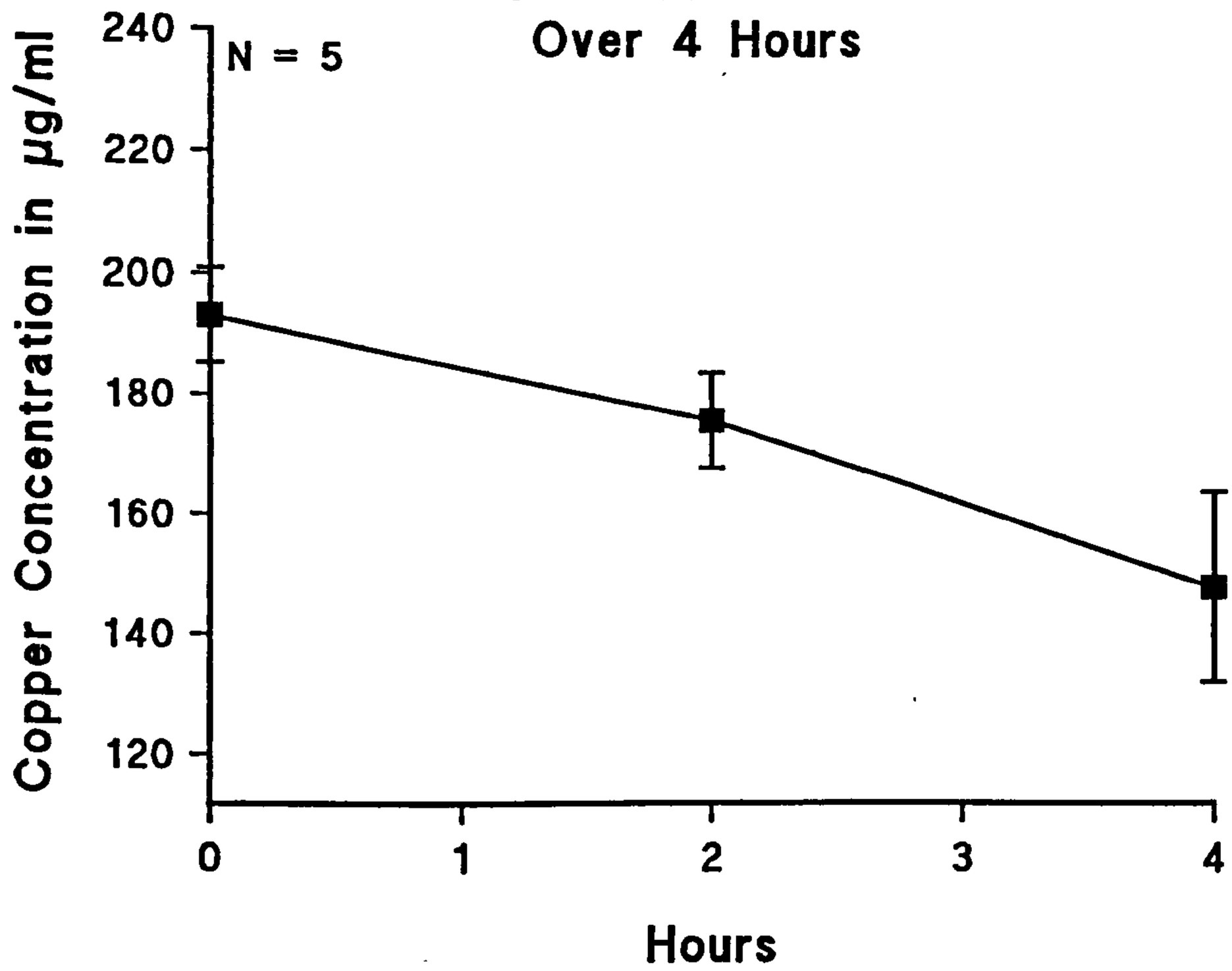
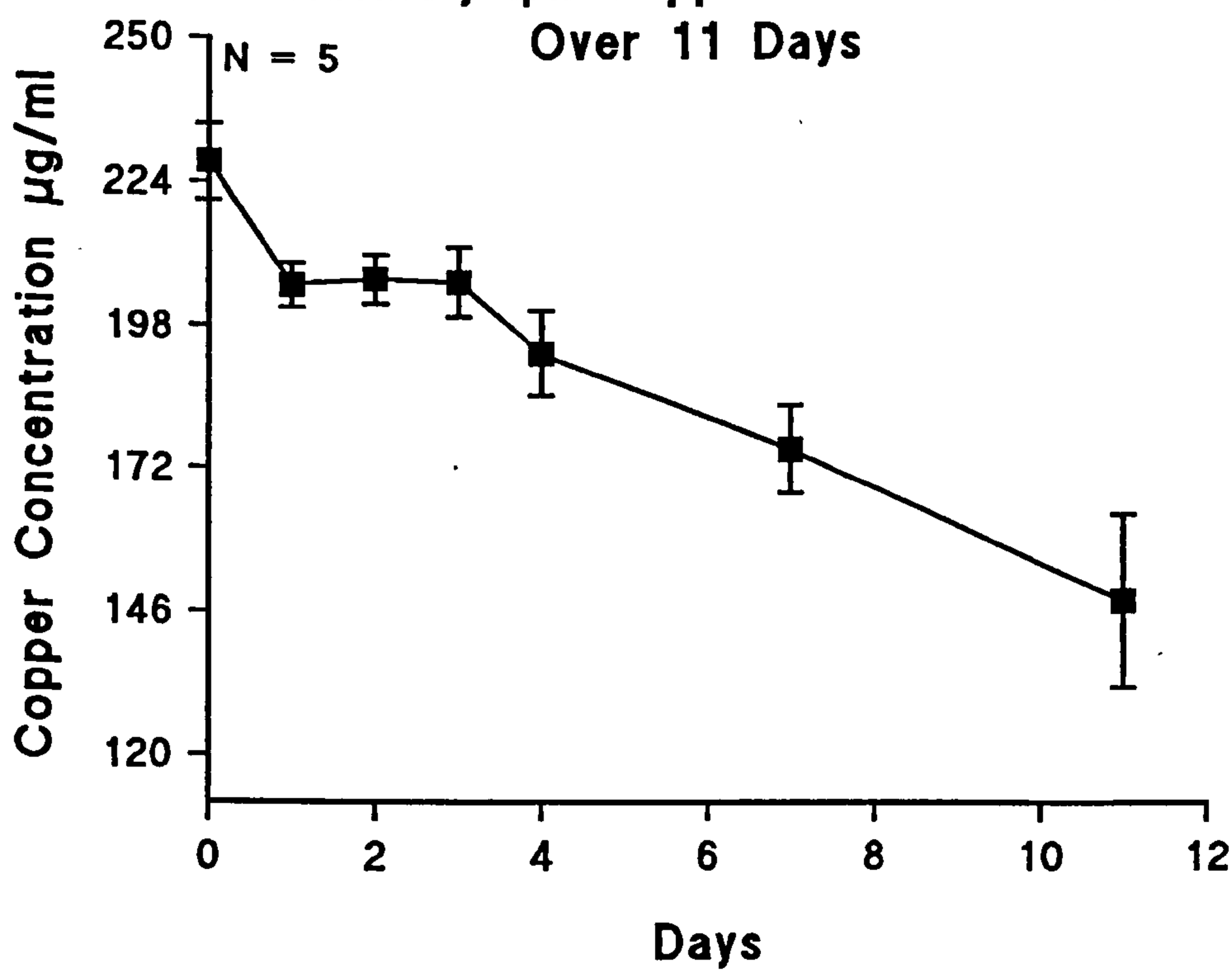


Figure 3.11



Discussion

The number of circulating haemocytes in *Eledone cirrhosa* increases in response to blood loss. The increase in haemocyte numbers/ml is associated with an increase in the percentage of haemocytes containing cytoplasmic granules as detected by the Giemsa blood stain. The concentration of copper in the haemolymph decreases and does not appear to be replaced whereas the protein concentration did not significantly change over seven sampling days.

Increased haemocyte counts have been demonstrated for other molluscs following injury (Sminia *et al.*, 1973), decrease in oxygen tension (Wolmorans & Yssel, 1988), increased temperature (Stumpf & Gilbertson, 1978), blood extraction (Sminia, 1972) and after bacterial challenge (Suresh & Mohandas, 1990). Haemocyte counts initially increase 4 h after surgery in the Pacific oyster *Crassostrea gigas* and this appears to be a stress response (Jones *et al.*, 1993). Blood sampling obviously induces stress in *E. cirrhosa*, as evidenced by the initial increase in blood count but it does decrease by day 8, before increasing again, suggesting that stress was not a major factor in increasing haemocyte numbers. One possible explanation could be the release of mature or maturing haemocytes from the white body or leucopoetic organ, and/or other stores, e.g. the posterior salivary glands (pers obs.), to compensate for blood loss. Production of amoebocytes in large numbers under specific pathological conditions has been demonstrated in *Biomphalaria glabrata* after infection with different *Echinostoma* species (Lie *et al.*, 1975). Jeong *et al.* (1983) further demonstrated that the amoebocyte producing organ of *B. glabrata* increased in size and an increased number

of amoebocytes were loosely arranged in zones of progressive maturation after infection with *Echinostoma* species. The bivalves *Sunetta scripta* and *Villorita cyprinoides* var. *cochinensis* demonstrated leucocytosis after both sham and bacterial injections (Suresh & Mohandas, 1990). Further, using *Lymnaea stagnalis*, Mohandas *et al.* (1992) suggested that after haemolymph extraction young and mature haemocytes were released from a 'reservoir' (Sminia *et al.*, 1983) into the haemolymph of the snail. No evidence for leucocytosis was demonstrated for *E. cirrhosa* after sampling, however when challenged with bacteria, (Chapter 8) massive haemocyte release was also indicated suggesting possible leucocytosis. Though invertebrate circulating haemocytes have been shown to proliferate (Sequira *et al.*, 1996; Peddie *et al.*, 1995), no evidence of such proliferation was observed with *E. cirrhosa* (pers. obs.).

A complex cycle of increasing and decreasing haemocyte numbers in *E. cirrhosa* is also indicated by the animals sampled on 3 occasions at different times over 10 days. The counts in set A^1 (fig. 6) sampled 1 day after the initial sample had increased to a very high level, however, set D^1 which was not sampled until 4 days after the initial sample had a reduced count and this was still low 5 days later (D^2). This sampling regime needs to be repeated at a wider range of sampling times.

The changes in the percentage of haemocytes containing granules and the changes in the histochemical properties of the haemocytes under the different sampling regimes would support the suggestion that haemocytes with different properties are being added to those already present in the blood of the octopod. Presumably these are new

haemocytes, being released from the white body, containing a higher proportion of cytoplasmic granules. Cowden and Curtis (1974, 1981) showed that mature haemocytes from *Octopus briareus* and *O. vulgaris* have a large number of irregular electron-dense inclusions in the cytoplasm upon release into the circulation. Interestingly haemocyte numbers/ml decreased significantly in *E. cirrhosa* after the third sample (day 2) was taken during the 24 day sample routine. However the percentage of haemocytes containing visible granules continues to increase until the fifth sample (day 5). Over the following 8 days the number of haemocytes/ml increases while the percentage of haemocytes with granules decreases. Mohandas *et al*, (1992) showed that haemocytes from *L. stagnalis* demonstrated differential staining after haemolymph collection following forced foot retraction. Acid phosphatase activity in *L. stagnalis* increased over 1-5 days following bleeding whereas peroxidase staining decreased. Lysosomal enzymes and peroxidase have been demonstrated to be associated with many invertebrate blood cells (Ratcliffe *et al*, 1985; Millar and Ratcliffe, 1994). With *Octopus*, a PAS positive reaction in the cytoplasm of mature haemocytes was obtained for *O. vulgaris* but not for *O. briareus* (Cowden, 1972). Since *E. cirrhosa* haemocytes are known to be involved in cellular defence functions (Stuart, 1968; Chapter 5, Chapter 6, Chapter 7) it is assumed that lysosomes and peroxisomes would be present in the haemocytes, particularly in those newly released. The concentration of haemolymph protein decreases initially during the first 4 h of sampling in *E. cirrhosa*. Over 7 days however it does not significantly depart from the day 0 value. Apart from haemocyanin, octopus haemolymph also contains

glycoproteins, lectins and agglutinins (Rögener *et al*,, 1986,1987). Busselen (1970) demonstrated that haemolymph glycoprotein levels in *Carcinus maenas* changed during blood sampling, causing an increase of the ratio of glycoprotein : haemocyanin concentrations. Further the glycoprotein concentration depended on the nutritional status of the animal. Djangmah (1970) also demonstrated that glycoprotein levels in *Crangon vulgaris* haemolymph were dependent on nutritional status with both the glycoprotein and haemocyanin being utilized as a food reserve during starvation.

The large variation observed for the protein concentrations obtained could be due to individual variation. Though all octopuses used were female, not all animals were of the same weight or maturity stage. Food was available in abundance in an attempt to reduce any effects on plasma protein due to nutritional stress. Large variation of the total protein concentration in the haemolymph has been demonstrated in the blue crab *Callinectes sapidus* (Horn & Kerr, 1963) and the shore crab *C. maenas* (Uglow, 1969a,b). However in both species sampling over a week caused a decrease in protein concentration accompanied by only a minor change in copper concentration. Horn and Kerr (1963) suggested that circulating apohaemocyanin molecules were being converted to haemocyanin by the addition of copper atoms.

The samples taken for protein determination in both the 4 h and 7 day sampling routines were also used to determine the copper concentration in the haemolymph of *E. cirrhosa*. The copper and protein levels decreased over the first hours of sampling. Interestingly the protein concentration does not significantly vary over 7 days (fig. 9) while the copper concentration decreases by 40% in the same samples (fig. 11). The

above results could therefore indicate that within the first few hours following sampling fluid moves into the blood reducing the haemocyanin concentration but that over a longer period the protein concentration is restored with a non-haemocyanin protein. Whether the protein is apohaemocyanin remains to be determined. It has been demonstrated that after blood loss, *O. vulgaris* is able to restore its blood volume (Wells & Wells, 1993), by increasing fluid uptake. Wells and Wells (1993) also showed no replacement of haemocyanin after the withdrawal of a large volume of blood from *O. vulgaris*. Controlled depletion and replacement of haemocyanin during nutritional distress, moulting and at different seasons have been shown for several crustaceans (Busselen, 1970; Djangmah, 1970; Djangmah & Grove, 1970). However the synthesis and breakdown of haemocyanin in cephalopods appears to be tightly controlled (Senozan *et al.*, 1988).

No correlation was found between weight, sex and haemocyanin concentration in the haemolymph of the cephalopod *Sepia officinalis*, and very little individual variation was shown in copper concentration (Senozan *et al,*, 1988). The copper concentration results presented for *E. cirrhosa* also show less individual variation than those found for protein even though the same samples were used for both determinations.

In conclusion, it would appear that loss of blood in *E. cirrhosa* is followed by an almost immediate increase in newly released circulating haemocytes. Further experiments are being undertaken to look at haemocyanin and also the other constituent proteins present in the haemolymph of *E. cirrhosa*. The haemocytes and

leucopoetic organs are also being extensively studied as is the relationship between blood loss, bacterial challenge and leucocytosis in *E. cirrhosa*.

Acknowledgements

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Chapter 4

Phagocytosis by Haemocytes from the Lesser Octopus

Eledone cirrhosa

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Abstract

Haemocytes from *Eledone cirrhosa* phagocytose formalized bacteria (*Vibrio anguillarum*). The phagocytic capabilities of *E. cirrhosa* haemocytes are affected by several factors, including the haemocyte culture medium, temperature, duration of the assay, and the bacterial pre-incubation conditions such as haemolymph concentration, temperature and the duration of pre-incubation.

Haemocytes will phagocytose in the absence of haemolymph and enhanced phagocytosis will occur in 100% haemolymph. After 2 hours at 15 or 20°C however, the number of haemocytes phagocytosing unopsonized bacteria is equivalent to the number engulfing 100% haemolymph opsonized bacteria. In addition, with a 30min incubation period, the number of phagocytosing haemocytes increases as the prior-opsonization concentration of haemolymph and the incubation temperature increase.

Key Words: *Eledone cirrhosa*; haemocytes; phagocytosis; opsonization.

Introduction

In vivo and *in vitro* investigations into the cellular activities of molluscs have demonstrated that, in a number of cases, the blood cells or haemocytes are avidly phagocytic and capable of recognising non-self (reviewed by Millar & Ratcliffe, 1994). The process of phagocytosis involves a number of recognizable stages, which include attraction, attachment, ingestion and killing of foreign organisms, and is influenced by a number of factors (reviews by Ratcliffe *et al.*, 1985; Millar & Ratcliffe, 1994). Variables which have been shown to affect phagocytic rates in molluscs include incubation temperature (Foley & Cheng, 1975), time and pH (Abdul-Salam & Michelson, 1980), the size of the particle presented for phagocytosis and the nature of the particles (reviewed by Bayne, 1983). Though phagocytosis will take place in the absence of opsonizing agents (Renwrantz & Stahmer, 1983; Tuan & Yoshino 1987; Fryer, *et al.*, 1989), several experiments have shown that soluble humoral factors or opsonins may be instrumental in non-self recognition (Prowse & Tait, 1969) and/or enhancement of phagocytosis (reviews by Jenkin, 1976; Ratcliffe *et al*, 1985).
The haemocyte culture medium has been shown to influence phagocytosis with, in the case of the Asian clam, *Corbicula fluminea*, the presence of divalent cations being necessary for both opsonin-independent and opsonin-dependent phagocytosis (Tuan *et al.* 1987). The process of opsonization also appears to be influenced by several other factors. Fryer and Bayne (1989), using *Biomphalaria glabrata*, showed that for this mollusc opsonization is a time-dependent process. Further, Tripp (1992), working

with *Mercenaria mercenaria* demonstrated that at low temperatures, opsonized particles were phagocytosed at a faster rate than unopsonized particles.

The octopus *Eledone cirrhosa* is benthic in habit, ranges in depth from sub-littoral to 770 m and encounters temperatures between 5 and 15°C (Boyle, 1983). The animal has a closed circulatory system and if wounded prevents blood loss by local vasoconstriction of the area surrounding the wound. The blood of the octopod does not clot and further blood loss is prevented by allowing seepage of blood through the wound until blood cells eventually plug the wound (Wells, 1978, 1983; Bayne, 1983). If the animal loses a large amount of blood a dilution of the blood constituents such as the respiratory pigment (haemocyanin) occurs which takes up to 2 hours to be reversed (Wells & Wells, 1993). There appears to be only one main type of blood cell or haemocyte in *E. cirrhosa*. The haemocyte matures in the white body, or leucopoetic organ, of the animal and is released into the closed circulatory system (Cowden & Curtis, 1974, 1981). Few cephalopod defense mechanisms have been elucidated (Ford, 1992). It is known that *E. cirrhosa* haemocytes will phagocytose erythrocytes only in the presence of haemolymph *in vitro* (Stuart, 1968). Also *in vivo* studies with *E. cirrhosa* (Stuart, 1968) and with *Octopus dofleini* (Bayne, 1973), demonstrate that it is mainly fixed phagocytes in certain organs which clear injected foreign particles, with haemocytes only removing a small fraction of them.

This paper investigates whether haemocytes from *E. cirrhosa* are capable of phagocytosing dead bacteria *in vitro* and whether temperature, time and haemolymph concentrations influence phagocytosis. Additional experiments were also performed to

determine whether bacterial pre-incubation (prior-opsonization) at different temperatures, times and haemolymph concentrations affected phagocytic rates.

Materials and Methods

Animals

Octopuses, *Eledone cirrhosa*, (Lamarck) were obtained from crab pots around the North Wales coast. The animals were brought into the aquarium at the University of Bangor and maintained in natural seawater at 10-12°C. After 48 h the animals were weighed, marked using a syringe and assigned to a particular tank. Five octopuses per tank were chosen at random for each set of experiments.

Haemolymph

Blood was withdrawn from the branchial blood vessel of each octopus as described in Chapter 2. The blood was centrifuged at 4°C for 5 min at 800g to remove the haemocytes. The resulting haemolymph from a number of individuals was pooled and frozen at -20°C. Before use the haemolymph was thawed and diluted to a final concentration of 0.1, 1 or 10% in sterile octopus saline (SOS)(NaCl, 2.367g/100ml; glucose, 1g/100ml;$CaCl_2$, 0.116g/100ml; KH_2PO_4, 0.0056g/100ml; KCl, 0.1089g/100ml; $MgSO_4.H_2O$, 0.503g/100ml; $MgCl_2$, 0.419g/100ml).

Haemocytes

From each animal 1ml blood samples were withdrawn into 10ml of ice cold marine anticoagulant (MA)(NaCl, 2.63g/100ml; glucose, 1.8g/100ml; tri-sodium citrate, 0.088g/ml; citric acid, 0.055g/100ml) containing ethylene glycol-bis(β-aminoethylether) N, N, N', N', - tetraacetic acid (EGTA) (0.029g/100ml). After a

blood count the haemocytes were centrifuged at 800g for 5 min at 4°C, and washed by resuspension in octopus Ringer (OR)(NaCl, 2.433g/100ml; glucose, 1.4g/100ml; EGTA, 0.015g/100ml; KCl, 0.082/100ml; KH_2PO_4, 0.004g/100ml) containing $CaCl_2$(0.0142g/100ml), $MgCl_2$ (0.0524g/100ml) and $MgSO_4$ (0.0629/100ml). A final haemocyte count was made before the haemocytes were washed for a second time and resuspended in SOS at 1 x 10^6 haemocytes/ml.

Bacteria

Vibrio anguillarum (MT275) were obtained from the Scottish Office, Agriculture and Fisheries Department, Marine Laboratory, Aberdeen. Formalized *V. anguillarum* were counted, washed twice by resuspension in SOS and centrifuged at 13000g for 10 min before resuspension at 8 x 10^8 cells/ml in the required treatments.

Transmission Electron Microscope (T.E.M.) Preparation

Five hundred µl of blood were withdrawn from the branchial blood vessel of the octopus and mixed directly with 500µl of washed bacteria. After 2 h incubation at 15°C the blood was centrifuged and the haemolymph removed. The pelleted haemocytes were fixed for 24 h at 4°C in 2.5% glutaraldehyde (in 0.1M sodium cacodylate buffer at pH 7.4). The haemocytes were washed in 0.1M sodium cacodylate buffer and secondarily fixed for 2h at room temperature in 1% osmium tetroxide before staining *en bloc* with 2% uranyl acetate over night. The pellet was then dehydrated through ethanol and propylene oxide and embedded in Spurr resin.

Cut sections (50nm) were mounted on 100 mesh pioloform coated copper grids and stained with lead citrate. Sections were viewed in a GEC Corinth 500 at 60 KV.

Phagocytosis Assay

Two phagocytosis experiments were performed to determine the effect of haemolymph concentration, temperature and time on haemocyte phagocytosis. Five animals were used for each experiment. The first experiment involved incubating haemocytes in 16 well, tissue culture slides (Nunc) for 2 h at different temperatures, but utilizing one pre-incubation temperature and time for the bacteria. The second experiment involved haemocyte incubations of 30 min only and utilized different temperatures, times and haemolymph concentrations for bacterial pre-incubations.

For the first experiment 50µl of the haemocyte suspension in SOS were put into each of the 16 well chambers of a tissue culture slide. Fifty microliters of either SOS or haemolymph diluted in SOS were added in duplicate, at half hour intervals, to selected wells. Bacteria were resuspended in either SOS or 100% haemolymph for 2 h at 15°C and washed twice before use. Fifty microliters of either SOS treated or haemolymph treated bacteria immediately followed the haemolymph additions, again in duplicate. Each well of the tissue culture slide therefore contained: 50µl of haemocytes in SOS, 50µl of either SOS or haemolymph diluted in SOS to 0.1, 1 or 10% concentration (final concentrations of 0.03, 0.33 or 3.33% respectively) and 50µl of bacteria resuspended in SOS after treatment. The assays were run at four temperatures (5, 10,

15 and 20°C). After 2 h the tissue culture slides were rinsed in SOS to remove unattached bacteria and the slide fixed by immersion in methanol for 3-5 min.

The second experiment involved the addition of 50µl of haemocytes in SOS at 1 x 10^6 haemocytes/ml, followed by 50µl of haemolymph diluted in SOS at 0, 0.1, 1 or 10% concentrations and 50µl of the different bacterial preparations added in duplicate to the tissue culture slides. The bacteria were washed and resuspended in haemolymph at concentrations of 0, 0.1, 1, 10 or 100%, using phosphate buffered saline pH 7.0 (PBS, Gibco, without Ca^{2+} and Mg^{2+}) as the diluent. Bacteria were incubated for 1, 10, 60 or 120 min at 5, 10, 15 or 20°C, before being washed twice and used in the assay. The slides were incubated at temperatures of 5, 10, 15 or 20°C. After 30 min the tissue slides were rinsed with SOS and the experiment stopped by immersion of the slide in methanol as previously.

All slides were then stained in Giemsa (Sigma), rinsed in Gurr buffer (BDH pH 6.8) and air dried before mounting using DPX.

Statistical Analysis

Analysis was performed by random counting of 200 haemocytes in each well. The haemocytes were counted under oil using a compound binocular microscope at 800x magnification. All slides were numbered and randomly selected to reduce observer bias. The number of haemocytes which had phagocytosed bacteria was expressed as a percentage of the haemocytes counted in each of the duplicate wells. The results for each of the duplicate wells were averaged and analysis of variance (ANOVA)

performed for the 2 experiments using the 5 replicates. In each case P values of < 0.05 were taken as being significant. The replicate means were calculated and Tukey's pairwise comparison was performed for each experiment using the calculated confidence interval estimation (CI estimation). The CI estimate allows 2 separate means to be statistically compared (Rice, 1988).

Reprint? NO.

Results.

Phagocytosis of the formalized Gram negative bacterium, *V. anguillarum*, by *E. cirrhosa* haemocytes occurs both in the presence and absence of haemolymph. Collected haemocytes were incubated with bacteria for 2 h before fixation for T.E.M.. Sections clearly indicated that *E. cirrhosa* haemocytes phagocytose and degrade bacteria (fig. 4.1).

From analysis of variance a number of significant conclusions were obtained. Phagocytosis by haemocytes following pre-incubation of the bacteria in 100% haemolymph was significantly greater than phagocytosis following SOS treatment ($F=594.85$, $P<0.0001$) (fig. 4.2a, b). Highly significant values were also obtained for the effect of incubation temperature ($F=155.09$, $P<0.0001$), and also for the duration of the assay ($F=178.9$, $P<0.0001$). The concentrations of haemolymph used in the assay medium did not have a significant effect ($F=0.32$, $P=0.814$) indicating that the rate of phagocytosis was statistically equivalent in assays containing 0, 0.1, 1 or 10% haemolymph. (Fig 4.3). W means.

Cross-wise comparisons of the percentage of haemocytes phagocytosing opsonized and unopsonized bacteria, temperature and assay duration were also highly significant, ($P<0.0001$), whereas cross-wise comparisons involving haemolymph concentration in the assay medium, confirmed that the haemolymph concentrations, in SOS, did not affect phagocytic rates. Haemolymph concentration was therefore not considered in further analysis, and results at each temperature and time were pooled.

Phagocytosis of bacteria pre-incubated in SOS was affected by temperature and time

Figure 4.1. Transmission electron micrograph of *Eledone cirrhosa* haemocytes (H). One haemocyte has engulfed a bacterium (*Vibrio anguillarum*) (B).

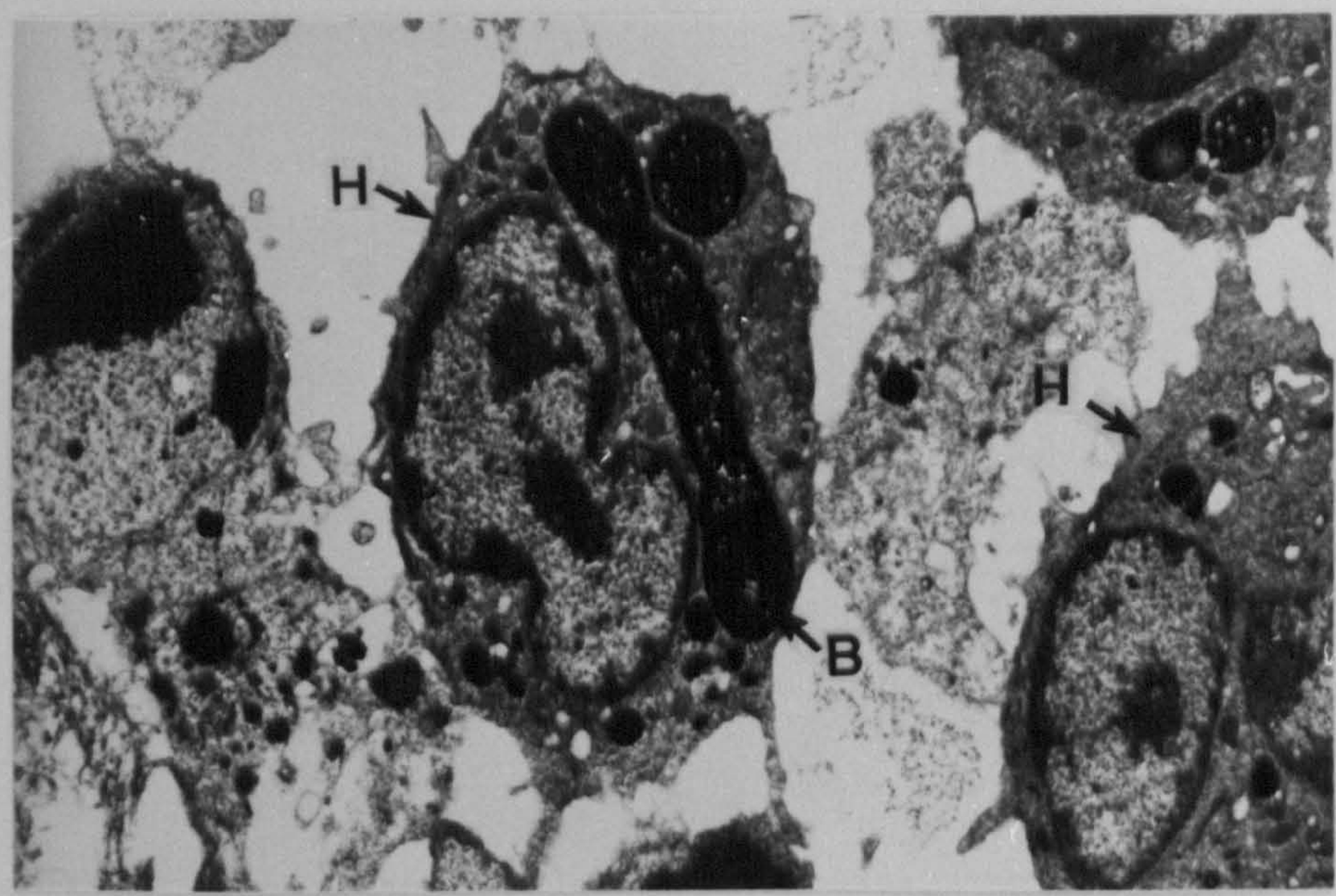
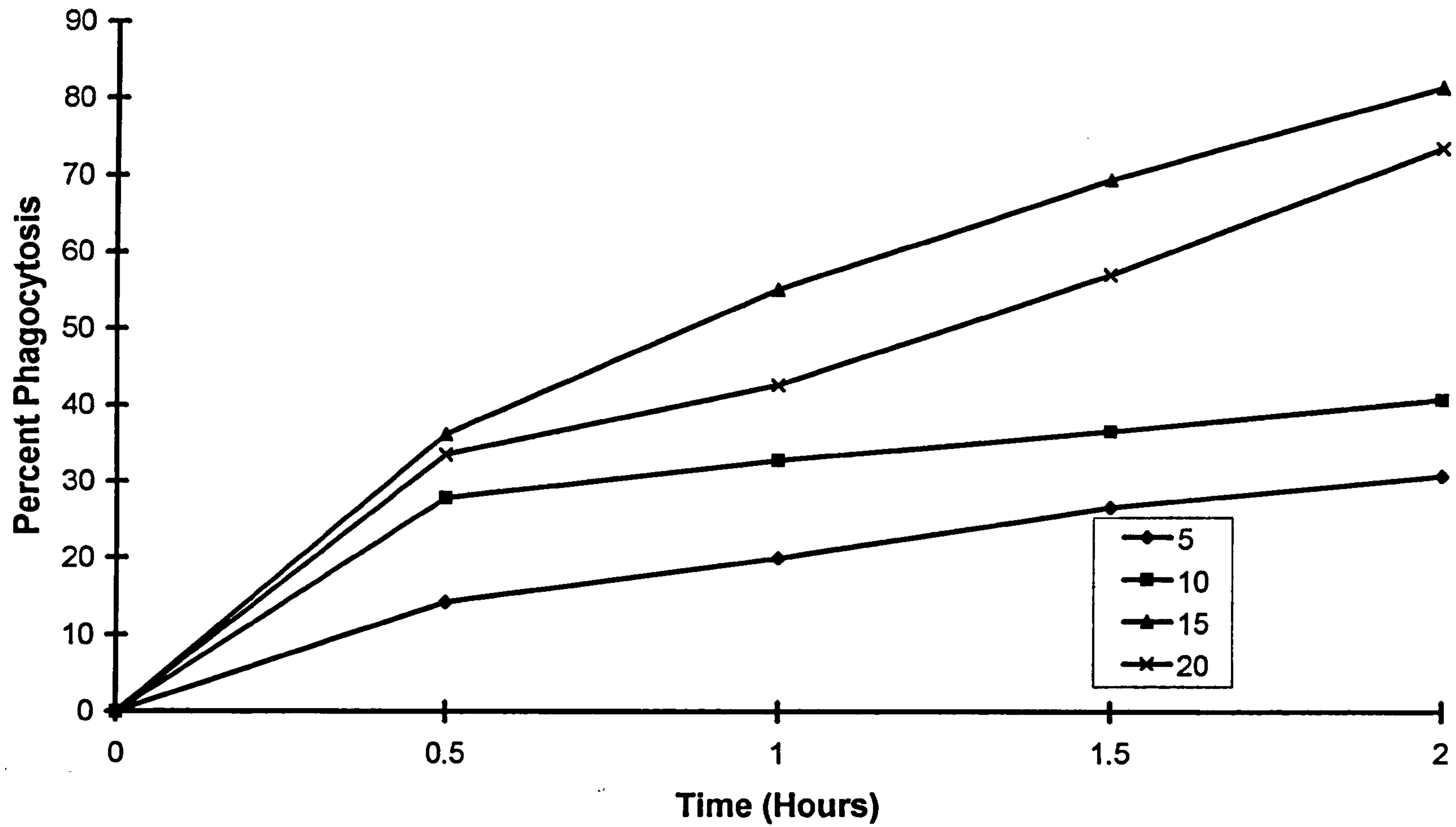


Fig. 4.1

4 μm

Figure 4.2(a) Phagocytosis by haemocytes of non-opsonized formalized *Vibrio anguillarum* at four temperatures over a 2 h incubation period. The bacteria were pre-treated with SOS for 2 h at 15°C. Tukeys CI estimate = 9.52.

Figure 4.2(a)

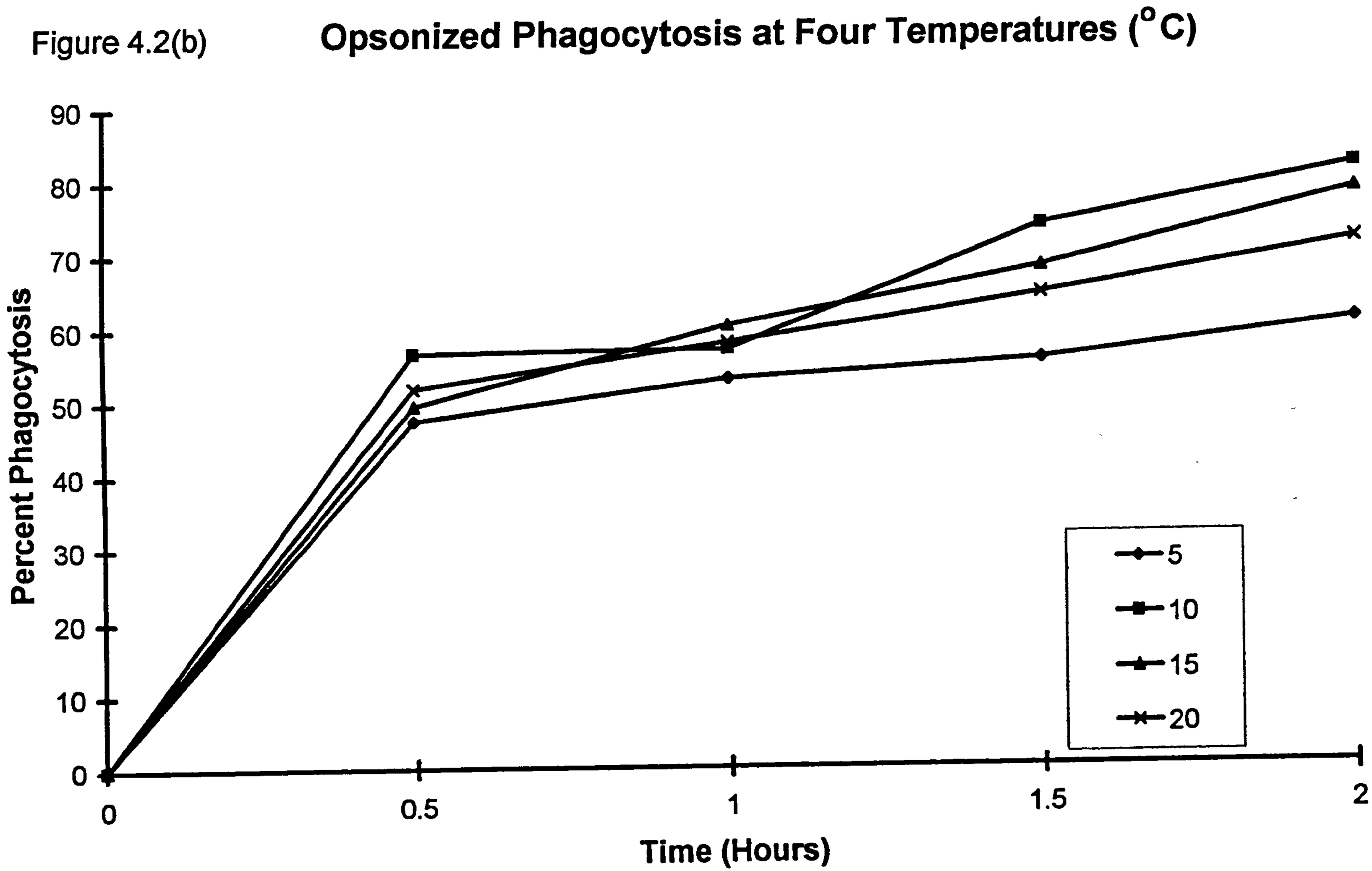

Non-Opsonized Phagocytosis at Four Temperatures (°C)
Percent Phagocytosis
90
80
70
60
50
40
30
20
10
0
0
0.5
1
1.5
2
Time (Hours)
5
10
15
20

(fig. 4.2a). At all temperatures the number of haemocytes engulfing bacteria increased over time. At 20°C there appeared to be fewer haemocytes phagocytosing than at 15°C, however statistically there was no difference between the means at the two temperatures. At 10°C there was a rapid increase in the number of haemocytes phagocytosing bacteria during the first 30 min followed by a slower rate of increase up to 2 h. At both 5 and 10°C significantly lower phagocytic rates were observed than at 15 and 20°C over the 2 h period. The mean number of haemocytes phagocytosing bacteria, pre-incubated in 100% haemolymph, over time were shown in Figure 4.2b. The haemocyte phagocytic rate again increased over the 2 h period but there were far smaller differences between the incubation temperatures. The phagocytic rates were again lower at 5°C than at the other temperatures. The maximum increase in phagocytosis at all temperatures occurred within the first 30 min.

As with the first experiment, the different concentrations of haemolymph in SOS (at 0, 0.1, 1 or 10%) used in the second assay were found to have little effect, so were removed from the pair wise comparison with no appreciable percentage error increase (0.027%) and the results pooled at each pre-incubation temperature and time. To simplify the pairwise comparison the assay temperature was not included as a main factor, but was added as an interacting factor. The results from the simplified model show that there were large statistically significant differences ($F=1083.35$ $P<0.0001$) between the haemolymph pre-incubation concentrations. The pre-incubation temperatures ($F=61.32$ $P<0.0001$), and the pre-incubation times ($F=725.24$ $P<0.0001$) were similarly significantly different. Pre-incubation of the bacteria in PBS alone at

Figure 4.2(b). Phagocytosis by haemocytes of opsonized formalized *Vibrio anguillarum* at four temperatures over a 2 h incubation period. The bacteria were pre-treated with 100% haemolymph for 2 h at 15°C. Tukeys CI estimate = 9.52.

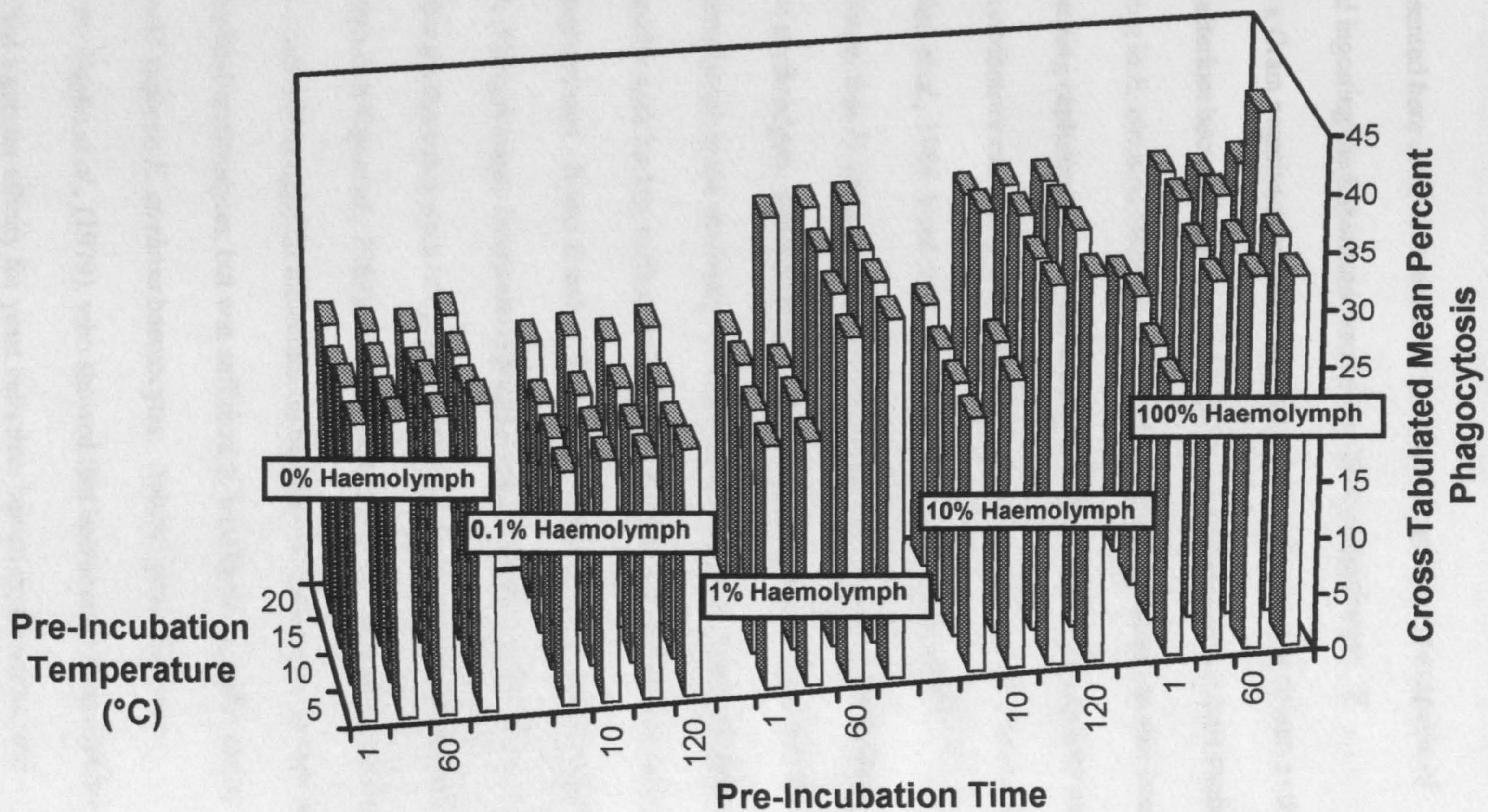
Figure 4.2(b)


Opsonized Phagocytosis at Four Temperatures (°C)
Percent Phagocytosis
90
80
70
60
50
40
30
20
10
0
0
0.5
1
1.5
2
Time (Hours)
5
10
15
20

different temperatures and time periods caused no significant differences in the phagocytic rate (fig. 4.3). Bacteria pre-incubated in 0.1% haemolymph in PBS at all pre-incubation temperatures and times were phagocytosed at a significantly lower rate than in PBS alone. Pre-incubation of the bacteria in 1% haemolymph showed initially the same lowered phagocytic rate as for 0.1% pre-incubation. However, pre-incubation of the bacteria in 1% haemolymph for 10 min at 20°C caused an enhanced phagocytic rate which also occurred at all temperatures at 60 and 120 min. Bacteria pre-incubated in 10% haemolymph for 1 min at 5, 10, 15 and 20°C and for 10 min at 5 and 10°C were statistically equivalent to the values determined in PBS alone. However, at 10 min following pre-incubation at 15 and 20°C more haemocytes were observed phagocytosing bacteria than at 5 or 10°C, or at 1 min at all temperatures. Pre-incubation of the bacteria in 100% haemolymph caused enhanced phagocytic rates after 10 min at all temperatures and time periods. Apart from one value incubated at 15°C for 120 min, the enhanced phagocytic rate was statistically equivalent to the enhanced rates found after bacterial pre-incubation in 1 and 10% haemolymph concentrations.

Figure 4.3. Phagocytosis of formalized *Vibrio anguillarum*. The haemocytes were incubated at different temperatures for 30 min only. The bacteria were pre-incubated in 0% haemolymph (i.e., PBS only), 0.1% haemolymph, 1% haemolymph, 10% haemolymph and 100% haemolymph concentrations. The bacterial pre-incubation temperatures were 5, 10, 15 and 20°C and the pre-incubation times were 1, 10, 60 and 120 min. Tukeys CI estimate = 3.1.

Figure 4.3 **Phagocytosis of Bacteria Pre-Incubated in Different Haemolymph Concentrations**



Discussion

The results presented here demonstrate that *E. cirrhosa* haemocytes are capable of recognizing and ingesting the formalized bacterium *Vibrio anguillarum*. *V. anguillarum* is a Gram negative commensal marine opportunist and was chosen as the experimental bacterium because it has been isolated from, and used in previous studies on wound healing in *E. cirrhosa* (Bullock *et al.*, 1987). This bacterium has also been implicated in causing cephalopod infections when the animals are held in captivity and is a common contributory cause of death at high aquarium temperatures (Lebovitz *et al.*, 1977; Hanlon *et al.*, 1984; Ford *et al.*, 1986; Hanlon & Forsythe, 1990). Stuart (1968) found that *E. cirrhosa* haemocytes required haemolymph for *in vitro* phagocytosis of erythrocytes. The data presented in this paper demonstrate that the presence of haemolymph is not necessary for ingestion of bacteria. However, this bacterium is smaller with far less surface area than an erythrocyte and as such may be more easily phagocytosed. It was found by Tyson and Jenkin (1974) that haemocytes from a crayfish (*Parachaeraps bicarinatus*) phagocytosed bacteria in the absence of haemolymph, but erythrocytes were not phagocytosed unless they were pre-treated with haemolymph (McKay *et al.*, 1969). Further Jenkin (1976), suggested that the concentration of certain recognition molecules on the crayfish haemocyte surface was not sufficient to bind erythrocytes, but was sufficient to bind bacteria, and a similar explanation could apply to *E. cirrhosa* haemocytes. Another possibility was demonstrated by Bayne *et al.*, (1979), who showed that haemocytes from *Mytilus californianus* had a greater affinity for yeast cells than human erythrocytes, and

suggested that phagocytosis of foreign particles was selective. Results from other molluscan species also demonstrate that surface antigenicity of the respective test particles has an effect on phagocytosis by haemocytes (Tripp & Kent, 1967; Anderson & Good,1976).

Tripp (1966) using the bivalve *M. mercenaria* concluded that haemolymph pre-treatment of erythrocytes caused increased phagocytosis. The same experiment showed however that if untreated erythrocytes were incubated with haemocytes for longer periods of time, the same levels of phagocytosis were achieved. With *E. cirrhosa* haemocytes at 15 and 20°C the phagocytic rate is higher at 30 min for 100% haemolymph treated bacteria compared to SOS treated bacteria, but after 2 h there was no difference in phagocytic rates between the 2 treatments. The data presented here also indicate that a higher percentage of haemocytes phagocytosed haemolymph treated bacteria at 5 and 10°C over 2 h than SOS treated bacteria. Tripp (1992) also showed that the haemocytes of *M. mercenaria* were avidly phagocytic in the absence of haemolymph, however at low temperatures, in the presence of haemolymph there was increased phagocytosis of yeast. Abdul-Salam and Michelson (1980) working with *Biomphalaria glabrata* also demonstrated that temperature has an effect on haemocyte phagocytosis. A phagocytic activity peak was evident at 30°C with inhibition of phagocytosis below 15°C. Low temperature inhibition (4°C) of phagocytic rates has also been demonstrated for haemocytes from the hard clam *M. mercenaria* with maximum rates occurring at 22 and 37°C (Foley & Cheng, 1975). With SOS treated bacteria, *E. cirrhosa* haemocytes demonstrate an activity peak with

about 70% of haemocytes phagocytosing after 2 h at 15 and 20°C. At 5°C only 14% of haemocytes contained bacteria, whereas if the bacteria were initially pre-incubated in haemolymph before addition to the assay, the phagocytic rate at 5°C increased to around 47%.

The results presented above indicate that the amount of haemolymph present in the bacterial pre-incubation medium has a dramatic effect on the number of haemocytes subsequently engulfing these bacteria within a 30 min period. Haemolymph concentrations of 0.1 and 1% in PBS, resulted in lower numbers of haemocytes phagocytosing compared to PBS alone. This inhibition changes to enhanced phagocytosis, at all higher pre-incubation concentrations. Further comparisons demonstrate that the temperature of the pre-incubation medium and particularly the duration of incubation are also important factors. The observed trends indicate that increasing the pre-incubation temperature decreases the pre-incubation time needed for enhanced phagocytosis to occur. Fryer and Bayne (1989) working on *B. glabrata* similarly demonstrated that phagocytosis was inhibited after short pre-incubation periods, whereas longer pre-incubation periods of 1 h resulted in enhanced levels. It was suggested by the authors that initial non-specific adsorption of a variety of plasma components (opsonins) occurred onto, in their case, the yeast surface. Longer exposure to the plasma allowed more of the opsonins to bind to the yeast surface. The results from the data presented here for the different pre-incubation haemolymph concentrations and durations of exposure seem to support this hypothesis. In addition it is possible that if the temperature is increased further, more of the available plasma

components would adhere onto the surface of the bacterium.

When haemocytes from *E. cirrhosa* were resuspended in SOS, as stated above, there is phagocytosis of the formalized bacterium *V. anguillarum*. In buffers containing either EDTA or EGTA, no phagocytosis of the same bacterium was evident (Malham, unpublished data). SOS contains Ca^{2+} and Mg^{2+} and it appears likely that the presence of these divalent ions has an effect on phagocytosis. Fryer and Adema (1993) showed that manipulated haemocytes from *B. glabrata* retained some phagocytic activity, but that addition of excess Ca^{2+} and Mg^{2+} to the haemocytes before the addition of the target particles enhanced their phagocytic rates. *E. cirrhosa* haemocytes were initially drawn into an anticoagulant buffer containing EGTA and washed in Octopus Ringer, also containing EGTA, before resuspension in EGTA-free-SOS, all of which could alter haemocyte behaviour and affect phagocytosis. *Corbicula fluminea* haemocytes (Tuan & Yoshino, 1987) also required extracellular Ca^{2+} or Mg^{2+} for both opsonin-dependent and -independent phagocytosis. The authors suggest that the opsonin possibly exists as a divalent cation-macromolecular complex due to the loss of enhanced phagocytosis after dialysis against EDTA and EGTA. Further, *Mytilus edulis* haemocytes phagocytosed yeast cells with high efficiency when calcium ions were present in the suspension medium, and gave similar results when haemolymph alone was added, but almost no phagocytosis was recorded with haemocytes in buffered saline (Renwrantz & Stahmer, 1983). When *V. anguillarum* was resuspended in SOS, *E. cirrhosa* haemolymph diluted in SOS, or in PBS alone, there was no change in the haemocyte phagocytic rate. However, when *V.*

anguillarum was resuspended in haemolymph diluted in PBS (≥ 1% haemolymph concentration) or in haemolymph alone, enhanced phagocytosis was observed. Haemolymph lectins have been shown to act as opsonins for haemocyte phagocytosis (e.g., Renwrantz, 1983; Renwrantz 1986; Smina & Van der Knapp, 1986, Vasta, 1991). Agglutination results from *Octopus maya* (Fisher & Dinuzzo, 1991) further support the role of lectins in recognition of non-self. Studies using the molluscs *Mytilus edulis* (Renwrantz & Stahmer, 1983) and *Lymnaea stagnalis* (Van Der Knapp, 1982) have demonstrated that molecules antigenically related to haemolymph lectins have been found in the cytoplasm and on the surface of haemocytes. Lectins, in particular C-type, are found in a number of invertebrates including *Octopus vulgaris*. These lectins are Ca^{2+} dependent, and these ions are required for ligand binding of the lectin (Rögener *et al.*, 1986). Stuart (1968) suggested a possible link between an opsonic factor and haemocyanin in *E. cirrhosa*. Also a lectin identified from the haemolymph of *O. vulgaris* has been shown to be similar to a haemocyanin subunit (Rögener *et al.*, 1985). The nature of the soluble factor causing enhanced phagocytosis in *E. cirrhosa* has not been studied, however the factor (s) must be present at a high concentration, since it is effective at a haemolymph concentration of 1% at 15 and 20°C.

In conclusion, *in vitro* phagocytosis of *Vibrio anguillarum* by haemocytes from *E. cirrhosa* is aided by a component of haemolymph and is affected by temperature, duration of the assay and pre-incubation of the bacterium with different haemolymph concentrations. Further studies to elucidate whether *E. cirrhosa* haemocytes are

capable of phagocytosing and digesting live microorganisms *in vitro* and *in vivo* are being pursued.

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Chapter 5

Migration of Haemocytes from the Lesser Octopus *Eledone cirrhosa* (Lam.)

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Abstract

Eledone cirrhosa haemocytes are attracted to certain blood preparations at low concentrations (0.1-10%). Haemocytes migrated to blood solutions which had contained live *Vibrio anguillarum* (a Gram-negative bacterium) for 2 h at 15° C. When the same experiments were performed with formalized *V. anguillarum* very little migration was observed. *E. cirrhosa* haemocytes also migrated to blood preparations containing lipopolysaccharide from the Gram-negative bacterium, *Escherichia coli* at 0.0625 - 0.25mg/ml.

Key Words: *Eledone cirrhosa*; Whole blood; Whole blood lysate; Haemolymph; Haemocyte lysate; Haemocytes; Migration; Bacteria; Lipopolysaccharide

Introduction

The primary cellular defence mechanism of most invertebrates is the recognition, ingestion and killing of invading micro-organisms by the blood cells (Millar & Ratcliffe, 1994). Recognition involves the detection, by the blood cell, of the invader and the directional movement towards the offending organism (Ratcliffe *et al.*, 1985). It has been demonstrated in a few invertebrates, e.g. insects (Stefano *et al.*, 1989), crustaceans (Smith & Söderhäll, 1986), and oligochaetes (Marks *et al.*, 1979) that chemotaxis/chemokinesis is involved in non-self detection. Chemotaxis is the activation by, or detection of, a chemical gradient by the blood cell and directional movement in that gradient whereas chemokinesis is an increase in random migration due to a chemical gradient and is not directional.

Molluscan studies have concentrated on the bivalves and gastropods. It is known that haemocytes from certain bivalves, e.g. *Mytilus edulis*, will migrate to, and infiltrate wounded areas (Bubel *et al.*, 1977). Haemocytes from *Mercenaria mercenaria* (Fawcett & Tripp, 1994), *Crassostrea virginica* (Cheng & Rudo, 1976; Cheng & Howland, 1979) and *Mytilus edulis* (Schneeweiß & Renwrantz, 1993) have also been shown to migrate *in vitro* towards bacteria or secreted bacterial products. Gastropod haemocytes from *Viviparus malleatus* migrated towards heat killed bacteria (Schmid, 1975) and in *Biomphalaria glabrata* haemocyte migration to *Schistosoma mansoni* sporocysts occurred in resistant snails (Basch, 1979) and was modulated by excretory-secretory products *in vitro* (Lodes & Yoshino, 1990). Molecules of bacterial origin also stimulate molluscan haemocyte migration. In *M. edulis*, lipopolysaccharide (LPS)

from various bacteria was found to be chemoattractive to haemocytes, whereas the formylated tripeptide N-formyl-methionyl-leucyl-phenylalanine (N-FMLP) stimulated random cell migration (Schneeweiß & Renwrantz, 1993). Fawcett and Tripp (1994) demonstrated that N-FMLP induced migration in *M. mercenaria* haemocytes and that migration was inhibited by use of the N-FMLP receptor antagonist. Howland and Cheng (1982), working with *C. virginica*, found that certain bacterial cell wall and cell envelope proteins of about 10,000Da induced haemocyte migration.

Octopus blood does not coagulate upon wounding, however the muscles around the wound vasoconstrict and the wound is eventually plugged by the blood cells (haemocytes) of the animal. There appears to be only one type of haemocyte detectable in *Eledone cirrhosa* (Cowden & Curtis, 1974, 1981) and it is released from the leucopoetic organs or white bodies located in the orbital sinuses behind the eyes (Wells, 1978, 1983). The haemocyte is capable of *in vitro* phagocytosis of mammalian red blood cells (Stuart, 1968) and bacteria (Chapter 4), and will rapidly infiltrate and migrate to wounded areas which quickly become infested with bacteria (Bullock *et al.*, 1987; Polglase *et al.*, 1983; Féral, 1988; reviewed by Ford, 1992). This paper attempts to determine whether *E. cirrhosa* haemocytes migrate to different blood preparations *in vitro*. Lipopolysaccharide (LPS) as well as bacteria were also added to the blood preparations, and to a buffer, to determine any effect on haemocyte migration.

Materials and Methods

Animals

Octopuses (*Eledone cirrhosa*) were obtained from around the North Wales and Isle of Anglesey coast lines. The animals were maintained in the aquarium at the University of Wales, Bangor at 12°C. The animals were weighed, sexed and marked (Chapter 3) within 48 h of being brought into the aquarium. The marked animals were returned to specific tanks (8 animals/tank) and allowed to recover for 24 h.

Haemocytes

Animals were randomly chosen from certain tanks, weighed, anaesthetized and blood sampled from the branchial blood vessel (Chapter 2). The sample was put directly into an ice cold marine anticoagulant (MA) (NaCl, 2.63g/100ml; glucose, 1.8g/100ml; tri-sodium citrate, 0.088g/100ml; citric acid, 0.055g/100ml) containing ethylene glycol-bis(β-aminoethylether) N, N, N', N', - tetraacetic acid (EGTA) (0.029g/100ml) to which blood was added (1ml blood : 10ml MA). A haemocyte count was taken and the blood centrifuged at 800g for 5 min at 4°C. The haemocytes were then resuspended in octopus Ringer (OR) (NaCl, 2.433g/100ml; glucose, 1.4g/100ml; EGTA, 0.015g/100ml; KCl, 0.082g/100ml; KH_2PO_4, 0.004g/100ml) at 1 x 10^6 haemocytes/ml before a final centrifugation and resuspension in OR containing $CaCl_2$ (0.0142g/100ml), $MgCl_2$ (0.0524g/100ml) and $MgSO_4$ (0.0629g/100ml (ORCM). The haemocytes were then held on ice before use in the chemotaxis assay.

Experimental Media

Four blood preparations, whole blood, whole blood lysate, haemolymph, haemocyte lysate and ORCM were used in the migration assay. Whole blood preparations were standardized by taking a blood count and adjusting the haemocyte number to 1 x 10^6 haemocytes/ml by the addition of extra pooled haemolymph. The whole blood preparation was pooled from several individuals and then held on ice until the various incubations and additions had been performed (see below). After the various incubations the whole blood preparation was centrifuged at 800g for 5 min at 4°C to remove the haemocytes and then centrifuged at 13000g for 10 min to remove incubated bacteria (see below). The resulting haemolymph was stored at -20°C until use.. Preparation of the whole blood lysate solution involved pooling standardized whole blood, freezing and thawing (x3), to ensure rupture of all the haemocytes, centrifuging at 13000g for 5 min to remove all cellular debris and storing at -20°C until required. For haemolymph, the sampled blood was centrifuged at 800g for 5 min at 4°C and the haemolymph decanted and frozen at -20°C until use. In order to obtain enough haemolymph and to negate individual variation between haemolymph samples, haemolymph from several individuals was pooled before use. The haemocyte lysate preparation involved putting the blood directly into MA, centrifuging and resuspending in ORCM at 1x 10^6 haemocytes/ml and then freezing at -20°C. Before use the haemocyte lysate solution was frozen and thawed x3 and centrifuged at 13000g for 5 min as for whole blood lysate and stored at -20°C.

The four blood preparations were each diluted in ORCM to 0, 0.1, 1, 10, 25, 50 and 100% concentrations (table 1). Lipopolysaccharide (LPS) (*E. coli* serotype 0127:B8) (Sigma) (0.25mg/ml) was added to each of the blood preparations and also to ORCM and then either serially diluted (x2) in the respective blood preparations or in ORCM (i.e. 100, 50 and 25% concentrations were used) (tables 2 & 3). After 2 h incubation at 15°C the different solutions (diluted blood preparations and LPS preparations) were frozen at -20 °C until use. Live and dead *Vibrio anguillarum* (MT275) (obtained from the Scottish Office Agricultural Environment and Fisheries Department, Aberdeen) were centrifuged at 13000g for 10 min and resuspended at 10^8 cells/ml in ORCM. The bacteria were then centrifuged again before resuspension in each of the blood preparations or in ORCM to 10^8 ,10^7 and 10^6 bacteria/ml (tables 2 & 3). After incubation for 2 h at 15°C the bacteria were removed by centrifuging at 1300g for 10 min and the solutions frozen at -20°C until use.

Table 1. Blood preparations were diluted in octopus Ringer, with added calcium and magnesium, to 0.1, 1, 10, 25, 50 and 100% concentration. The preparations were used in the determination of haemocyte migration.

Blood Preparation	Percentage of Blood Preparation in ORCM*					
Whole Blood	0.1	1.0	10.0	25.0	50.0	100.0
Whole Blood Lysate	0.1	1.0	10.0	25.0	50.0	100.0
Haemolymph	0.1	1.0	10.0	25.0	50.0	100.0
Haemocyte Lysate	0.1	1.0	10.0	25.0	50.0	100.0

* = Octopus Ringer with Calcium and Magnesium.

Table 2. Live or dead *V. anguillarum* and lipopolysaccharide were added to 100% blood preparations. After 2 h incubation the bacteria were removed. The preparations were used in the determination of haemocyte migration.

Blood Preparation	Live or Dead Bacteria/ml			Lipopolysaccharide (mg/ml)		
Whole Blood	10^6	10^7	10^8	0.625	1.25	2.50
Whole Blood Lysate	10^6	10^7	10^8	0.625	1.25	2.50
Haemolymph	10^6	10^7	10^8	0.625	1.25	2.50
Haemocyte Lysate	10^6	10^7	10^8	0.625	1.25	2.50

Table 3. Live and dead *V. anguillarum* and lipopolysaccharide were added to 25, 50 and 100% blood preparations. After a 2 h incubation the bacteria were removed. The preparations were used in the determination of haemocyte migration.

Solutions	Live or Dead Bacteria/ml			Lipopolysaccharide (mg/ml)		
25% Whole Blood	10^6	-	-	0.625	-	-
50% Whole Blood	-	10^7	-	-	1.25	-
100% Whole Blood	-	-	10^8	-	-	2.50
25% Whole Blood Lysate	10^6	-	-	0.625	-	-
50% Whole Blood Lysate	-	10^7	-	-	1.25	-
100% Whole Blood Lysate	-	-	10^8	-	-	2.50
25% Haemolymph	10^6	-	-	0.625	-	-
50% Haemolymph	-	10^7	-	-	1.25	-
100% Haemolymph	-	-	10^8	-	-	2.50
25% Haemocyte Lysate	10^6	-	-	0.625	-	-
50% Haemocyte Lysate	-	10^7	-	-	1.25	-
100% Haemocyte Lysate	-	-	10^8	-	-	2.50
ORCM* Buffer	10^6	10^7	10^8	0.625	1.25	2.50

* = Octopus Ringer with Calcium and Magnesium.

Migration Assay

Thirty microlitres of one of the blood solutions (experimental media) or the control (ORCM), were added, in triplicate, to the wells in the bottom part of a 48 well migration chamber (Neuro Probe, MD, USA). The blood solutions used were the four blood preparations diluted in ORCM (table 1), the four blood preparations that had contained live or dead bacteria (tables 2 & 3), and the blood preparations containing LPS (tables 2 & 3). A 3μm pore size polyvinylpyrrolidone-free polycarbonate filter (Millipore, Bedford, MA, USA) was placed over the bottom of the lower chamber followed by a silicon gasket and the top part of the chamber. Forty-three microlitres of the haemocytes in ORCM at 1×10^6 haemocytes/ml (held on ice) were added to the wells in the top part of the migration chamber. The migration chamber was then sealed in a moist environment at 15°C for 90 min. The assay was terminated by removing the filter paper from the chamber and carefully scraping any haemocytes from the upper side of the filter. The filter paper was then fixed in methanol for 2 min and air dried. The paper was stained in Giemsa, rinsed in Gurr buffer (pH 6.8), dried and mounted using DPX. The number of migrating haemocytes (those in the pores or on the underside of the filter) were counted in triplicate in each well using a $1mm^2$ field of view at 400x magnification.

Each set of experimental media was run 5 times. At the end of each run the chamber was sterilized before the addition of the same but fresh media to the bottom of each chamber. For each set the haemocytes added to the top of the chamber originated from a different octopus.

Analysis

Means and standard errors were taken of the 5 replicates of each set of experiments. The control values in each case were subtracted from the experimental values and the results plotted. Analysis of Variance (ANOVA) and Student's t-test were used to determine significant differences between the different blood preparations and the various experimental media ($P < 0.05$).

Positive migration was indicated where more haemocytes were traversing the filter than the control value for each set of replicates ($N = 5$). Negative migration values were obtained when less haemoyctes traversed the filter than the control.

Results

Eledone cirrhosa haemocytes showed both positive and negative migration from a suspension in ORCM through the 3μm pore sized membrane to various blood preparations. Significant haemocyte migration ($P < 0.05$) was also recorded when live or dead bacteria (*Vibrio anguillarum*) or LPS were added to the blood preparations. Dilution of the four blood preparations (whole blood, whole blood lysate, haemolymph and haemocyte lysate) affects the number of haemocytes traversing the 3μm pore filter (fig. 5.1a & b). Analysis of Variance (ANOVA) results showed that blood preparations at low concentrations (0-10%), and the type of blood preparation used, significantly ($P<0.0001$) affected haemocyte migration. A low concentration (fig. 5.1a) of haemocyte lysate (0.1% at 1×10^5 haemocytes/ml) attracted fairly high numbers (60 haemocytes/mm^2) ($P<0.05$) of haemocytes across the filter. Increasing the haemocyte lysate concentration however decreased ($P<0.05$) the number of migrating haemocytes, whereas with the whole blood lysate and whole blood preparation the number of haemocytes crossing the membrane was greatest ($P<0.05$) at 10% concentration (137.83 and 77.2 haemocytes/mm^2 respectively). At whole blood and whole blood lysate concentrations of 25 to 100% the number of haemocytes which traversed the filter decreased ($P<0.05$) compared to the number at 10%. Both haemolymph and haemocyte lysate preparations at 25 - 100% (fig. 5.1b) showed no difference compared to the controls in the number of haemocytes crossing the filter to the preparations.

Figure 5. 1a. The number of haemocytes/mm^2 which migrated towards various blood preparations diluted in ORCM buffer to 0.1, 1 and 10% concentrations. Each value represents the mean of 5 individual animals. Error bars are the standard errors of the mean.

Figure 5. 1b. The number of haemocytes/mm^2 which migrated towards various blood preparations diluted in ORCM buffer to 25, 50 and 100% concentrations. Error bars are the standard errors of the mean.

Figure 5.1.a. Haemocyte Migration to Blood Preparations (Diluted in ORCM)

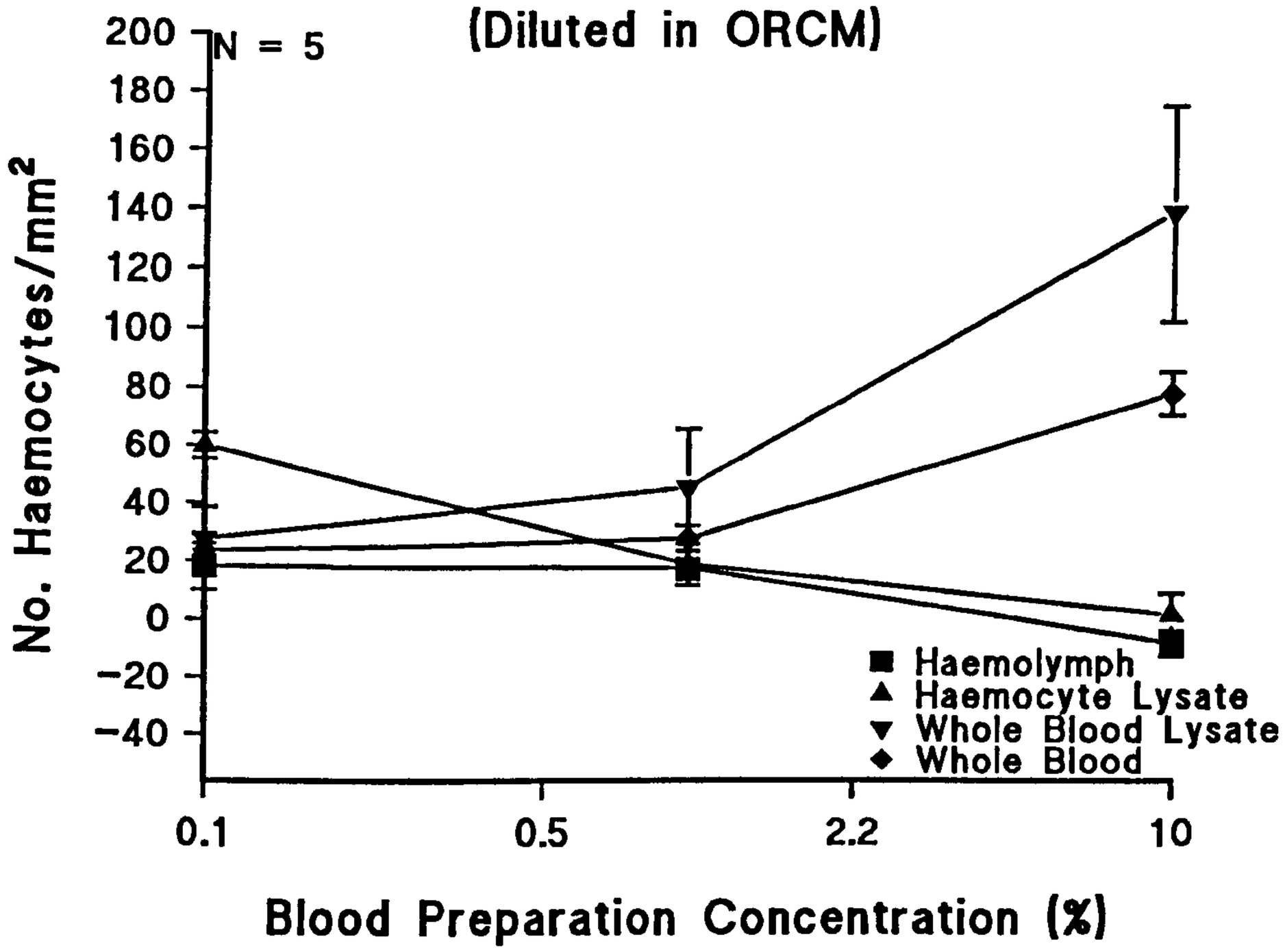
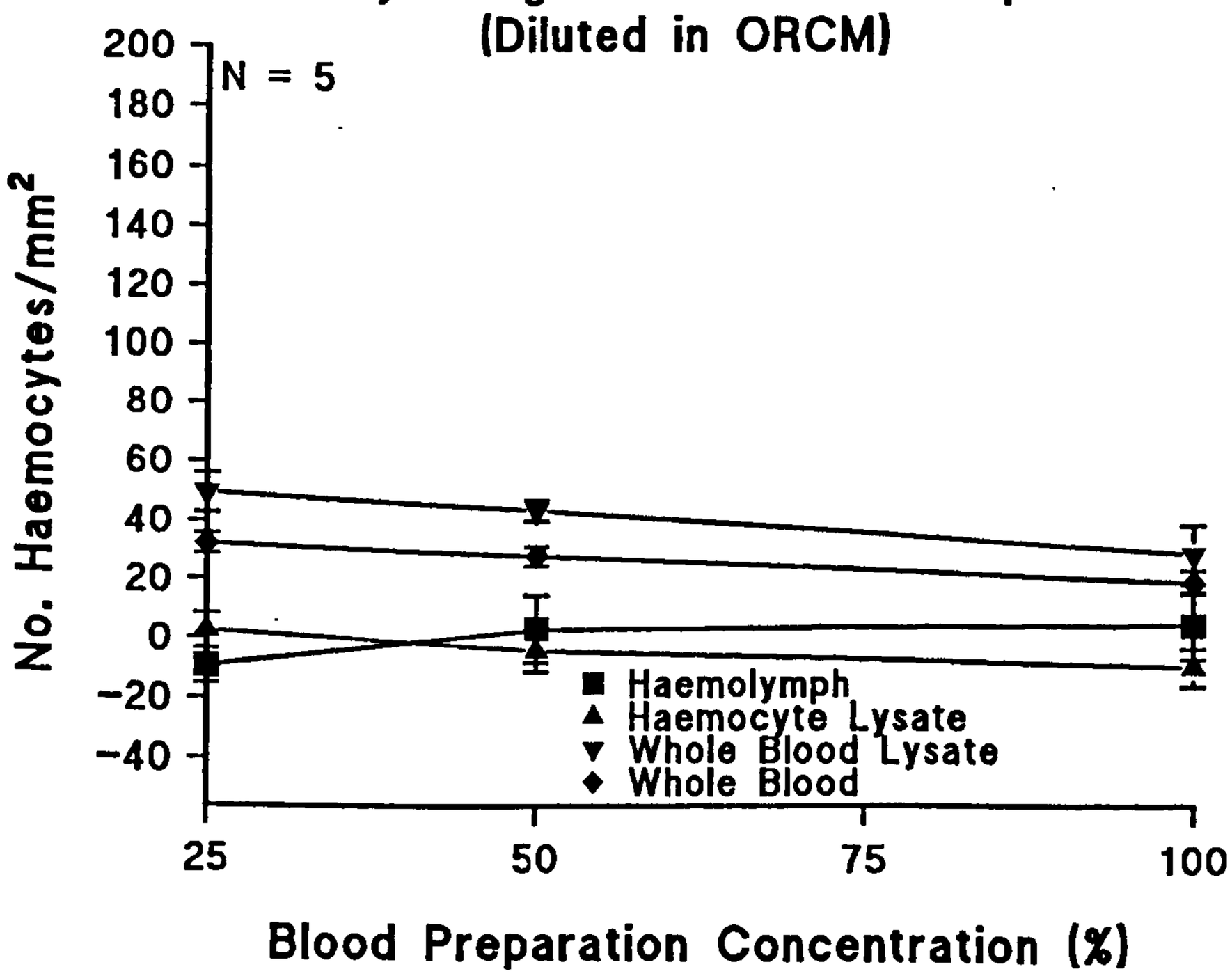


Figure 5.1.b. Haemocyte Migration to Blood Preparations (Diluted in ORCM)



The effects of the blood preparations which had contained live and dead bacteria (*V. anguillarum*) on the number of haemocytes crossing the filter to the blood preparations are shown in Figure 5.2 (a&b) and Figure 5.3 (a&b). ANOVA results demonstrated that haemocyte migration is significantly ($P<0.0001$) affected by the concentration of live bacteria previously incubated in the blood preparation, as well as by the blood preparation used to incubate the live bacteria. The different blood preparations in which dead bacteria had previously been incubated also demonstrated highly significant ($P<0.0001$) ANOVA values. The pre-incubation of live bacteria in whole blood (fig. 5.2 a&b) or whole blood diluted in ORCM, caused significantly ($P<0.05$) higher numbers of haemocytes to migrate through the filter compared to the other blood preparations. The number of haemocytes which migrated decreased ($P<0.05$) as the concentration of bacteria decreased for both the 100% whole blood lysate preparation (fig. 5.2a) and for the lysate diluted with ORCM to 50% (10^7 bacteria/ml) (fig. 5.2b).

The pre-incubation of bacteria at low concentrations (1×10^6 bacteria/ml) in 100% haemolymph and whole blood preparations caused fairly high ($P<0.05$) numbers of haemocytes to cross the filter (75.5/mm^2 and 102.56/mm^2 respectively compared to 1×10^7 bacteria/ml). However when haemolymph was diluted with ORCM to 50 and 25% concentrations the number of migrating haemocytes decreased ($P<0.05$) and became less than the control value at a bacterial concentration of 1×10^6/ml.

Haemolymph pre-incubated with dead bacteria at 10^6 and 10^7 cells/ml (fig.5.3a&b) was significantly ($P<0.05$) less attractive to haemocytes compared to the control and all the

Figure 5. 2a. The number of haemocytes/mm^2 which migrated towards various blood preparations at 100% concentrations in which live bacteria at 10^6, 10^7 and 10^8 cells/ml were incubated for 2 h, after which the bacteria were removed and the supernatant used in the assay. Error bars are the standard errors of the mean.

Figure 5. 2b. The number of haemocytes/mm^2 which migrated towards various blood preparations diluted to 25, 50 and 100% concentrations in ORCM buffer in which live bacteria at 10^6, 10^7 and 10^8 cells/ml respectively were incubated for 2 h before removal of the bacteria and use of the preparations in the assay. Error bars are the standard errors of the mean.

Figure 5.2.a. Haemocyte Migration to Preparations which had contained Live Bacteria

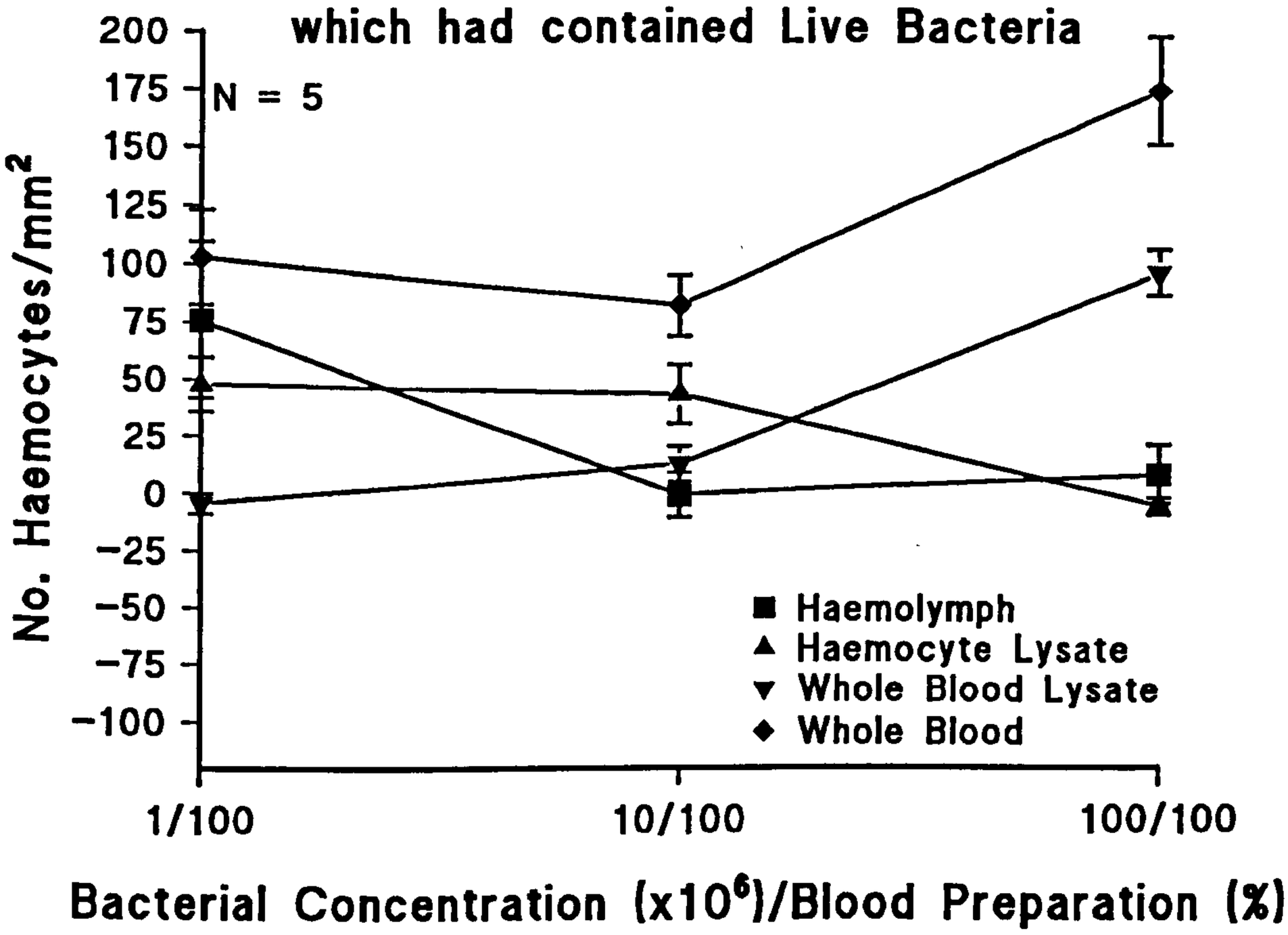
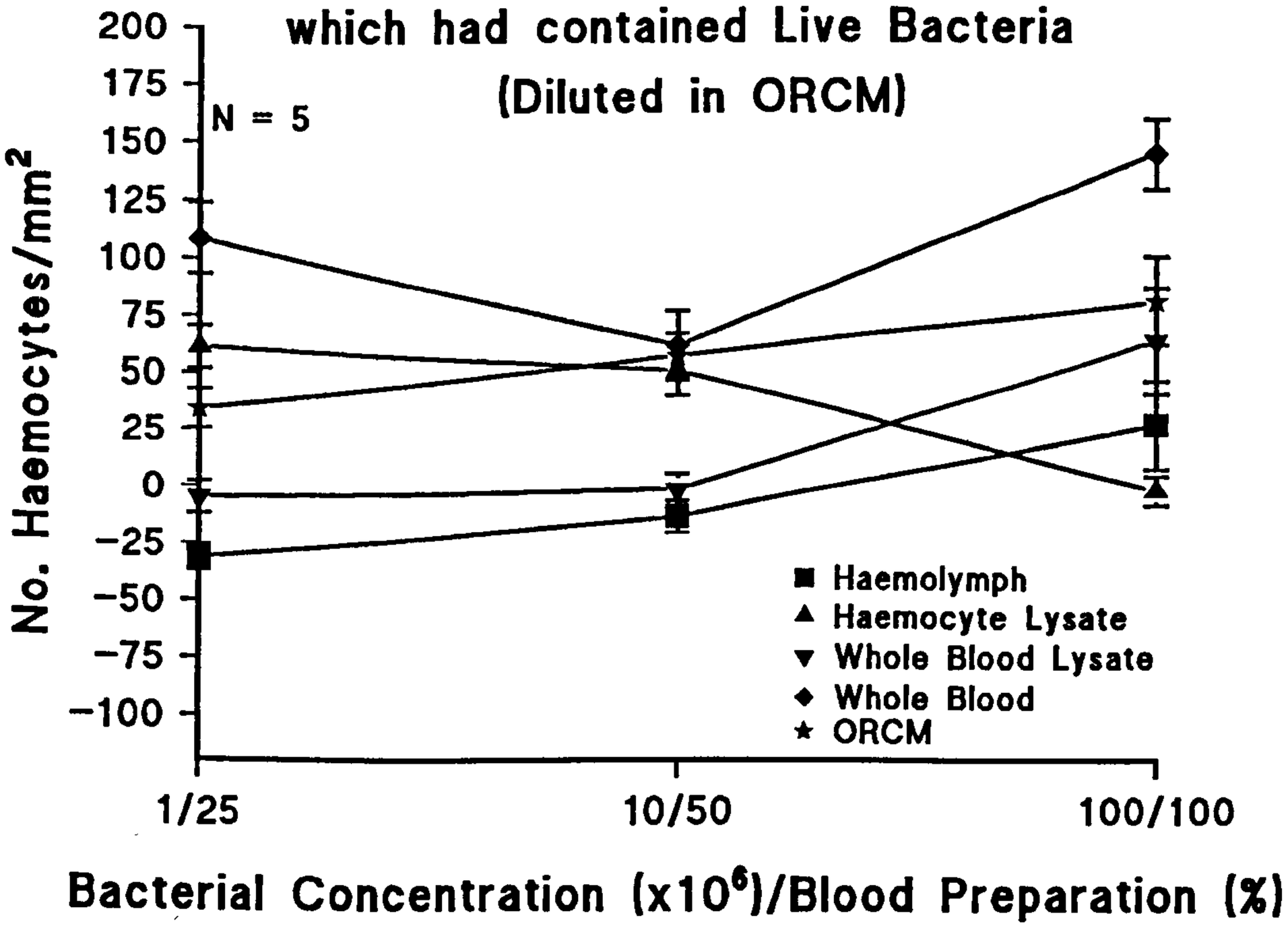


Figure 5.2.b. Haemocyte Migration to Preparations which had contained Live Bacteria (Diluted in ORCM)



other blood preparations. Whole blood lysate and whole blood at bacterial concentrations of 10^6 and 10^7 bacteria/ml when diluted to 50 and 25% concentrations with ORCM, caused between 50 - 75 haemocytes/mm^2 to cross the filter. At a bacterial concentration of 1 x10^8 cells/ml with all preparations at 100% concentration a small amount of haemocyte migration was evident (fig. 5.3a), whereas the same concentration of bacteria (1x10^8 cells/ml) in the ORCM diluted preparations (fig. 5.3b) showed almost no significant migration.

A comparison of solutions which had originally contained live or dead bacteria indicated that less haemocyte migration occurred when dead bacteria were incubated in the blood preparations than when live bacteria were used. In particular, the pre-incubation of dead bacteria with a 100% concentration of whole blood caused a significant ($P<0.05$) decrease in the number of haemocytes crossing the filter in both the 100% blood preparation and the 50 and 25% concentrations ORCM diluted blood preparation when compared to the pre-incubation of live bacteria in these media. Interestingly, undiluted haemolymph in which live bacteria had been incubated at 1 x 10^6/ml attracted haemocytes but when dead bacteria were used the solution was repellent at all concentrations (figs. 5.2a & 5.3a).

LPS addition to the blood preparations also affected haemocyte migration (fig. 5.4a&b). ANOVA results showed that both the blood preparations used and the concentration in both the LPS experiments, significantly ($P<0.0001$) affected haemocyte migration. Apart from haemolymph, which remained significantly ($P<0.05$) but constantly high, (fig. 5.4a), the other blood preparations caused an increase in the

Figure 5. 3a. The number of haemocytes/mm^2 which migrated toward various blood preparations, at 100% concentrations, in which dead bacteria at 10^6, 10^7 and 10^8 cells/ml were incubated for 2 h, after which the bacteria were removed and the preparations used in the assay. Error bars are the standard errors of the mean.

Figure 5. 3b. The number of haemocytes/mm^2 which migrated toward various blood preparations diluted to 25, 50 and 100% concentrations in ORCM buffer in which dead bacteria at 10^6, 10^7 and 10^8 cells/ml respectively were incubated for 2 h before removal of the bacteria and use of the preparations in the assay. Error bars are the standard errors of the mean.

Figure 5.3.a. Haemocyte Migration to Preparations which had contained Dead Bacteria

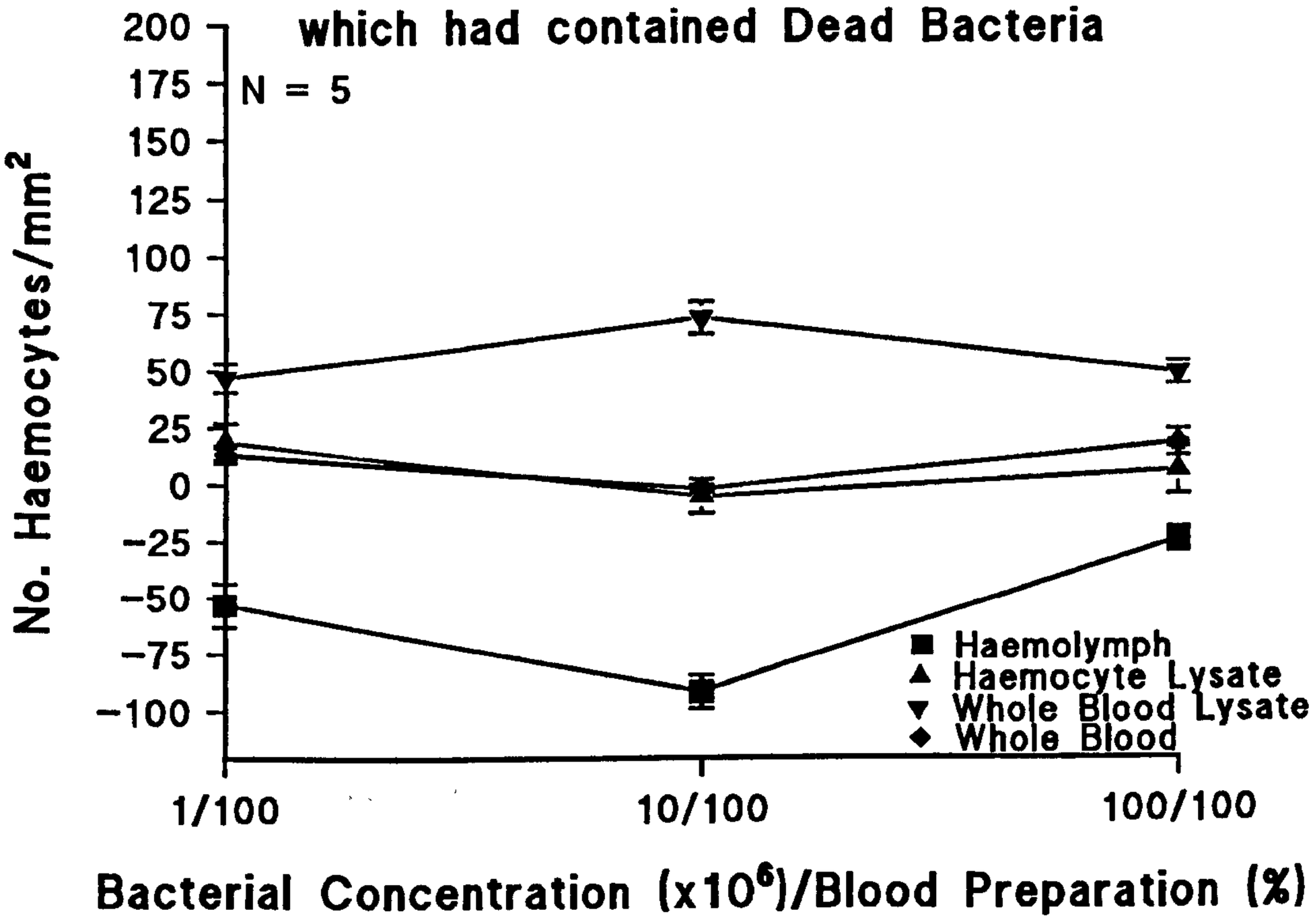
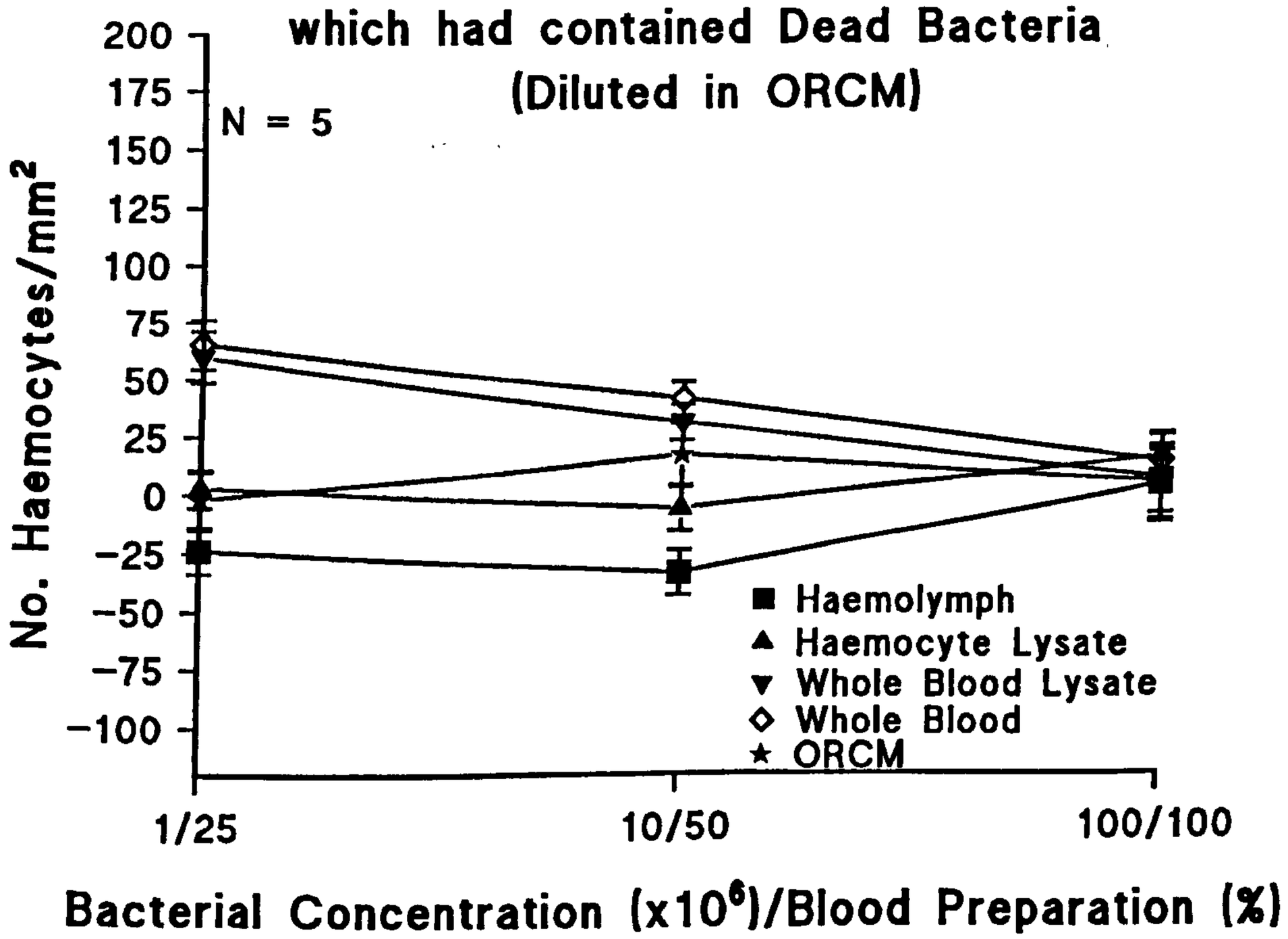


Figure 5.3.b. Haemocyte Migration to Preparations which had contained Dead Bacteria (Diluted in ORCM)



number of haemocytes passing through the membrane as the LPS concentration increased (fig. 5. 4a). Dilution of the haemolymph to 50 and 25% concentrations in ORCM also caused the number of migrating haemocytes to decrease with decreasing LPS concentrations (fig. 5.4b). When comparing the blood preparations, haemocyte lysate caused the highest number of haemocytes to migrate in both the 100% undiluted and 50 and 25% ORCM diluted preparations. ORCM with LPS (fig. 5.4b) caused haemocytes to migrate at significantly ($P<0.05$) higher levels at 0.0625 and 0.125mg/ml (133.8/mm^2 and 154.4/mm^2 respectively) compared to the blood preparations. At a LPS concentration of 0.025mg/ml in ORCM however, the number of haemocytes crossing the membrane decreased.

With all blood preparations pre-incubation with live bacteria or the addition of LPS caused increased haemocyte migration, while there was either no difference or a decrease for dead bacteria. Pre-incubation with live bacteria, generally, prompted increased migration at concentrations of 1×10^6 and 1×10^7 cells/ml for haemocyte lysate and whole blood.

Figure 5. 4a. The number of haemocytes/mm^2 which migrated toward various blood preparations, at 100% concentration, containing LPS at 0.0625, 0.125 and 0.25mg/ml. Error bars are the standard errors of the mean.

Figure 5. 4b. The number of haemocytes/mm^2 which migrated toward various blood preparations, diluted to 25, 50 and 100% concentrations in ORCM buffer, containing LPS at 0.0625, 0.125 and 0.25mg/ml respectively. Error bars are the standard errors of the mean.

Figure 5.4.a. Haemocyte Migration to Preparations containing LPS

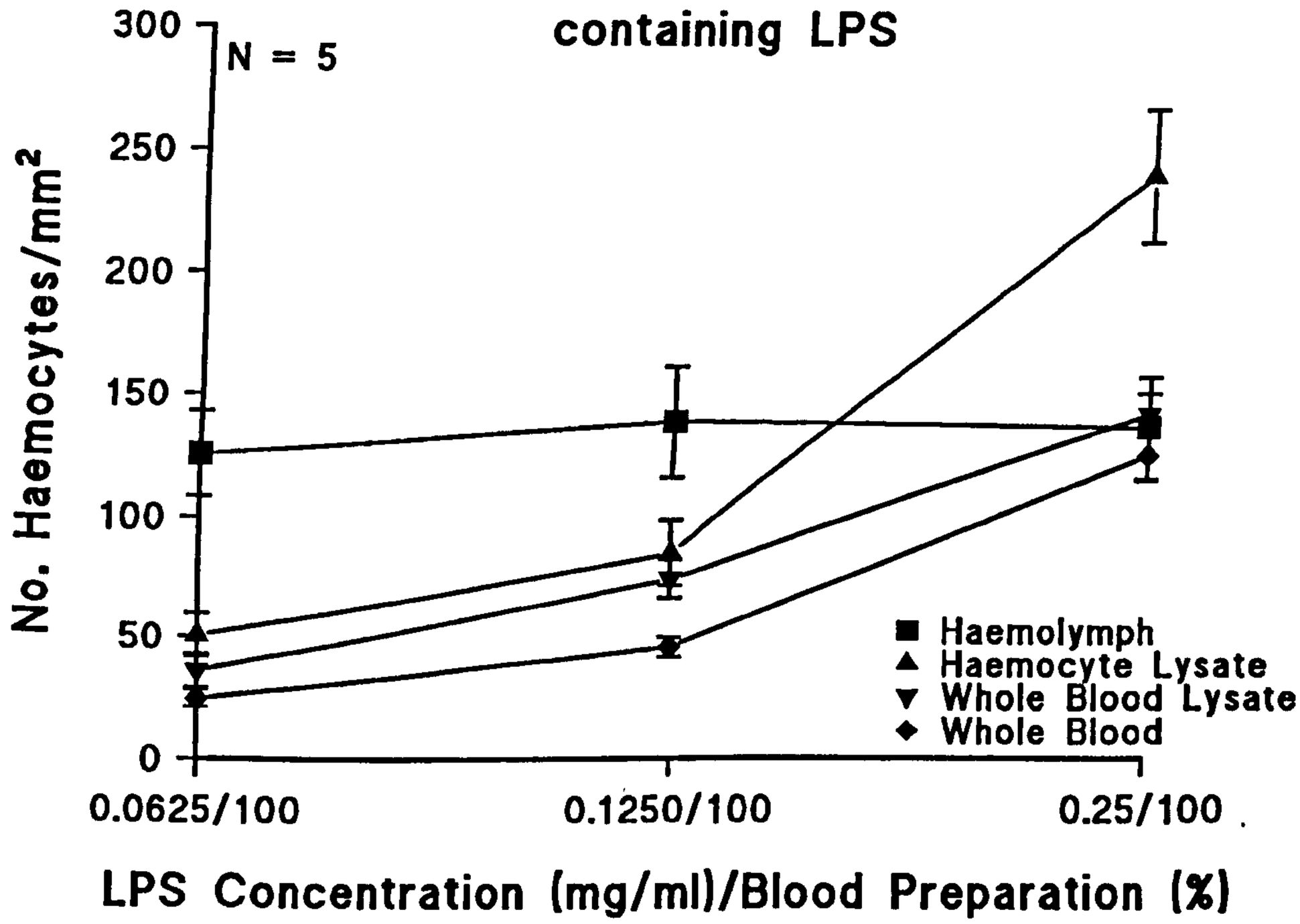
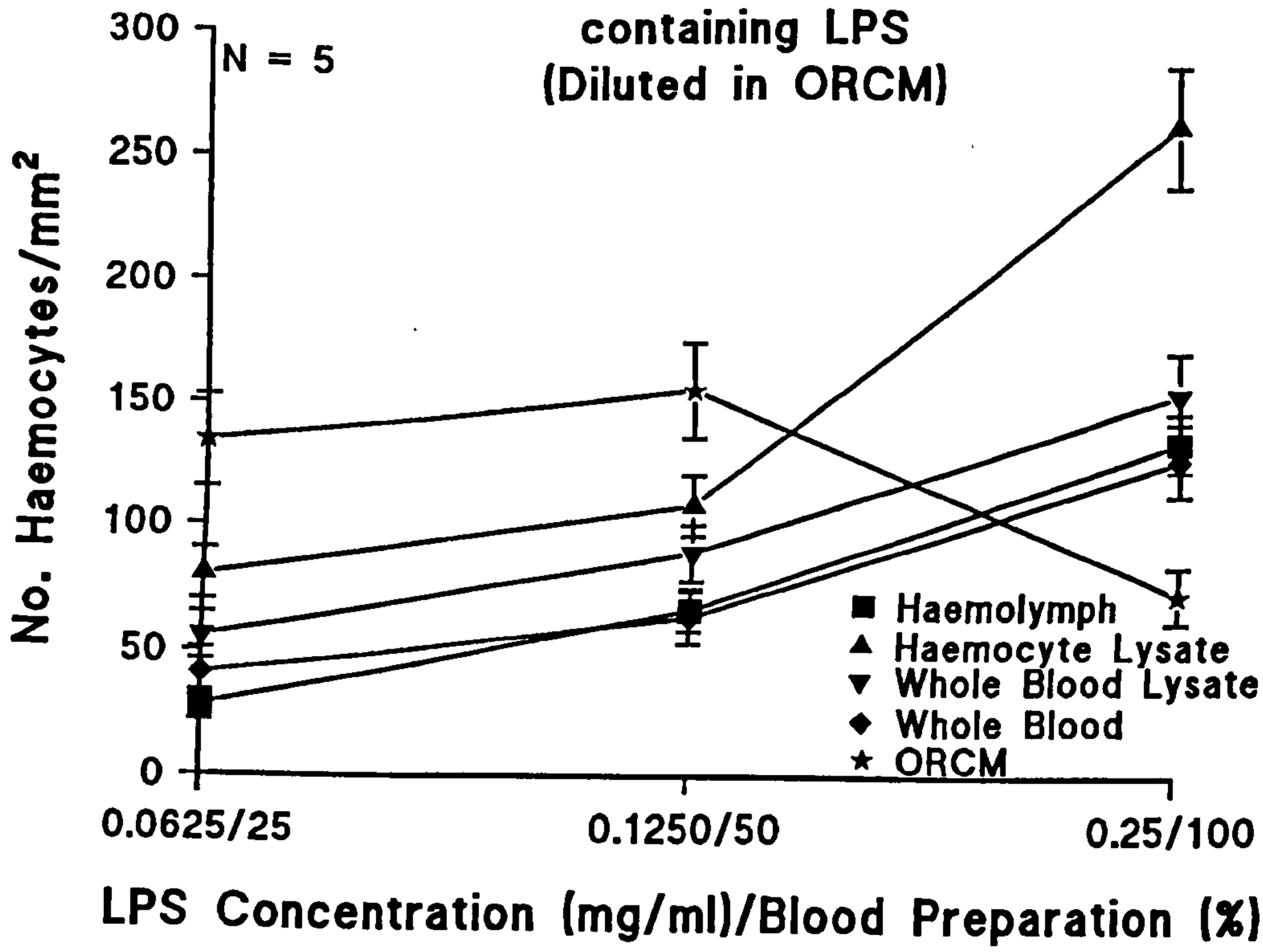


Figure 5.4.b. Haemocyte Migration to Preparations containing LPS (Diluted in ORCM)



Discussion

It has been demonstrated that *Eledone cirrhosa* haemocytes will positively migrate towards certain chemical stimuli, in particular factors induced by the presence of LPS and by pre-incubation with live bacteria, *Vibrio anguillarum*.

Negative migration in the form of fewer haemocytes passing through the 3μm pores of the filter, compared to controls, has also been shown in particular for haemolymph in which dead bacteria had been incubated.

Vibrio species are known to be fatal to cephalopods in captivity (Hanlon & Forsythe, 1990). These bacteria are found naturally on the skin of octopuses and when this soft bodied animal squeezes through rocks in its natural environment it could easily sustain injuries in which these opportunistic bacteria would rapidly multiply.

Interestingly, it is low concentrations of blood (0.1 - 10%) which prove attractive to haemocytes. Specifically haemocyte lysate is attractive at a 0.1% concentration but whole blood lysate and whole blood attract high haemocyte numbers at 10% concentration. There appear to be no previously published studies on haemocyte migration to different blood preparations apart from that inferred from the effects of injury in molluscs (Tripp, 1961; Pauley & Sparks, 1967; DesVoigne & Sparks, 1969; Pauley & Krassner, 1972; Sminia *et al.*, 1973; Bubel *et al.*, 1977; Bayne *et al.*, 1979; Sparks & Morado, 1988), the effect of haemocyte lysate supernatant on insect haemocytes (Takle & Lackie, 1986) or the effect on, among others, the earthworm coelomocytes by grafting experiments (Marks *et al.*, 1979). Presumably the release of certain constituents by haemocytes themselves attracts naive haemocytes. However in

high concentrations of haemocyte lysate (>1%) and haemolymph (>1%) the blood preparations appear to become either too concentrated and prevent haemocyte migration or is the normal value and therefore does not stimulate migration respectively. Further, since both whole blood and whole blood lysate are highly attractive for haemocytes, even at 10% concentration, while undiluted haemolymph has little attraction, it appears that a chemoattractant could be released from *E. cirrhosa* haemocytes when placed in diluted haemolymph or released from cell breakdown.

The response of haemocytes to blood preparations in which a Gram-negative bacterium (*V. anguillarum*) has been pre-incubated depends on whether the bacteria are alive or dead. Whole blood in which live bacteria have been incubated caused high numbers of haemocytes to migrate, which is significantly ($P<0.05$) increased above the migration found for 100% whole blood and whole blood lysate without bacteria. The results indicate again that combined components from the haemolymph and either whole or lysed haemocytes moderate haemocyte migration when using a high bacterial concentration. Studies with insects have suggested that insect immunocytes will secrete chemotactic substances upon contact with a foreign antigen and haemolymph factors such as lysozyme are also chemoattractants (Gupta, 1991). Interestingly both the haemocytes and haemolymph of *E. cirrhosa* display lysozyme and antiprotease activity (Chapter 8).

When formalin killed *V. anguillarum* were incubated in the blood preparations the results indicate that haemocytes are not attracted to preparations in which dead

bacteria are incubated, possibly indicating that dead bacteria do not induce the release of chemoattractants. However, there is evidence for some attraction when dead bacteria are incubated in whole blood lysate and whole blood (diluted in ORCM). Indeed, negative chemotaxis was detected when bacteria had been pre-incubated in haemolymph. Several authors working on molluscs have determined that migration of haemocytes occurs to live but not to dead bacteria. Cheng and Howland (1979), using *Crassostrea virginica* haemocytes, and Fawcett and Tripp (1994), using *Mercenaria mercenaria* haemocytes, demonstrated that live Gram negative and Gram positive bacteria were chemoattractant but dead bacteria were not. However, haemocytes from the gastropod *Viviparus malleatus* were attracted towards heat killed *Staphylococcus aureus*, though the presence of an 'agglutinin' was necessary before migration would occur (Schmid, 1975). Lastly, Kumazawa *et al.* (1990) demonstrated that haemocytes from the marine gastropod *Nerita albicilla* were attracted to both live and dead *Vibrio parahaemolyticus* without agglutinins being necessary. With *E. cirrhosa* it is highly unlikely that any agglutinin effect on haemocyte migration would be apparent since to prevent cell aggregration *E. cirrhosa* haemocytes were suspended in ORCM which would inhibit any agglutinating or opsonizing activity present in the haemolymph, (Chapter 4).

Fawcett and Tripp (1994) and Cheng and Howland (1979) suggest that chemoattractant molecules are emitted by living vegetative cells of certain Gram-positive and Gram-negative bacteria, and indicate that there are several possible attractants. Investigations by Howland and Cheng (1982) showed that the

chemoattractant for *C. virginica* haemocytes was a protein of approximately 10,000Da associated with the cell wall components of *Bacillus megaterium* and the cell envelope components of *Escherichia coli*. However, Fawcett and Tripp (1994) and Schneeweiß and Renwrantz (1993) have demonstrated haemocyte migration for *M. mercenaria* and haemocyte activation for *Mytilus edulis* haemocytes, respectively, using N-formyl-methionyl-leucyl-phenylalanine (N-FMLP) an oligopeptide isolated from Gram-negative bacterial cultures (Marasco *et al.*, 1984).

E. cirrhosa haemocytes are attracted to LPS, isolated from *E. coli*, in both buffer and blood preparations. Dilution of each of the blood preparations in buffer caused the migration of haemocytes to haemolymph to decrease, but increased the overall migration of haemocytes to haemocyte lysate, whole blood lysate and whole blood (fig. 5. 1.a&b). Overall the maximum number of migrating haemocytes, (about 172 haemocytes/mm^2), occurred to LPS at 0.25mg/ml in haemocyte lysate (1 x 10^6 haemocytes/ml). Experiments using higher concentrations of LPS produced no significant haemocyte migration (unpublished data). Though Fawcett and Tripp (1994) state that *M. mercenaria* haemocytes were not attracted to LPS, Hughes *et al.* (1991) clearly demonstrated that haemocytes from *M. edulis* were attracted to bacterial products. LPS from Gram negative bacteria (*Serratia marcescens* and *E. coli*) stimulated chemotaxis in *M. edulis* whereas formylated peptides appeared to induce chemokinesis (Schneeweiß & Renwrantz, 1993). Substances which induce chemokinesis (random haemocyte movement) were not specifically investigated in the experiments on *E. cirrhosa* reported here. The optimum assay incubation time found

for *E. cirrhosa* haemocytes was 90 min, with shorter assay termination times giving unreliable and unreproducible results (pers. obs.). However as found by Schneeweiß and Renwrantz (1993) with *M. edulis*, there was large individual variation among replicates. Individual variation was reduced as much as possible with *E. cirrhosa* by pooling the blood solutions and subtracting control values away from experimental values. It proved impossible to investigate whether *E. cirrhosa* haemocytes were displaying chemotaxis or chemokinesis as investigated by Schneeweiß and Renwrantz (1993) with *M. edulis*. The addition, to naive haemocytes from *E. cirrhosa*, of the solutions used to determine migration caused the haemocytes to clump together (unpub. data) and appeared to negate the effect of EGTA in the ORCM buffer which was used to keep the haemocytes separate and viable (Chapter 2). Low concentrations of calcium and magnesium were also added to the ORCM buffer to facilitate haemocyte movement. The second set of experiments was performed in order to try to quantify the effect of the buffer on the blood solutions. However, the results obtained, with the differing solutions used in the determination of haemocyte migration, prove that *E. cirrhosa* haemocytes do migrate in response to these stimulants.

In conclusion, *E. cirrhosa* haemocytes are capable of migrating to low concentrations of blood preparations such as whole blood lysate and whole blood. The haemocytes will also migrate to whole blood in which live bacteria and dead bacteria had been incubated before use in the assay. Finally, the haemocytes will recognise and move towards LPS. Further experimentation is required to determine the nature of the

chemoattractants demonstrated in this paper, and whether other chemoattractants such as N-FMLPs will also cause *E. cirrhosa* haemocytes to migrate.

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Chapter 6

In Vitro Cellular Bacteriostatic Activity and Nitro Blue Tetrazolium Reduction in the Lesser Octopus *Eledone cirrhosa* (Lam.)

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Abstract

Eledone cirrhosa haemocytes are capable of inhibiting the growth of the bacteria *V. anguillarum, V. parahaemolyticus* and *Aeromonas salmonicida in vitro.* Haemocyte growth inhibition of the bacteria varies with the type and concentration of bacteria used, the incubation temperature (5-20°C), and the duration of the experiment (0, 3 and 6h). Superoxide dismutase inhibitable nitroblue tetrazolium reduction showed, that exposure of haemocytes to dead *V. anguillarum* caused a higher reduction compared to live *V. anguillarum*. Lipopolysaccharide also caused formazan deposition.

Key Words: *Eledone cirrhosa*; Haemocytes; Bacteriostatic activity; Reactive oxygen intermediates.

Introduction

Circulating invertebrate blood cells are involved in a number of innate defence functions. In particular, phagocytosis has been studied and demonstrated in numerous invertebrates and involves the recognition, ingestion and killing of invading microorganisms (Ratcliffe *et al.*, 1985; Millar & Ratcliffe, 1994). Cellular bactericidal activity has been demonstrated in a few invertebrates, e.g., insects (Anderson *et al.*, 1973), crustaceans (White *et al.*, 1985; Chisholm & Smith, 1992; 1995; Schnapp & Smith, 1996), ascidians (Findlay & Smith, 1995, 1996), bivalves (Nottage & Birkbeck, 1990) and echinoderms (Plytycz & Seljelid, 1993; Stabili *et al.*, 1996). Cellular bactericidal activity involves the ingestion and killing of foreign invaders which can stimulate the intracellular release of various killing factors. Lysozyme, peroxidase and Reactive Oxygen Species (ROS) are all part of the battery of killing factors used by haemocytes against microorganisms (Ratcliffe *et al.*, 1985). The production of different Reactive Oxygen Intermediates (ROIs), e.g., superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-) and myeloperoxidase (MPO), has been demonstrated in a number of invertebrates, e.g., with gastropods (Dikkeboom *et al*, 1987, 1988; Shozawa *et al.*, 1989; Adema *et al.*, 1991) and with bivalves (Anderson *et al*, 1992; Pipe, 1992; Takahashi *et al*, 1993; Greger, 1996). Release of ROS after membrane stimulation following phagocytosis, causes an increase in oxygen utilization, which is termed the respiratory burst. Biochemically the respiratory burst involves the change of molecular oxygen into superoxide anion by the use of the enzyme NADPH oxidase (Chakravarti & Chakravarti, 1987) and the

activation of the hexose monophosphate shunt pathway. The superoxide anion is then detoxified either spontaneously, or via the enzyme SuperOxide Dismutase (SOD) to H_2O_2 which is then converted to molecular oxygen and water. Some invertebrates, however, appear unable to generate ROS, e.g. *Mercenaria mercenaria* (Cheng, 1976). Two methods are used to detect O_2^- production. Intracellular ROS production is detected by the reduction by O_2^- of the redox dye nitroblue tetrazolium (NBT) to formazan. Extracellular ROS release is detected by the reduction of ferricytochrome C by O_2^- (Secombes, 1990).

It has been demonstrated that *Eledone cirrhosa* haemocytes are attracted to certain stimulants (Chapter 5) and will phagocytose carbon, red blood cells (Stuart, 1968) and dead bacteria (Chapter 4). Haemocytes also contain various granules which stain with acid phosphatase, indicating lysosomal activity, and stain with DiAminoBenzidine (DAB) indicating peroxidase (hence peroxisome) activity (Chapter 3). Lysozyme activity has also been demonstrated in the haemocytes of *E. cirrhosa* and the activity is affected by bacterial challenge (Chapter 8). Live bacteria injected into *Octopus dofleini* (Bayne, 1973) and *E. cirrhosa* (Chapter 8) are quickly cleared from the circulation suggesting that the bacteria are either sequestered into fixed phagocytes in certain organs, and/or that the circulating haemocytes phagocytose and kill the bacteria. This paper investigates the effect of incubating live bacteria with *E. cirrhosa* haemocytes and determines whether bacteria or lipopolysaccharide cause the production of intracellular ROIs using the NBT reduction assay.

Materials and Methods

Animals

The octopuses were obtained from crab pots around the north coast of Anglesey. The animals were brought into an aquarium at the University of Wales at Bangor and maintained at 10-12°C. After 48 h the animals were weighed, sexed and marked using a syringe (1% alcian blue in octopus Ringer (OR) (NaCl, 2.433g/100ml; glucose, 1.4g/100ml; EGTA, 0.015g/100ml; KCl, 0.082g/100ml; KH_2PO_4, 0.004g/100ml)) (Chapter 3) and returned to specific tanks.

Haemocytes

Five marked animals were chosen at random. The animals were then weighed, anaesthetised and bled from the branchial blood vessel (Chapter 2). The collected blood was immediately transferred into marine anticoagulant (MA) (NaCl, 2.63g/100ml; glucose, 1.8g/100ml; tri-sodium citrate, 0.088g/100ml; citric acid, 0.055g/100ml) containing ethylene glycol-bis(β-aminoethylether) N, N, N', N', -tetraacetic acid (EGTA) (0.029g/100ml). Generally 1 ml of blood was added to 10 ml of ice cold MA. Blood counts were performed on the individual samples obtained and the blood was then centrifuged at 800g for 5 min at 4°C. The haemocytes were resuspended at 1 x 10^6 haemocytes/ml in OR. The haemocytes were then centrifuged again and resuspended in OR containing calcium and magnesium (ORCM) ($CaCl_2.6H_2O$, (0.0142g/100ml), $MgCl_2$ (0.0524g/100ml) and $MgSO_4.7H_2O$, (0.0629g/100ml)) and held on ice until use.

Bacterial Cultures

The cellular bacteriostatic assay used three bacteria, *Vibrio anguillarum* (MT275), *Vibrio parahaemolyticus* (MT295) and *Aeromonas salmonicida* (MT004), obtained from the Scottish Office, Agricultural Environment and Fisheries Department, Marine Laboratory, Aberdeen. The two *Vibrio* species were grown on 3% Tryptic Soya Broth (Gibco) (TSB) containing 2% NaCl, whereas the *Aeromonas* bacterium was grown on 3% TSB only. Before use the bacteria were counted, using Norris Powell Diluent (NPD) (0.5% formalin, sodium dodecyl sulphate, adjusted to pH 7.3 with Na_2HPO_4) and HCl, centrifuged at about 13000g for 10 min and resuspended in their respective growth media at 1×10^8 bacteria/ml.

Cellular Bacteriostatic Assay

The cellular bacteriostatic assay used to establish the bacteriostatic capacity of *E. cirrhosa* haemocytes was based on that described by Secombes (1990). Serial dilutions were made of the bacteria from a concentration of 1×10^8 cells/ml. One hundred microlitres of the haemocytes at 1×10^6 haemocytes/ml were added to the wells of a 96 well flat bottom plate (Dynatech) followed by 20µl of each of the bacterial dilutions in triplicate. Blanks containing ORCM (100µl) and TSB (20µl ± NaCl) were incorporated on each plate. The plates were immediately centrifuged at 800g for 5 min at 4°C and then placed in incubators set at 5, 10, 15 or 20°C and incubated for 6 h. The remaining bacteria, at the required dilutions, were stored at the same temperature as the 96 well plates.

After 3 h a further set of plates was set up using the bacteria which had been serially diluted and allowed to multiply for the 3 h. Twenty microlitres of the stored bacteria at the required incubation temperature were added in triplicate to 100µl of haemocytes at the same temperature. These plates were also incubated at 5, 10, 15 or 20°C but for 3 h only.

After 6 h incubation, 20µl of the remaining stored bacteria, which had been serially diluted and allowed to multiply, were added to haemocytes at the same temperature to both 3 and 6 h plates. This last bacterial addition was the control (0 h) for the experiment.

After the addition all plates were immediately centrifuged at 800g for 5 min at 4°C (i.e. 0 h). The experiment was then stopped by removal of the supernatant and addition of 50µl of 0.1% Tween 20 (Sigma) (0.1% Tween in sterile octopus saline (NaCl, 2.367g/100ml; glucose, 1g/100ml;$CaCl_2.6H_2O$, 0.116g/100ml; KH_2PO_4, 0.0056g/100ml; KCl, 0.1089g/100ml; $MgSO_4.7H_2O$, 0.503g/100ml; $MgCl_2$, 0.419g/100ml)) to lyse the haemocytes. The plates were then left for 5 min before the addition of 100µl of 3% TSB (± NaCl depending on the bacteria used). All plates were then incubated at 15°C for 16 h, to allow the bacteria to grow, before the addition of 10µl of 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, (MTT) (Sigma) (5mg/ml in distilled water) (detects live bacteria). The plates were then incubated for a further 15 min in the dark and the optical density values read at 600nm in a multiscan spectrophotometer. This procedure was used for all 3 bacteria, using the same incubation periods (0, 3 and 6 h) and the same incubation temperatures.

NBT Reduction Assay

The NBT reduction assays used one species of bacterium, *V. anguillarum*, or lipopolysaccharide (LPS) (*E. coli*, Sigma 0127:B8). Live and formalized *V. anguillarum* were centrifuged and resuspended at dilutions of 10^9, 10^8, 10^7 and 10^6 in ORCM. LPS was diluted in ORCM to 1, 0.5, 0.25, 0.125 and 0.063mg/ml.

A modified form of the assay used by Secombes (1990) was used to test reactive oxygen production by octopus haemocytes. One hundred microlitres of *E. cirrhosa* haemocytes in ORCM at 1 x 10^6 haemocytes/ml, were added to triplicate wells of 96 well plates. The plates were centrifuged at 800g for 5 min and the supernatants removed. One hundred microlitres of the stimulants, live and dead *V. anguillarum* and LPS, were then each added in triplicate to the wells and the plates were incubated at 15°C for 1 h. ORCM was added to haemocytes in triplicate wells on each plate to act as an unstimulated haemocyte control. Superoxide dismutase (SOD) (Sigma) controls were also incorporated for each of the stimulants at a final concentration of 300μg/ml. After 1 h the plates were centrifuged again and the supernatant containing the stimulant was removed. The haemocytes were then washed with octopus buffer (x2) before the addition of 100μl of NBT (Sigma) (0.3% in ORCM), followed by a further incubation of 1 h at 15°C. The NBT solution was then removed from the wells and absolute methanol was added. The haemocytes were washed (x2) with 70% methanol, to remove any extracellular NBT, followed by the addition of 120μl KOH (2M) and 140μl of dimethyl sulphoxide (DMSO) to rupture the haemocytes and release the intracellular NBT. After a further 10 min incubation the optical densities of the plates

were recorded at 620nm on a multiscan spectrophotometer with KOH/DMSO used as a blank for each stimulant.

Statistical Analysis

Analysis for the cellular bacteriostatic assay involved subtracting the blank from all values. The triplicate means for each of the 5 replicates were averaged and the mean and standard error values for the control, 3 and 6 h incubations for the 5 replicates at each temperature were plotted against the initial bacterial concentration. Analysis of variance (ANOVA) and Student t-tests were used to determine significant differences between the incubation time periods, the incubation temperatures and the different bacteria used.

For the NBT assay, the blank was subtracted from all values, and the control values were also subtracted from the optical density values obtained from the wells given stimulants. Means and standard errors of all of the triplicated 5 replicates were calculated and the results plotted. ANOVA was performed to determine the effect of concentration for each stimulant.

Results

Cellular Bacteriostatic Assay

Haemocytes from *Eledone cirrhosa* demonstrated an ability to inhibit the growth of the bacteria *Vibrio anguillarum, Vibrio parahaemolyticus* and *Aeromonas salmonicida in vitro*. Analysis of Variance (ANOVA) performed on the cellular bacteriostatic results produced highly significant ($P<0.0001$) values for each parameter tested. This growth inhibition by the haemocytes depended on the species and concentration of bacteria used ($P<0.0001$ in both cases). Other factors which affect haemocyte killing are the incubation temperature and the duration of the exposure of bacteria to the haemocytes ($P<0.0001$ for both parameters).

At 5°C (fig. 6.1a) very little growth or growth inhibition of *V. anguillarum* was observed at bacterial concentrations of 1.25×10^7 bacteria/ml and below. During incubations at both 3 and 6 h, haemocytes inhibited bacteria at 1×10^8 bacteria/ml. At 10°C (fig. 6.1b) and 20°C (fig. 6.1d) bacterial inhibition by haemocytes occurred across the whole bacterial dilution range with very little difference between the 3 and 6 h results at 10°C. At 15°C (fig. 6.1c) incubation of *V. anguillarum* with *E. cirrhosa* haemocytes for 6 h causes significantly ($P<0.05$) greater inhibition than when incubated for 3 h.

Generally both 3 and 6 h incubations of live *V. parahaemolyticus* with *E. cirrhosa* haemocytes at 5 and 10°C produced similar levels of growth inhibition (figs. 6.2a & 6.2b respectively). However at bacterial concentrations of $> 6.25 \times 10^6$ bacteria/ml the 6 h and 3 h incubations at 10 and 15°C showed no significant difference from the

Figure 6. 1.a. Haemocytes from *Eledone cirrhosa* at 1 x 10^6 haemocytes/ml incubated with live *Vibrio anguillarum* at 5°C for 0. 3 or 6 h. Each point represents the mean from 5 replicates. Error bars are standard errors of the mean.

Figure 6. 1.b. Haemocytes from *E. cirrhosa* incubated with live *V. anguillarum* at 10°C for 0. 3 or 6 h. Each point represents the mean from 5 replicates. Error bars are standard errors of the mean.

Figure 6.1.a.

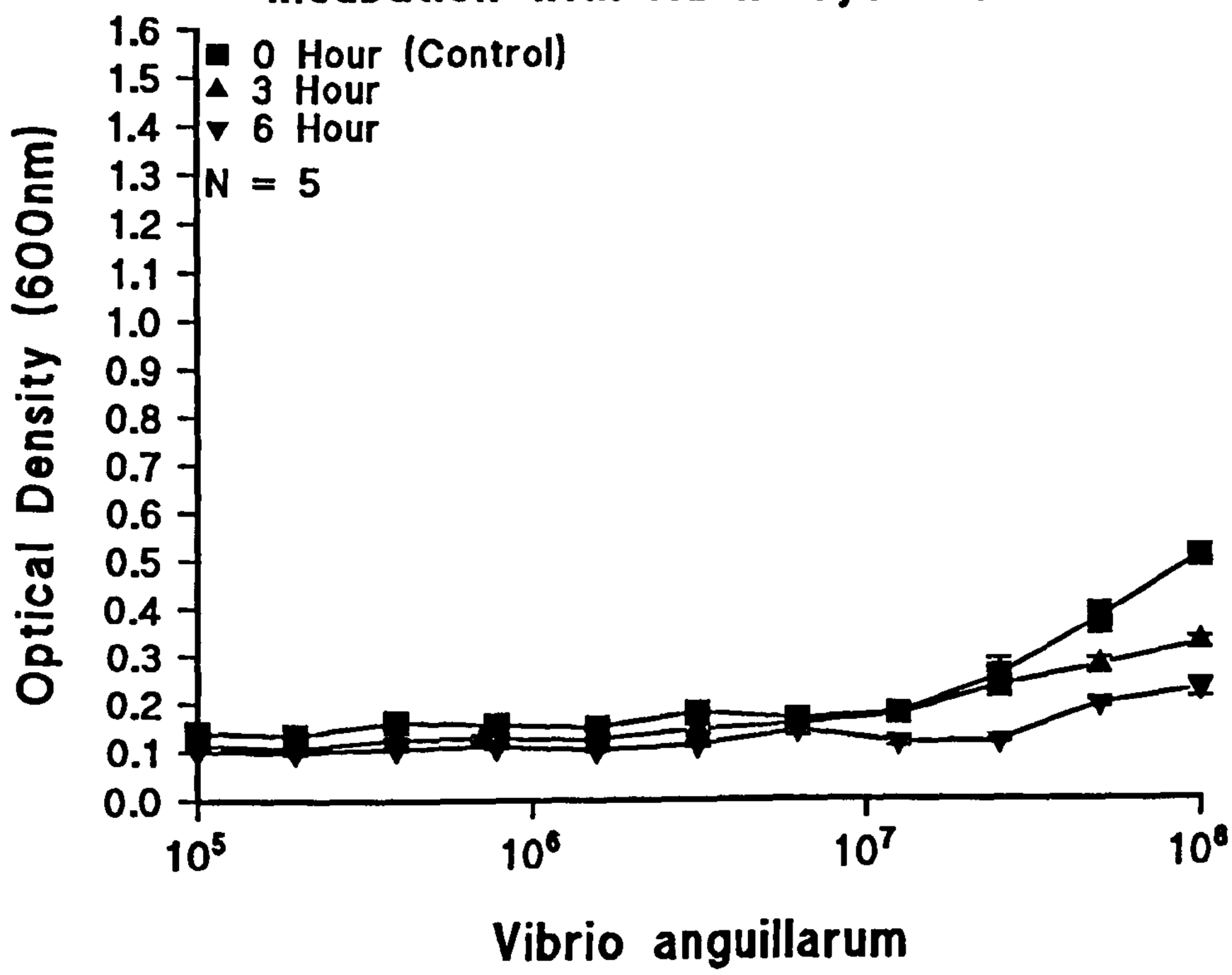


Figure 6.1.b.

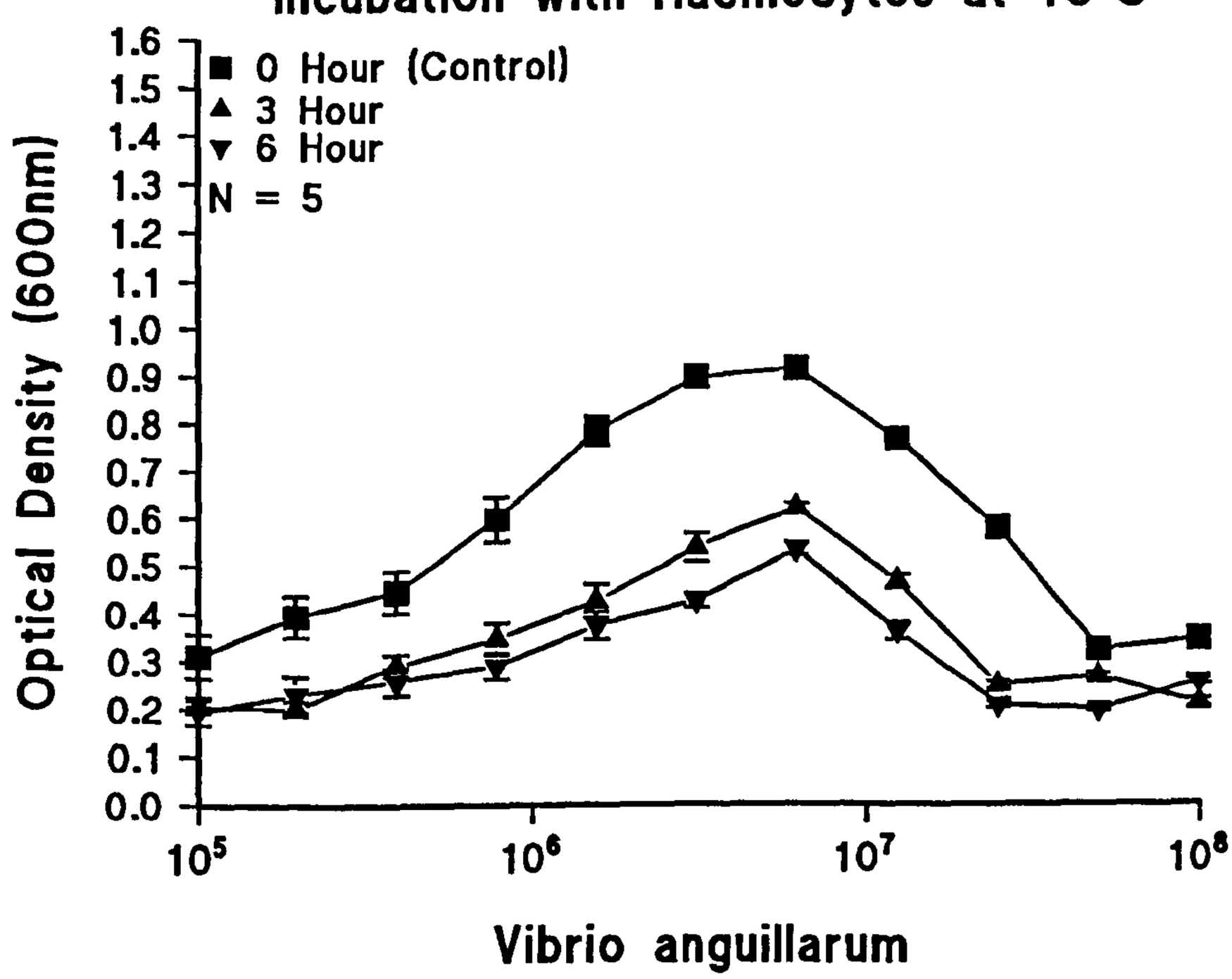


Figure 6. 1.c. Haemocytes from *E. cirrhosa* incubated with live *V. anguillarum* at 15°C for 0. 3 or 6 h. Each point represents the mean from 5 replicates. Error bars are standard errors of the mean.

Figure 6. 1.d. Haemocytes from *E. cirrhosa* incubated with live *V. anguillarum* at 20°C for 0. 3 or 6 h. Each point represents the mean from 5 replicates. Error bars are standard errors of the mean.

Figure 6.1.c. Incubation with Haemocytes at 15°C

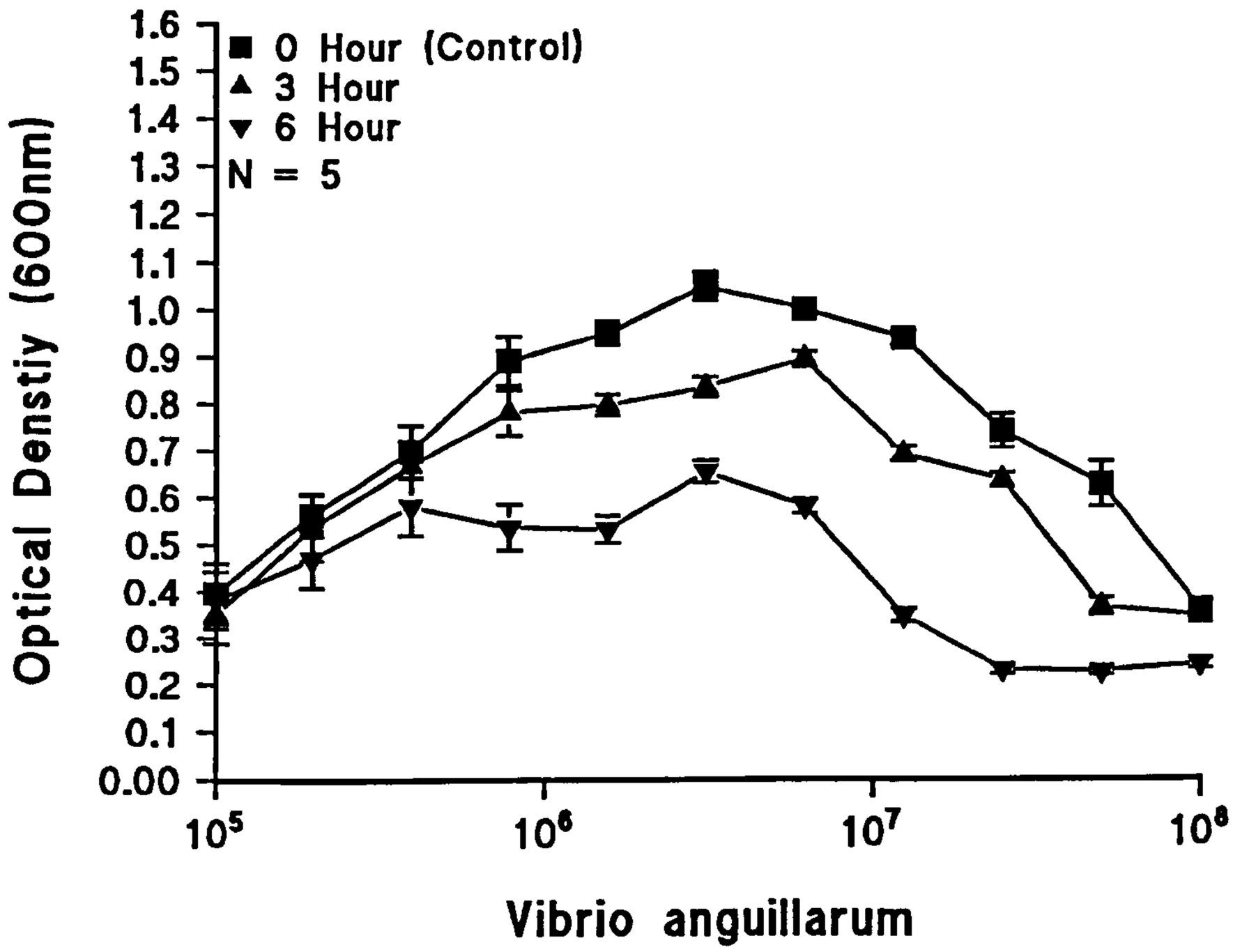


Figure 6.1.d. Incubation with Haemocytes at 20°C

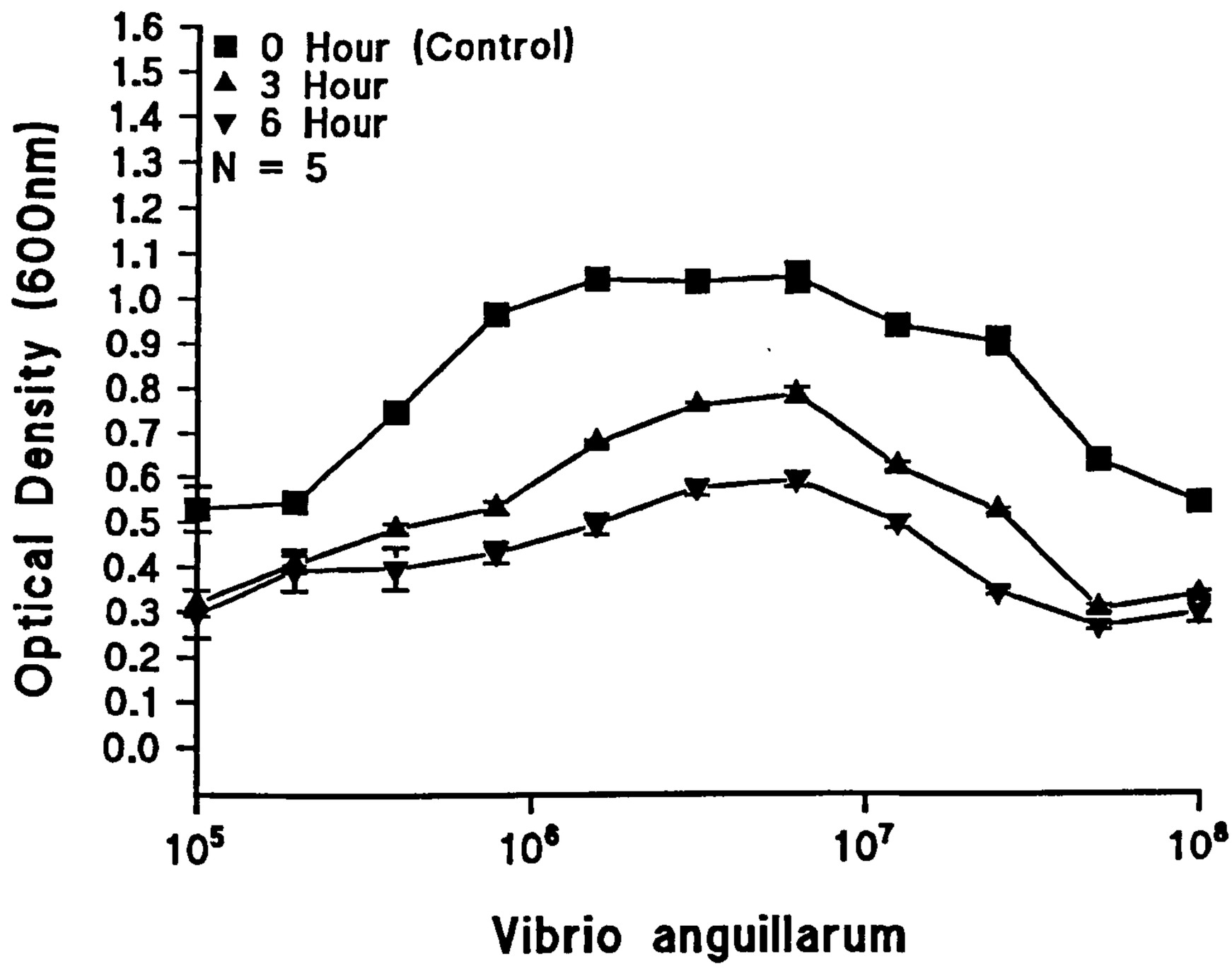


Figure 6. 2.a. Haemocytes from *E. cirrhosa* incubated with live *Vibrio parahaemolyticus* at 5°C for 0. 3 or 6 h. Each point represents the mean from 5 replicates. Error bars are standard errors of the mean.

Figure 6. 2.b. Haemocytes from *E. cirrhosa* incubated with live *V. parahaemolyticus* at 10°C for 0. 3 or 6 h. Each point represents the mean from 5 replicates. Error bars are standard errors of the mean.

Figure 6.2.a. Incubation with Haemocytes at 5°C

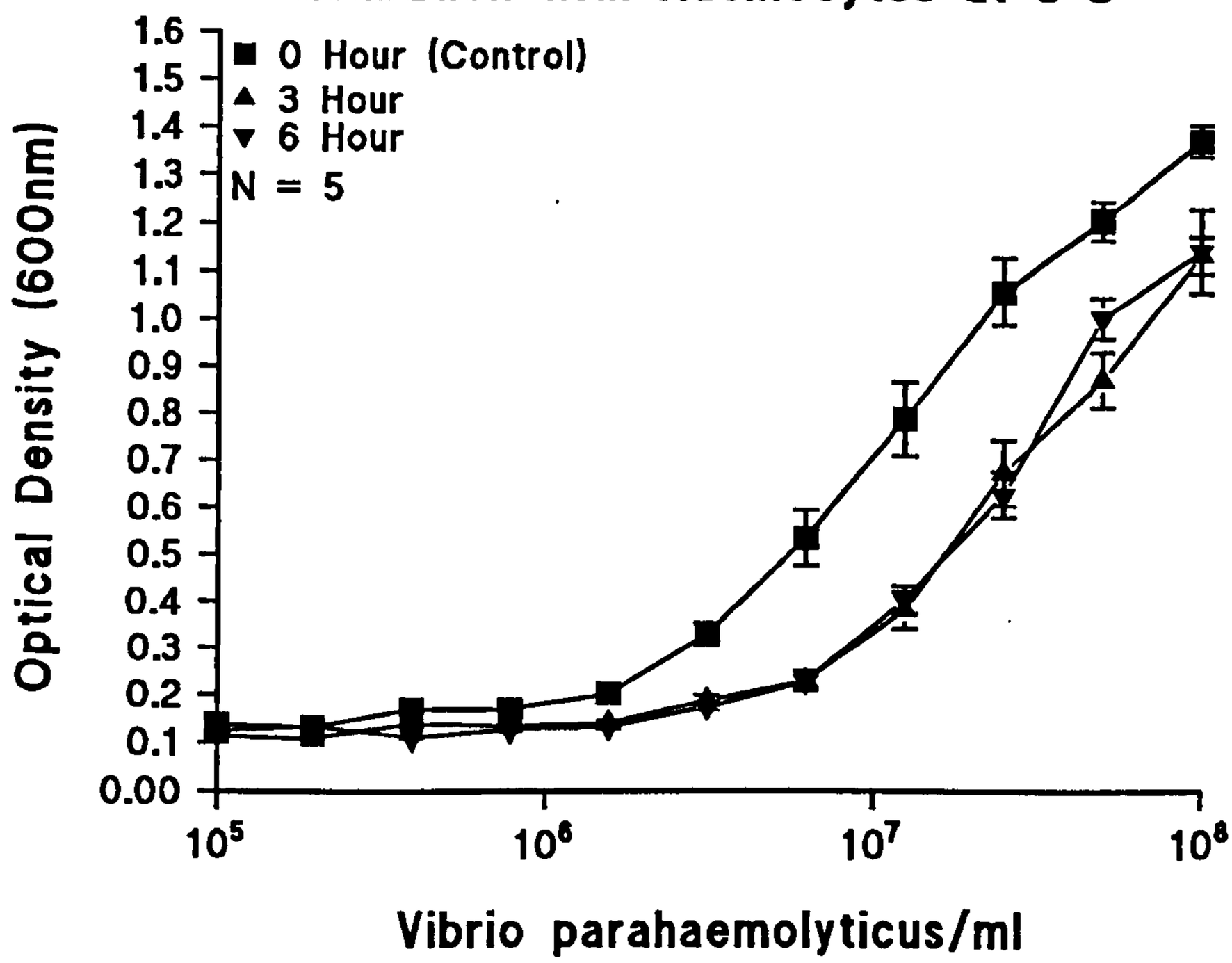


Figure 6.2.b. Incubation with Haemocytes at 10°C

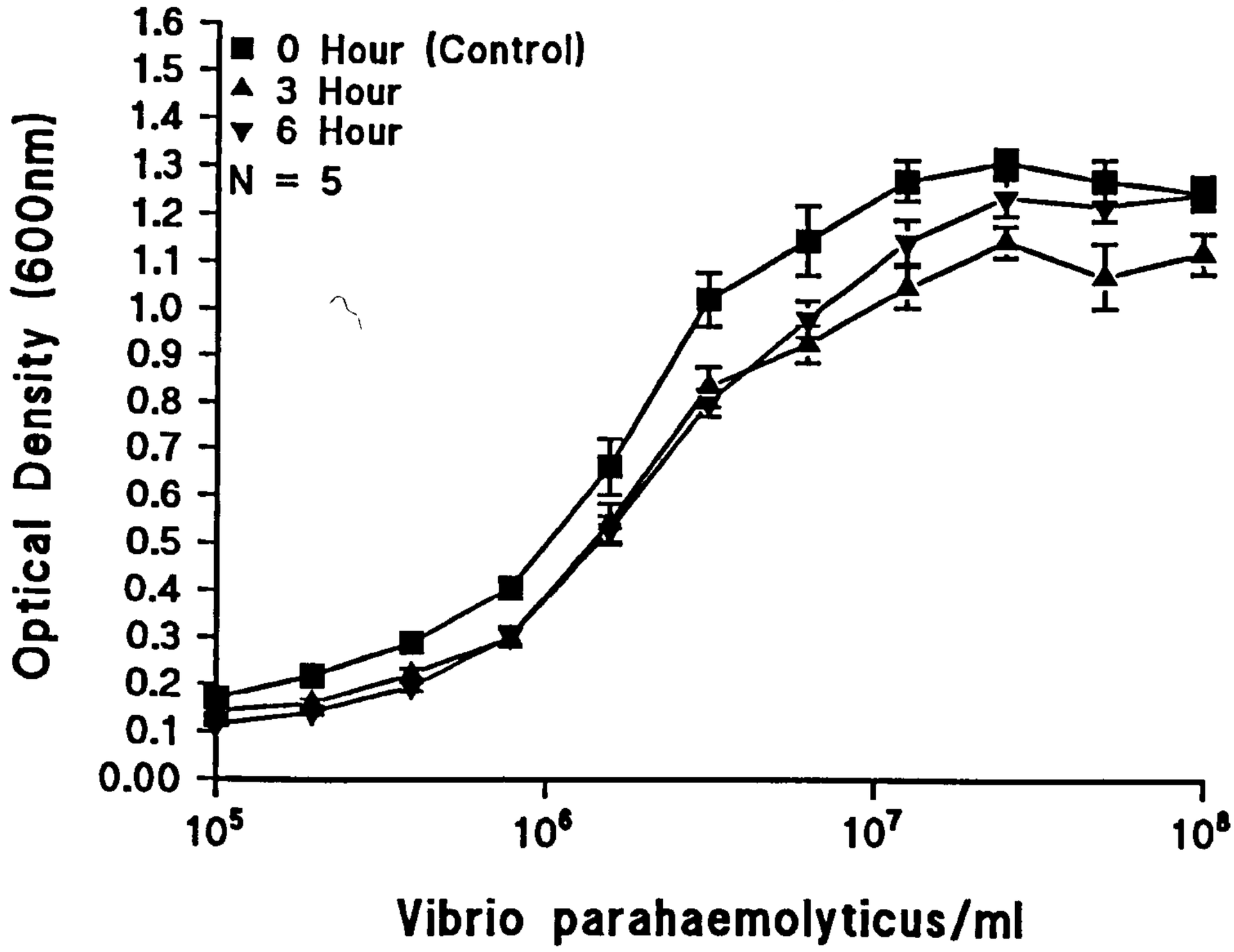


Figure 6. 2.c. Haemocytes from *E. cirrhosa* incubated with live *V. parahaemolyticus* at 15°C for 0. 3 or 6 h. Each point represents the mean from 5 replicates. Error bars are standard errors of the mean.

Figure 6. 2.d. Haemocytes from *E. cirrhosa* incubated with live *V. parahaemolyticus* at 15°C for 0. 3 or 6 h. Each point represents the mean from 5 replicates. Error bars are standard errors of the mean.

Figure 6.2.c. Incubation with Haemocytes at 15°C

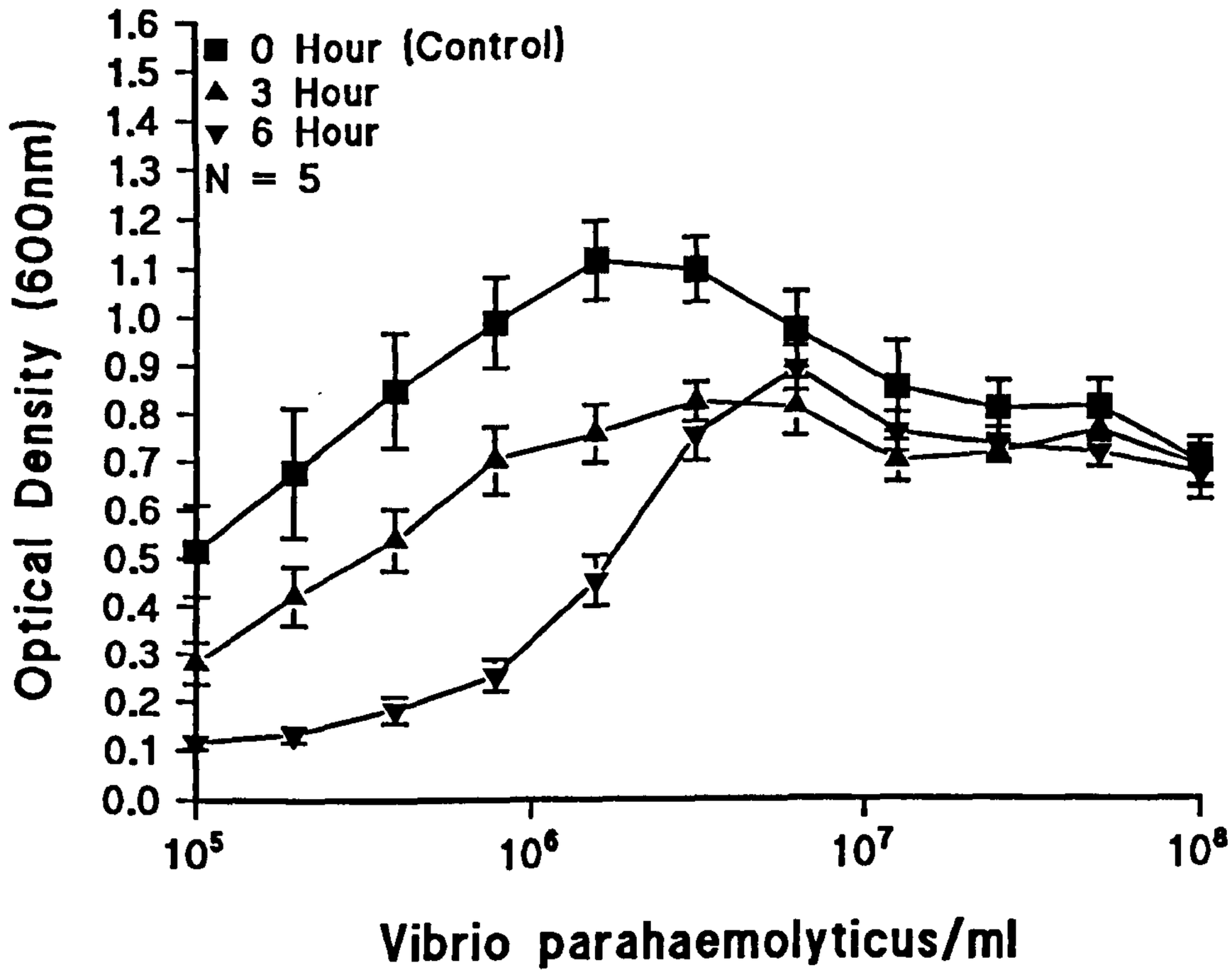
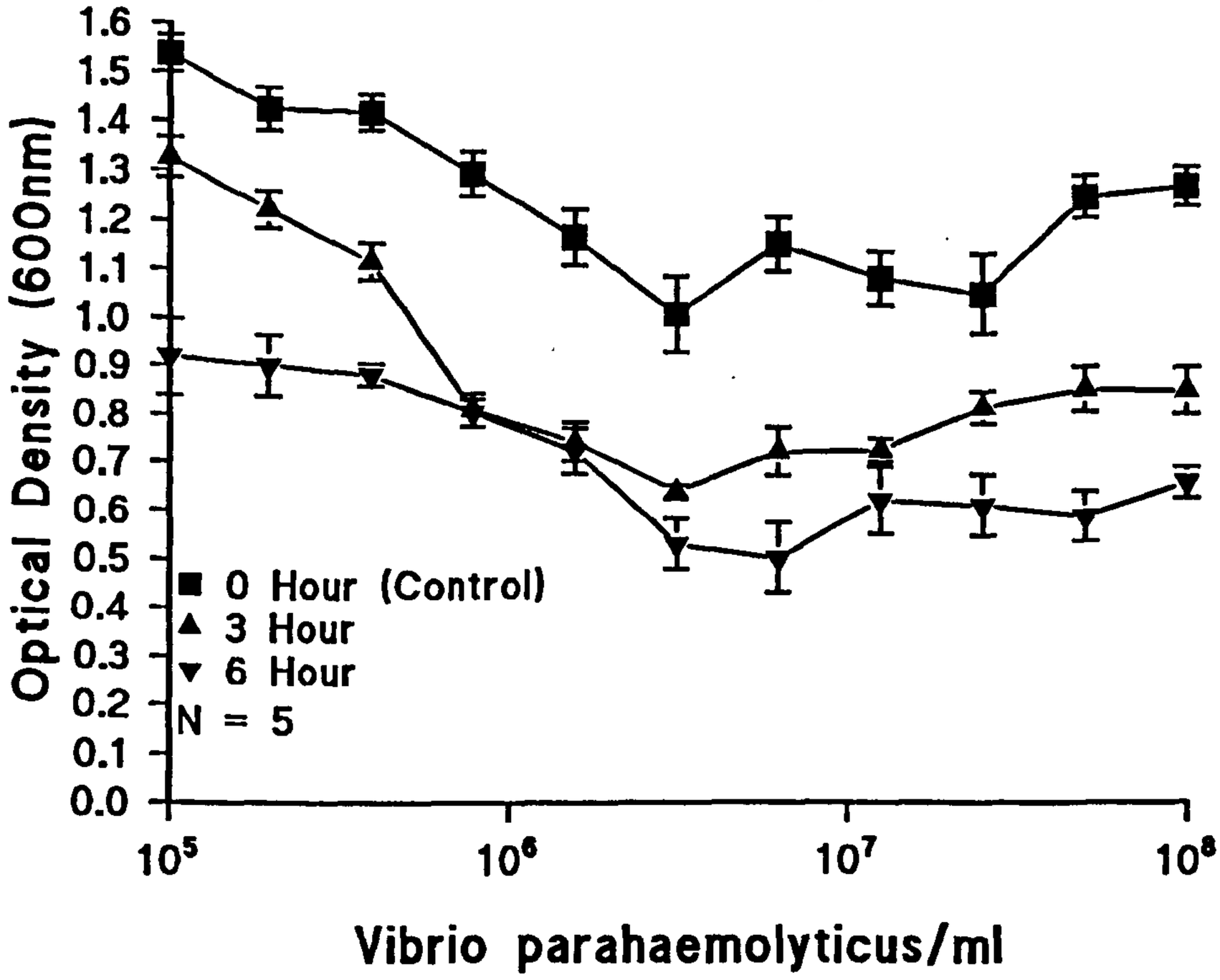


Figure 6.2.d. Incubation with Haemocytes at 20°C



control values. Low concentrations of bacteria (<3.125 x 10^6/ml) at 15°C (fig. 6.2c) were significantly ($P<0.05$) inhibited by *E. cirrhosa* haemocytes. Though growth inhibition was indicated at 20°C (fig. 6.2d) for both 3 and 6 h, the plots for both the control, 3 and 6 h incubations were erratic.

Very little significant inhibition of *A. salmonicida* occurred at 5°C (fig. 6.3a). Generally, at 10 and 15°C (figs. 6.3b & 6.3c respectively) 6 h incubations of the bacteria with the haemocytes indicated decreased growth, however at high bacterial concentrations (>2.5 x 10^7/ml) at 15°C significant ($P<0.05$) growth inhibition was detected after 3 h incubation only (fig. 6.3c). At 20°C significant ($P<0.05$) bacterial inhibition occurred at <6.25 x 10^6 bacteria/ml at both 3 and 6 h compared to the control (fig. 6.3d).

Overall *E. cirrhosa* haemocytes reduced the growth of *V. parahaemolyticus* more than *A. salmonicida* at 5°C, whereas at 10°C they significantly reduced the growth of *V. anguillarum*. Very little growth of *V. anguillarum* occurred ar 5°C. At 15°C *V. parahaemolyticus* at low concentrations were inhibited but *V. anguillarum* were inhibited over the middle range of concentrations tested. Very little inhibition of *A. salmonicida* occurred except at 20°C when low concentrations were inhibited. With all three bacteria inhibition of growth tended to be greater at 20°C.

NBT Reduction Assay

Dead *V. anguillarum* (fig. 6.4) at all concentrations tested, gave the strongest positive results with NBT reduction compared to the other 3 stimulants used. ANOVA results

Figure 6. 3.a. Haemocytes from *E. cirrhosa* incubated with live *Aeromonas salmonicida* at 5°C for 0. 3 or 6 h. Each point represents the mean from 5 replicates. Error bars are standard errors of the mean.

Figure 6. 3.b. Haemocytes from *E. cirrhosa* incubated with live *A. salmonicida* at 10°C for 0. 3 or 6 h. Each point represents the mean from 5 replicates. Error bars are standard errors of the mean.

Figure 6.3.a. Incubation with Haemocytes at 5°C

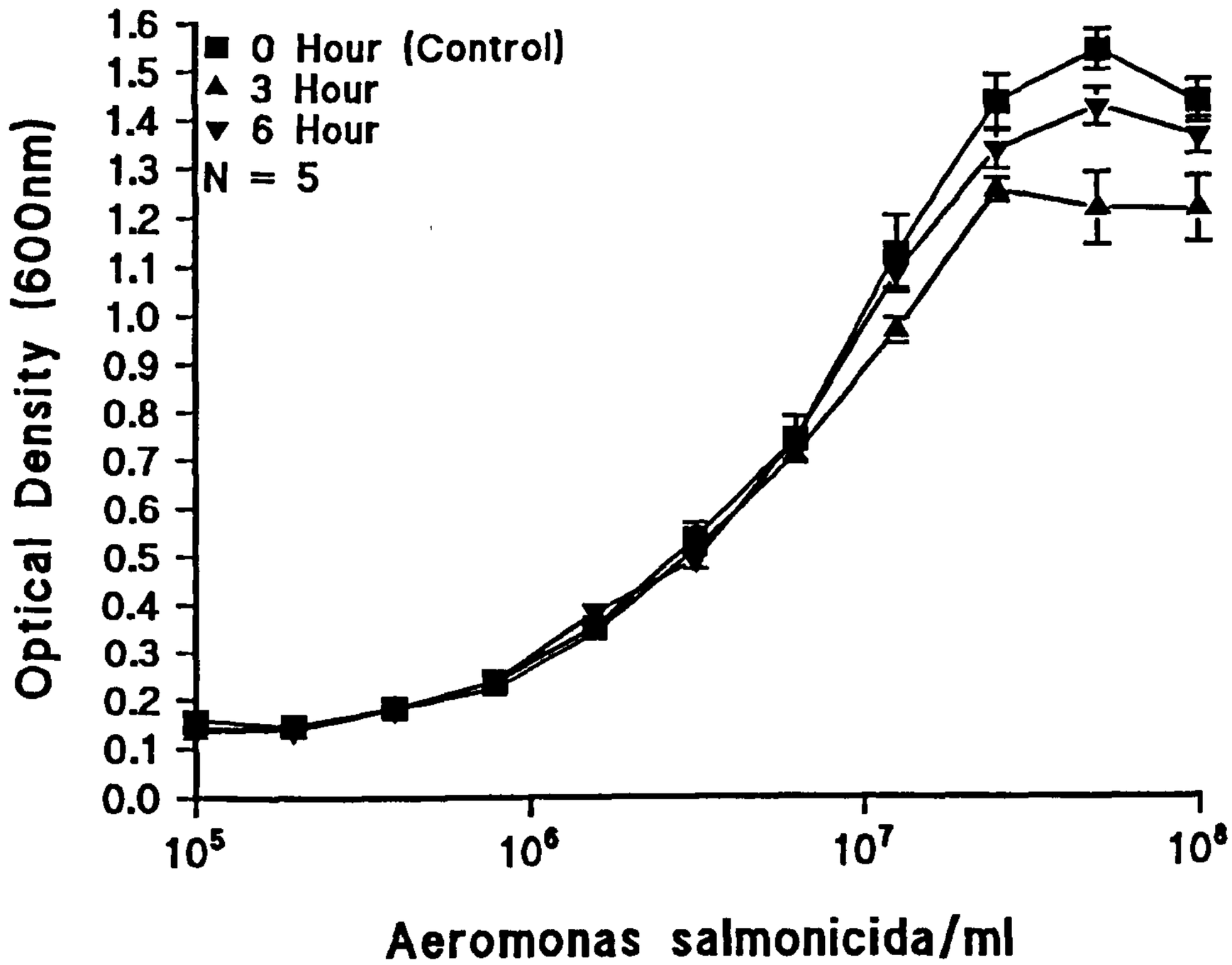


Figure 6.3.b. Incubation with Haemocytes at 10°C

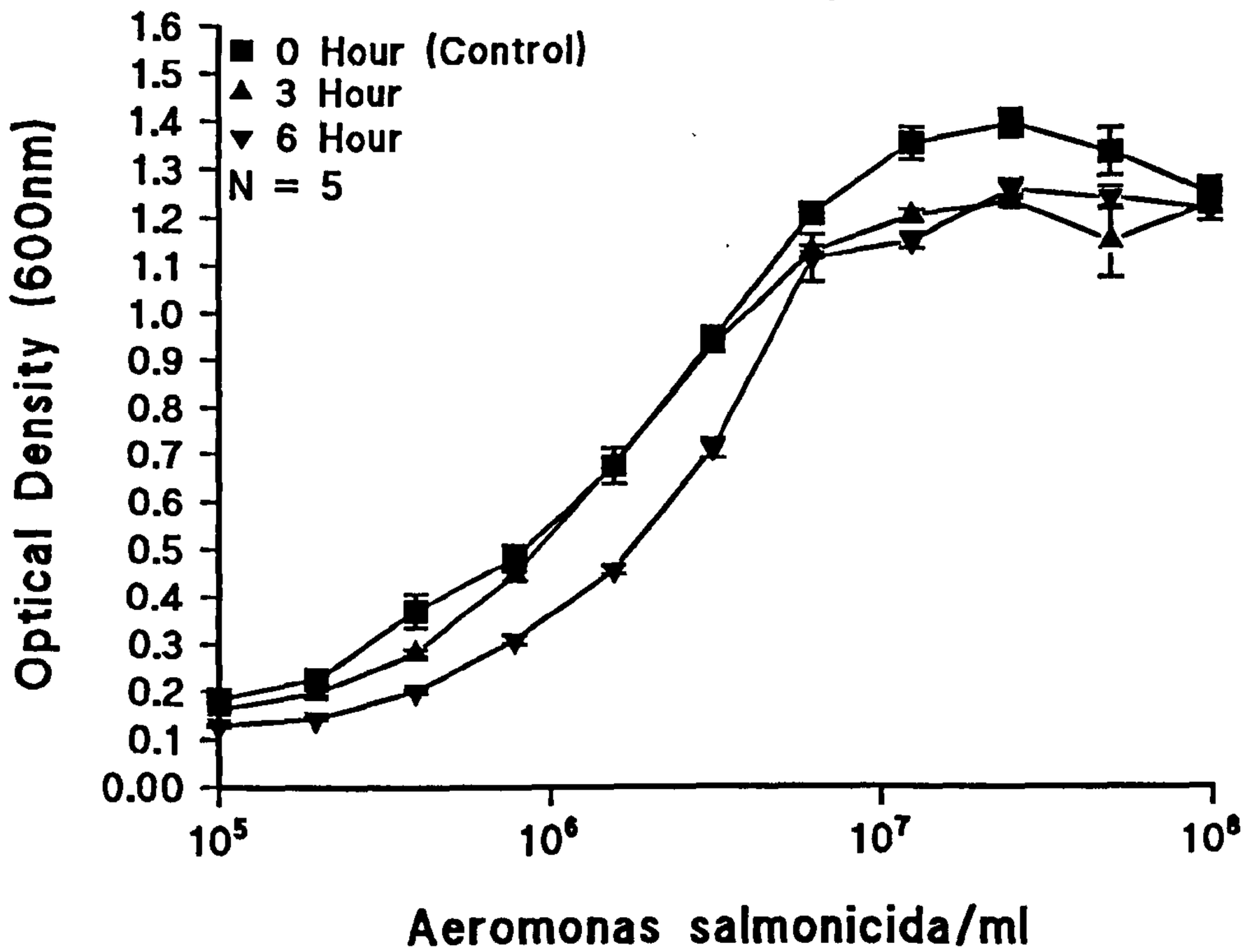


Figure 6. 3.c. Haemocytes from *E. cirrhosa* incubated with live *A. salmonicida* at 15°C for 0. 3 or 6 h. Each point represents the mean from 5 replicates. Error bars are standard errors of the mean.

Figure 6. 3.d. Haemocytes from *E. cirrhosa* incubated with live *A. salmonicida* at 20°C for 0. 3 or 6 h. Each point represents the mean from 5 replicates. Error bars are standard errors of the mean.

Figure 6.3.c.

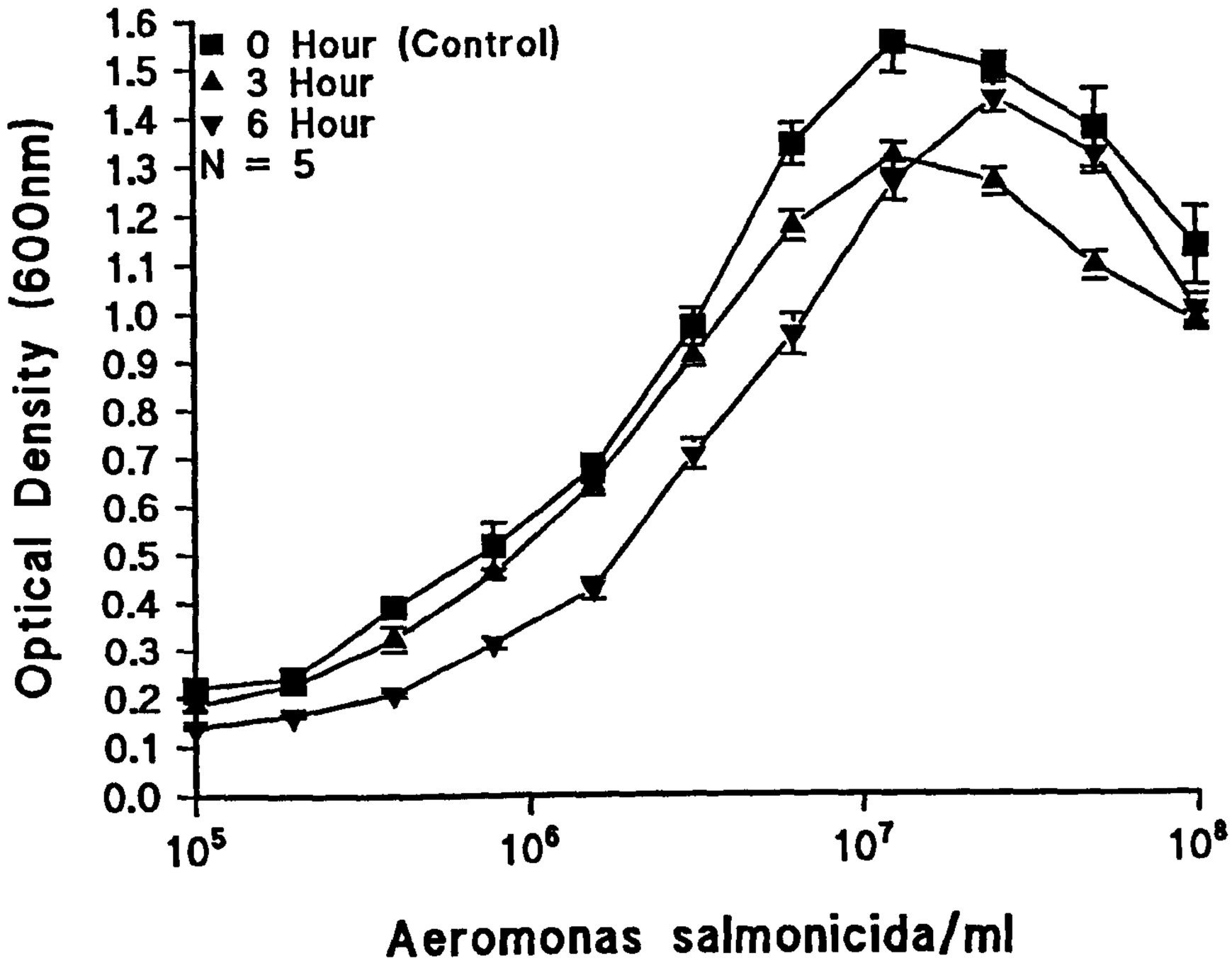


Figure 6.3.d.

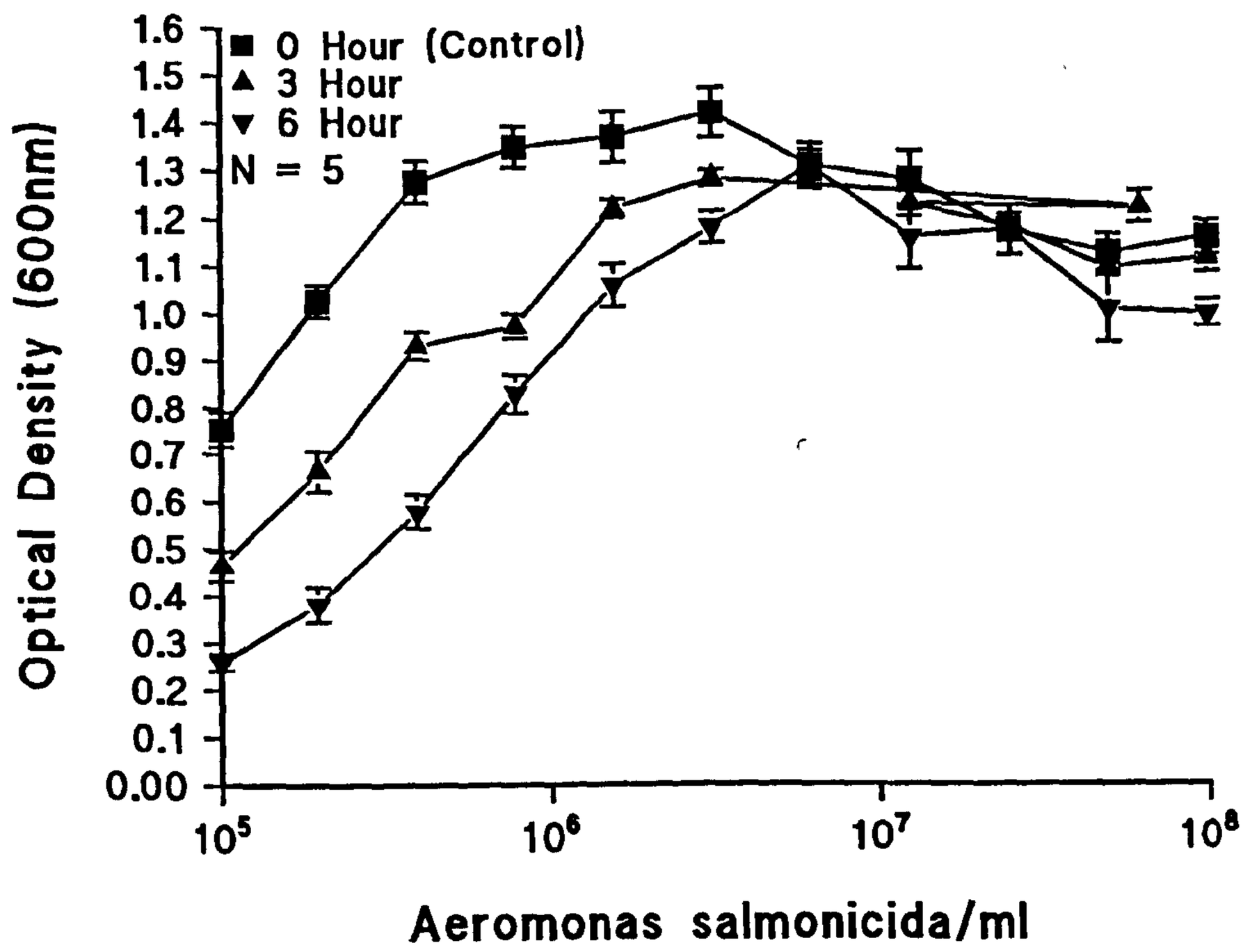
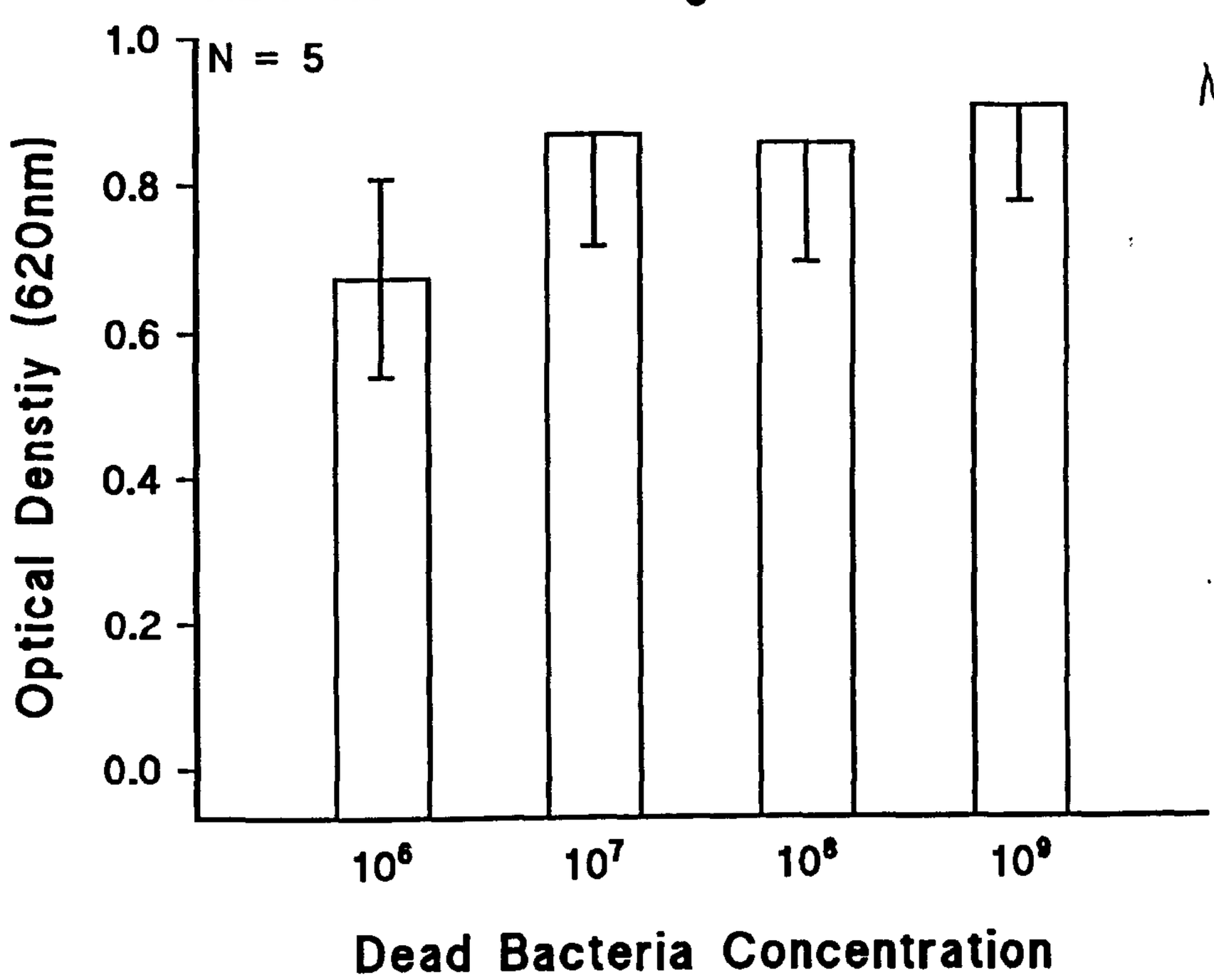


Figure 6. 4. Nitroblue tetrazolium reduction detected at 620nm using *E. cirrhosa* haemocytes at 1 x 10^6 haemocytes/ml stimulated with dead *V. anguillarum* at 10^6, 10^7, 10^8 and 10^9 bacteria/ml. Each bar represents the mean of 5 replicates. Error bars are standard errors of the mean.

Figure 6. 5. Nitroblue tetrazolium reduction detected at 620nm using *E. cirrhosa* haemocytes at 1 x 10^6 haemocytes/ml stimulated with live *V. anguillarum* at 10^6, 10^7, 10^8 and 10^9 bacteria/ml. Each bar represents the mean of 5 replicates. Error bars are standard errors of the mean.

Figure 6.4



No T
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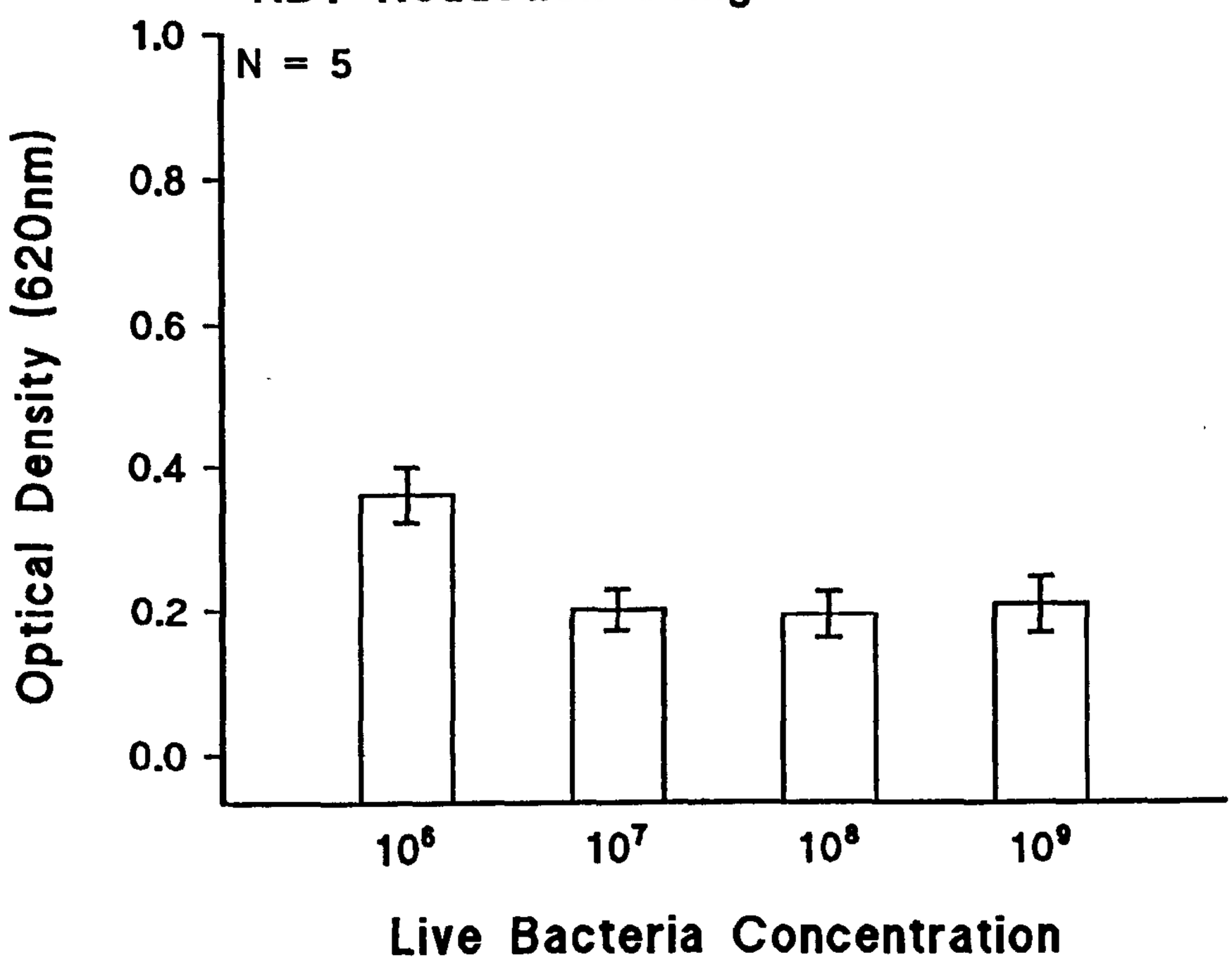
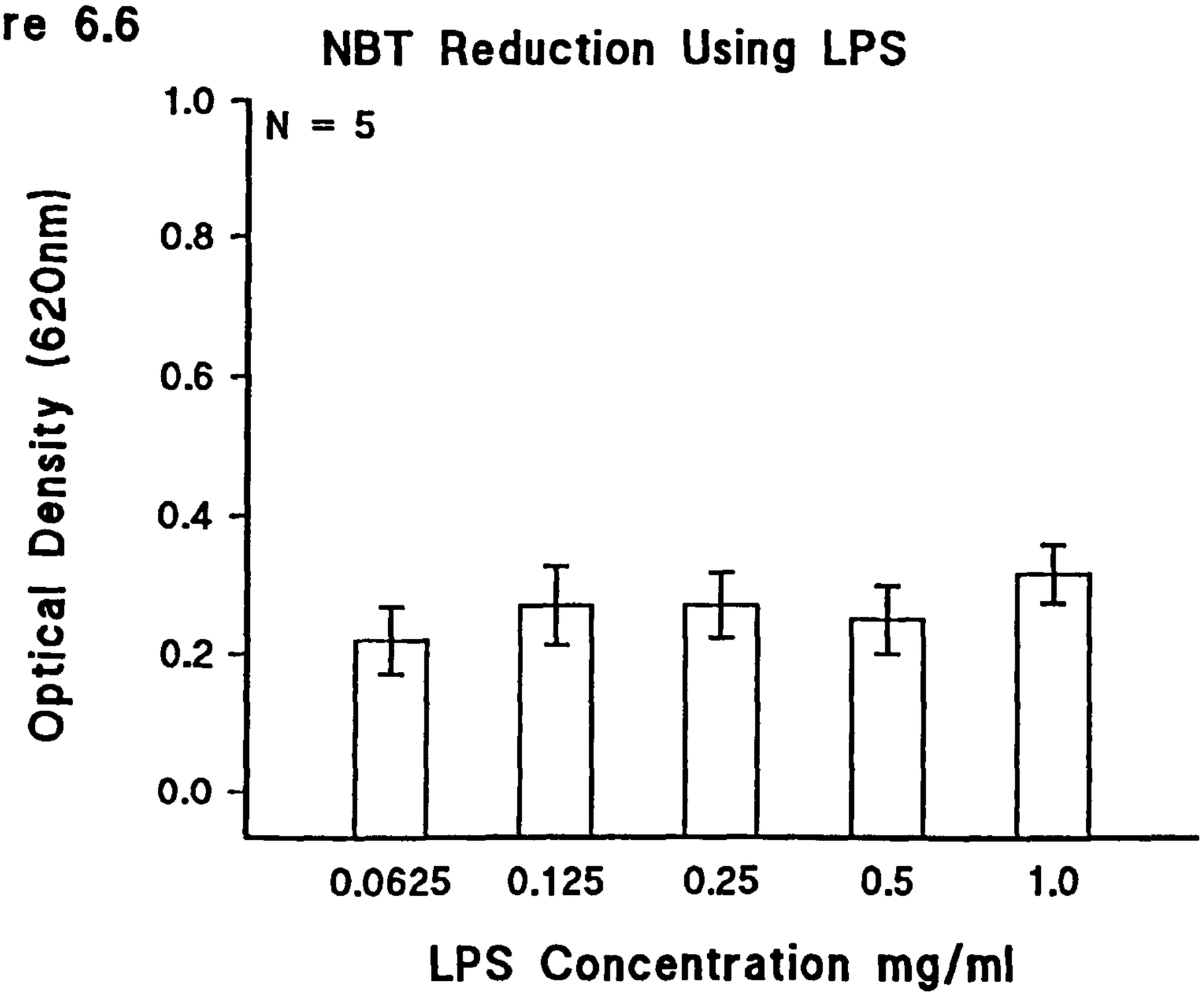
Figure 6.5



showed that incubation with dead bacteria significantly ($P<0.0001$) affected the haemocytes. NBT reduction was also significant ($P<0.05$) for live *V. anguillarum* (fig. 6.5) at 10^6 bacteria/ml and for LPS (fig. 6.6), but no differences were detected between the LPS concentrations used.

Figure 6. 6. Nitroblue tetrazolium reduction detected at 620nm using *E. cirrhosa* haemocytes at 1 x 10^6 haemocytes/ml stimulated with lipopolysaccharide at 0.0625, 0.125, 0.25, 0.5 and 1.0 mg/ml. Each bar represents the mean of 5 replicates. Error bars are standard errors of the mean.

Figure 6.6


NBT Reduction Using LPS
N = 5
Optical Density (620nm)
1.0
0.8
0.6
0.4
0.2
0.0
0.0625
0.125
0.25
0.5
1.0
LPS Concentration mg/ml

Discussion

The bacteria *Vibrio anguillarum, V. parahaemolyticus* and *Aeromonas salmonicida* are inhibited in their growth by haemocytes from *Eledone cirrhosa in vitro*. The amount of inhibition depends on the bacterium and the bacterial concentration present, as well as the incubation temperature and duration.

V. anguillarum, V. parahaemolyticus and *Aeromonas* (sp) have been cultured from ulcerated skin obtained from cephalopods held in captivity (Hanlon & Forsythe, 1990). Abrasions to the skin of cephalopods occur by contact with other cephalopods or by contact with the retaining tank wall. The bacteria, found naturally on the skin of cephalopods, are opportunistic and quickly invade and multiply in any wound causing ulcers which can be fatal. The octopod *E. cirrhosa*, will die from mantle ulcerations at temperatures above 17°C (pers. obs.).

Bayne (1973) showed that specialized tissues in *Octopus dofleini* removed injected live bacteria from the circulation rather than circulating haemocytes phagocytosing the bacteria. In *Carcinus maenas*, however, injected bacteria were killed following removal from the circulation by haemocyte clumps lodged in blood sinuses (White *et al*, 1985). It has been shown that *E. cirrhosa* haemocytes phagocytose *in vitro* dead (Chapter 4), and *in vivo* injected live, *V. anguillarum* (Chapter 9), thereby removing them from the circulatory system (Chapter 8). Studies on *E. cirrhosa* haemocytes have also demonstrated the presence of lysozyme activity (Chapter 8) which could aid in inhibiting growth of the bacteria. The haemocyte bacteriostatic results indicate that *E. cirrhosa* haemocytes are capable of inhibition of the growth of both *V. anguillarum*

and *V. parahaemolyticus* at temperatures up to 20°C. However, at 15°C there is no significant inhibition of *V. parahaemolyticus* at high bacterial concentrations. It is possible that the reduced growth of *V. anguillarum* at 10 - 20°C is due to culture confinement over the time period, and could be an artefact of the assay due to the rapid growth of this bacterium at these temperatures (Secombes, pers. comm.).

Haemocytes from the cockroach *Blaberus craniifer* were shown to phagocytose and destroy some bacteria, the pathogenicity being dependent on the bacterial strain and the concentration (Anderson *et al*, 1973). Chisholm and Smith (1992) demonstrated an antibacterial factor(s) present in the haemocyte lysate solution of *C. maenas*. They later showed (Chisholm and Smith, 1995) that similar antibacterial activity was present in the haemocyte lysate solutions from a variety of crustaceans. Though the antibacterial activity varied between species it was operative mainly against Gram-negative bacteria and was stable to between 65-70°C. Antibacterial proteins from the haemocyte lysate supernatants of *C. maenas* have, however, recently been characterized and were shown to be also active against Gram-positive bacteria (Schnapp & Smith, 1996). Findlay and Smith (1995) showed that haemocyte lysate supernatants from the solitary ascidian *Ciona intestinalis* displayed more effective antibacterial activity against Gram-positive than Gram-negative bacteria and that the bacteriolytic enzyme lysozyme was not present. The two proteins responsible for the antibacterial activity (Findlay & Smith, 1996) in *C. intestinalis* have also been characterized. Using haemocyte lysate solutions, the bivalves *Ostrea edulis* and

Crassostrea gigas have been shown to display inducible antibacterial activity against both Gram-negative and Gram-positive bacteria (Roch *et al.*, 1996). Using haemocytes from *Mytilus edulis*, Nottage and Birkbeck (1990) showed that large numbers of *V. alginolyticus* (2 isolates) were toxic to the bivalves' haemocytes as was the culture filtrate. The pathogenic virulent form of *V. alginolyticus* was demonstrated to have a higher cytotoxic effect than the 'environmental isolate' of the bacterium. Low *Vibrio* numbers were, however, killed by *M. edulis* haemocytes which produced reactive oxygen intermediates when stimulated by the bacteria. The bacteriolytic lysosomal enzyme, lysozyme, has been detected in both the haemocytes and haemolymph of *E. cirrhosa* (Chapter 8). It is possible that part of the inhibition reported here, with the 3 bacteria used, could be due to the action of lysozyme. Further work with *E. cirrhosa* is at present underway to determine whether the growth inhibition detected with the bacteria used is in fact killing of the bacteria by the haemocytes e.g. by phagocytosis or whether other secreted antibacterial factors are present.

The addition of live *V. anguillarum* to haemocytes from *E. cirrhosa* induces a small stimulatory effect on the haemocytes, as indicated by NBT reduction. However, the addition of dead *V. anguillarum* to *E. cirrhosa* haemocytes shows a significantly ($P<0.05$) higher reduction of NBT compared to all the other stimulants studied. The fact that dead *V. anguillarum* shows high SOD inhibitable NBT reduction leads to the possibility that ROS are produced during phagocytosis, if so this would be the first

demonstrable case of ROS production in cephalopods. LPS also stimulates a small amount of NBT reduction indicating possible haemocyte membrane stimulation. However, preliminary experiments with *E. cirrhosa* which used the soluble membrane elicitor phorbol 12-myristate 13-acetate (PMA) in conjunction with NBT, produced no reduction, possibly due to the concentrations used (0.125 - 1.0µl/ml PMA) (unpub. data). *Lymnaea stagnalis* haemocytes when stimulated with particulate agents and with PMA showed higher responses to the particulate agents (Dikkeboom *et al.*, 1987). Before and after stimulation with yeast cells, haemocytes from the Pacific oyster *Crassostrea gigas* also showed an ability to reduce NBT to solid formazan. Though phagocytosis increased NBT reduction by *C. gigas* haemocytes, it was temperature dependent (Anderson *et al.*, 1992). Both types of coelomocyte from the earthworm *Eisenia foetida* were also shown to produce ROI's after stimulation with zymosan (Valembios & Lassègues, 1995). Pipe (1992) further demonstrated the release of superoxide anions from *M. edulis* haemocytes using both particulate and soluble stimulants. However, Takahashi *et al.* (1993) working with the Pacific oyster *C. gigas*, and Bell and Smith (1993) working with *Carcinus maenas* were able to stimulate the respective haemocytes to produce O_2^- using PMA with ferricytochrome C. Preliminary experiments with *E. cirrhosa* haemocytes using ferricytochrome C to measure extracellular ROI production, produced no reduction using the same stimulants e.g. bacteria, LPS or PMA (unpub. data). When haemocytes from the tiger shrimp *Penaeus monodon* were stimulated with various agents, ß-glucan had the strongest stimulating effect (as shown using NBT reduction) followed by zymosan and

then PMA (Song & Hsieh, 1994). However, the addition of PMA caused hypochlorite (OCl^-) production and MPO activity, leading the authors (Song and Hsieh, 1994) to suggest that different stimulants affect different stages of the phagocytic process.

In conclusion, *E. cirrhosa* haemocytes are capable of inhibiting the growth of certain bacteria over a range of concentrations and temperatures. Part of the bacterial growth inhibiting mechanism involves the production of superoxide anions detectable by SOD inhibitable NBT reduction using bacterial and LPS stimulants. It is possible that the superoxide anions are retained within the haemocyte and not released extracellularly since experiments using ferricytochrome C produced no result (unpub. data). Further PMA did not stimulate *E. cirrhosa* haemocyte membranes to produce ROS although it is possible that the concentrations used were too high (unpub. data).

Work is continuing with *E. cirrhosa* haemocytes to determine whether other reactive oxygen intermediates are produced (e.g., H_2O_2). Since haemolymph bacteriostatic activity has also been established for *E. cirrhosa* (Chapter 7) it is apparent that the octopod is able to deal effectively with invading microorganisms. Further work will try to elucidate the nature of the killing mechanisms detected in the haemocytes.

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Chapter 7

Agglutination and Humoral Bacteriostatic Activity in the Lesser Octopus *Eledone cirrhosa* (Lam.)

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Abstract

Haemolymph from *Eledone cirrhosa* is able to significantly inhibit the growth of the bacteria *Vibrio anguillarum, V. parahaemolyticus* and *Aeromonas salmonicida*. At 15 and 20°C with *V. anguillarum* and at 20°C with *V. parahaemolyticus* at high bacterial concentrations (1 x 10^9 cells/ml) growth is less inhibited.

V. anguillarum, V. parahaemolyticus and *A. salmonicida* are agglutinated by *E. cirrhosa* haemolymph. Agglutination activity is generally not affected by temperatures between 5, and 20°C. *A. salmonicida* showed significantly lower $\log_2$ titre values than either *V. anguillarum* or *V. parahaemolyticus* at all temperatures.

Key Words: *Eledone cirrhosa*; Haemolymph; Agglutination; Bacteriostatic activity.

Introduction

Humoral immunity in invertebrates encompasses a wide range of defence activities such as agglutination, lytic factors and antimicrobial agents (Millar & Ratcliffe, 1994; Smith & Chisholm, 1992; Ractliffe *et al.*, 1985). Agglutination, or the aggregation of foreign particles, is perhaps the most widely studied of the serum defence activities and has been demonstrated in most invertebrates. Agglutinins appear to vary in structure and function between the different invertebrate classes but most are proteins with the ability to bind certain carbohydrate moieties. Some agglutinins are referred to as lectins, or carbohydrate binding proteins, and can be free in the serum or bound to the surface of blood cells. In a variety of invertebrates some agglutinins are involved in enabling, or enhancing, the phagocytosis of particles by the blood cells, they are then referred to as opsonins (Ratcliffe *et al.*, 1985; Renwrantz, 1986; Sminia & Van der Knaap, 1986; Millar & Ratcliffe, 1994). Invertebrates, in general, possess several agglutinins enabling a variety of particulate materials (e.g. bacteria and erythrocytes) to be recognised by one animal.

In molluscs, agglutinins have been detected in the cell free haemolymph of various gastropods (e.g. Prowse & Tait, 1969; Stanislowski *et al.*, 1976; Stein & Basch, 1979; Harm & Renwrantz, 1980; Jeong *et al.*, 1981; Renwrantz & Stahmer, 1983; Boswell & Bayne, 1984), bivalves (e.g. Bayne *et al.*, 1979; Suzuki & Mori, 1990; Fisher & DiNuzzo, 1991; Olafsen *et al.*, 1992; Tripp, 1992) and cephalopods (Stuart, 1968; Rögener *et al.*, 1986; Fisher and DiNuzzo, 1991). Agglutinins have also been found in the gastropod albumen gland (Renwrantz & Mohr, 1978; Stein & Basch, 1979; Jeong

et al., 1981) and cephalopod embryos (Marthy, 1974) as well as in the 'skin slime' of cephalopods (Marthy, 1974; Renwrantz & Uhlenbruck, 1974).

The ability of humoral components to kill invading organisms is demonstrated by antimicrobial and lytic factors. In particular antibacterial activity has been detected in the cell free haemolymph of a number of invertebrates (e.g., Anderson & Chain, 1982; Valembois *et al.*, 1982; Kubo *et al.*, 1984; Vaillier *et al.*, 1985; Lassègues *et al.*, 1989; Nottage & Birkbeck, 1990: Xylander & Nevermann, 1990; Stabili *et al.*, 1996), in mucus from the sea hare *Aplysia kurodai* (Yamazaki *et al.*, 1990) and from the hemichordate *Saccoglossus ruber* (Millar & Ratcliffe, 1987), in whole body homogenates of the hemichordate *S. ruber* (Millar & Ratcliffe, 1987), in haemocytes from *Eledone cirrhosa* (Chapter 6) and in haemocyte lysate supernatants from various crustaceans (Chisholm & Smith, 1992,1995; Findlay & Smith, 1995). Natural and induced antibacterial components have been demonstrated in invertebrates, with most studies concentrating on insects where several bactericidal factors, e.g. lysozyme and antibactericidal proteins such as cecropins and attacins, have been isolated and identified (Okada & Natori, 1983; Millar & Ratcliffe, 1994; Morishima *et al.*, 1995). Induced antibacterial activity can also depend on the bacterium used, as in the spiny lobsters *Panulirus interruptus* (Evans *et al.*, 1969) and *P. argus* (Evans *et al.*, 1968) where the induced bactericidal effect was due to the use of a Gram negative bacterium normally associated with the lobsters.

There have been some cephalopod studies on haemolymph agglutination and bactericidal activity. Bayne (1973) suggested that no bactericidal factors existed in the

haemolymph from *Octopus dofleini*. However, Russo and Tringall (1983) using the octopod *O. vulgaris* demonstrated that haemagglutinating and antibacterial activity were present in the haemolymph. The antibacterial activity was effective against the bacteria *Serratia marcescens* and *Salmonella typhimurium* as detected by a decrease in the percentage viable bacteria in counts after incubation in haemolymph. Fisher and DiNuzzo (1991) showed that bacterial agglutinins were also present in the haemolymphs of 3 other cephalopods; the octopus, *Octopus maya*, the cuttlefish, *Sepia officinalis*, and the squid *Sepioteuthis lessoniana*.

Previous work with the octopod *E. cirrhosa* has demonstrated that the haemocytes can migrate towards (Chapter 5) and affect the growth of bacteria (Chapter 6). Further the phagocytic properties of haemocytes are increased by pre-opsonization of particles with haemolymph (Chapter 4) and in some cases these haemocytes will only ingest foreign objects after pre-opsonization (Stuart, 1968). The haemolymph of *E. cirrhosa* also exhibits lysozyme activity which is unaffected by bacterial challenge (Chapter 8). The aim of this paper is to broadly investigate whether the cell-free haemolymph from *E. cirrhosa* has any effect on dead or live bacteria at various temperatures.

Materials and Methods

Animals

The Lesser Octopus *Eledone cirrhosa* (Lam.) were brought into the aquarium at the University of Wales at Bangor from the surrounding area. Within 48 h of arrival the animals were weighed, marked with alcian blue, using a syringe, (Chapter 3) and sexed. The marked animals were assigned to particular tanks and allowed to recover for 24 h. The animals were kept at 10-12°C and food in the form of *Carcinus maenas* was always available.

Haemolymph Collection

The animals were weighed, anaesthetised and bled from the branchial blood vessel (Chapter 2) and the volume of blood obtained was recorded. The blood was then centrifuged at 800g for 5 min at 4°C and the cell-free haemolymph removed. For the agglutination assay, haemolymph from different individuals was frozen separately at -20°C. The haemolymph for the bacteriostatic activity assay was pooled from a number of individuals and frozen at -20°C.

Bacteria

Vibrio anguillarum, (MT275), *V. parahaemolyticus*, (MT295), and *Aeromonas salmonicida*, (MT004) were all obtained from the Scottish Office Agricultural Environment and Fisheries Department, Torry, Aberdeen. *V. anguillarum* and *V. parahaemolyticus* were grown on 3% tryptic soya broth (Gibco) (TSB) with 2%

NaCl, whereas *A. salmonicida* was grown in TSB only. Quantitative bacterial counting in the assays was performed in triplicate using Norris Powell Diluent, (NPD) (0.5% formalin, sodium dodecyl sulphate, adjusted to pH 7.3 with Na_2HPO_4), HCl and a haemocytometer. For the agglutination assays, the 3 bacteria were formalised after counting.

Haemolymph Bacteriostatic Assay

The 3 live bacterial species were washed, centrifuged and resuspended (x2) at 1×10^9 bacteria/ml in TSB or TSB + NaCl as appropriate. Fifty µl of each bacterium were serially diluted in 50µl of the respective TSB growth medium in a 96 well flat bottom microtitre plate (Dynatech). Fifty µl of TSB were also added to the control wells and 100µl of TSB were added in triplicate to each plate to act as a blank. Fifty, 25 or 5µl of octopus haemolymph were added to the experimental wells in triplicate. Where 25 and 5µl of haemolymph were used a further 25 or 45µl respectively of TSB were added. The final haemolymph concentrations was therefore 5, 25 or 50% respectively. Haemolymph was found to interfere with the optical density values obtained and so triplicate haemolymph blanks at the same concentrations as the experimental wells were incorporated on each plate. The readings obtained from the haemolymph blank wells were subtracted from the experimental wells prior to analysis.

After the addition of haemolymph and TSB the 96 well plates were shaken and then incubated at 5, 10, 15 or 20°C for 4 h, shaking hourly, before the addition of 10µl of 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, (MTT) (Sigma)

(5mg/ml in distilled water) to each well to quantify the surviving bacteria. The plates were then shaken and after a further 15 min incubation in the dark, endpoints were read at 600nm on a multiscan spectrophotometer.

Agglutination Assay

A bacterial count was made of the formalised bacteria. The bacteria were then centrifuged at 13000g for 10 min and resuspended (x2) in phosphate buffered saline pH7.0 (PBS, Gibco, without Ca^{2+} and Mg^{2+}) at 8 x 10^8 cells/ml. Serial dilutions of 100µl of haemolymph from each of 10 individuals was performed, in triplicate, in 100µl of PBS using round bottom 96 well microtitre plates (Dynatech). One hundred µl of each of the bacterial suspension were then added to each well. The assays were incubated at 5, 10, 15 or 20°C for 16 h before being read. In each case the $\log_2$ titre at the last dilution able to give visible agglutination was recorded.

Statistical Analysis

For the agglutination results the triplicate $\log_2$ titres for each individual were averaged and then means and standard errors were taken of the ten individuals for each bacterium at each temperature.

For the haemolymph bacteriostatic results the blank value was taken away from the controls (no haemolymph added) and the haemolymph blank was taken away from the experimental wells, for each plate. For each temperature and each bacterial species the means for the triplicated values obtained for 0 (control), 5, 25 and 50µl haemolymph

additions were calculated. One way analysis of variance (ANOVA) was performed to determine whether, the bacteria used, the bacterial concentration, the amount of haemolymph added and the incubation temperature affected the experimental results. Student t-tests were then performed to determine statistical differences between the amount of haemolymph added, the temperature and the bacterium used at each concentration.

Results

Cell-free haemolymph from *Eledone cirrhosa* can agglutinate and inhibit the growth of the bacteria *Vibrio anguillarum*, *V. parahaemolyticus* and *Aeromonas salmonicida*. Analysis of variance (ANOVA) for these results demonstrated that various factors significantly ($P<0.0001$) influenced the action of haemolymph on the bacteria. Significant factors were; the bacterial species used, the bacterial concentration present, the amount of haemolymph added to the bacteria and the incubation temperature used for the assay.

Increasing the incubation temperature over the range 5 - 20°C caused an increase ($P<0.05$) in the number of *V. anguillarum* (figs. 7.1a-d) present after 4 h, except at the highest initial bacterial concentrations, as indicated by the control samples at the 4 temperatures. Generally a haemolymph concentration of 50% caused a significant reduction ($P<0.05$) in the number of surviving bacteria, compared to the control bacterial samples, after 4 h at the 4 temperatures. At 10°C (fig. 7.1b) 25% haemolymph caused significant ($P<0.05$) bacterial inhibition at bacterial concentrations above 6.25×10^7. After 4 h at 15°C (fig. 7.1c) and 20°C (fig. 7.1d) the growth of bacteria was reduced ($P<0.05$) in the presence of 25% haemolymph at bacterial concentrations between 1.56×10^7 and 5×10^8/ml.

The effect of different concentrations of *E. cirrhosa* haemolymph on *V. parahaemolyticus* cultures at 4 temperatures is shown in figures 7.2a - 7.2d. As demonstrated by the control values bacterial growth increased ($P<0.05$) as the incubation temperature increased. At 5°C (fig. 7.2a) and 10°C (fig. 7.2b) the number

Figure 7. 1.a. Amounts of haemolymph (0, 5, 25 and 50μl) added to the assay with live *Vibrio anguillarum* at 5°C. Each point represents the mean of triplicated incubations using pooled haemolymph. The error bars are standard errors.

Figure 7. 1.b. Amounts of haemolymph (0, 5, 25 and 50μl) added to the assay with live *V. anguillarum* at 10°C. Each point represents the mean of triplicated incubations using pooled haemolymph. The error bars are standard errors.

Figure 7.1.a.

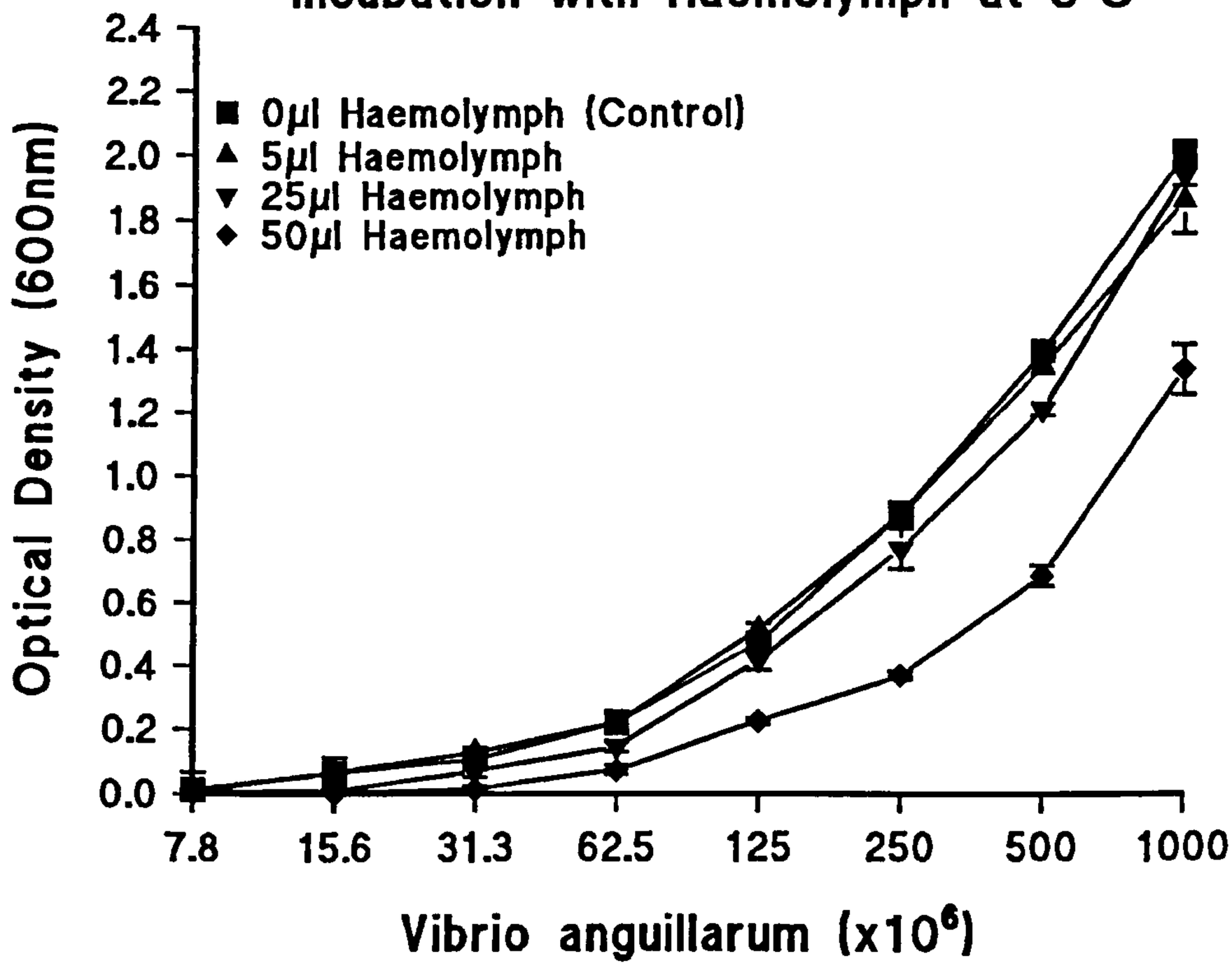


Figure 7.1.b.

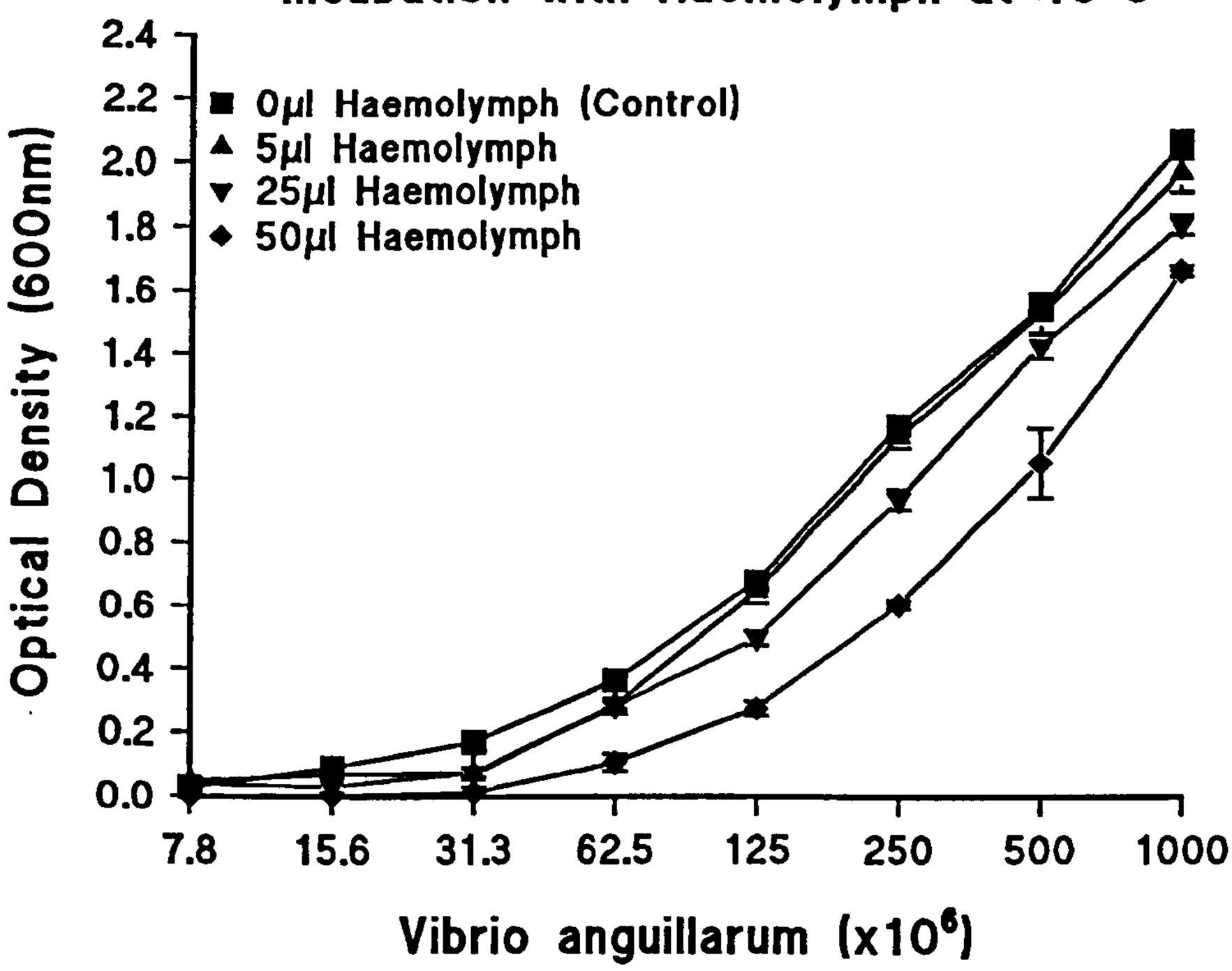


Figure 7. 1.c. Amounts of haemolymph (0, 5, 25 and 50μl) added to the assay with live *V. anguillarum* at 15°C. Each point represents the mean of triplicated incubations using pooled haemolymph. The error bars are standard errors.

Figure 7. 1.d. Amounts of haemolymph (0, 5, 25 and 50μl) added to the assay with live *V. anguillarum* at 20°C. Each point represents the mean of triplicated incubations using pooled haemolymph. The error bars are standard errors.

Figure 7.1.c.

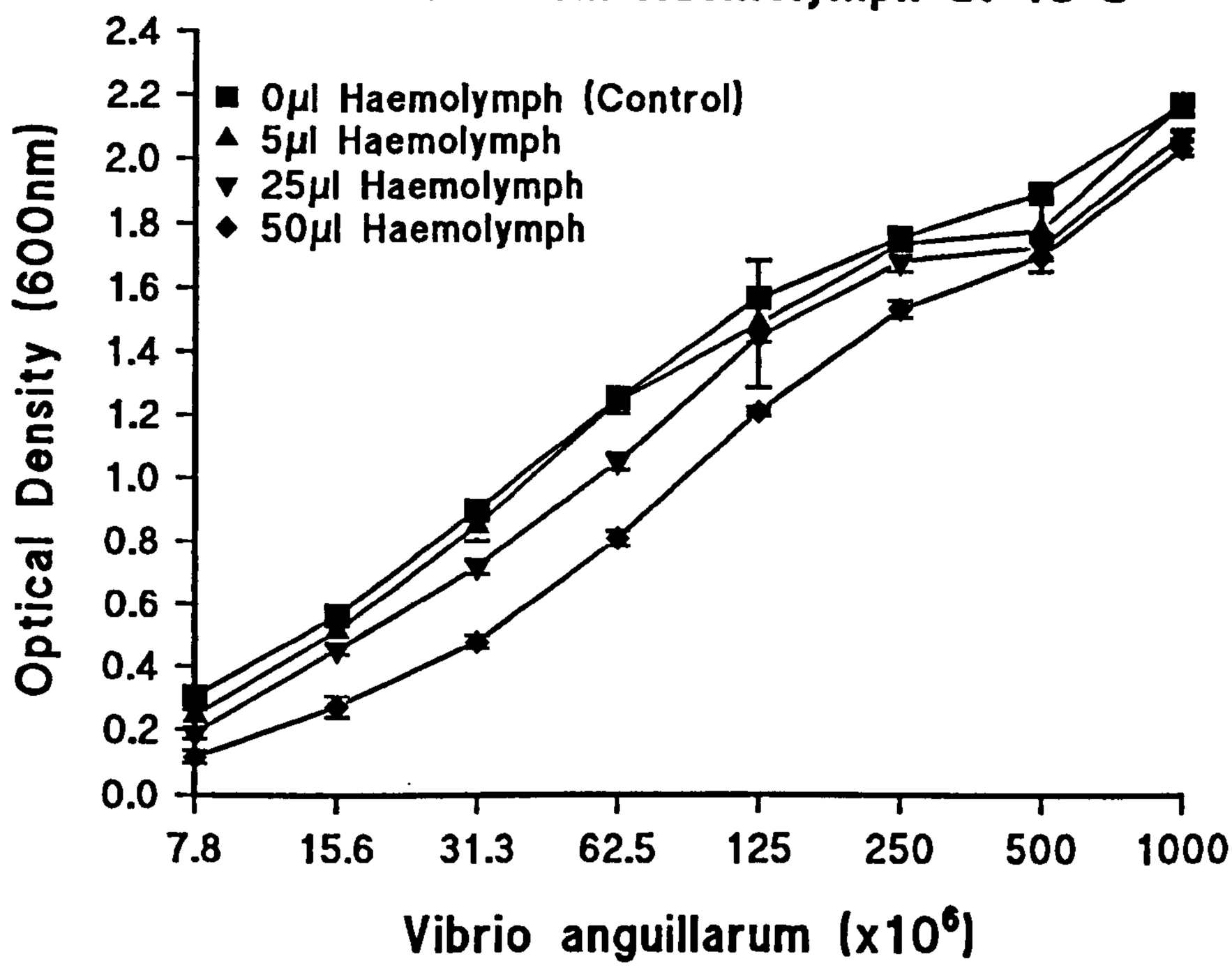


Figure 7.1.d.

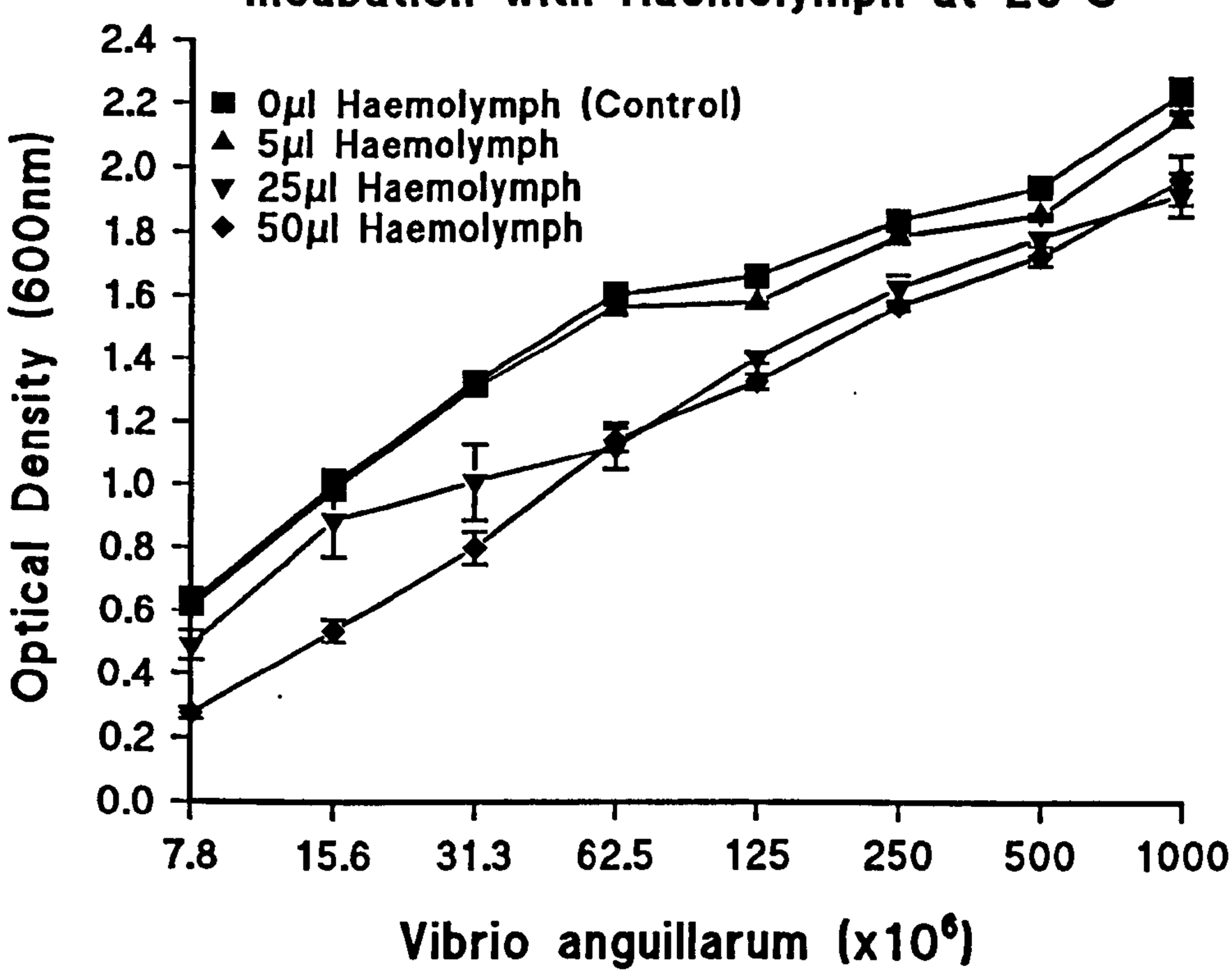


Figure 7. 2.a. Amounts of haemolymph (0, 5, 25 and 50μl) added to the assay with live *Vibrio parahaemolyticus* at 5°C. Each point represents the mean of triplicated incubations using pooled haemolymph. The error bars are standard errors.

Figure 7. 2.b. Amounts of haemolymph (0, 5, 25 and 50μl) added to the assay with live *V. parahaemolyticus* at 10°C. Each point represents the mean of triplicated incubations using pooled haemolymph. The error bars are standard errors.

Figure 7.2.a.

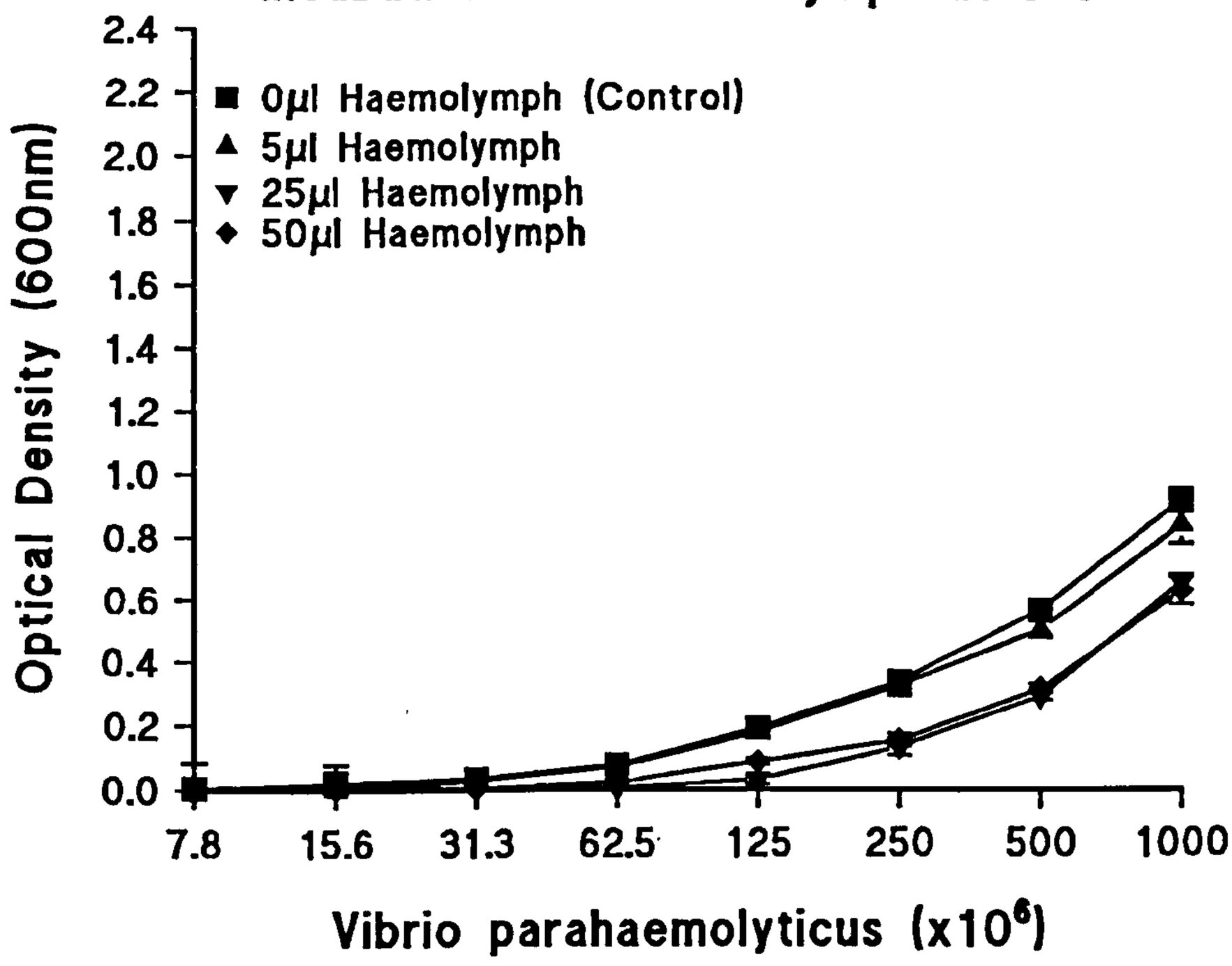
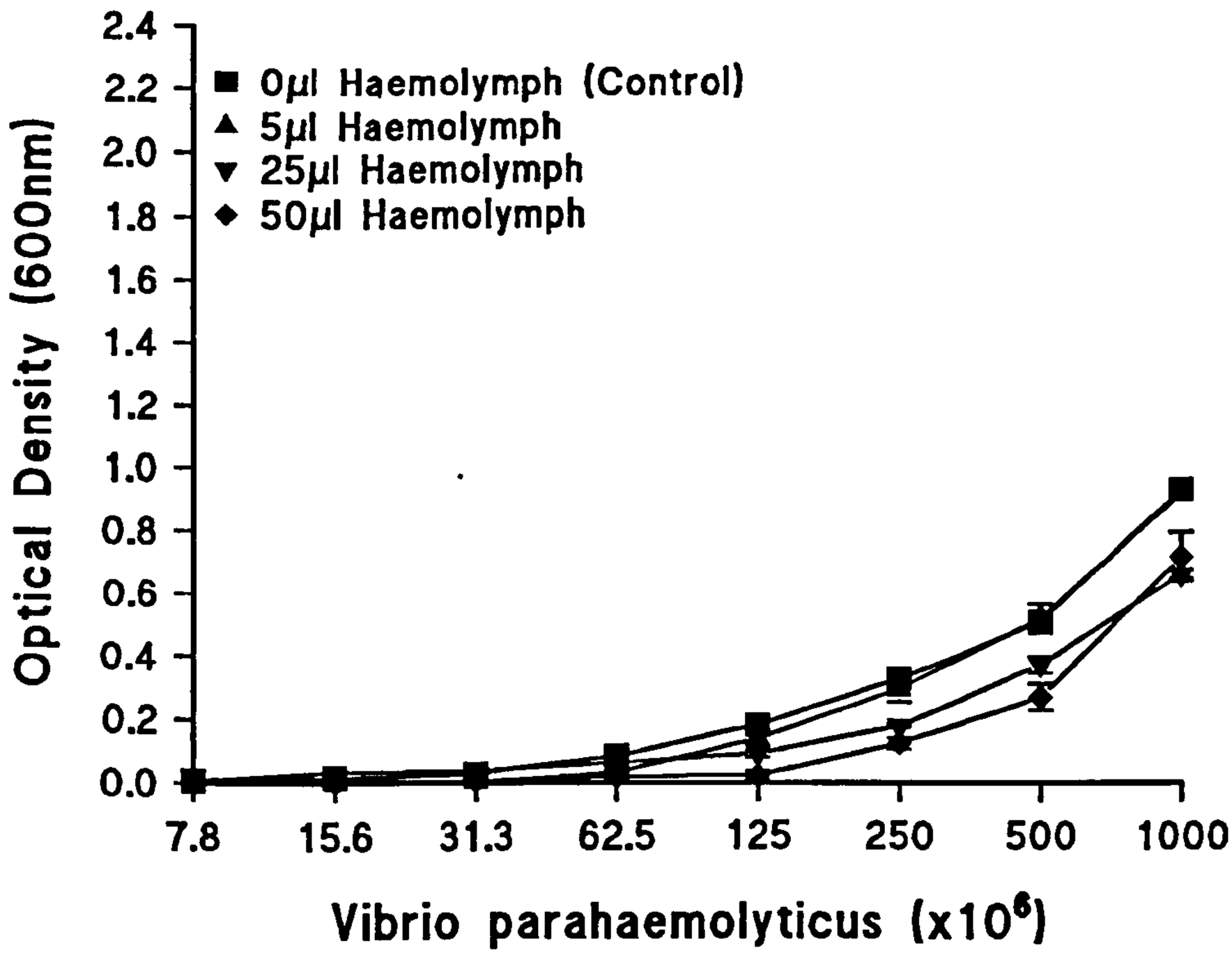


Figure 7.2.b.



of live bacteria detected was significantly ($P<0.05$) reduced compared to the control by the addition of either 50 or 25µl haemolymph. At 15°C (fig. 7.2c), however, a haemolymph concentration of 50% caused a greater reduction ($P<0.05$) in bacterial growth than a haemolymph concentration of 25% at bacterial concentrations above 6.25×10^7. Incubation of 25 and 50µl of haemolymph with *V. parahaemolyticus* cultures at 20°C (fig. 7.2d) also caused reduced ($P<0.05$) bacterial growth at initial bacterial concentrations below 2.5×10^8 and 5×10^8 bacteria/ml respectively. A small but significant ($P<0.05$) reduction in the bacterial concentration present was also detected when 5µl of haemolymph was added to 1×10^9 bacteria/ml at 15°C and between 6.25 and 12.5×10^7 bacteria/ml at 20°C.

A. salmonicida grows more slowly than either *V. anguillarum* or *V. parahaemolyticus* between 5 and 20°C (figs. 7.3a-d) but *E. cirrhosa* haemolymph again demonstrated a moderating effect on the growth of the bacteria. High initial bacterial concentrations were significantly ($P<0.05$) affected by both 25 and 50% haemolymph concentrations at 5 (fig. 7.3a) and 10°C (fig. 7.3b). Lower bacterial concentrations at 15°C (fig.7.3c) and 20°C (fig. 7.3d) also showed decreased ($P<0.05$) growth after the addition of 25 or 50µl of haemolymph. Fifty µl of haemolymph significantly ($P<0.05$) reduces bacterial growth compared to the control and compared to 25µl haemolymph at higher bacterial concentrations of 1×10^9 at 15°C and above 1.25×10^8 at 20°C.

Figure 7. 2.c. Amounts of haemolymph (0, 5, 25 and 50μl) added to the assay with live *V. parahaemolyticus* at 15°C. Each point represents the mean of triplicated incubations using pooled haemolymph. The error bars are standard errors.

Figure 7. 2.d. Amounts of haemolymph (0, 5, 25 and 50μl) added to the assay with live *V. parahaemolyticus* at 20°C. Each point represents the mean of triplicated incubations using pooled haemolymph. The error bars are standard errors.

Figure 7.2.c.

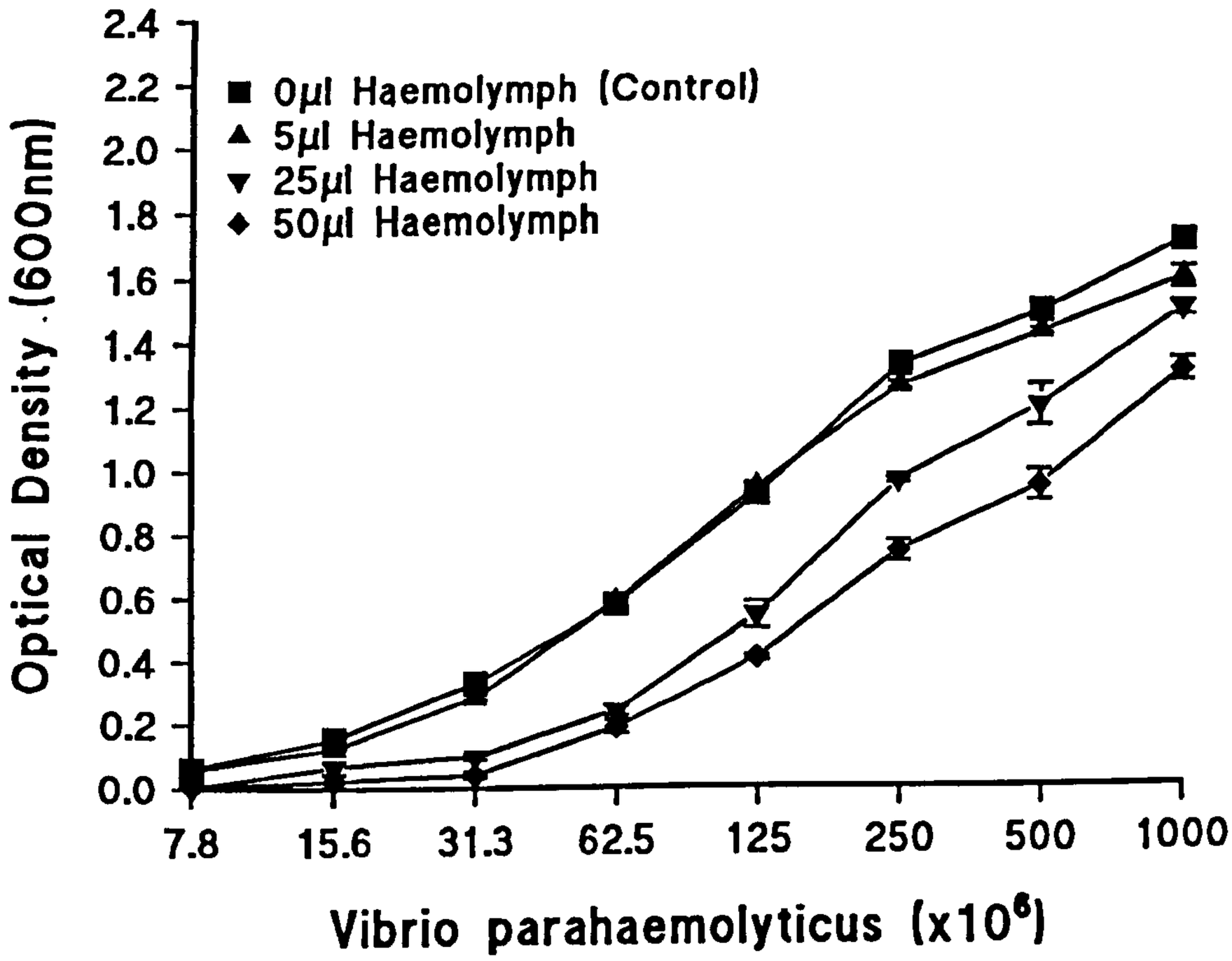


Figure 7.2.d.

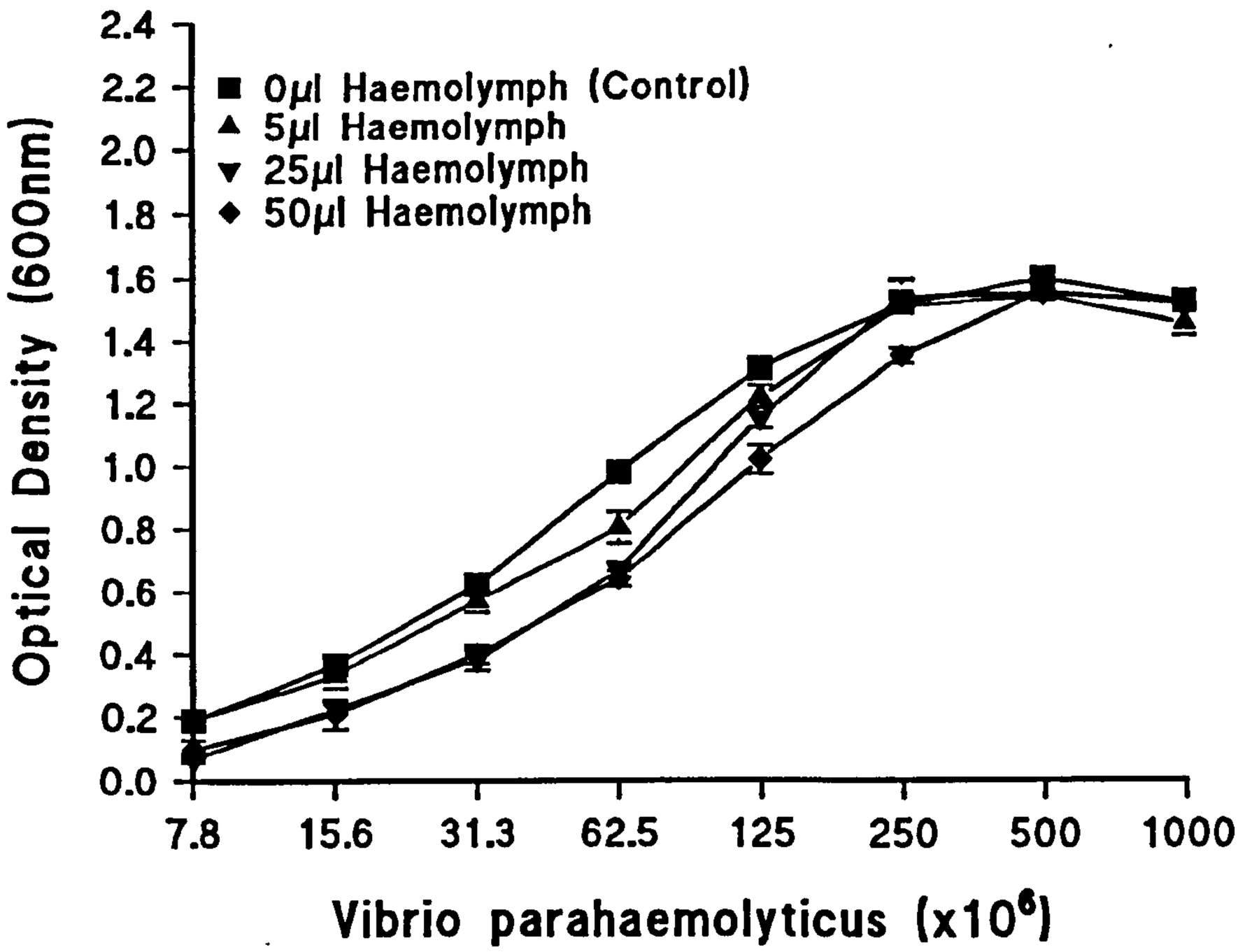


Figure 7. 3.a. Amounts of haemolymph (0, 5, 25 and 50μl) added to the assay with live *Aeromonas salmonicida* at 5°C. Each point represents the mean of triplicated incubations using pooled haemolymph. The error bars are standard errors.

Figure 7. 3.b. Amounts of haemolymph (0, 5, 25 and 50μl) added to the assay with live *A. salmonicida* at 10°C. Each point represents the mean of triplicated incubations using pooled haemolymph. The error bars are standard errors.

Figure 7.3.a.

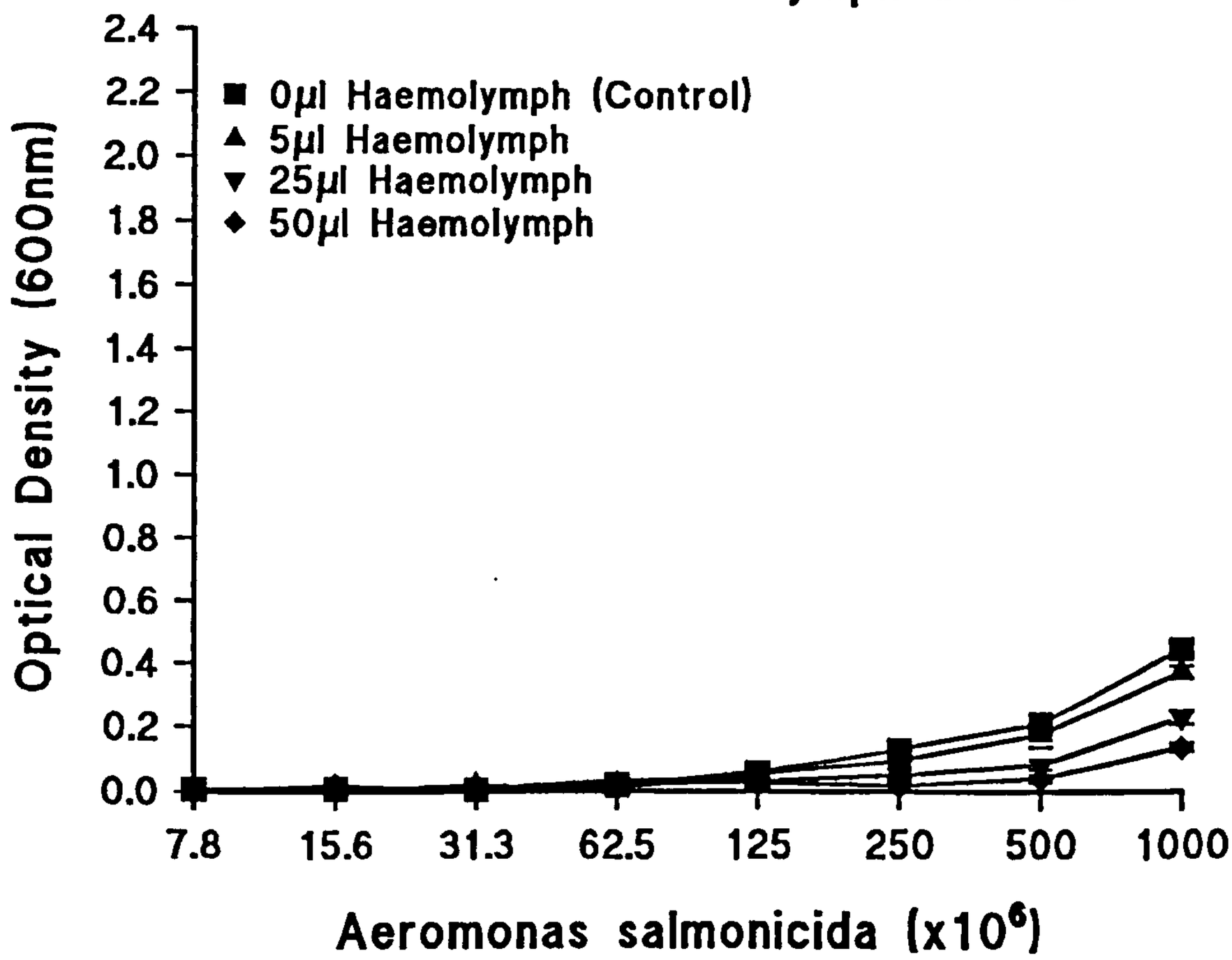


Figure 7.3.b.

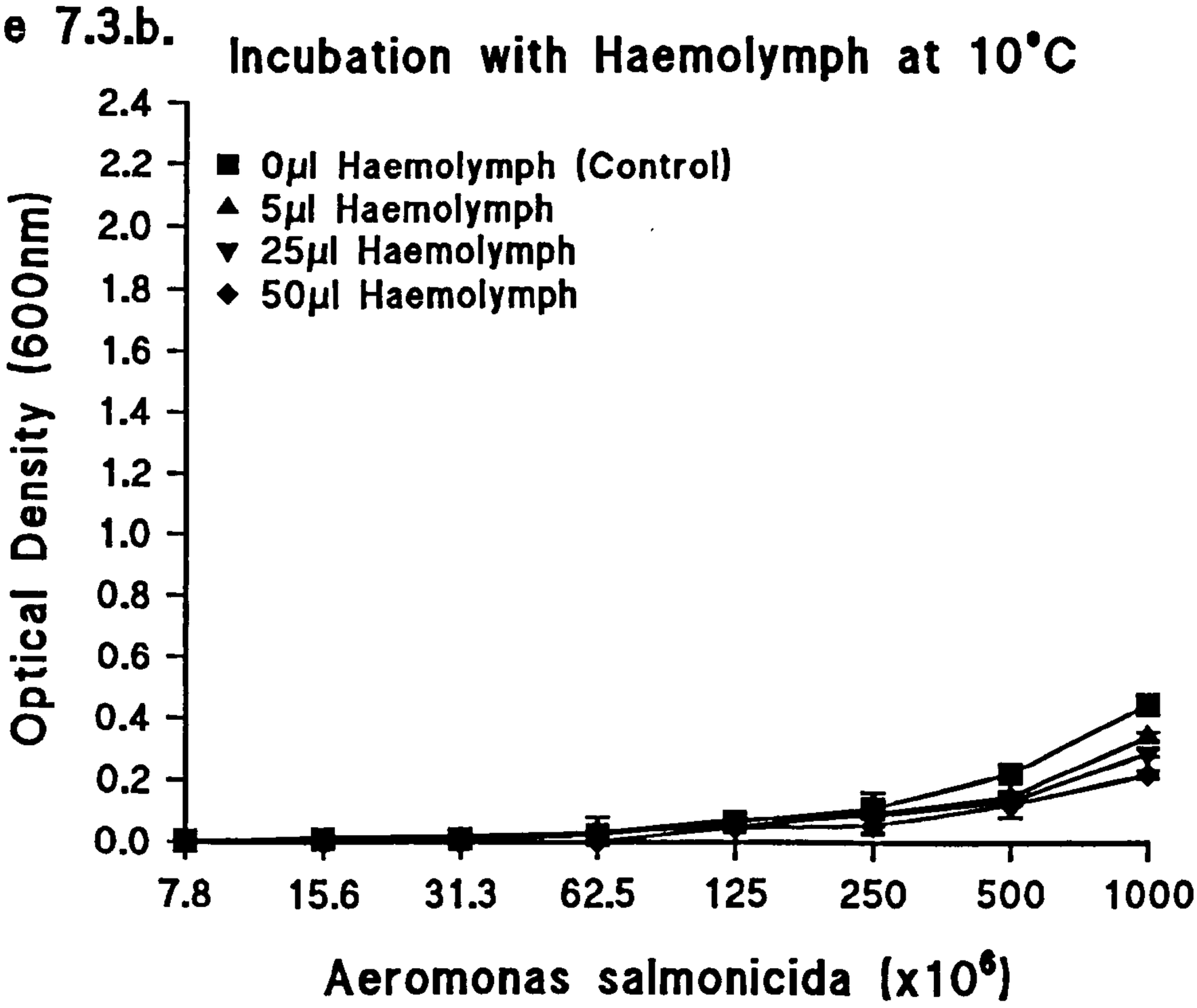


Figure 7. 3.c. Amounts of haemolymph (0, 5, 25 and 50μl) added to the assay with live *A. salmonicida* at 15°C. Each point represents the mean of triplicated incubations using pooled haemolymph. The error bars are standard errors.

Figure 7. 3.d. Amounts of haemolymph (0, 5, 25 and 50μl) added to the assay with live *A. salmonicida* at 20°C. Each point represents the mean of triplicated incubations using pooled haemolymph. The error bars are standard errors.

Figure 7.3.c.

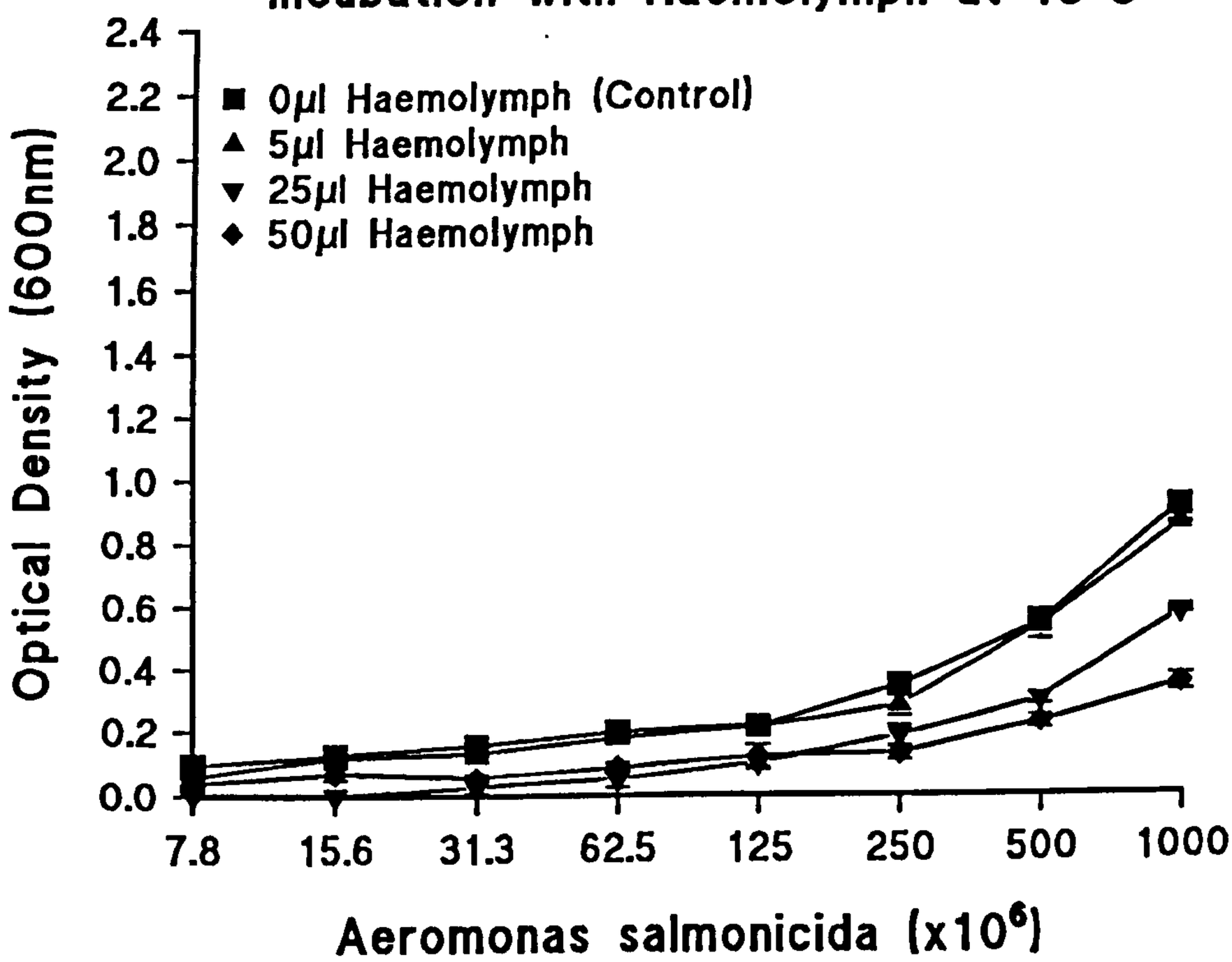
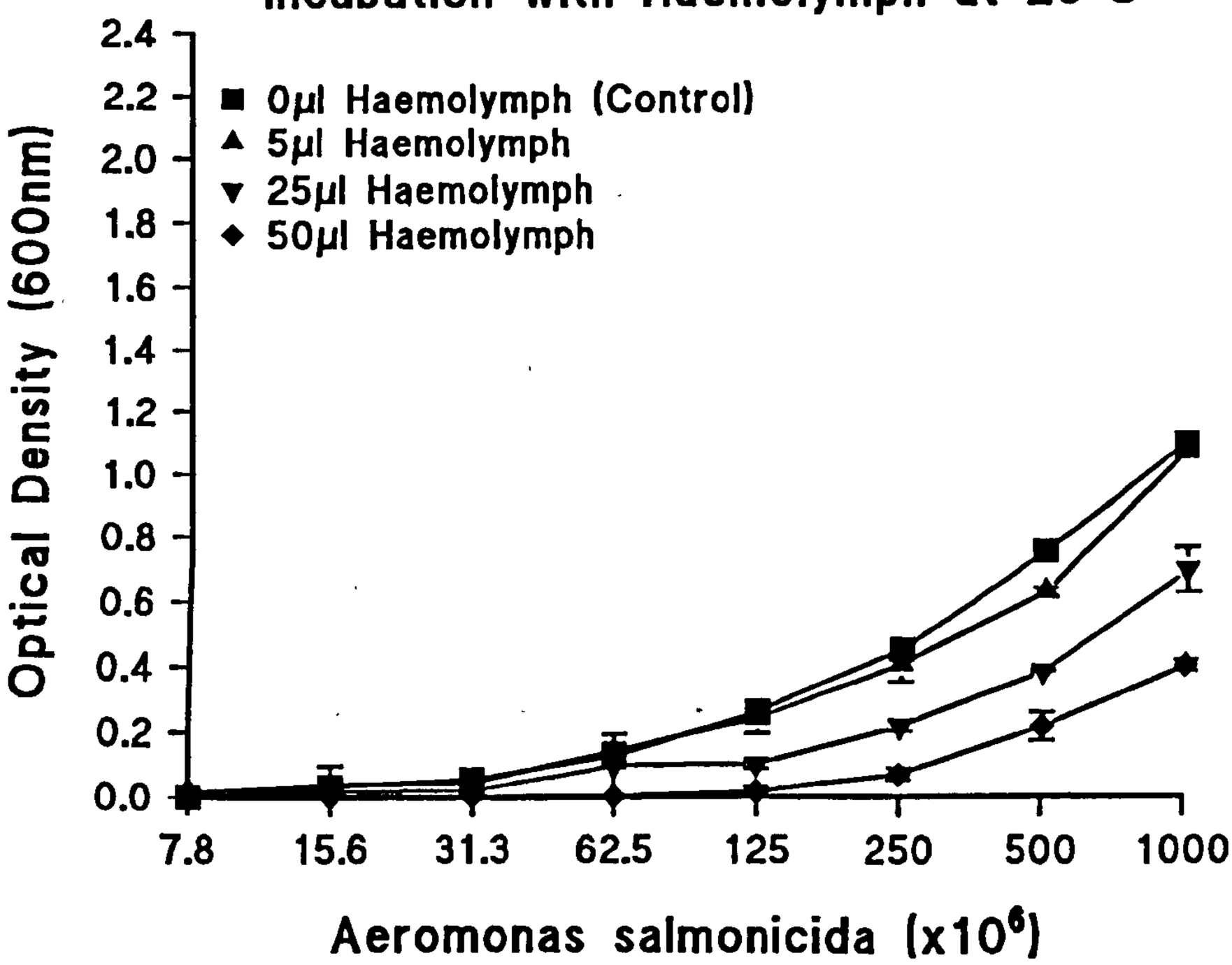


Figure 7.3.d.



The agglutination results for *E. cirrhosa* haemolymph were affected by the bacterial species used and slightly by the incubation temperature (table 1). Significantly ($P<0.05$) higher agglutination titres ($P<0.05$) were obtained for both *V. angullairum* and *V. parahaemolyticus* as compared to *A. salmonicida*.

Table 1. Log_2 titres of the Agglutination experiments using *Vibrio anguillarum*, *Vibrio parahaemolyticus* and *Aeromonas salmonicida* at 5, 10, 15 and 20°C. Each mean log_2 titre represents the mean of 10 individuals.

1.a. *Vibrio anguillarum*

Incubation Temperature	Mean Log_2 Titre	Standard Error
5°C	7.0	0.00
10°C	5.8	0.20
15°C	6.4	0.25
20°C	6.8	0.20

1.b. *Vibrio parahaemolyticus*

Incubation Temperature	Mean Log_2 Titre	Standard Error
5°C	6.4	0.25
10°C	6.2	0.37
15°C	5.6	0.25
20°C	6.8	0.27

1.c. *Aeromonas salmonicida*

Incubation Temperature	Mean Log_2 Titre	Standard Error
5°C	4.2	0.20
10°C	4.8	0.20
15°C	4.8	0.20
20°C	3.2	0.20

Discussion

Cell-free haemolymph from *Eledone cirrhosa* possesses antibacterial factors which inhibit the growth of the bacteria *Vibrio anguillarum*, *V. parahaemolyticus* and *Aeromonas salmonicida*. Agglutinating properties are also present as demonstrated by the aggregating ability of *E. cirrhosa* haemolymph for dead *V. anguillarum*, *V. parahaemolyticus* and *A. salmonicida*.

V. anguillarum, *V. parahaemolyticus* and various *Aeromonas* (sp) are opportunistic marine bacteria and have been implicated in causing skin ulcers in various laboratory maintained cephalopods. These Gram negative bacteria are a normal constituent of the skin flora and secondarily invade wound sites when they can cause death of the infected animal, particularly at high temperatures (Hanlon & Forsythe, 1990; Ford, 1992). The haemolymph from *E. cirrhosa* is able to inhibit the growth of high concentrations of *V. anguillarum* and *V. parahaemolyticus* at temperatures below 15°C. However, at 20°C *V. parahaemolyticus* is not inhibited at high bacterial concentrations and the number of viable *V. anguillarum* detected at high concentrations at 15°C is only slightly below the control values. Nevertheless, the bacterium *A. salmonicida*, a freshwater bacterial pathogen of fish which also grows in seawater but has not been identified as pathogenic to *E. cirrhosa*, is affected by 25 and 50% haemolymph concentrations at 20°C.

Bayne (1973) using the bacterium *Serratia marcescens*, suggested that haemolymph from the octopus *Octopus dofleini* contained no bactericidal capacity. However Russo & Tringall (1982) indicated that bactericidal activity against the bacteria *S. marcescens*

and *Salmonella typhimurium* was present in the haemolymph of *Octopus vulgaris*. It is possible that the two octopods demonstrate differences in their ability to kill these bacteria and it would be interesting to determine whether *O. dofleini* haemolymph demonstrates any bactericidal activity towards marine Gram negative bacteria.

At present it is unknown whether haemolymph from *E. cirrhosa* produces a bactericidal (killing) or a bacteriostatic (growth inhibiting) effect on live bacteria. It is known that lysozyme is present in unchallenged haemolymph from *E. cirrhosa* (Chapter 8), and heating of the haemolymph to 80°C for 30min did not conclusively remove the bacteriostatic capacity of the plasma (unpub. data). It is possible that some of the reduction of growth is due to lysozyme, isolated and characterised in a number of invertebrates (Jollés & Jollés, 1975; Jollés & Jollés, 1984; Lassalle *et al.*, 1988; Fenouil & Roch, 1991; Sun *et al.*, 1991), but further work would be needed to determine this.

Humoral antibacterial activity has been detected in a variety of marine invertebrates. Anderson & Chain (1982) demonstrated the presence of a naturally occurring antibacterial factor in the coelomic fluid of the marine annelid *Glycera dibranchiata*. The study showed that it was probably due to a protein which did not display lytic properties against the bacteria, and also that lysozyme was not present in the coelomic fluid. Further study of *G. dibranchiata* antibacterial activity demonstrated a 2 stage killing process involving binding of the bactericidal factor (a glycoprotein) (Chain & Anderson, 1983a) to the bacterium and then killing of the bacterium (Chain & Anderson, 1983b,c). Antibacterial activity against, in particular, Gram negative marine

bacteria, was also detected in mucus and whole body homogenate from the hemichordate *Saccoglossus ruber* (Millar & Ratcliffe, 1987). However, though the nature of the bactericidal activity in *S. ruber* was not elucidated, the preliminary data suggested that the active factor was not proteinaceous. Stabili *et al.* (1996) has also demonstrated the presence of antibacterial activity in naive haemolymph from the sea urchin *Paracentrotus lividus*. In crustaceans, Noga *et al.* (1994) showed that the antibacterial activity detected in the haemolymph of the blue crab *Callinectes sapidus* was active against over 50% of the bacteria isolated from the shell of the crab. A true bactericidal effect has also been detected in the haemolymph of the bivalve, *Mytilus edulis* against the Gram negative bacterium *Vibrio alginolyticus* (Nottage & Birkbeck, 1990).

Bacteriostatic or growth inhibiting activity has mainly been demonstrated using the earthworm *Eisenia fetida andrei*. The antibacterial activity, shown to be directed only against highly pathogenic soil bacteria from the worm's environment, consisted of two lipoproteic molecules which were also demonstrated to be involved in haemolytic activities (Valembois *et al.*, 1982). The antibacterial activity was also shown to be active against sensitive species of both Gram positive and Gram negative bacteria and the authors suggested that lysozyme (which acts primarily against Gram positive (Salton, 1957) but also against Gram negative bacteria (Miller, 1969)) was not involved. Further work demonstrated that the bacteriostatic activity found in the coelomic fluid of the earthworm was mediated by three different proteins (Vaillier *et al.*, 1985) and the activity could be induced by injection with pathogenic bacteria

(Lassègues *et al.*, 1989). A bacteriostatic factor 'aplysianin P' has also been isolated from the purple fluid of the sea hare *Aplysia kurodai* and is capable of inhibiting nucleic acid synthesis (Yamazaki *et al.*, 1990).

As found with other cephalopods, the cell free haemolymph from *Eledone cirrhosa* displays both bacterial and erythrocyte agglutination properties (Russo & Tringall, 1983; Marthy, 1974; Stuart, 1968). All three of the bacteria used in this study were agglutinated with the highest activities against *V. anguillarum* and *V. parahaemolyticus.* Stuart, (1968) indicated that no agglutination of unsensitised particles (erythrocytes or bacteria) occurred with *E. cirrhosa* serum, the bacteria used by Stuart (1968) were *Escherichia coli, Salmonella typhi* and *Staphylococcus aureus.* Preliminary experiments for this paper demonstrated that reliable agglutination results for unsensitised bacteria were produced only when PBS was used as the diluent buffer for the haemolymph. Other buffers studied were a marine anticoagulant and an octopus Ringer, which both contained ethylene glycol-bis(β-aminoethylether) N, N, N', N', - tetraacetic acid (EGTA), an octopus Ringer with added calcium and magnesium and sterile octopus saline which contains high quantities of calcium and magnesium (unpub. data). Haemagglutinating activity as demonstrated by Russo & Tringall (1983) for *O. vulgaris* was found to be partially dependent on the divalent cation content of the haemolymph. Removal of calcium from the haemolymph of *O. vulgaris* inhibited the haemagglutination activity.

A lectin has been isolated from the haemolymph of *O. vulgaris* (Rögener *et al.*, 1985) and its composition is similar to 'a subunit' of the haemolymph respiratory pigment,

haemocyanin (Rogener *et al.*, 1986). Investigations into haemolymph from *Sepia officinalis* and *Loligo vulgaris* revealed the presence of glycoproteins with blood group A like specificity (Renwrantz & Uhlenbruck, 1974). Rögener *et al.* (1987), using haemolymph from *O. vulgaris,* demonstrated the presence of a glycoprotein with blood group A-like properties and suggested it could be involved in neutralizing bacterial lectins.

In conclusion, *E. cirrhosa* haemocytes display both cellular bacteriostatic activity (Chapter 6) and phagocytosis (Chapter 4), which is enhanced by pre-opsonization of the bacteria with haemolymph, suggesting a combined humoral and cellular response to bacterial pathogens. Experiments are currently underway to determine the nature of the growth inhibition effect on live bacteria and to elucidate the haemolymph components involved.

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Chapter 8

Lysozyme and Antiprotease Activity in the Lesser Octopus *Eledone cirrhosa* (Lam.) (Cephalopoda).

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Abstract

Antiprotease and lysozyme activities were detected in various tissue samples and in the haemocytes and haemolymph of *Eledone cirrhosa*. Injection of live *Vibrio anguillarum* caused an increase in lysozyme activity in the branchial heart over 48 h and a decrease in the lysozyme activity of haemocytes over 24 h. The haemocytes from control PBS injected animals demonstrated increased lysozyme levels within 4 h after injection whereas it decreased after the injection of live bacteria in PBS. Both PBS alone and PBS with bacteria did not affect the lysozyme activity of the haemolymph. Bacterial injections had no effect on the antiprotease activity of the tissue samples but increased the antiprotease activity of the haemocytes compared to the control haemocytes in the 4 h sample. Haemolymph antiprotease activity decreased at a greater rate following bacterial injection than in control PBS injected animals.

Haemocyte numbers/ml increased for both the control and bacterial injected animals with a greater increase demonstrated for the bacterial injected animals in the 4 h sample. Concomittant with the increase in the numbers of circulating haemocytes live *V. anguillarum* were cleared from the circulation of *E. cirrhosa* in about 4 h.

Key Words: *Eledone cirrhosa*; Haemocytes; Haemolymph; Tissues; Lysozyme; Antiprotease.

Introduction

Antimicrobial defence mechanisms against various potentially pathogenic organisms have been demonstrated in a variety of invertebrates such as insects, crustaceans and annelids (Ratcliffe *et al.*, 1985; Millar & Ratcliffe, 1994). Lysosomal enzymes which form part of the cellular and humoral antimicrobial defence of invertebrates, appear to act non-specifically against a wide range of foreign invaders. Lysozyme, one of the most common of these enzymes, is bacteriolytic and destroys certain pathogenic agents by breaking the 1-4 bonds between N-acetylglucosamine and N-acetylmuramic acid present in the cell walls of certain bacteria and fungi (Millar & Ratcliffe, 1994; Ratcliffe *et al.*, 1985). Lysozyme has been identified and characterized in a variety of invertebrates for example, annelids (Lassalle *et al.*, 1988; Çotuk & Dales, 1984), echinoderms (Jollés & Jollés, 1975; Jollés & Jollés, 1984) and insects where the lysozyme gene has been isolated and characterized for the giant silk moth *Hyalophora cecropia* (Sun *et al.*, 1991). Infection or injury is known to induce lysozyme synthesis, apparently in the haemocytes of crustaceans (Fenouil & Roch, 1991), annelids (Lassalle *et al.*, 1988; Çotuk & Dales, 1984) and in insects (Zachary & Hoffmann, 1984) where synthesis also occurs in the fat body (Morishima *et al.*, 1995). Studies on molluscs such as gastropods (Bayne, 1983; Cheng, 1983) and bivalves (Cheng, 1983; Takahashi *et al.*, 1986) have demonstrated that various lysosomal enzymes, e.g. lysozyme, aminopeptidase and lipase, are present in both the blood cells and haemolymph of these molluscs and contribute to their antimicrobial system. Cheng *et al.* (1975) working on *Mercenaria mercenaria* demonstrated that lysozyme was

present in certain haemocytes (granulocytes) and was released into the haemolymph during phagocytosis by, or degranulation of, the granulocytes. Lysozyme activity has also been detected in the digestive diverticula, gill, mantle and crystalline style of the bivalves *Crassostrea virginica* and *Mytilus edulis* (Takahashi *et al.*, 1986).

Additionally, both gastropods (Cheng *et al.*, 1977) and bivalves (Cheng *et al.*, 1975) have elevated levels of lysozyme activity after *in vivo* exposure to various agents. Antiproteases or proteinase inhibitors aid in the defence of various organisms by regulating and inhibiting the activities of potentially destructive proteases. There are several classes of protease inhibitors which only inhibit specific proteases. However, there is one family of inhibitors, the α-macroglobulins, and in particular α_2-Macroglobulin (α_2M), which will inhibit proteases of various classes. In general antiproteases inhibit the action of proteases either by binding to the active site of the proteinase or for α_2M, by "trapping" the proteinase to prevent protein hydrolysis although the active site is still accessible by low molecular weight substances (Laskowski & Kato, 1980; Travis & Salvesen, 1983). Interestingly, in mammals proteases bound to α_2M complexes are removed from the blood system by secondary lysosomes following internalisation by endocytosis (Van Leuven, 1984).

Antiproteases are present in invertebrates (Boigegrain *et al.*, 1994) and in particular α_2M activity has been demonstrated (Quigley &Armstrong, 1994). In gastropods α_2M occurs in the plasma of *Biomphalaria glabrata* (Bender *et al.*, 1992) and acts by inhibiting cysteine proteinase from *Schistosoma mansoni* (Fryer *et al.*, 1996). α_2Macroglobulin activity also occurs in the plasma of the gastropod *Busycon*

canaliculatum and the bivalve *Spisula solidissima* (Armstrong & Quigley, 1992). In cephalopods antiprotease activity was detected in the skin of *Loligo vulgaris* (Tschesche & Von Rücker, 1973) and in the plasma of *Loligo pealii* (Armstrong & Quigley, 1992).

Most of the other work concerning invertebrate antiproteases originates from arthropods. In the horseshoe crab *Limulus polyphemus*, α_2M activity was detected in the haemolymph (Quigley & Armstrong, 1983; Enghild *et al.*, 1990; Armstrong & Quigley, 1991) and in the haemocytes (Armstrong & Quigley, 1985) and appears to be involved in suppression of proteases released from aggregated blood cells at wound sites (Armstrong *et al.*, 1990). α_2-M has been isolated from the haemolymph of the American lobster *Homarus americanus* (Spycher *et al.*, 1987) and α_2-Macroglobulin-like activity has also been antigenically characterized in both the haemocytes and haemolymph of *Penaeus japonicus* (Bachère *et al.*, 1995). Additionally, haemocyte degranulation, or exocytosis, appears to be the source of the plasma antiprotease activity in the horseshoe crab *Limulus polyphemus* (Armstrong & Quigley, 1985; Armstrong *et al.*, 1990). Various antiproteases have also been identified in the crayfish *Pacifastacus leniusculus* i.e. α_2-M and another serine protease inhibitor are found in the haemocytes and haemolymph and these aid in the regulation of coagulation and in prophenoloxidase activation (Aspán *et al.*, 1990; Hergenhahn *et al.*, 1988; Hergenhahn & Söderhäll, 1985; Häll & Söderhäll, 1982, 1983). Protease inhibitors have also been found in mucus from the skin of *Branchiostoma lanceolatum* (Möck & Renwrantz, 1987).

Cephalopods have a closed circulatory system containing one type of blood cell (haemocyte) and plasma (haemolymph). Blood coagulation does not occur after wounding, instead blood flow eventually ceases by constriction of the muscles around the wound and the formation of a plug of haemocytes (Polglase *et al.*, 1983). It has also been demonstrated that lysosomal enzymes such as acid phosphatase occur in the branchial heart appendage of the cuttlefish *Sepia officinalis* (Schipp *et al.*, 1971). It is known from previous work with octopods that live bacteria injected into the octopod *Octopus dolfeini* are removed from the circulation in about 2 hours (Bayne, 1973), and work with *O. vulgaris* has demonstrated the presence of α_2M in the cell free haemolymph (Thøgersen *et al.*, 1992). Experiments with the octopod *Eledone cirrhosa* have demonstrated that the haemocytes will phagocytose (Chapter 4) and restrict the growth of live bacteria (Chapter 6) and that the haemolymph of *E. cirrhosa* will opsonize (Chapter 4), agglutinate and inhibit the growth of live bacteria (Chapter 7). This paper aims to determine whether lysozyme and antiprotease activity exist in various tissues and in the haemolymph of *E. cirrhosa*. Additionally the *in vivo* effect of live bacteria on these lysozyme and antiprotease activities will be determined.

Materials and Methods

Animals

Octopus (*Eledone cirrhosa* (Lam.)) were obtained from commercial crab pots situated around the coast of the Isle of Anglesey. The animals were brought into an aquarium at the University of Wales, Bangor, weighed and marked using a syringe containing 1% alcian blue (Chapter 3) in octopus Ringer (OR) ((NaCl, 2.433g/100ml; glucose, 1.4g/100ml; EGTA, 0.015g/100ml; KCl, 0.082/100ml; KH_2PO_4, 0.004g/100ml). The animals were maintained at 12°C for 48 h before experimentation.

Haemolymph and Haemocyte Collection

Animals were sampled from the branchial blood vessel as described in Chapter 2. One hundred µl of the blood were added to 900µl of marine anticoagulant (MA) (NaCl, 2.63g/100ml; glucose, 1.8g/100ml; tri-sodium citrate, 0.088g/100ml; citric acid, 0.055g/100ml) containing ethylene glycol-bis(β-aminoethylether) N, N, N', N', -tetraacetic acid (EGTA) (0.029g/100ml)and a blood count taken. A further 100µl of blood were added to 900µl of phosphate buffered saline (pH 7.0) (PBS) (Gibco) and blood sterility checked. The remaining blood was separated into 2 aliquots, centrifuged at 800g for 5 min at 4°C and the haemolymph removed and stored immediately at -70°C. The separated haemocytes were washed and centrifuged in OR (Chapter 2). The OR was removed and the haemocytes frozen at -70°C.

Bacteria

The Gram-negative bacterium *Vibrio anguillarum* (MT275) was obtained from the Scottish Office Agricultural Environment and Fisheries Department, Torry, Aberdeen. The bacterium was cultured in tryptic soya broth (Gibco) containing 2% sodium chloride for 48 h. Triplicate bacterial counts were taken and the bacterial concentration adjusted to 1 x 10^6 bacteria/ml. The bacteria were centrifuged at 13000g for 10 min and washed twice in sterile PBS before resuspension to 500µl in PBS.

Inoculation and Experimentation

Five control and 5 experimental animals were chosen at random. Both sets of animals were maintained separately under sterile conditions and waste seawater treated to kill any escaping bacteria. The control and experimental animals were sampled from the branchial blood vessel at 0 h and blood counts taken. Immediately following the 0 h sample the experimental animals were injected with 500µl of bacteria in PBS, and the control animals injected with 500µl of PBS, alone into the branchial blood vessel. All animals were sampled again after 4 h and 24 h, and blood counts recorded. After 48 h the experiment was stopped and duplicate tissue samples were taken from all animals and rinsed in PBS before storage at -70°C for use in both the lysozyme and antiprotease assays.

Blood Sterility

Blood samples at 0, 4 and 24 h from both the control and experimental animals, collected in PBS, were further diluted, twice to a final dilution of 1:1000 in PBS. Twenty µl of each of the samples were then placed in triplicate on tryptic soya agar (Gibco) (TSA plates), incorporating blanks on each plate, which were then incubated at 15°C for 48 h. After 48 h bacterial colonies on all plates were counted and the number of colonies present recorded. In order to check the viability of the bacteria, 20µl samples of the bacteria in PBS were plated out on TSA, as for the blood samples, at the same time that the animals were injected with the bacteria.

Lysozyme Assay

Tissue samples from both experimental and control animals were weighed and homogenised in a known amount of phosphate/citrate buffer (pH 5.8) ($Na_2HPO_4.2H_2O$, 4.45g/250ml distilled H_2O; citric acid, 2.1g/100ml distilled H_2O; NaCl, 0.09g/100ml buffer). Washed haemocytes at a known concentration were homogenized in the same buffer and all samples (haemocytes, haemolymph and tissue) centrifuged at 13000g for 10 min. Supernatants were removed and stored at -70°C until use in the assay.

Fifty µl of a hen egg white lysozyme (Sigma) (5ug/ml of buffer) standard were serially diluted in duplicate in sterile 96 well flat bottom plates (Dynatech). Fifty µl of each

sample was added in triplicate to the 96 well plates as well as 50 µl aliquots of buffer, as blanks. One hundred and fifty µl of freeze dried *Micrococcus lysodeikticus* (Sigma) (0.075g/100ml of buffer) was added to each well. The plates were read on a multiscan spectrophotometer for 5 min at 15 sec intervals at 450nm using negative kinetics.

Antiprotease Assay

Tissue samples from both sets of animals were weighed, and both the tissues and haemocyte pellets were homogenised in a known amount of PBS. All samples, including haemolymph, were centrifuged at 13000g for 10 min and the supernatants frozen at -70°C until required. Samples were serially diluted in 25µl PBS in round bottom 96 well microtitre plates (Dynatech) and 20µl of each dilution transferred to 96 well flat bottom microtitre plates. PBS was used for the blanks and for the standards which were incorporated on these flat bottom plates. Ten µl of trypsin standard (Sigma) (5mg/100ml PBS) was added to each well and the plate incubated for 5 min at room temperature. Two hundred µl BAPNA (5.2mg benzoylarginine-p-nitroaniline/ml dimethylformamide added to 20ml Tris.$CaCl_2$ buffer (Tris 6.057g/100ml distilled H_2O, $CaCl_2$ 0.694g/100ml distilled H_2O added together and adjusted to pH 7.8 with conc. HCl)) was added to each well and the plate read on a spectrophotometer, using kinetic analysis, at 405nm for 15 min at 30 sec intervals.

Analysis

The means and standard errors of the replicate blood counts were calculated for each sampling period. Student t-tests were used to compare the mean blood counts at the different sampling times and between the experimental and control animals.

Lysozyme activity per well was recorded and activity calculated /mg for the tissue samples and /10^6 haemocytes/ml for the haemocyte samples. The means and standard errors for the lysozyme replicates were determined for each sample (tissue, haemocytes and haemolymph). Student t-tests were used to establish the significance of the differences between the control and experimental animals for each set of samples.

The 85% inhibition value for antiprotease activity in units of trypsin inhibited /µg were calculated for all the samples. Means and standard errors were calculated for the replicates and the results of the control and experimental samples compared using Student t-tests for each set of samples.

Results

Blood Counts

The number of haemocytes/ml increased between the 0 h and 4 h samples (fig. 8.1) and remained at an equivalent level in the 24 h sample. Significantly more ($p<0.05$) haemocytes were detected after 4 h in the bacterial injected animals compared to the control animals.

Blood Sterility

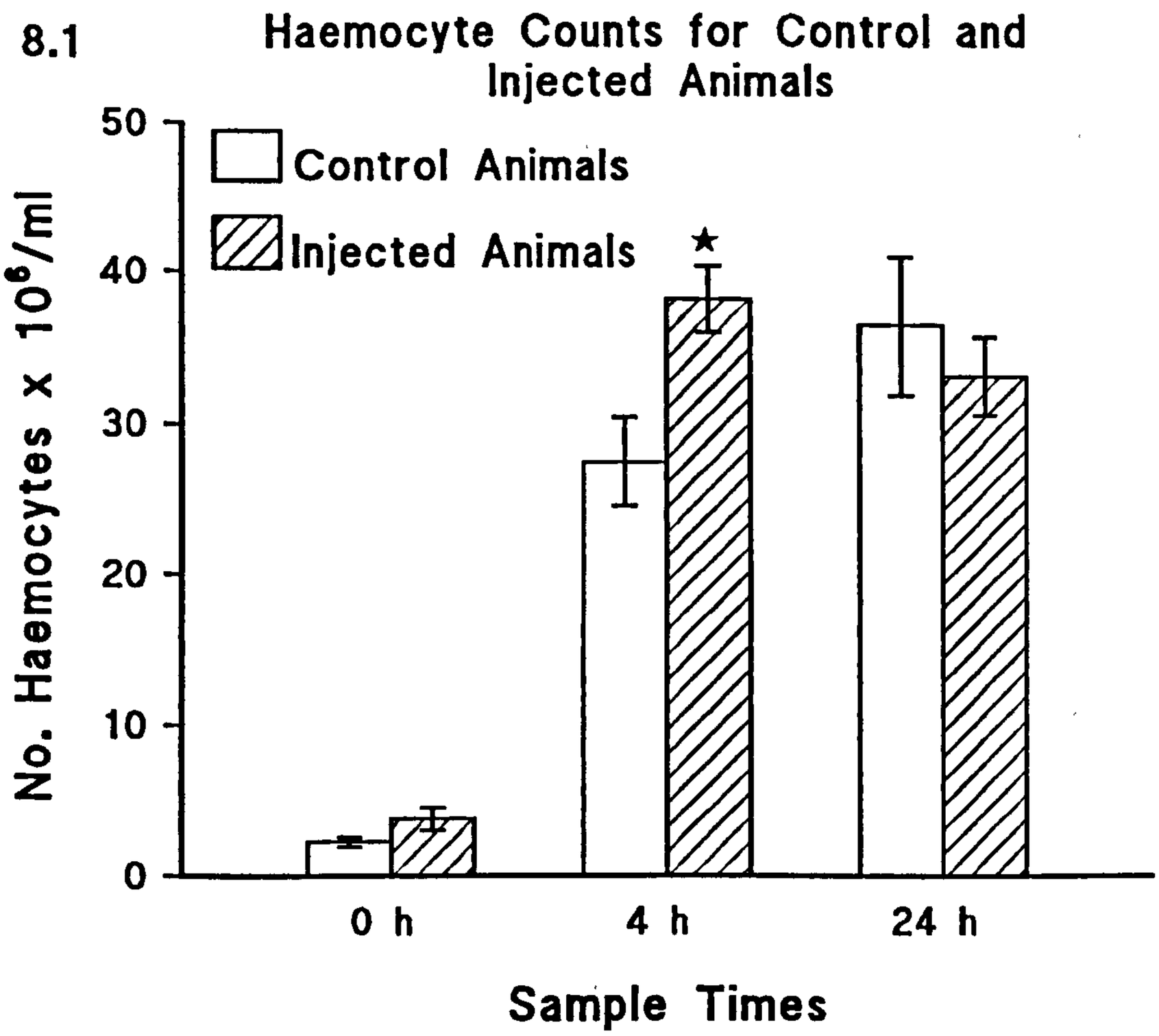
No bacteria were present in the blood of *E. cirrhosa* before injection of either PBS or PBS containing bacteria (table 1). One bacterial colony was observed at the first dilution (1:10) for 1 of the animals, which had been injected with bacteria, on the plates made from the 4 h sample. Bacterial colonies where the bacteria in PBS were plated out to determine viability proved impossible to count at the dilutions chosen due to the large number of colonies present (data not shown). No bacterial colonies grew from the 24 h sample TSA plates in either the control or bacterial injected animals.

Table 1. The mean number of bacterial colonies present in the blood of *Eledone cirrhosa* over a 24 h sampling period for both control (sham injected) and experimental (live *Vibrio anguillarum* injected) animals. The mean was taken from 5 animals.

Mean Number of Bacterial Colonies						
Sample Time	Control (1:10)	Infected (1:10)	Control (1:100)	Infected (1:100)	Control (1:1000)	Infected (1:1000)
0 h	0	0	0	0	0	0
4 h	0	0.067	0	0	0	0
24 h	0	0	0	0	0	0

Figure 8. 1. The number of haemocytes/ml in blood from both control and injected animals over a 24 h sampling period. The bars are the means of 5 animals and the error bars are the standard errors of the mean. *=$P<0.05$ compared to control animals.

Figure 8.1


Haemocyte Counts for Control and Injected Animals
Control Animals
Injected Animals
No. Haemocytes x 10⁶/ml
50
40
30
20
10
0
0 h
4 h
24 h
Sample Times

Lysozyme Assay

Lysozyme activity was detected in all tissue samples (fig. 8.2). The branchial heart appendage and anterior salivary gland had a significantly higher ($p<0.05$) activity/mg of tissue than the other samples. Only the branchial heart tissue from the bacterial injected animals had a significantly higher ($p<0.05$) lysozyme activity than in the control animals. Lysozyme activity increased in the haemocyte samples from control animals within 4 h (fig. 8.3) but significantly ($p<0.05$) decreased over the 24 h sampling period in the animals injected with bacteria. Haemolymph lysozyme levels (fig. 8.4) showed no change over the sampling period for either the control or bacterial injected animals.

Antiprotease Assay

Tissue samples from both control and bacterial injected animals indicate variable amounts of antiprotease activity (units of trypsin inhibited/μg) (fig. 8.5). The standard error bars indicate large variation between individuals. Antiprotease activity was observed in varying amounts in the branchial heart appendage, branchial heart, the optic lobe and in the posterior salivary glands and anterior salivary gland. There was no significant difference in antiprotease activity in the tissue samples collected from the control and bacterial injected animals. Haemocyte antiprotease activity (fig. 8.6) was significantly ($p<0.05$) higher in samples taken 4 h after bacterial injection compared to samples taken 4 h after the control injection. Both control injected and bacterial injected animals had a significantly ($p<0.05$) lower antiprotease activity in the 4 and 24

Figure 8. 2. Lysozyme activity/mg (tissue wet weight) present in the tissues of both control and bacterial injected animals after 48 h. The bars represent the means of 5 animals and the error bars are the standard errors of the mean. Gill=Branchial Gill; Gland=Branchial Gland; BH=Branchial Heart; BHA=Branchial Heart Appendage; WB=White Body; OL=Optic Lobe; PSG=Posterior Salivary Gland; ASG= Anterior Salivary Gland. *=$P<0.05$ compared to control animals.

Figure 8.2 Lysozyme Activity in Tissues from Control and Injected Animals

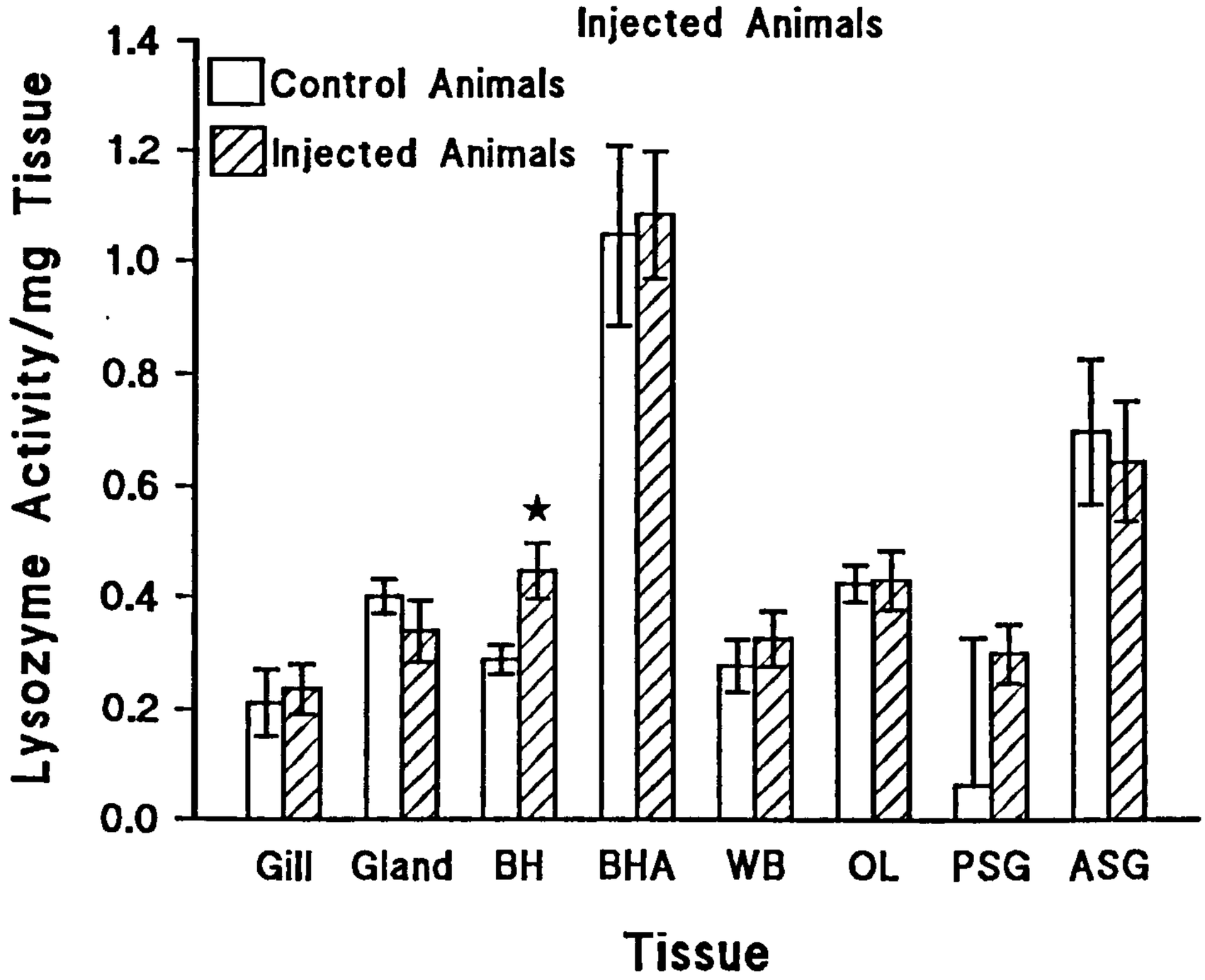


Figure 8. 3. Lysozyme activity/ml in a suspension at 10^6 /ml of haemocytes from control and bacterial injected animals over a 24 h sampling period. The bars represent the means of 5 animals and the error bars are the standard errors of the mean.

Figure 8. 4. Lysozyme activity/ml present in the haemolymph of control and bacterial injected animals over a 24 h sampling period. The bars represent the means of 5 animals and the error bars are the standard errors of the mean.

Figure 8.3 Lysozyme Activity in Haemocytes from Control and Injected Animals

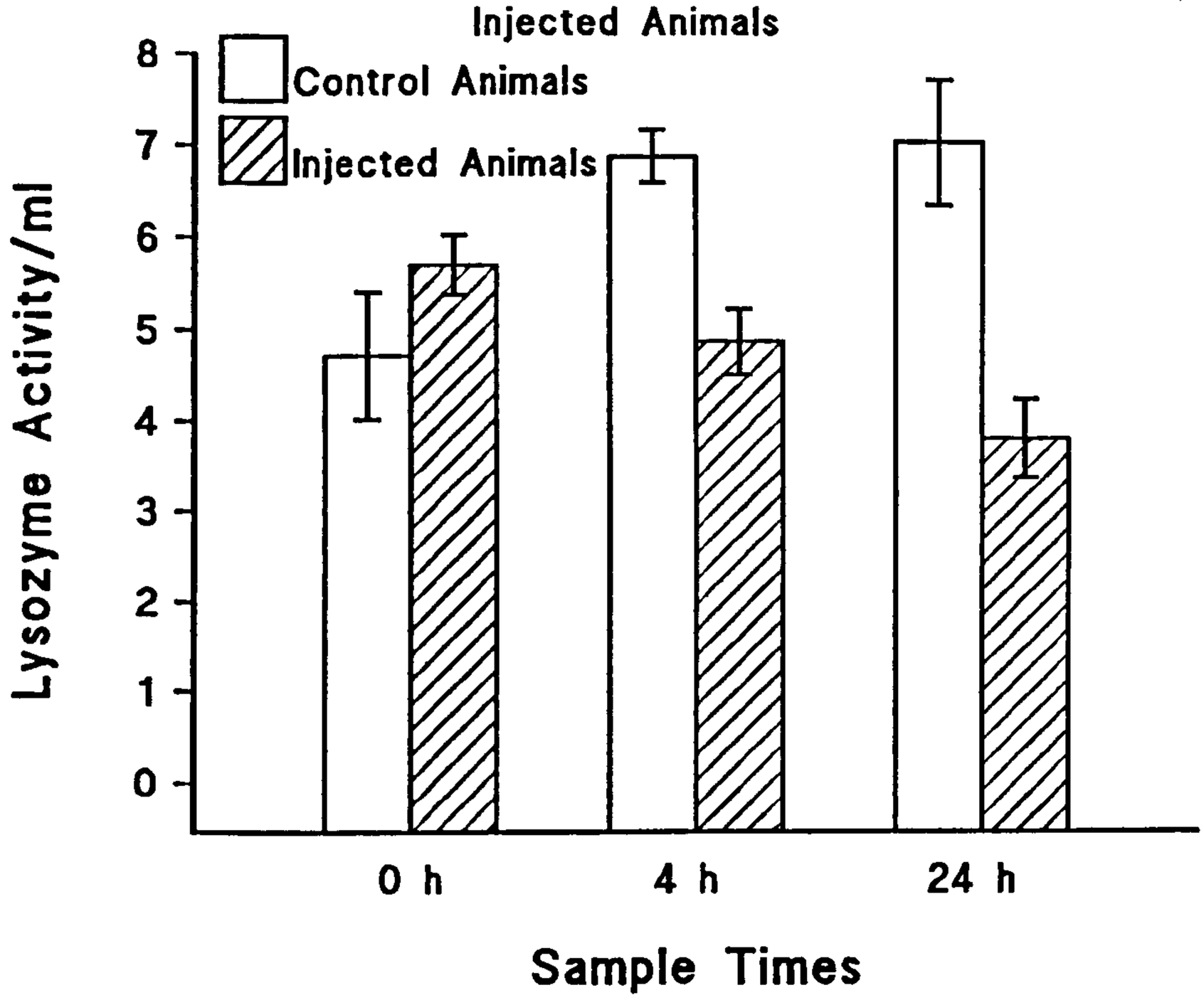


Figure 8.4 Lysozyme Activity in Haemolymph from Control and Injected Animals

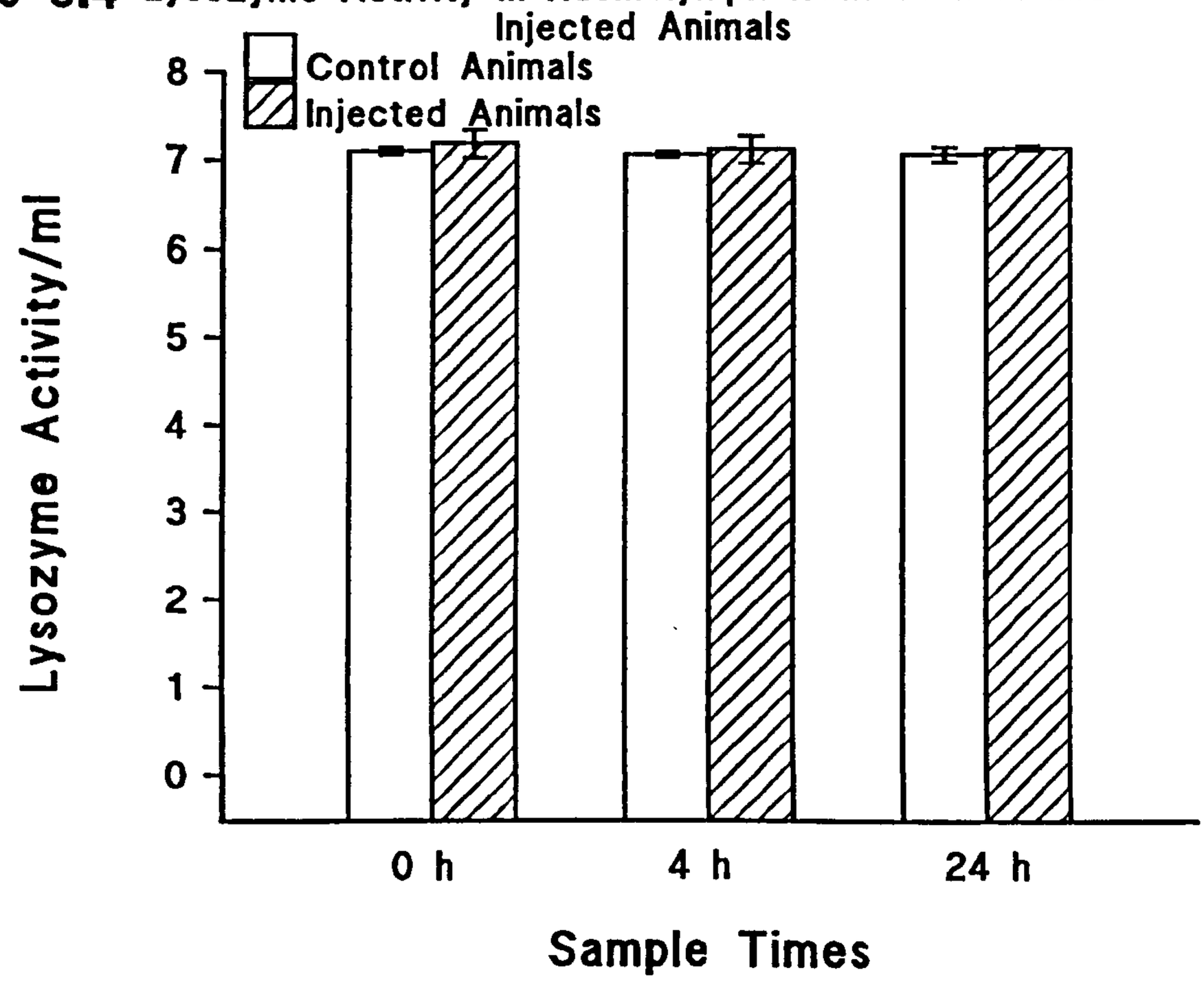
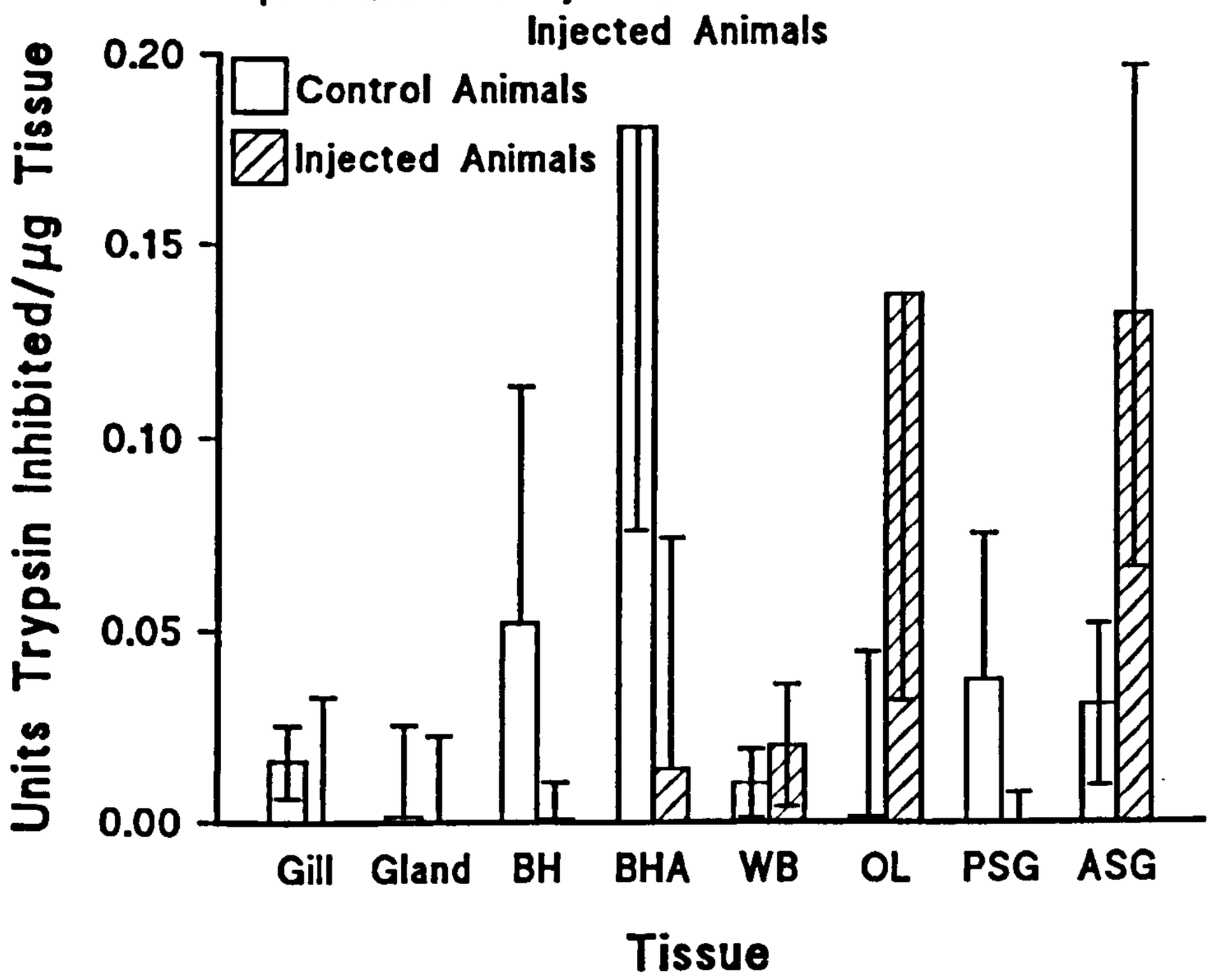


Figure 8. 5. Antiprotease activity (units of trypsin inhibited/µg tissue wet weight) present in the tissues of both control and bacterial injected animals after 48 h. The bars represent the means of 5 animals and the error bars are the standard errors of the mean. Gill=Branchial Gill; Gland=Branchial Gland; BH=Branchial Heart; BHA=Branchial Heart Appendage; WB=White Body; OL=Optic Lobe; PSG=Posterior Salivary Gland; ASG= Anterior Salivary Gland.

Figure 8.5 Antiprotease Activity in Tissues from Control and Injected Animals



h samples compared to 0 h. In haemolymph samples (fig. 8.7) the amount of antiprotease present was significantly ($P<0.05$) less in the bacterial injected animals than in control injected animals and the 4 and 24 h samples had significantly ($p<0.05$) less activity than the 0 h samples.

Figure 8. 6. Antiprotease activity (units of trypsin inhibited/10^6 haemocytes/ml) present in the haemocytes of control and bacterial injected animals over a 24 h sampling period. The bars represent the means of 5 animals and the error bars are the standard errors of the mean. *=P<0.05 compared to control animals.

Figure 8. 7. Antiprotease activity (units of trypsin inhibited/μl) present in the haemolymph of control and bacterial injected animals over a 24 h sampling period. The bars represent the means of 5 animals and the error bars are the standard errors of the mean.

Figure 8.6 Antiprotease Activity in Haemocytes from Control and Injected Animals

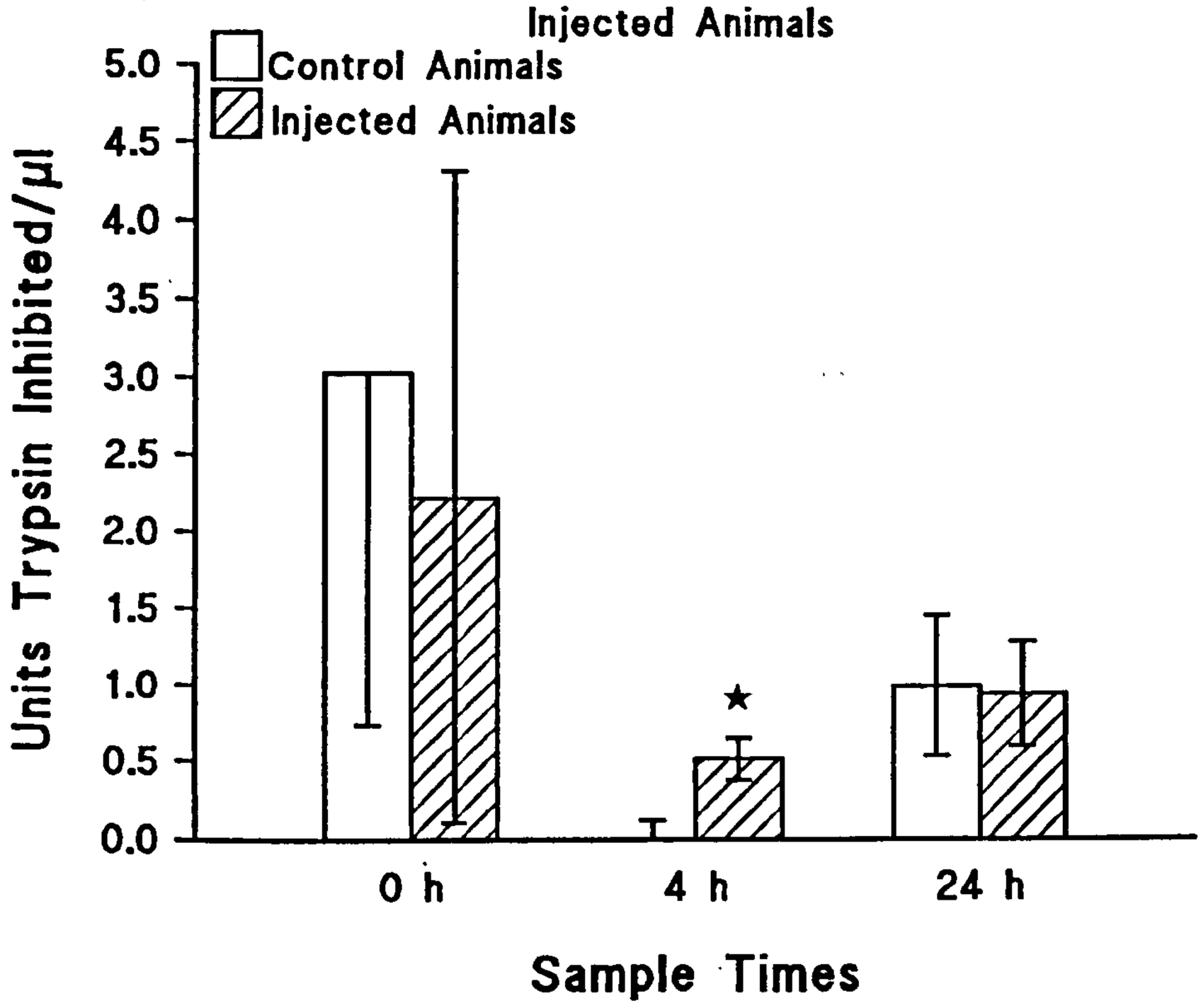
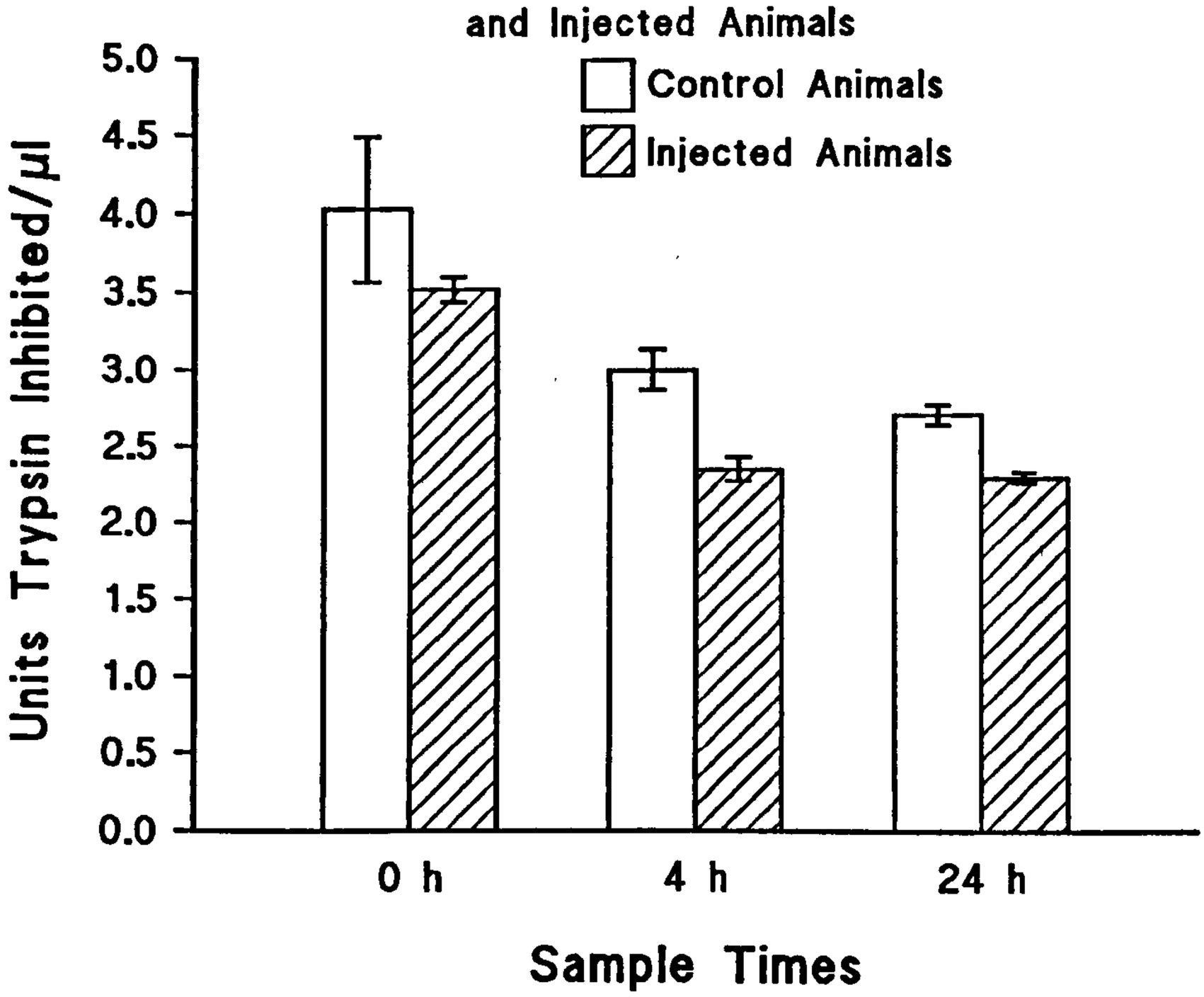


Figure 8.7 Antiprotease Activity in Haemolymph from Control and Injected Animals



Discussion

Live bacteria, *Vibrio anguillarum*, injected into the circulatory system of *Eledone cirrhosa* were mostly removed within 4 hours. Over the same time period the number of haemocytes/ml present in the blood increases. The haemocyte, haemolymph and various tissue samples display both lysozyme and antiprotease activity in control animals. However, the injection of live bacteria generally reduces the lysozyme and antiprotease activity of the haemocytes and the antiprotease activity of the cell free haemolymph.

As indicated by Bayne (1973) with *Octopus dolfeini* practically all of the live bacteria injected into *E. cirrhosa* were either cleared from the circulation or rendered non-viable within 4 hours. It appears possible that haemocytes play an important role in removing foreign organisms from the circulation of *E. cirrhosa*. As with other invertebrates (Millar & Ratcliffe, 1994; Ratcliffe *et al.*, 1985; Bayne, 1983) the haemocytes from *E. cirrhosa* phagocytose (Chapter 4) and affect bacterial growth (Chapter 6). Further, the number of haemocytes/ml increase in both the control and bacterial injected *E. cirrhosa* with a greater increase in the bacterial injected animals at the 4 hour sample (fig. 1). Recent work on the effects of repeatedly sampling blood from *E. cirrhosa* also demonstrated increased blood counts after sampling (Chapter 3). Additionally haemocytes are involved in the clearance of bacteria from the circulation (Chapter 9) and the increase in haemocytes demonstrated for this paper may indicate possible leucocytosis. Prospectively as demonstrated with the bivalves *Sunetta scripta* and *Villorita cyprinoides* var. *cochinensis* (Suresh & Mohandas, 1990) and the

gastropods *Biomphalaria glabrata* (Lie *et al.*, 1975; Jeong *et al.*, 1983) and *Patella vulgata* (Cooper-Willis, 1979) *in vivo* exposure to pathogenic organisms can cause the release of large numbers of haemocytes. This release however may be dose and/or time dependent since other authors working on gastropods such as Bayne and Kime (1970), Renwrantz *et al.* (1981) and Van der Knapp (1982) showed a decrease in the number of circulating haemocytes after injections of large numbers of bacteria (Ratcliffe *et al.*, 1985).

Though lysozyme is known to act primarily on Gram positive bacteria (Salton, 1957) it will also act on Gram negative bacteria (Miller, 1969) and in this instance the Gram negative bacterium *V. anguillarum* was injected into *E. cirrhosa*. *V. anguillarum* is known to be pathogenic to cephalopods in captivity and infections can be fatal (Hanlon & Forsythe, 1990). All tissue samples taken from the octopus demonstrated lysozyme activity with the branchial heart appendage and anterior salivary gland having the highest activities. It is possible that the activity detected in the anterior salivary gland could be secreted as a constituent of the saliva during feeding. The branchial heart appendage of octopods is involved in ultrafiltration of the haemolymph (Martin & Aldrich, 1970; Schipp & Hevert, 1981) and lysozyme activity in this instance could aid in the degredation or inactivation of certain molecules. Of particular interest is the increase in lysozyme activity, 48 hours after infection, in the branchial heart, which is involved in the circulation of blood and which in *Sepia officinalis* contains lysosomal activity (Schipp *et al.*, 1971), suggesting a possible synthesis site for circulating lysozyme. Lysozyme activity has been demonstrated in the mantle mucus of the oyster

(McDade & Tripp, 1967), the digestive gland and headfoot of *Biomphalaria glabrata* (Kassim & Richards, 1978a, b) and the kidney of *Planorbarius corneus* (Ottaviani, 1991). As with other molluscs (Cheng, 1983) *E. cirrhosa* exhibits lysozyme activity in both the haemolymph and haemocytes. While the level of activity in the haemolymph does not change over the 48 hours of the experiment, the haemocyte activity level in the control injected animals increases, while in the bacterial injected animals it shows decreasing activity. Cheng *et al.* (1977) demonstrated that the *in vivo* challenge of *Biomphalaria glabrata* with dead bacteria caused initially elevated serum lysozyme activity levels followed by depressed levels. Additionally, the initial level (0 h sample) of lysozyme activity in the haemolymph of *E. cirrhosa* was greater than that found in the haemocytes. Similar findings of higher lysozyme activity in the haemolymph were demonstrated for the oyster *Crassostrea virginica* (Rodrick & Cheng, 1974) and the clam, *Mercenaria mercenaria* (Cheng *et al.*, 1975), leading the authors to suggest that serum lysozyme originated in the respective haemocytes. Foley and Cheng (1977) confirmed the release of lysozyme from certain *M. mercenaria* haemocytes into the haemolymph via the process of degranulation, particularly during phagocytosis. *E. cirrhosa* haemocytes phagocytose bacteria (Chapter 4) and newly released haemocytes contain numerous cytoplasmic granules some of which could contain lysozyme (Chapter 3). However, the white body or leucopoetic organ from which new haemocytes are supposedly released, demonstrated significantly less lysozyme activity than the haemocytes obtained from the circulation for both sets of animals. The reduced lysozyme activity in the white body might suggest that new haemocytes

containing lysozyme from both control and experimental animals had already been released over the 48 hour sampling period, or that enzymatically active lysozyme synthesis occurs in the haemocytes after their release from the white body. It is also possible that lysozyme is produced elsewhere, secreted into the haemolymph and pinocytosed by the haemocytes, or that only a small part of the white body consists of mature haemocytes containing a full lysozyme complement.

As stated by most workers who have determined lysozyme levels in invertebrates, (Cheng, 1983; Hardy *et al.*, 1976; Möck *et al.*, 1992; Takahashi *et al.*, 1986), the optimum pH for lysozyme detection, when using a phosphate/citric acid buffer is between 4 and 6.2 depending on the animal (e.g., pH 5.0 for the soft clam *Mya arenaria* (Cheng & Rodrick, 1974), pH 5.5 for *Mercenaria mercenaria* (Cheng *et al.*, 1975) and pH 6.2 for the earthworm *Eisenia foetida* (Çotuk & Dales, 1984)). Though lysozyme activity in *E. cirrhosa* was detected in different samples at various pH's (between 4 and 7) (pers. obs.) the buffer pH of 5.8 was chosen for 2 main reasons. Firstly it gave the most consistent results and secondly the haemolymph did not immediately precipitate upon addition of the substrate containing the buffer as it did at more acid pH's.

The antiprotease activity detected in the salivary glands (posterior and anterior) could well be involved in the regulation of the powerful proteases associated with the saliva (Grisley & Boyle, 1987). The branchial heart appendage, branchial heart and the optic lobe exhibit some antiprotease activity. The white body, which is a possible leucopoetic organ (Cowden & Curtis, 1974, 1981), exhibits a very low level of

antiprotease activity when compared to the haemocyte samples at 24 hours. The amount of antiprotease activity in the haemolymph decreases at a faster rate in the bacterial injected animals than in the control animals.

Recent work has demonstrated that injected active trypsin associates with α_2-M in *Limulus polyhemus* and after clearance is degraded to small peptides (Melchoir *et al.*, 1995). Additionally, the same experiment demonstrated that the blood cells in this animal were also involved in the clearance of trypsin α_2-M complex. Melchior *et al.* (1995) suggested that as with vertebrates, the protease α_2-M complex is endocytosed by blood cells and degraded by secondary lysosomes. Additionally α_2-M and agglutinins/lectins also appear to be functionally related (Bachére *et al.*, 1995). Both *L. polyhemus* (Armstrong &Quigley, 1991; Quigley & Armstrong, 1994) and *Homarus americanus* (Spycher *et al.*, 1987) have thioester-containing peptide sequences similar to mammalian α_2-M suggesting evolutionary conservation of this molecule from invertebrates, such as arthropods (Spycher *et al.*, 1987; Armstrong & Quigley, 1991; Spycher & Painter, 1991; Quigley & Armstrong, 1994) and molluscs (Armstrong & Quigley, 1992; Bender *et al.*, 1992; Fryer *et al.*, 1996), to mammals (Sottrup-Jensen *et al.*, 1990).

Since haemolymph from *Octopus vulgaris* (Thøgersen *et al.*, 1992) and *Loligo pealii* (Armstrong & Quigley, 1992) have been shown to contain α_2M like activity it is possible that some of the detected antiprotease in *E. cirrhosa* could also be α_2M.

Further work however is needed to characterize the antiprotease activity found in *E. cirrhosa* and to determine the role it plays in octopod immunity.

In conclusion, both lysozyme and antiprotease activity are present in the haemocytes and haemolymph of *E. cirrhosa*. Work is continuing with *E. cirrhosa* in order to determine whether other lysosomal enzymes are present and also to characterise the antiprotease activity detected.

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Chapter 9

Histological and Ultrastructural Observations on the Fate of Graphite and Bacteria Injected into the Lesser Octopus *Eledone cirrhosa* (Lam.) (Cephalopoda).

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Abstract

The octopus *Eledone cirrhosa* was challenged with live *Vibrio anguillarum* for 4 h and 48 h and with colloidal graphite for 4 h. The fixed tissues (branchial gill, branchial heart, branchial heart appendage, white body and haemocyte pellets) were examined histologically and ultrastructurally to determine which tissues were involved in the clearance of the injected particles. The tissues were also compared to control tissues to identify any major morphological changes resulting from bacterial or graphite challenge.

Colloidal graphite was found to aggregate in blood vessels and caused a morphological change in the nuclei of some circulating haemocytes 4 h after the injection of the graphite. Evidence of bacteria in the circulating haemocytes were obtained in sections from all the tissues sampled from the octopus. Forty-eight hours after bacterial challenge the nuclei of some of the haemocytes demonstrated morphological changes. Bacteria were seen in the branchial heart, branchial heart appendage and white body cells.

Key Words: *Eledone cirrhosa*; Bacteria; Colloidal graphite; Haemocytes; Branchial Heart; Branchial Heart Appendage; White Body.

Introduction

Various *in vivo* studies have demonstrated that invertebrates are able to recognise and remove from their circulation foreign biotic and abiotic particles. Methods of removal vary between the invertebrate classes but include such processes as phagocytosis by free and fixed phagocytes, nodule formation and encapsulation (Ratcliffe *et al.* 1985; Millar & Ratcliffe, 1994).

Research using molluscs has shown that in gastropods injected particles are removed by circulating and fixed phagocytes and also by aggregation of the particles in specific organs such as the digestive gland, the foot and the kidney (Bayne, 1973a; Crichton *et al.*, 1973; Killby *et al.*, 1973; Renwrantz, *et al.*, 1981; Bayne 1983). Experimentally introduced particles are also removed from bivalves by circulating phagocytes (haemocytes) (Sparks & Morado, 1988; Suresh & Mohandas, 1990). Cephalopods will also remove experimentally introduced particles from their circulation. Stuart (1968) showed that varying quantities of injected colloidal carbon were removed by specific organs such as the gill and posterior salivary gland of *Eledone cirrhosa*, where supposedly fixed phagocytes occur. Further Bayne (1973b) injected carbon and live bacteria into *Octopus dofleini* and these were rapidly removed from the circulation with the carbon accumulating in the gills. Cephalopods have a closed circulatory system as in vertebrates (Browning, 1979; Wells, 1983; Shadwick & Nilsson 1990). The blood consisting of haemolymph (plasma) and haemocytes (blood cells), is pumped around the animals body by 3 hearts, the central systemic heart and a branchial heart associated with each gill. The branchial hearts and the associated branchial heart

appendage are involved in the circulation (Wells, 1978; Wells, 1983; Wells & Smith, 1987) and ultrafiltration (Schipp *et al.*, 1971; Witmer & Martin, 1973; Schipp & Hevert, 1981) of the blood of the octopus. The haemocytes in cephalopods are thought to originate from the leucopoetic organ, or white body, located in the orbital sockets behind the eyes. The haemocytes are apparently held there in clusters undergoing maturation, before release into the circulation as mature haemocytes containing various granules in their cytoplasm (Cowden, 1972; Cowden & Curtis, 1974, 1981). Interestingly encapsulation reactions have been recorded in some cephalopods (Jullein, 1940) and the formation of 'cellular aggregrates' after dissociation of the white body in *O. birareus* has been reported (Cowden & Curtis, 1973).

Previous work from this laboratory has demonstrated that *E. cirrhosa* will clear live *Vibrio anguillarum* from the circulation and that the number of circulating haemocytes as well as lysozyme activity, detected in the branchial heart, increased in response to bacterial challenge (Chapter 8). This paper aims to conduct a preliminary investigation into certain tissues and the haemocytes of *E. cirrhosa* to determine their involvement in the response to colloidal graphite and bacterial challenge.

Materials and Methods

Animals

Octopuses were collected from commercial crab pots situated around the north side of the Isle of Anglesey. The animals were brought into the aquarium at the University of Wales, Bangor and immediately weighed, marked at the base of the arm using a 21 guage 1½" needle (Chapter 3) containing 1% alcian blue in octopus Ringer (OR) (NaCl, 2.433g/100ml; glucose, 1.4g/100ml; EGTA, 0.015g/100ml; KCl, 0.082g/100ml; KH_2PO_4, 0.004g/100ml) and assigned to tanks. The animals were maintained at 12°C for 48 h before experimentation.

Haemocyte Sampling

Blood was sampled from the branchial blood vessel (Chapter 2). A haemocyte count was taken and the blood immediately divided into 2 Eppendorfs and centrifuged at 800g for 4 min at 4°C. The haemolymph was removed and the pellet of cells fixed either for histological or Transmission Electron Microscope (TEM) analysis.

Graphite

Colloidal graphite (Agar Scientific) (pH 10) was diluted (1:4) in sterile phosphate buffered saline (pH 7) (PBS) (Gibco) and the pH altered to pH 7.0 using sodium hydroxide. Five hundred µl of the solution were loaded into sterile 2 ml syringes and the syringes frozen at -20°C until use.

Bacteria

Vibrio anguillarum (MT275) was obtained from the Scottish Office Agricultural Environment and Fisheries Department, Torry, Aberdeen. The Gram negative bacterium was cultured in tryptic soya broth (Gibco) containing 2% sodium chloride. Triplicate bacterial counts were taken and the bacterial concentration adjusted to 1 x 10^6 bacteria /ml. The bacteria were centrifuged at 13000g for 10 min and washed twice in sterile PBS before resuspension to 500µl in PBS.

Inoculation and Experimentation

Five control and 15 experimental animals were chosen at random. All sets of 5 animals were maintained separately under sterile conditions and waste seawater was treated to kill any escaping bacteria. All the control and experimental animals were sampled from the branchial blood vessel at 0 h and blood counts taken. Immediately following the 0 h sample 10 of the experimental animals were injected with 500µl of bacteria in PBS, and the 5 control animals injected with 500µl of PBS only, into the branchial blood vessel. The last set of 5 animals were injected with the prepared colloidal graphite in PBS. The control animals and 5 of the bacteria injected animals were sampled after 4 h and after 24 h and the duplicate haemocyte samples fixed for histology or TEM preparation. Five of the bacteria injected, and all the carbon injected, animals were sacrificed 4 h after injection and duplicate tissue samples were taken. After 48 h the experiment was stopped and duplicate tissue samples were taken from the remaining bacterial infected animals and from the control animals. Tissue samples were taken

from the branchial gill, the branchial heart, the branchial heart appendage and the white body and fixed for either histology or TEM.

Histology Preparation

Tissue samples and the haemocyte pellets were fixed for 48 hours in 10% phosphate buffered formalin. The samples were washed in distilled water and dehydrated in alcohol before infiltration in Historesin (Leica) for 7 days at 4°C. Samples were embedded in Historesin and sections cut, put on glass slides and mounted with DPX after staining.

Staining

The Historesin sections were stained either with Ehrlich's haematoxylin and eosin (H & E), toluidine blue (1% toluidine blue in 1% $Na_2B_4O_7.10H_2O$) or with a polychrome stain (Blackstock, unpublished) (1% Alcian blue in 3% acetic acid, 1% periodic acid, Schiff's reagent, iron alum (2.5%), Heidenhain's haematoxylin, acid fuchsin).

TEM. Preparation

All tissue samples including the haemocyte pellets were fixed for 24 h at 4°C in 2.5% glutaraldehyde (in 0.1M sodium cacodylate buffer at pH 7.4). The samples were washed in 0.1M sodium cacodylate buffer and secondarily fixed for 2h at room temperature in 1% osmium tetroxide before staining *en bloc* with 2% uranyl acetate overnight. The samples were then dehydrated through ethanol and propylene oxide and embedded in Spurr resin. Cut sections (50nm) were mounted on 100 mesh

pioloform coated copper grids and stained with lead citrate. Sections were viewed in a GEC Corinth 500 at 60 KV.

Results

Branchial Gill. (Figures 9.1-9.5)

The haemocyte (H) (fig. 9.1) in the gill lamellae of a control animal has rather indistinctly stained granules, however, 4 h after the injection of bacteria (fig. 9.2) distinct granules (G) and large dark stained masses surrounded by an unstained halo, which appear to be ingested bacteria, are also present in the cytoplasm. Histological sections taken (fig. 9.3) 48 h after the injection of bacteria indicated the presence of large masses resembling phagocytosed bacteria (B), as well as sites of possible bacterial degredation (D), indicated by unstained areas in the cytoplasm of the haemocytes (H). A TEM section of a haemocyte (fig. 9.4) from the gill, 48 h after bacterial challenge, showed bacteria being degraded (D), indicated by the particulate nature of the bacterium surrounded by a double membrane, in the cytoplasm. Four hours after colloidal graphite (C) injection an histological section (fig. 9.5) of a gill lamella indicated the accumulation of graphite in a blood vessel.

Branchial Heart. (Figures 9.6-9.16)

Histological sections of the branchial heart of a control animal (fig. 9.6) showed large cells each with a large vacuole (V) containing unknown large particles of differentially stained material. Haemocytes (H), with a variety of cytoplasmic granules (G), were present in blood vessels (BV) (fig. 9.6). A TEM section of the branchial heart from a control animal (fig. 9.7) showed characteristic pore cells (P) (after Sminia *et al.*, 1972) each with a large prominent electron-dense vacuole (V) of unknown material. The

Figure 9.1. Polychrome stained section of a gill lamella of a control *E. cirrhosa*.

BV = Blood vessel; H = Haemocyte with small cytoplasmic granules; E = Gill epithelium.

Figure 9.2. Polychrome stained section of part of the gill lamella of *E. cirrhosa* 4 h after bacterial injection.

H = Haemocyte; G = Cytoplasmic granules in the haemocyte; B = Bacteria in the haemocyte.

Figure 9. 3. Polychrome stained section of the gill of *E. cirrhosa* 48 h after bacterial injection.

H = Haemocyte; B = Bacteria in the haemocyte; D = Evidence of bacterial degradation in the haemocyte.

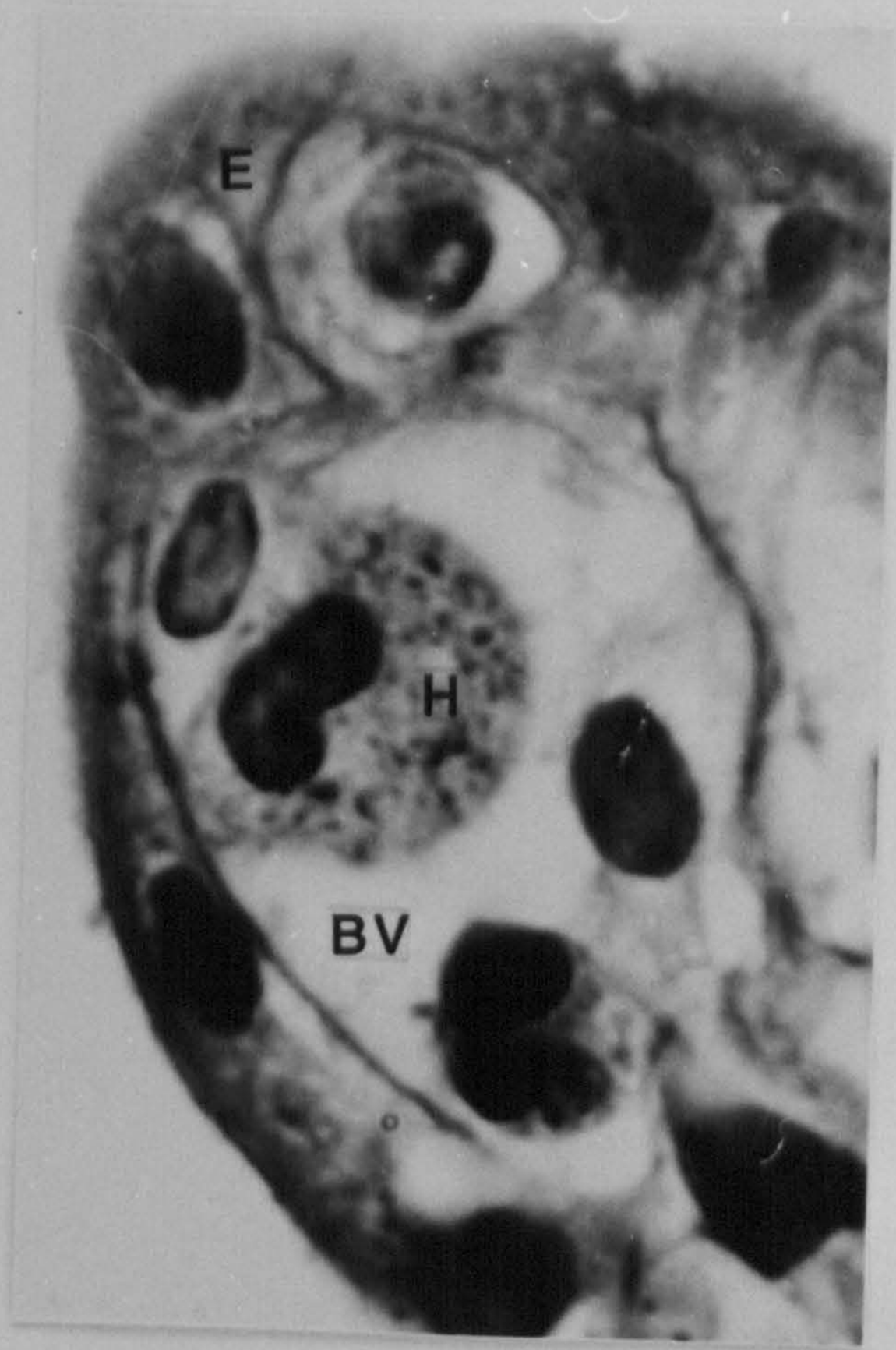


Fig. 9.1

5 μm

Fig. 9.2

5 μm

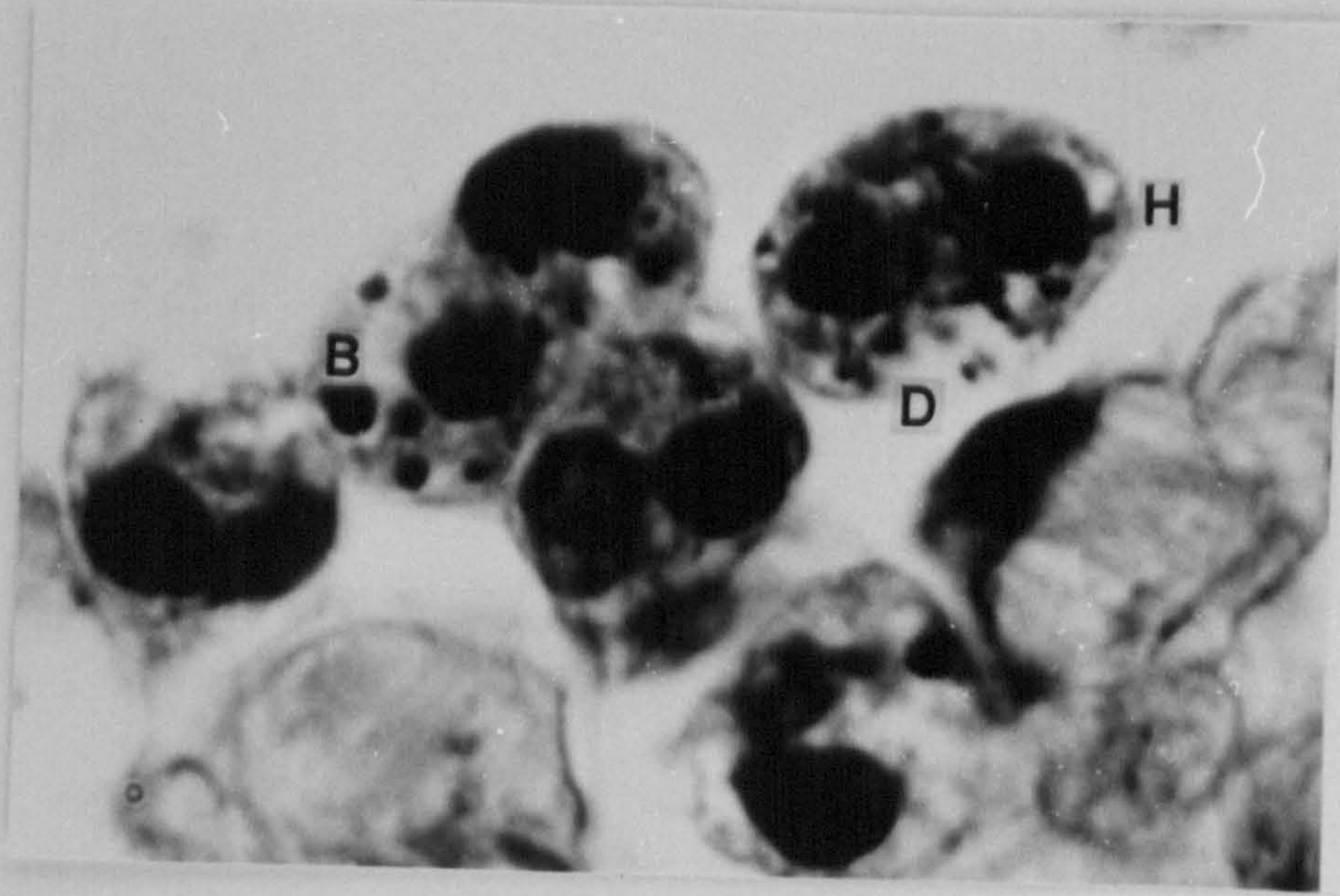


Fig. 9.3

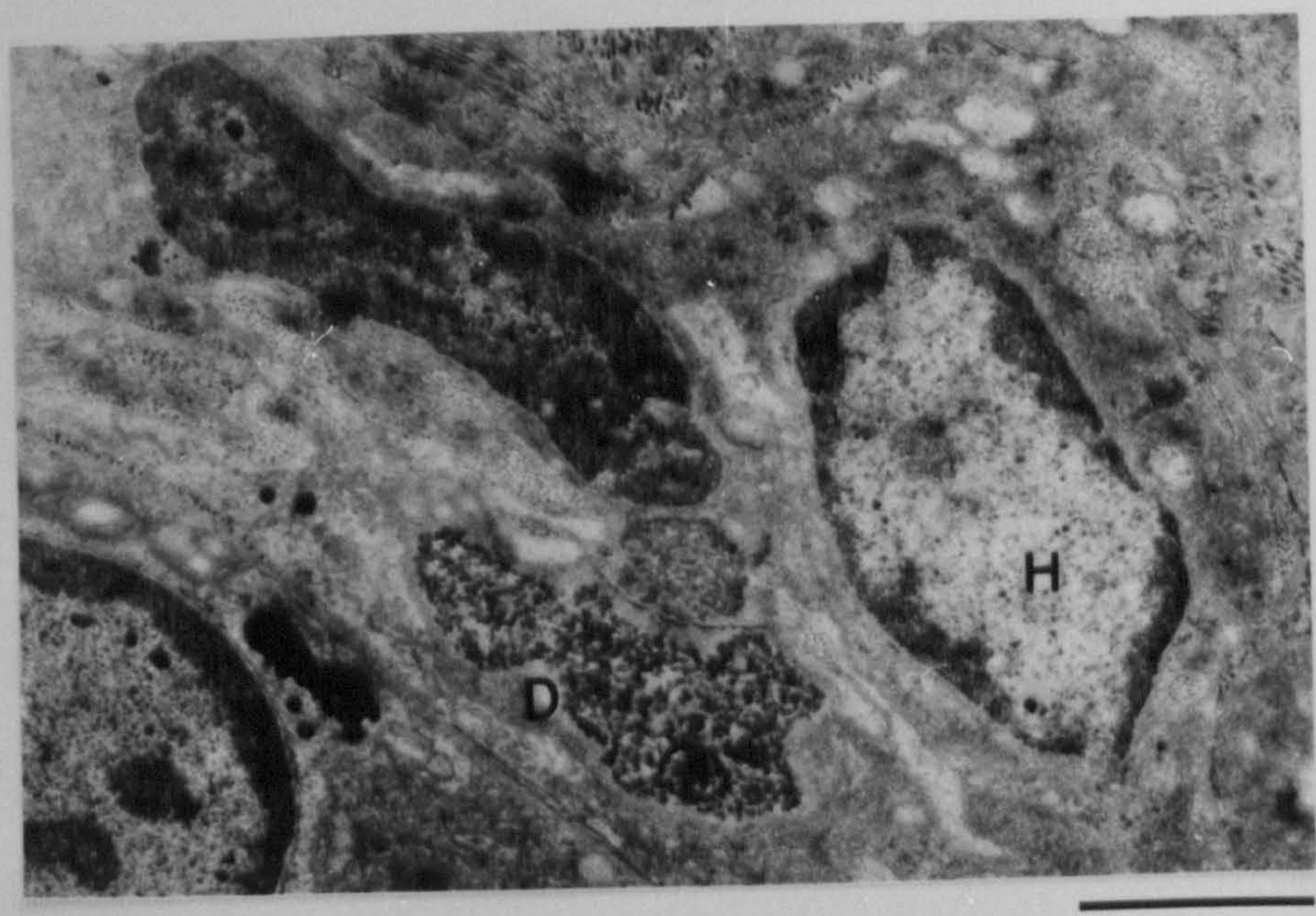
5 μm

Figure 9. 4. TEM section of a haemocyte in the gill of *E. cirrhosa* 48 h after bacterial injection.

H = Haemocyte; D = Possible bacterial degradation in the haemocyte.

Figure 9. 5. H&E section of gill lamellae of *E. cirrhosa* 4 h after the injection of colloidal graphite.

C = Graphite aggregated in a blood vessel.



2 μm

Fig. 9.4

5 μm

Fig. 9.5

vacuole (V) of the pore cell (P) (fig. 9.7) contained areas of darker electron-dense material which were difficult to distinguish from a surrounding matrix of similar electron-density. A TEM section of a haemocyte (H) (fig. 9.8) next to a pore cell (P) in a control animal indicated the heterogeneity of the electron-dense granules (G) present in the cytoplasm. Four hours after the injection of bacteria the prominent vacuole (V) in the pore cells (P) of the branchial heart had become finely granular in appearance (fig. 9.9). A TEM section taken 4 h post-injection (fig. 9.10) showed that this large vacuole (V), contained several small accumulations of electron-dense material. Clear vacuoles (CV) were also present in these pore cells (P) (fig. 9.9) which suggest that breakdown of the contents had occured resulting in a distinct double membrane surrounding the translucent vacuole containing indistinguishable contents. A haemocyte (H) next to a pore cell (P), 4 h post-injection (fig. 9.11), showed a variety of electron-dense granules (G), a pleomorphic nucleus and a translucent area (CV) surrounded by a double membrane which possibly contained breakdown products of phagocytosed material. Forty-eight hours after injection with bacteria (fig. 9.12) the large vacuole (V) in the pore cell (P) has become indistinct, however granules which resemble phagocytosed bacteria (B), shown by particles enclosed in medium stained vacuoles, and clear vacuoles (CV), which could indicate bacterial breakdown, were present in the cytoplasm. The normally bilobed nucleus (N) of some haemocytes (H) (fig. 9.13) had become pleomorphic, 48 h post-challenge, and only a few small granules (G) were present in the cytoplasm. Colloidal graphite (C) was found in a blood vessel (BV) of the branchial heart 4 h after graphite injection (fig. 9.14) and the

Figure 9. 6. Polychrome stained section of the branchial heart of a control animal.

H = Haemocyte; G = Cytoplasmic granules in the haemocyte; BV = Blood vessel with haemocytes; CN = Nucleus of pore cell from a branchial heart; V = Large vacuole of pore cell with differentially stained contents.

Figure 9. 7. TEM section of a pore cell in the branchial heart of a control animal.

P = Pore cell in a branchial heart; V = Large vacuole of a pore cell which contains dark granules difficult to distinguish from a matrix of similar electron density; CN = Nucleus of pore cell.

Figure 9. 8. TEM section of a haemocyte containing various granules from the branchial heart of a control animal.

H = Haemocyte; G = Cytoplasmic granules of the haemocyte; P = Pore cell.

Figure 9. 9. Polychrome stained section of the branchial heart 4 h after the injection of bacteria.

V = Large vacuole of a pore cell which now contains less granular material.

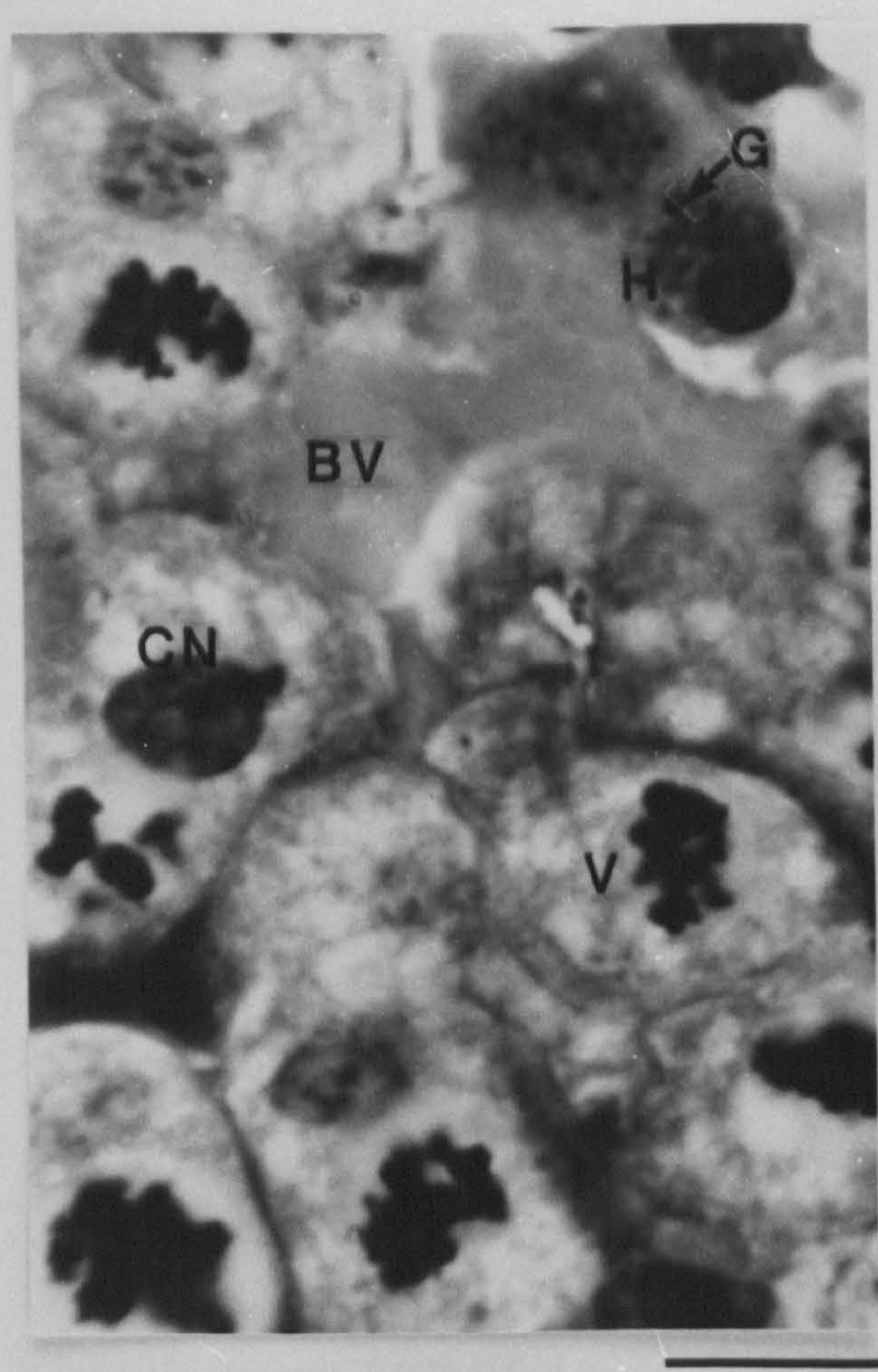


Fig. 9.6 4 µm

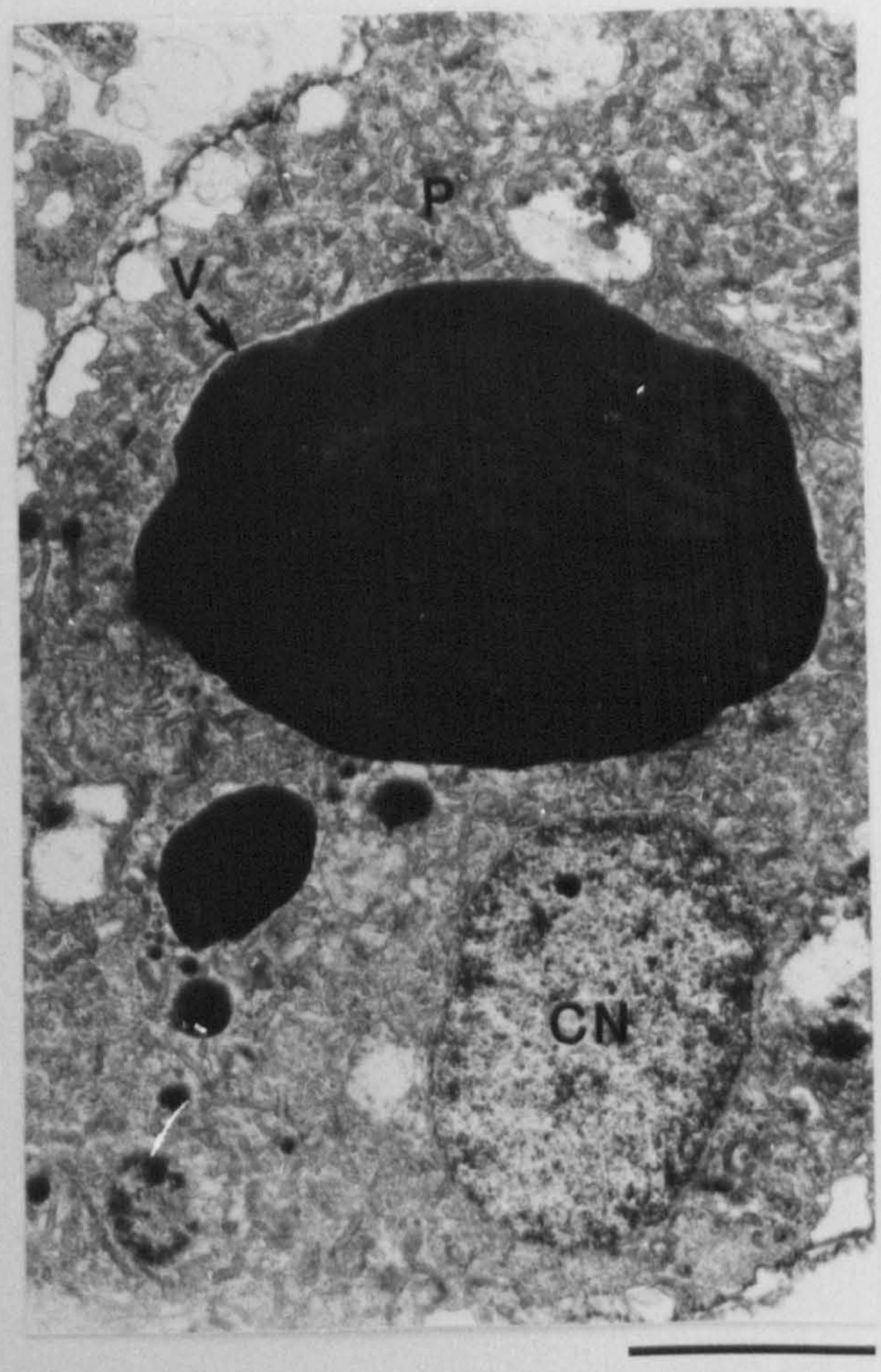


Fig. 9.7 2 µm

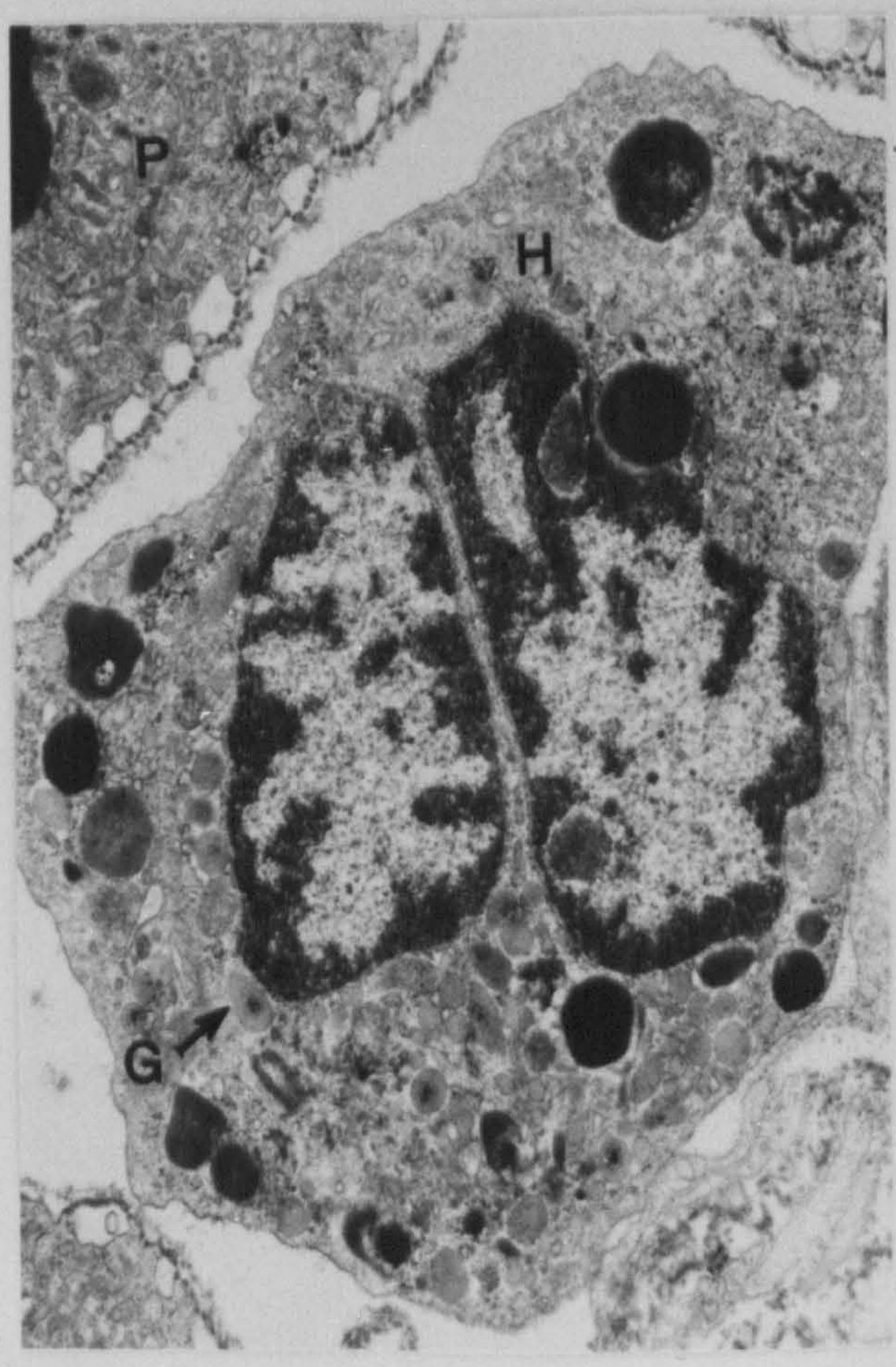


Fig. 9.8 2 µm

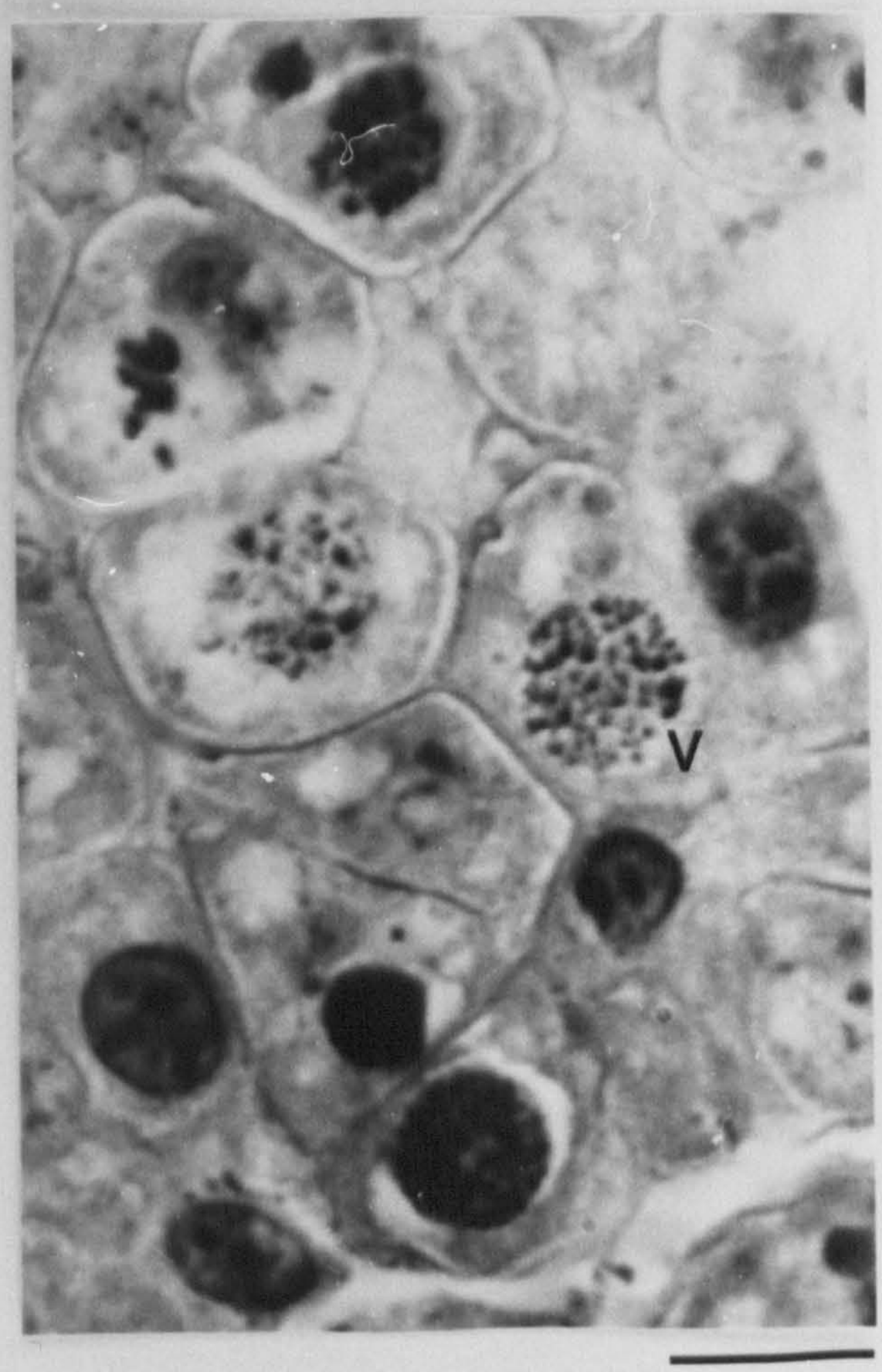


Fig. 9.9 5 µm

Figure 9. 10. TEM section of the branchial heart of an experimental animal 4 h after bacterial injection.

P = Pore cell; V = Large vacuole of a pore cell which now contains electron-dense granules in a clear matrix; CV = Small clear vacuoles.

Figure 9. 11. TEM section of a haemocyte in the branchial heart of an experimental animal 4 h after bacterial injection.

H = Haemocyte; N = Nucleus of haemocyte; G = Various electron-dense cytoplasmic granules; D = Possible bacterial breakdown in the cytoplasm of the haemocyte.

Figure 9. 12. Polychrome stained section of the branchial heart of an experimental animal 48 h after bacterial challenge.

V = Smaller vacuole of a pore cell with less contrast between the granules and the matrices; CV = Clear vacuoles; B = Vacuoles containing bacteria in the cytoplasm of a pore cell.

Figure 9. 13. TEM section of a haemocyte in the branchial heart 48 h after bacterial injection.

H = Haemocyte; G = Small granules in the cytoplasm of the haemocyte; N = Nucleus of the haemocyte which is now pleomorphic.

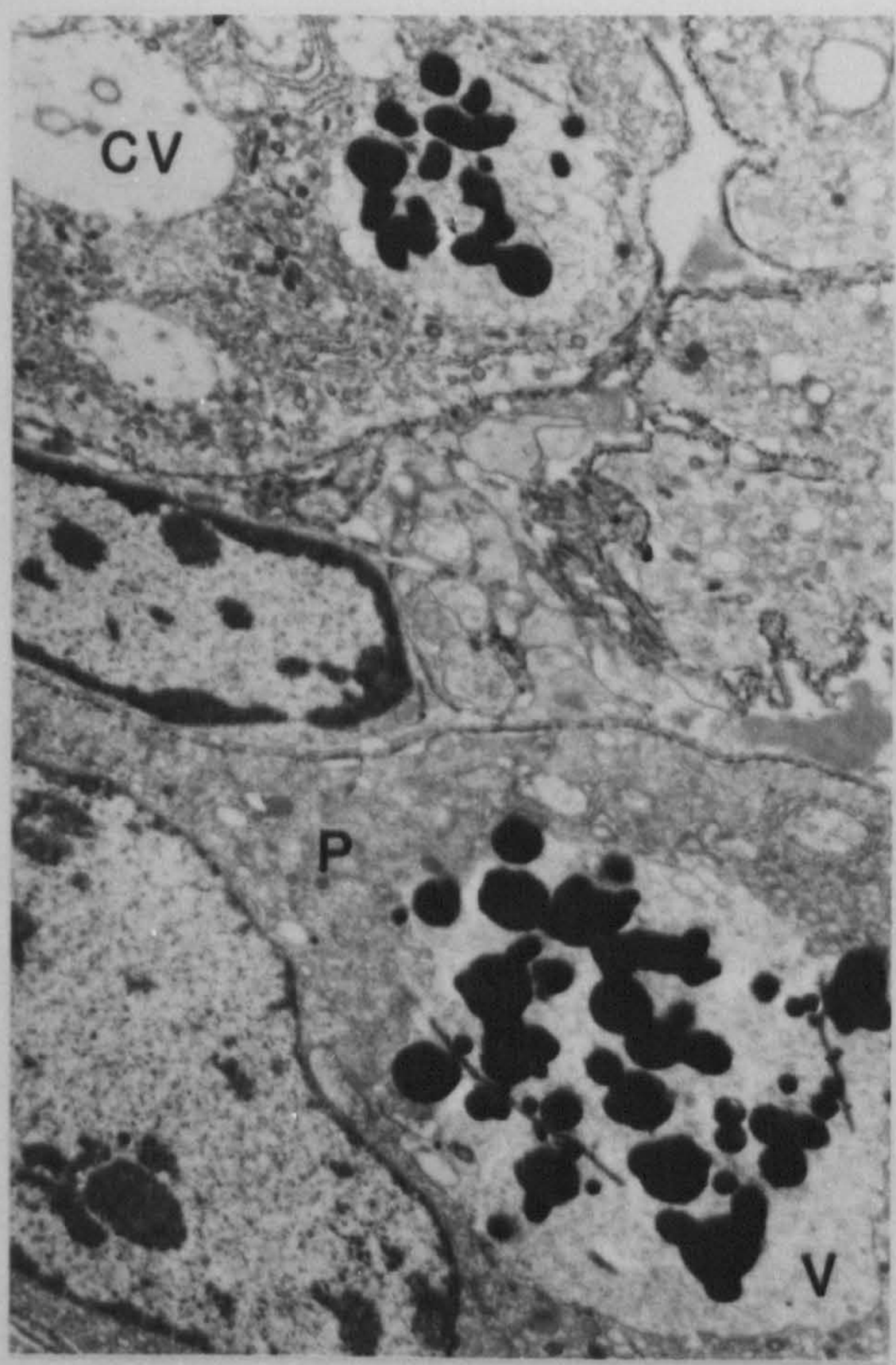


Fig. 9.10 4 μm

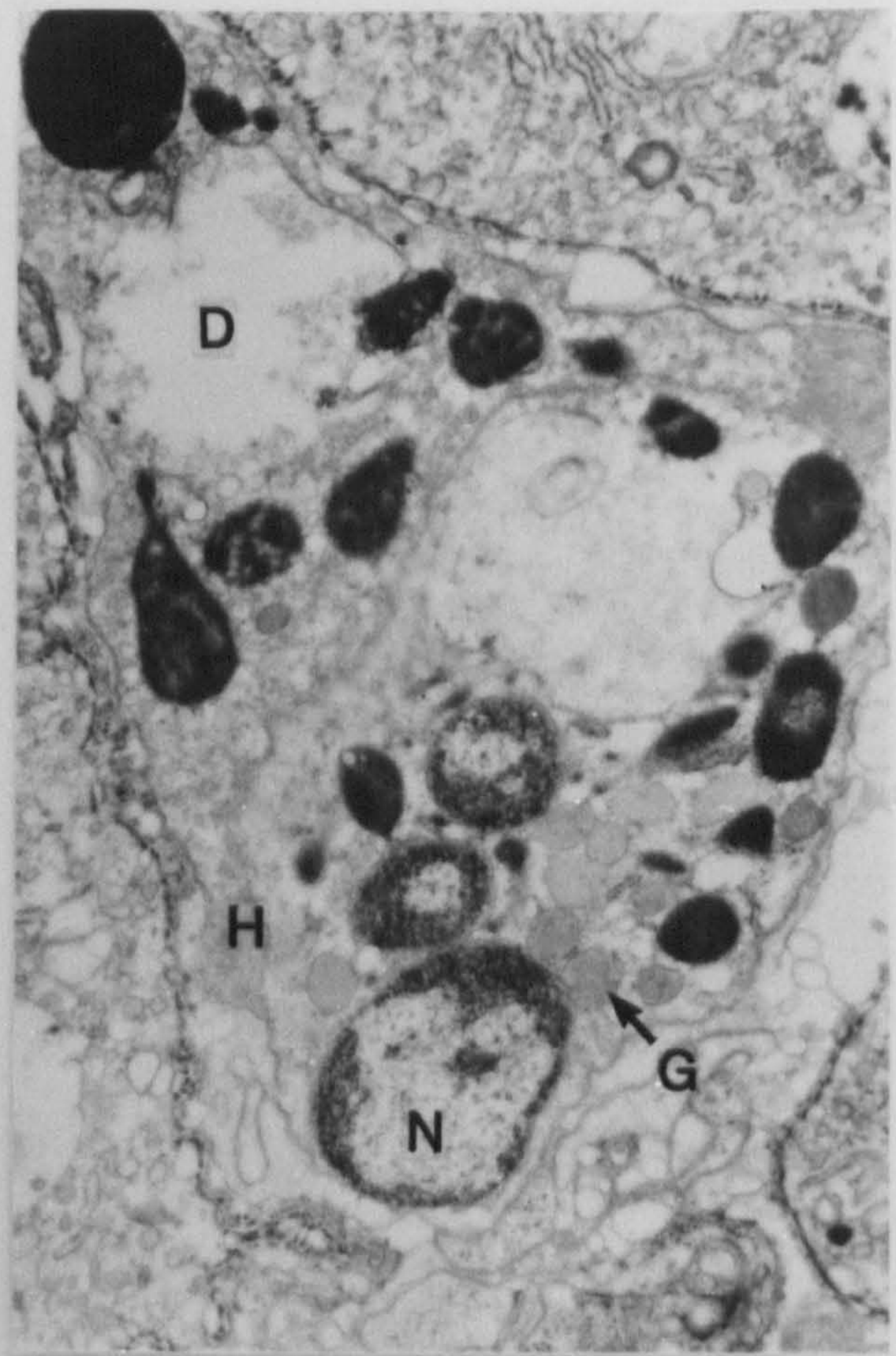


Fig. 9.11 2 μm

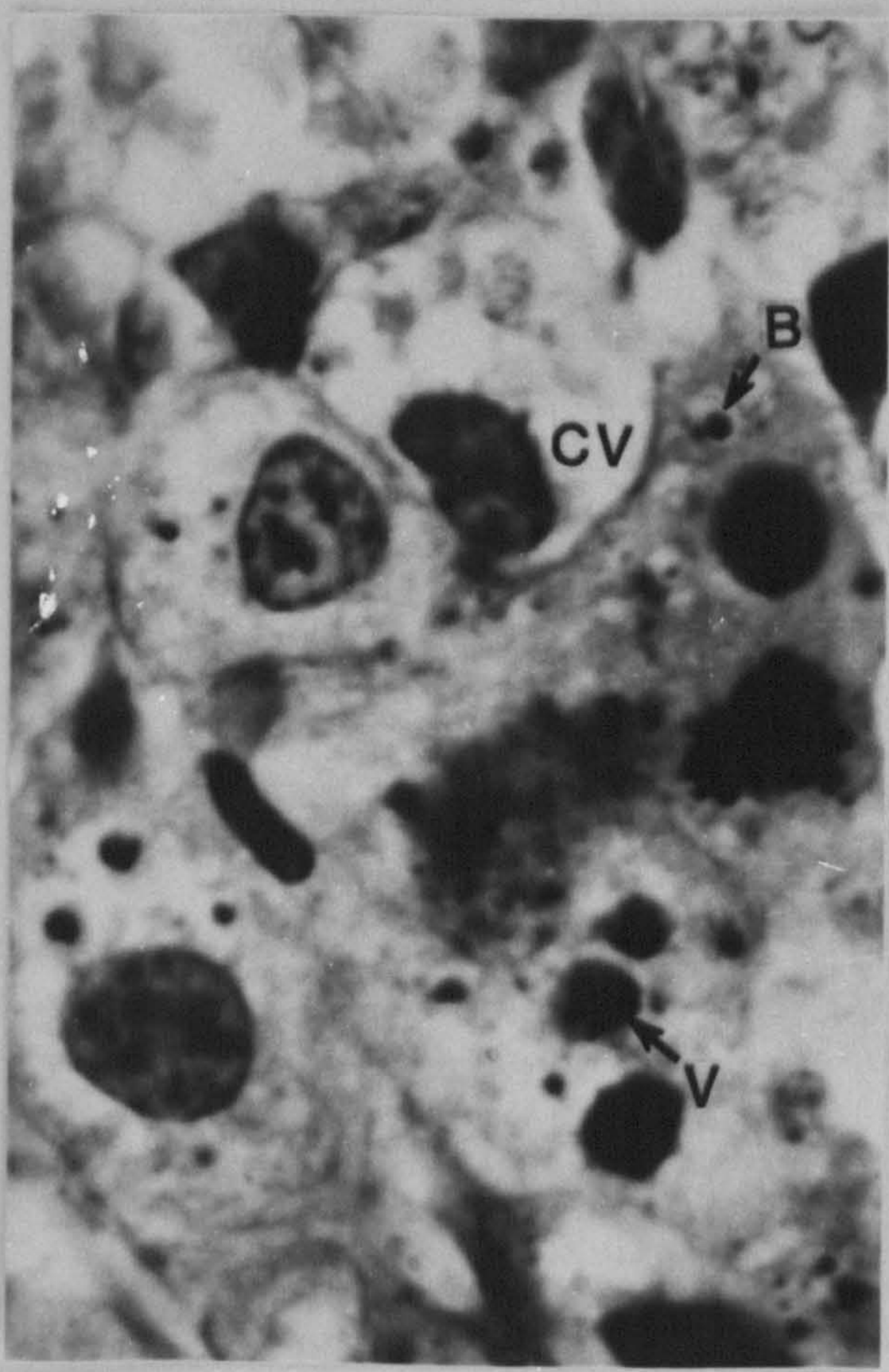


Fig. 9.12 5 μm

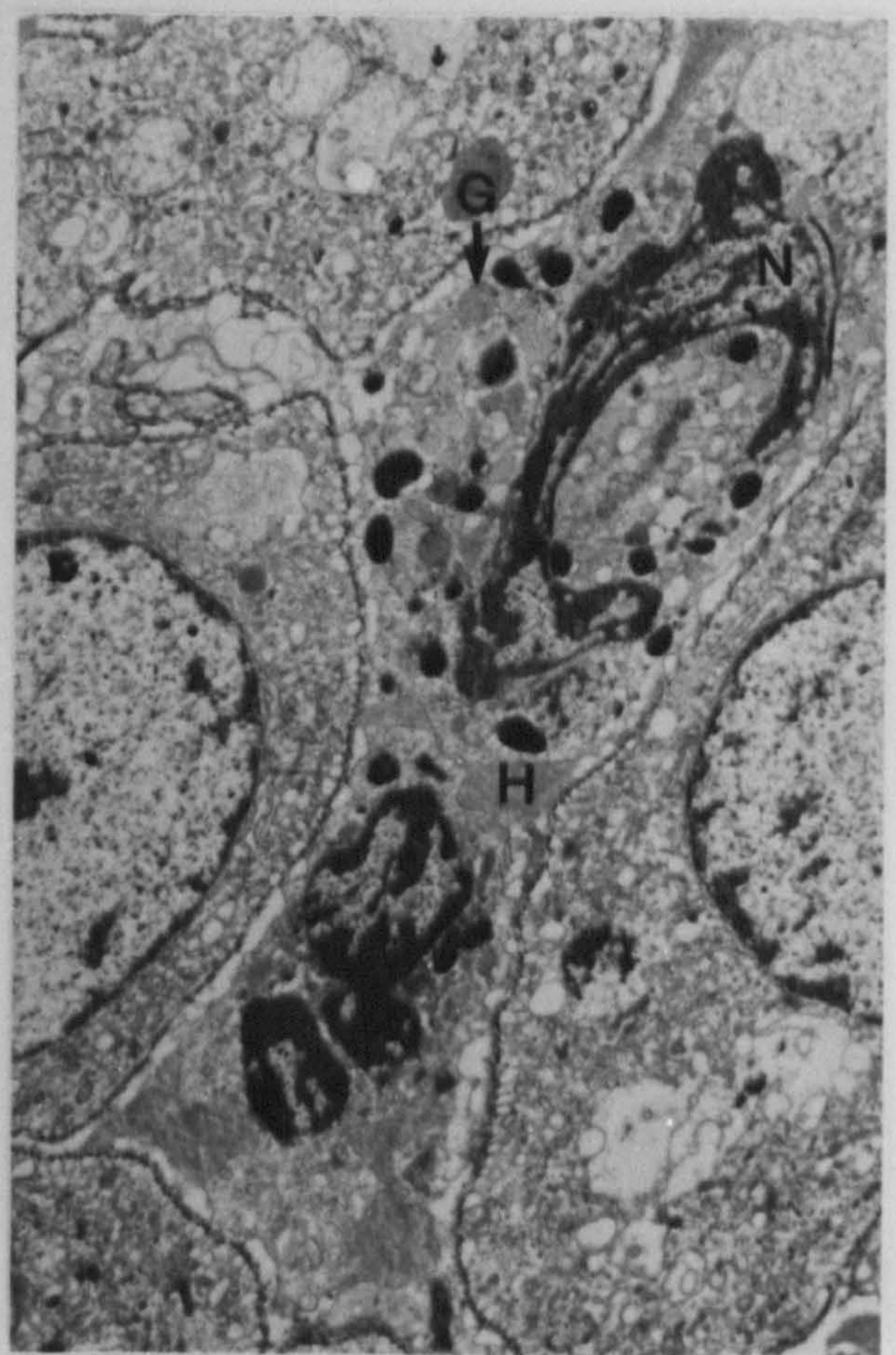


Fig. 9.13 4 μm

large vacuole (V) (fig. 9.15) of the pore cells (P) showed small accumulations of electron-dense material. It is possible that the clear areas (CV) (fig. 9.15) represent accumulations of graphite, which would be translucent when viewed using the TEM. The nuclei (N) of some haemocytes (H) (fig. 9.16) were pleomorphic 4 h after the injection of graphite and the cytoplasm contained small granules (G) of varying density.

Branchial Heart Appendage. (Figures 9.17-9.22)

Circulating haemocytes (H) in the branchial heart appendage of a control animal (fig. 9.17) showed a few small granules present in the cytoplasm. The branchial heart appendage of the control animal contained various large clear blood spaces (BS). The circulating haemocytes (H) seen in a blood vessel (BV) 4 h post-injection (fig. 9.18) demonstrated a few cytoplasmic granules (G), however particles which resemble phagocytosed bacteria (B), indicated by a darker stained material surrounded by a lighter stained matrix, were visible in the cells of the appendage. A TEM section demonstrated an haemocyte (H) (fig. 9.19) containing cytoplasmic granules (G) and possible phagocytosed bacteria (B), a blood vessel (BV) and podocyte-type processes (PD) (after Witmer & Martin, 1973; Schipp & Hevert, 1981). Haemocytes (H) present in the branchial heart appendage 48 h after bacterial injection (fig. 9.20) contained various granules (G), however, a few also contain vacuoles of darker stained material which resemble phagocytosed bacteria (B). The haemocyte (H) (fig. 9.21) in the TEM section 48 h post-challenge showed electron-dense granules (G), clear

Figure 9. 14. H&E section of the branchial heart 4 h after colloidal graphite injection. BV = A blood vessel; C = Graphite visible in the blood vessel; P = Pore cell; V = Large vacuole of a pore cell which now contains granules.

Figure 9. 15. TEM section of the branchial heart 4 h after colloidal graphite injection. P = Pore cell; V = Large vacuole of a pore cell which now contains small accumulations of electron-dense material in an electron-lucent matrix; CV = Clear vacuoles which may contain colloidal graphite.

Figure 9. 16. TEM section of a haemocyte from the branchial heart 4 h after colloidal graphite injection.

H = Haemocyte; G = Small granules of varying density in the cytoplasm of the haemocyte; N = Nucleus of haemocyte which is now pleomorphic.

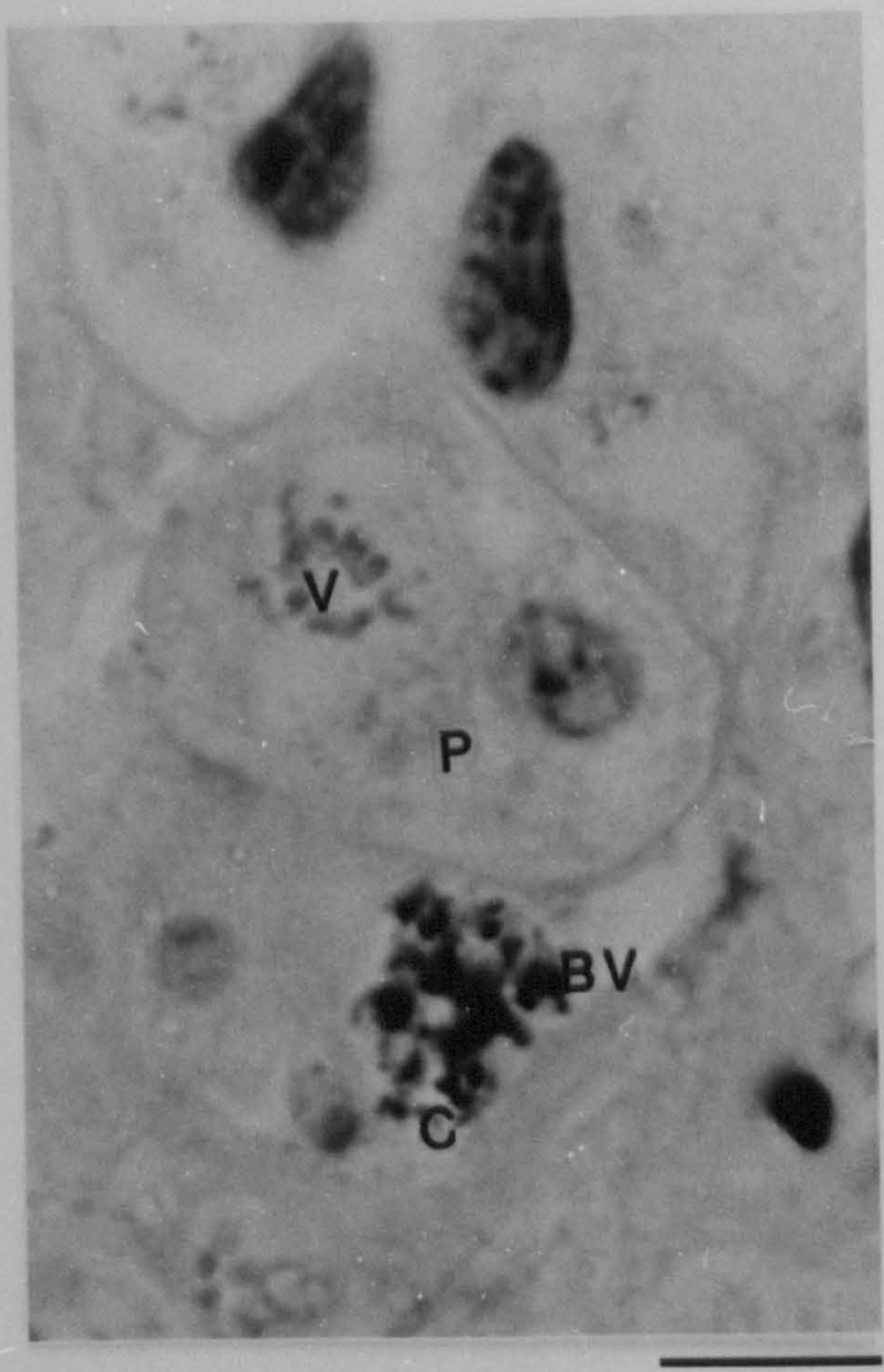


Fig. 9.14 5 µm

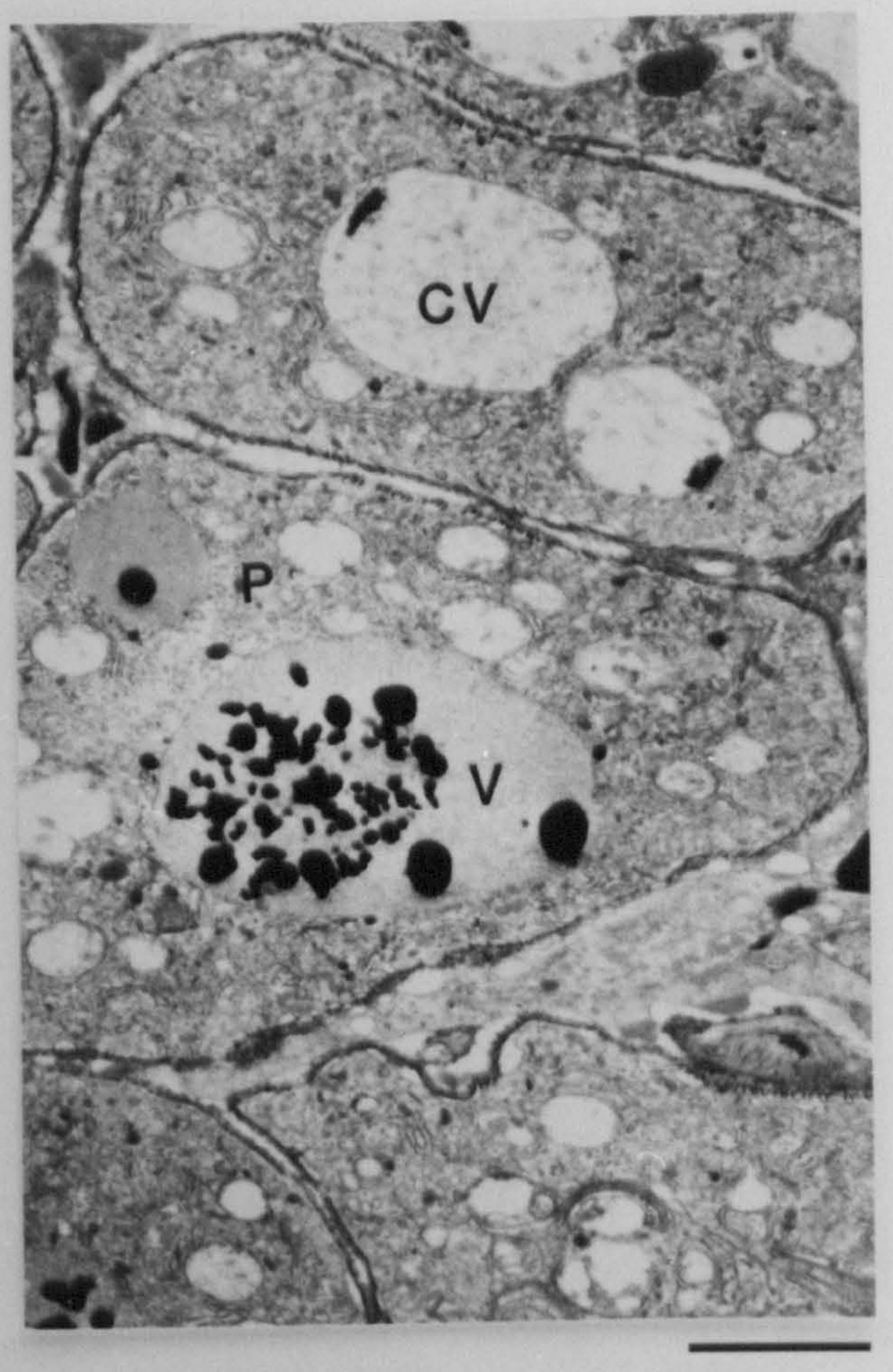


Fig. 9.15 2 µm

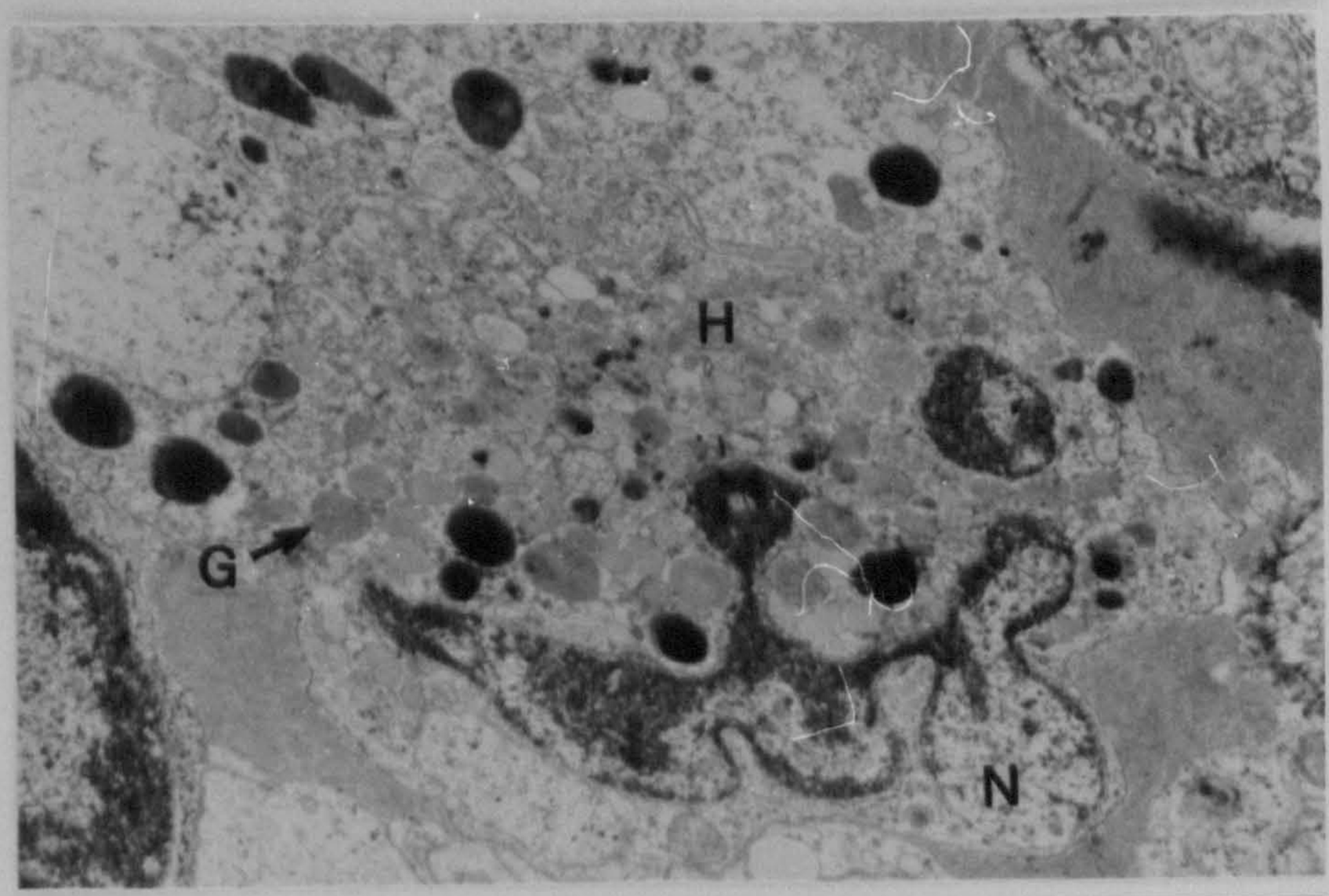


Fig. 9.16 2 µm

Figure 9. 17. Polychrome stained section of the branchial heart appendage of a control animal.

H = Circulating haemocytes; G = Small cytoplasmic granules of an haemocyte; BS = Blood spaces.

Figure 9. 18. Polychrome stained section of the branchial heart appendage 4 h after injection with bacteria.

H = Circulating haemocytes; G = Granules in the cytoplasm of an haemocyte; BV = A blood vessel containing haemocytes; B = Possible bacteria.

Figure 9. 19. TEM section of the branchial heart appendage 4 h after bacterial challenge.

H = An haemocyte; G = Granules in the cytoplasm of the haemocyte; B = Possible bacteria; PD = Podocyte processes surrounding a blood vessel; BV = Blood vessel.

Figure 9. 20. Polychrome stained section of the branchial heart appendage 48 h after injection of bacteria.

H = Haemocytes; G = Cytoplasmic granules of the haemocyte; B = Bacteria in some vacuoles.

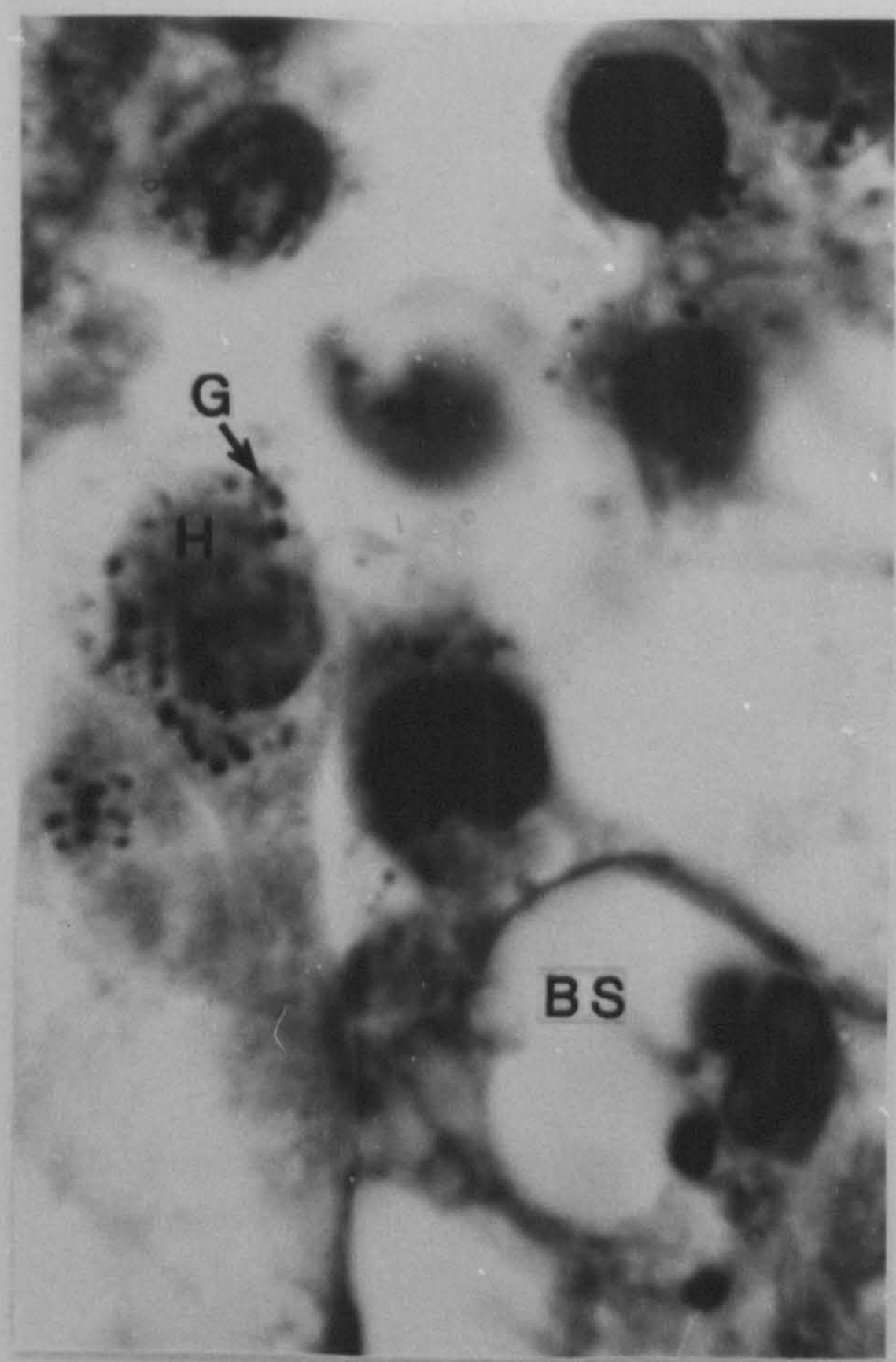


Fig. 9.17 5 μm

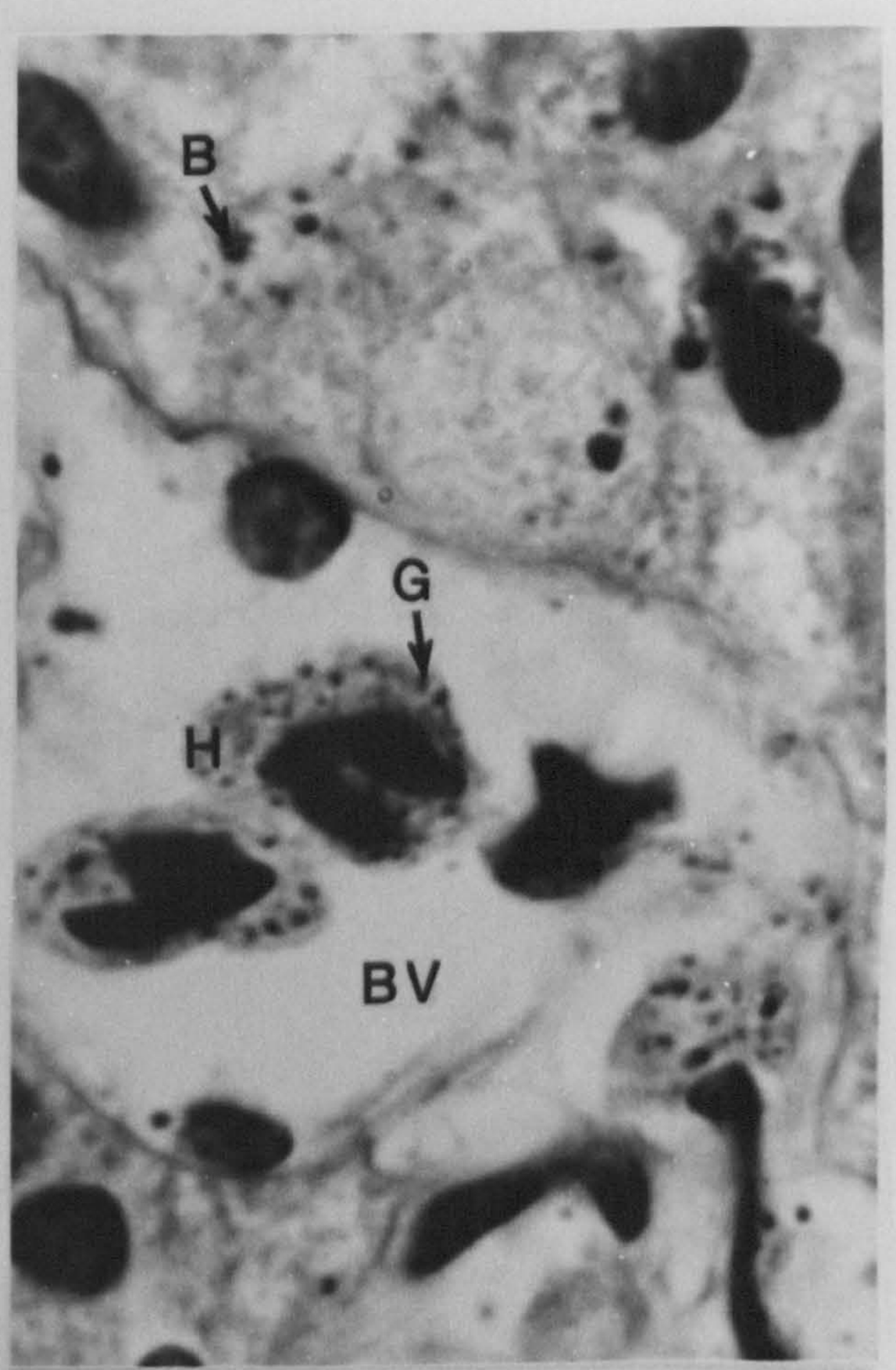


Fig. 9.18 5 μm

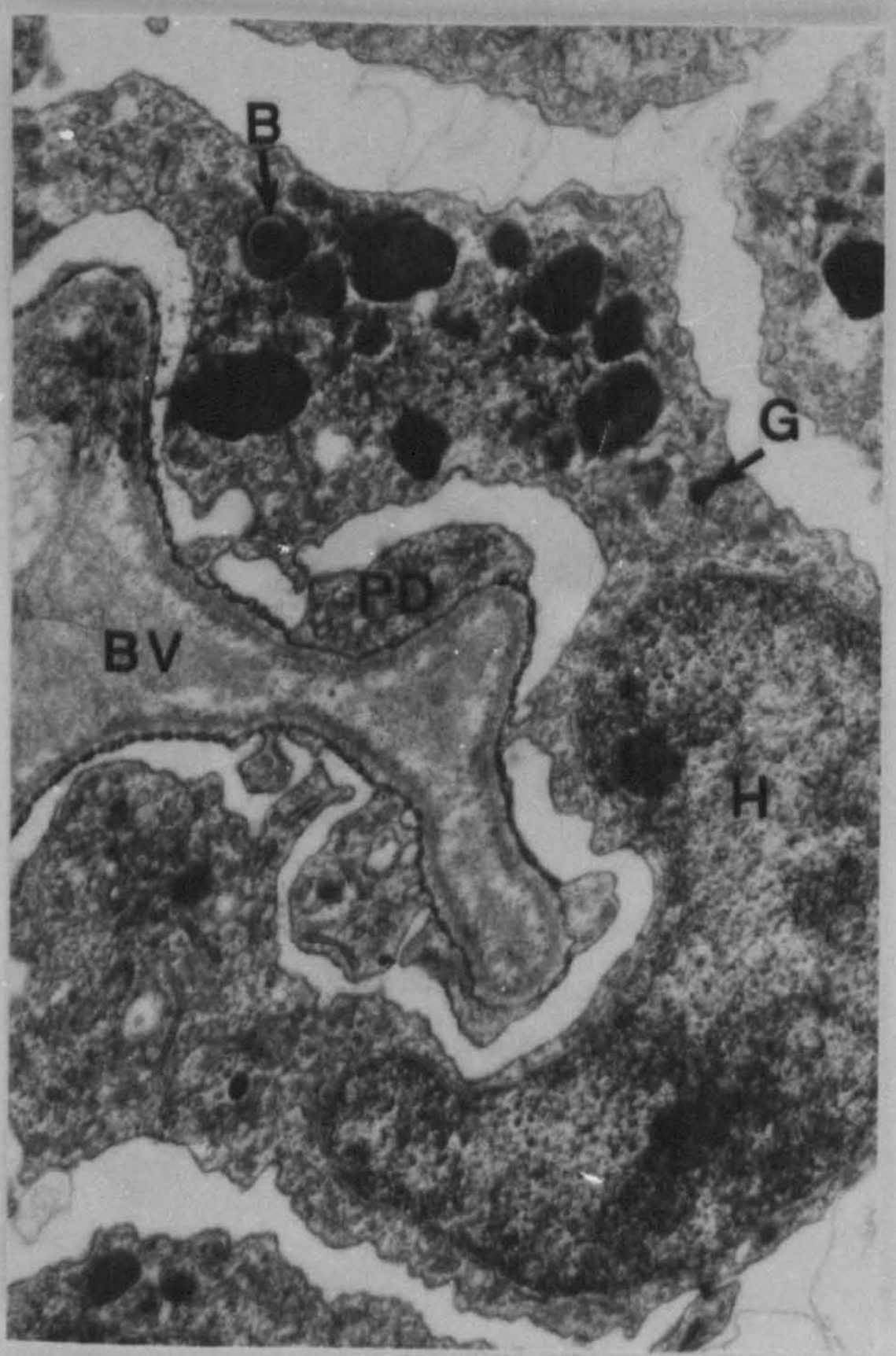


Fig. 9.19 2 μm

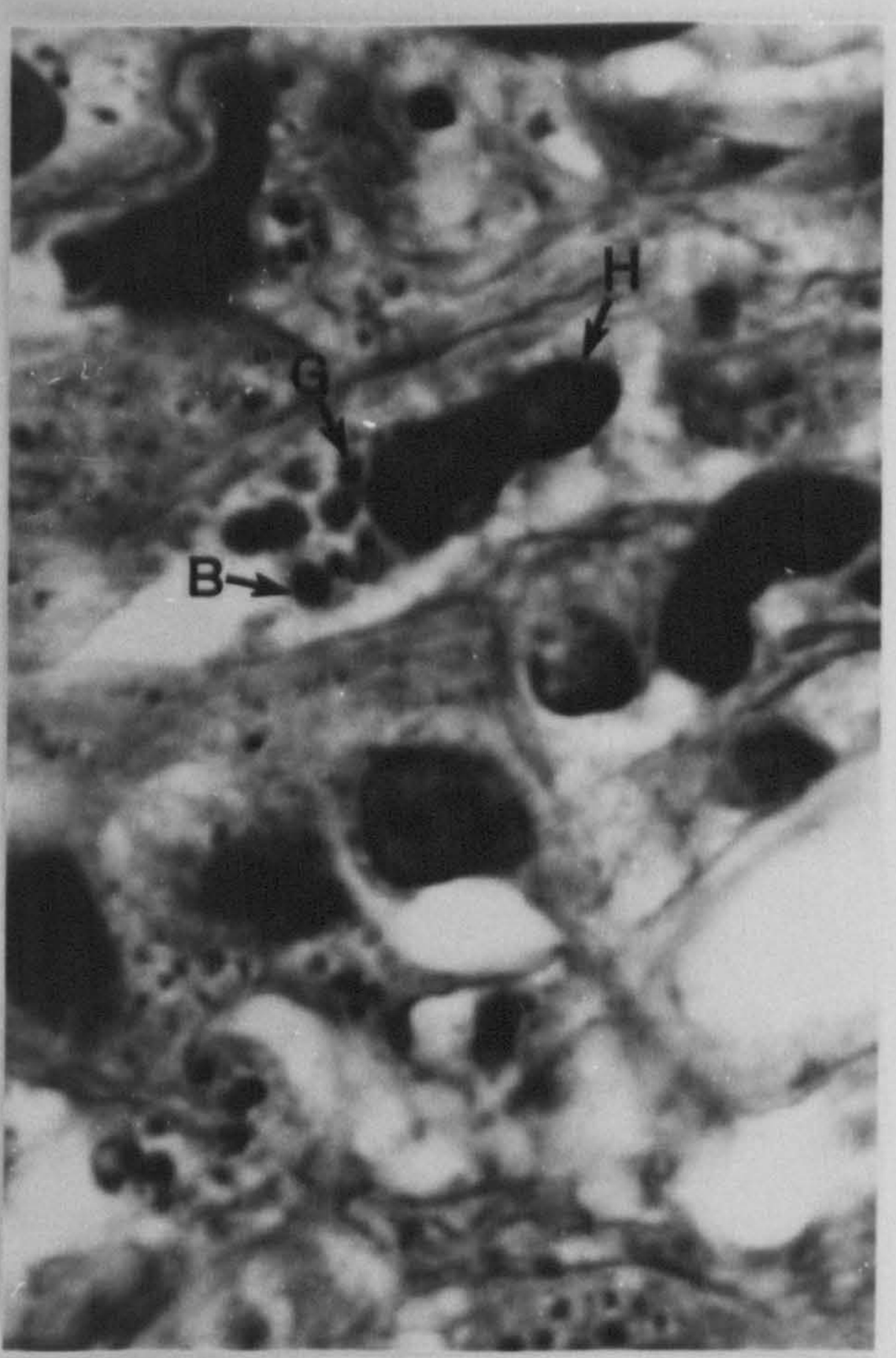


Fig. 9.20 5 μm

vacuoles (CV) which contain possible bacterial breakdown products (D), indicated by the particulate nature of the vaucoles, and also phagocytosed bacteria (B) in the cytoplasm. The haemocyte (H) (fig. 9.21) also contains a few cytoplasmic vacuoles which are crystalline (M) in appearance. Colloidal graphite (C) was found aggregrated in a blood vessel (BV) (fig. 9.22) 4 h post-injection.

White Body. (Figures 9.23-9.28)

Histological sections taken from a control animal (fig. 9.23) showed free mature circulating haemocytes (H) which contained a variety of granules, and attached maturing haemocytes (MH) with large granules (LG) in the cytoplasm (after Cowden, 1972; Cowden and Curtis 1974, 1981). Large granules (LG) and granules which resemble phagocytosed bacteria (B) (fig. 9.24) were visible in the attached maturing haemocytes (MH) 4 h post-injection. The circulating haemocytes (fig. 9.24) contained numerous small granules (G) as well as granules which contain possible bacteria (B). Forty-eight hours after bacterial injection (fig. 9.25) the maturing haemocytes (MH) contain granules (LG), areas (CV) where material (possibly bacterial) breakdown has occurred and granules which resemble phagocytosed bacteria (B). The circulating haemocytes contain small granules (G) as well as large darkly stained particles, possibly bacteria (B), in the cytoplasm. A TEM section of the white body 48 h post-injection showed an haemocyte (H) which contained small cytoplasmic granules (G) (figs. 9.26 & 9.27), and bacteria (B) (fig. 9.26) surrounded by a double membrane,

Figure 9. 21. TEM section of the branchial heart appendage 48 h after the injection of bacteria.

H = Haemocyte; G = Granules in the cytoplasm of the haemocyte; CV = Vacuoles containing bacteria or bacterial breakdown products; D = Bacterial breakdown products; B = Bacteria in the process of digestion; M = A crystalline material present in some vacuoles.

Figure 9. 22. H&E stained section of the branchial heart appendage 4 h after colloidal graphite injection.

BV = A blood vessel; C = Graphite aggregated in a blood vessel.

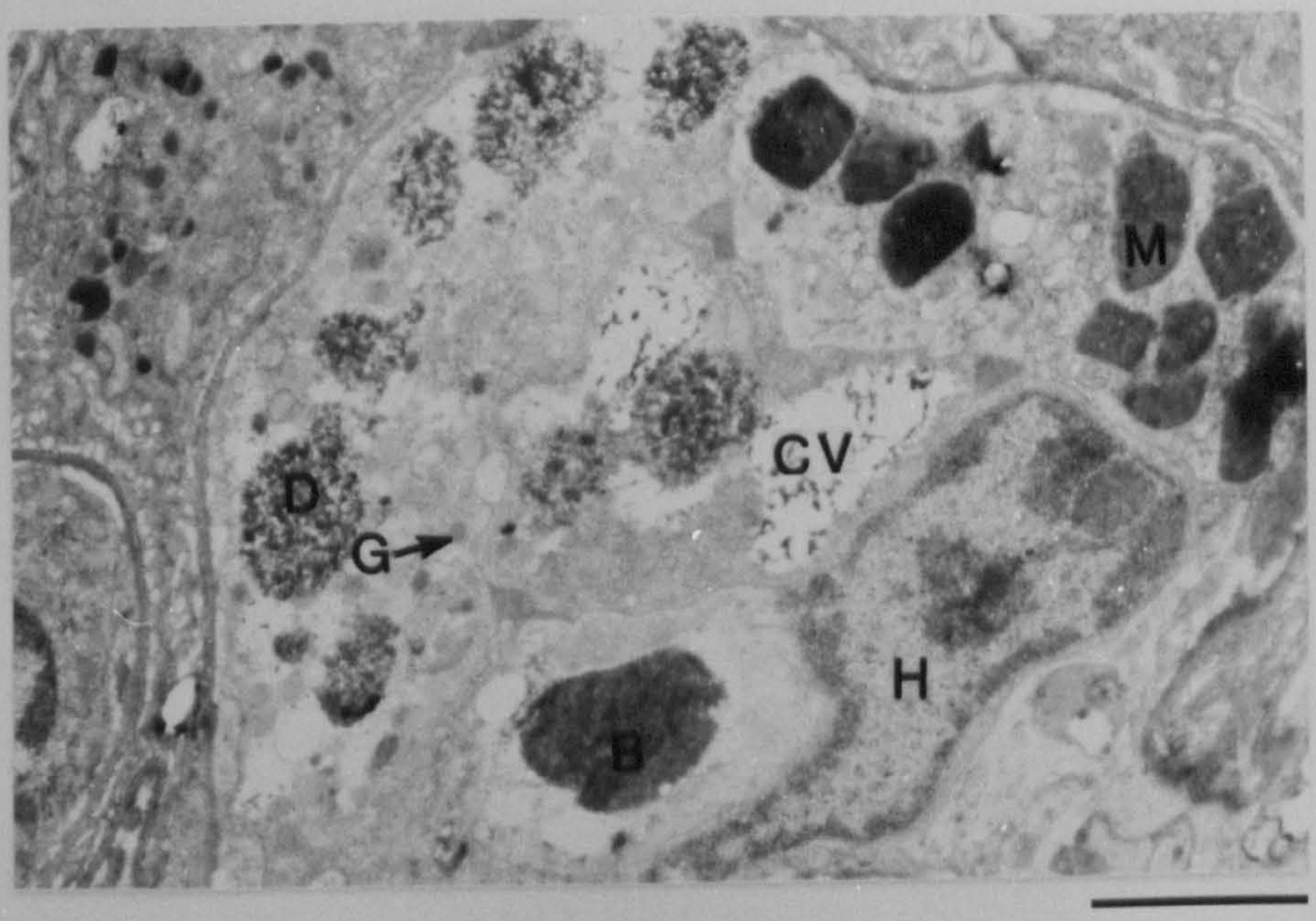


Fig. 9.21

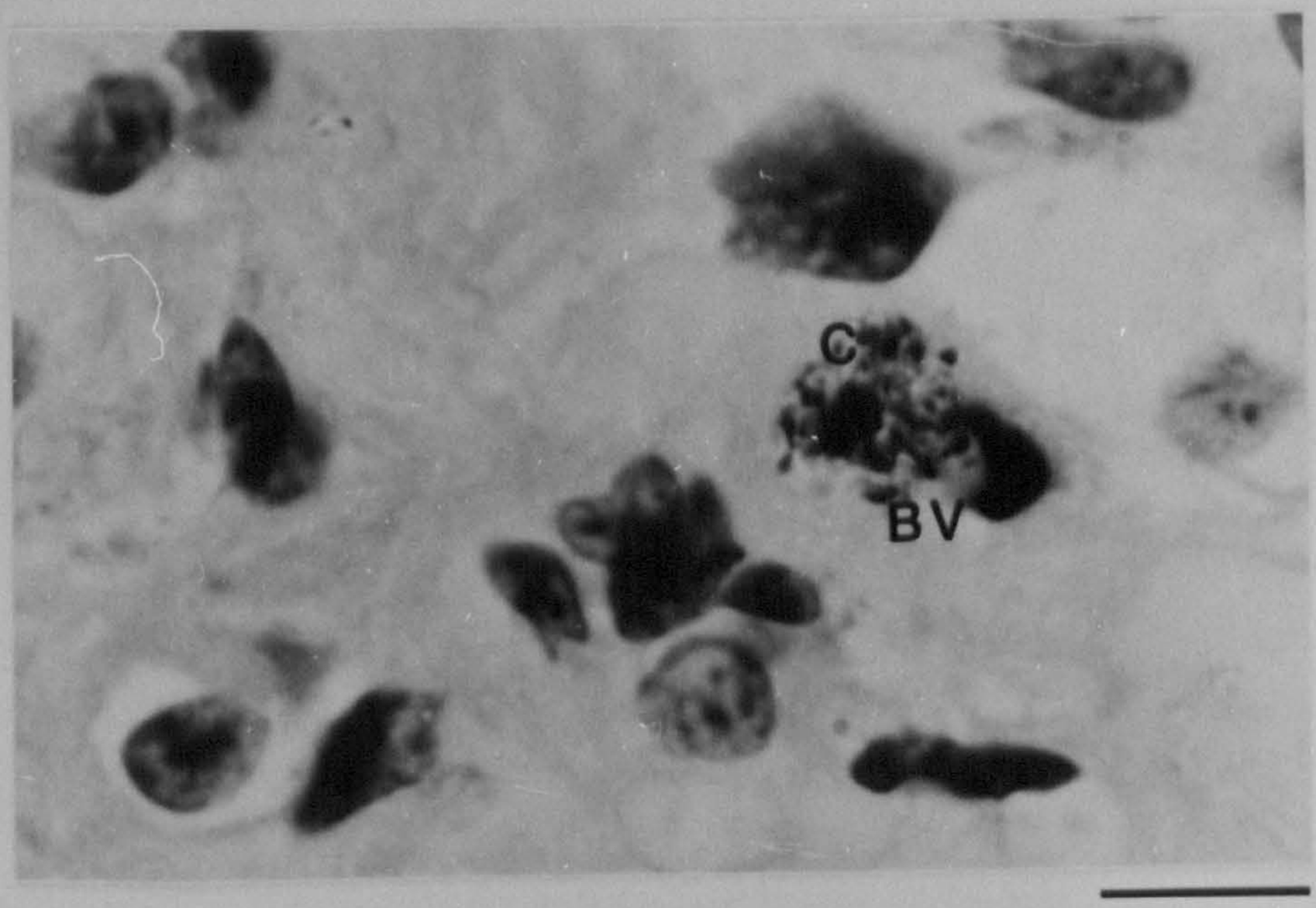


Fig. 9.22

Figure 9. 23. Polychrome stained section of the white body of a control animal.

H = Circulating haemocyte; G = Granules in the cytoplasm of the haemocytes; MH = Maturing haemocytes; LG = Large granules in the cytoplasm of maturing haemocytes.

Figure 9. 24. Polychrome stained section of the white body of an experimental animal 4 h after bacterial injection.

H = Circulating haemocytes; G = Cytoplasmic granules of the haemocytes; MH = Maturing haemocytes; LG = Granules of the maturing haemocytes; B = Possible phagocytosed bacteria in the granules of the maturing and circulating haemocytes.

Figure 9. 25. Polychrome stained section of the white body 48 h after bacterial injection.

H = Circulating haemocytes; G = Granules in the cytoplasm of the haemocytes; B = Phagocytosed bacteria in the cytoplasm of the haemocytes; MH = Maturing haemocytes; LG = Granules in the cytoplasm of maturing haemocytes, only a few are present; CV = Clear vacuoles containing degraded bacteria.

Figure 9. 26. TEM section of the white body of an experimental animal 48 h after the injection of bacteria.

N = Nucleus of an haemocyte; B = Degrading bacteria in the cytoplasm of the haemocyte; G = Cytoplasmic granules of the haemocyte.

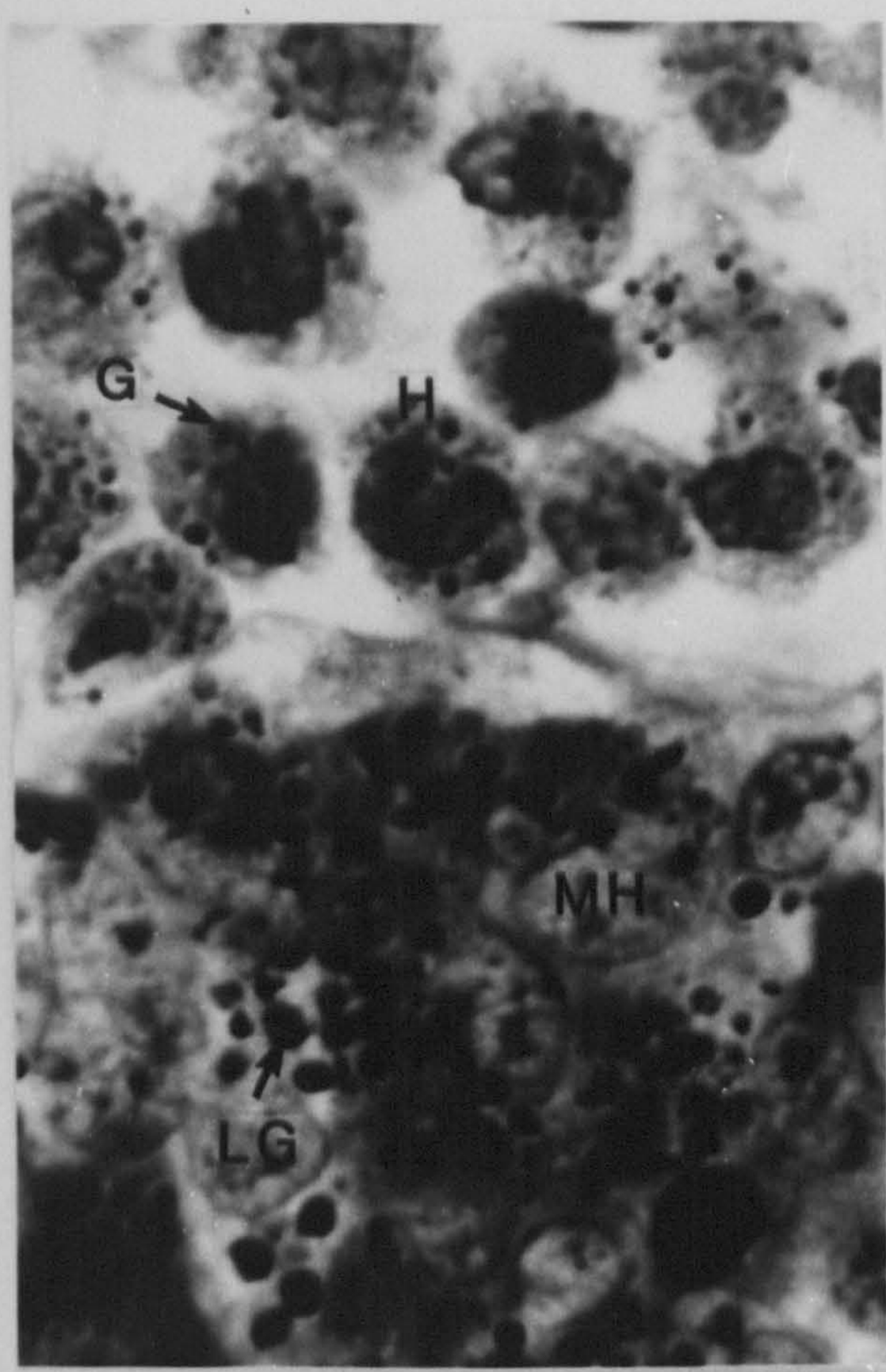


Fig. 9.23 6 μm

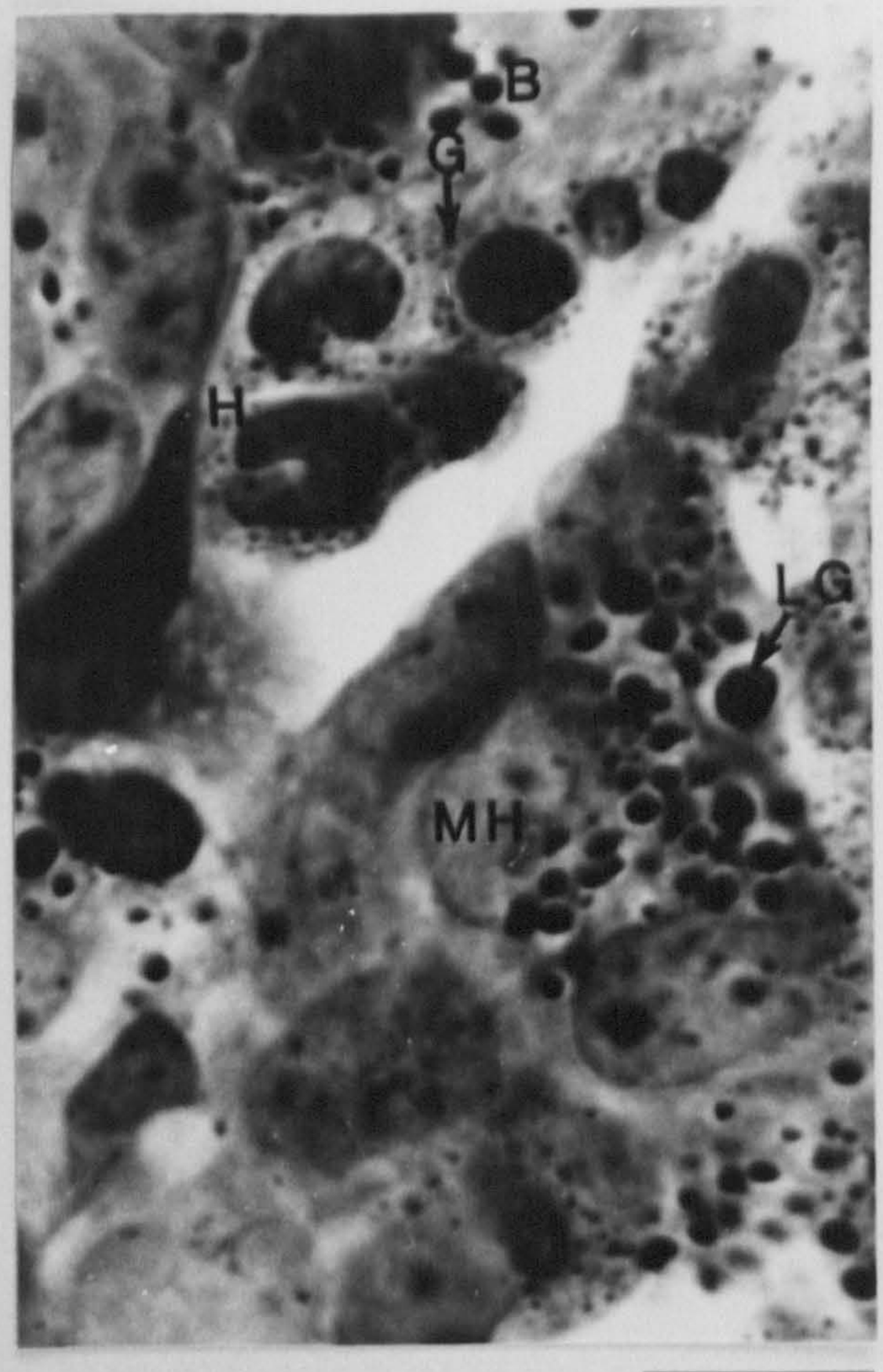


Fig. 9.24 6 μm

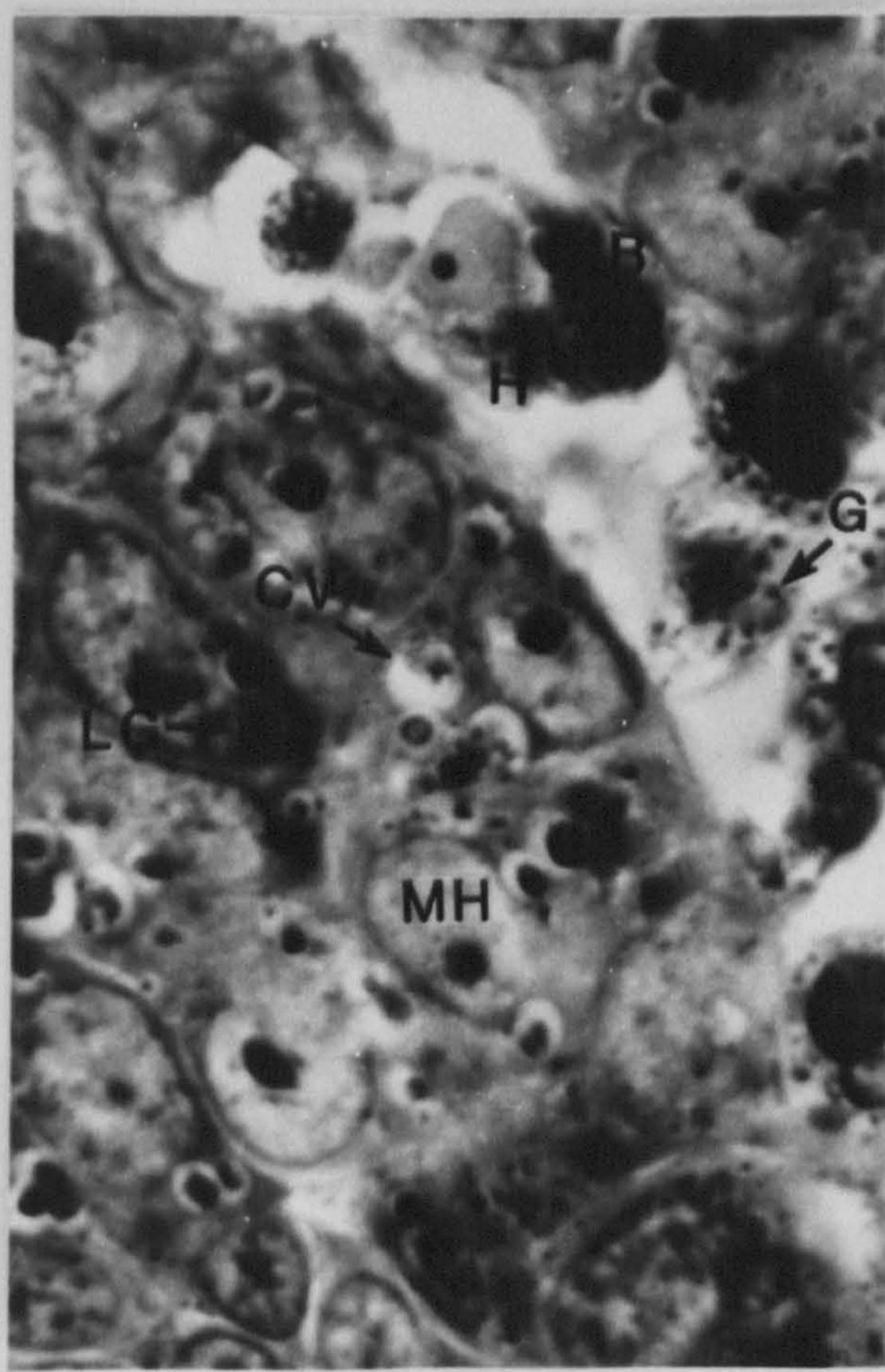


Fig. 9.25 6 μm

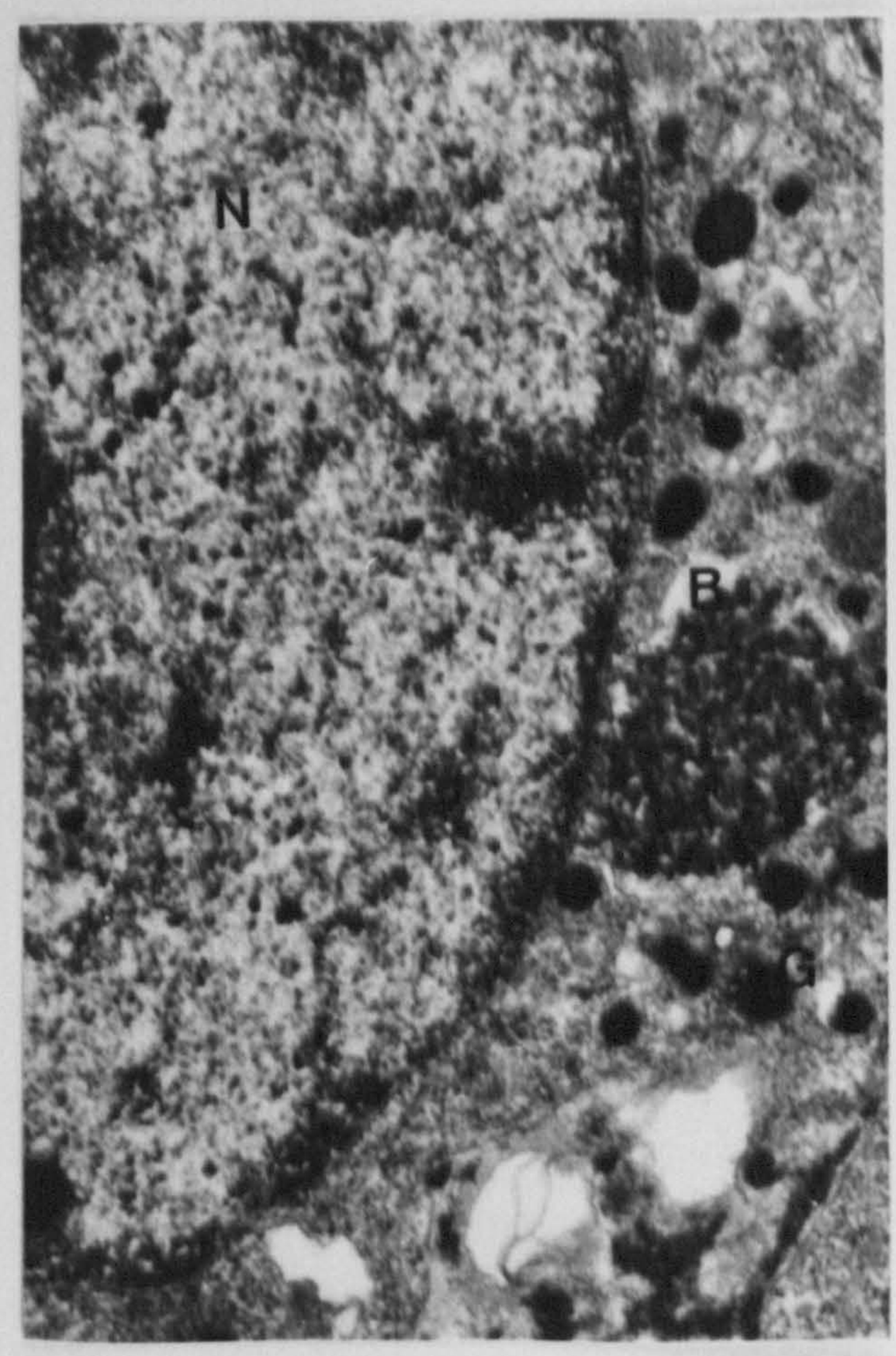


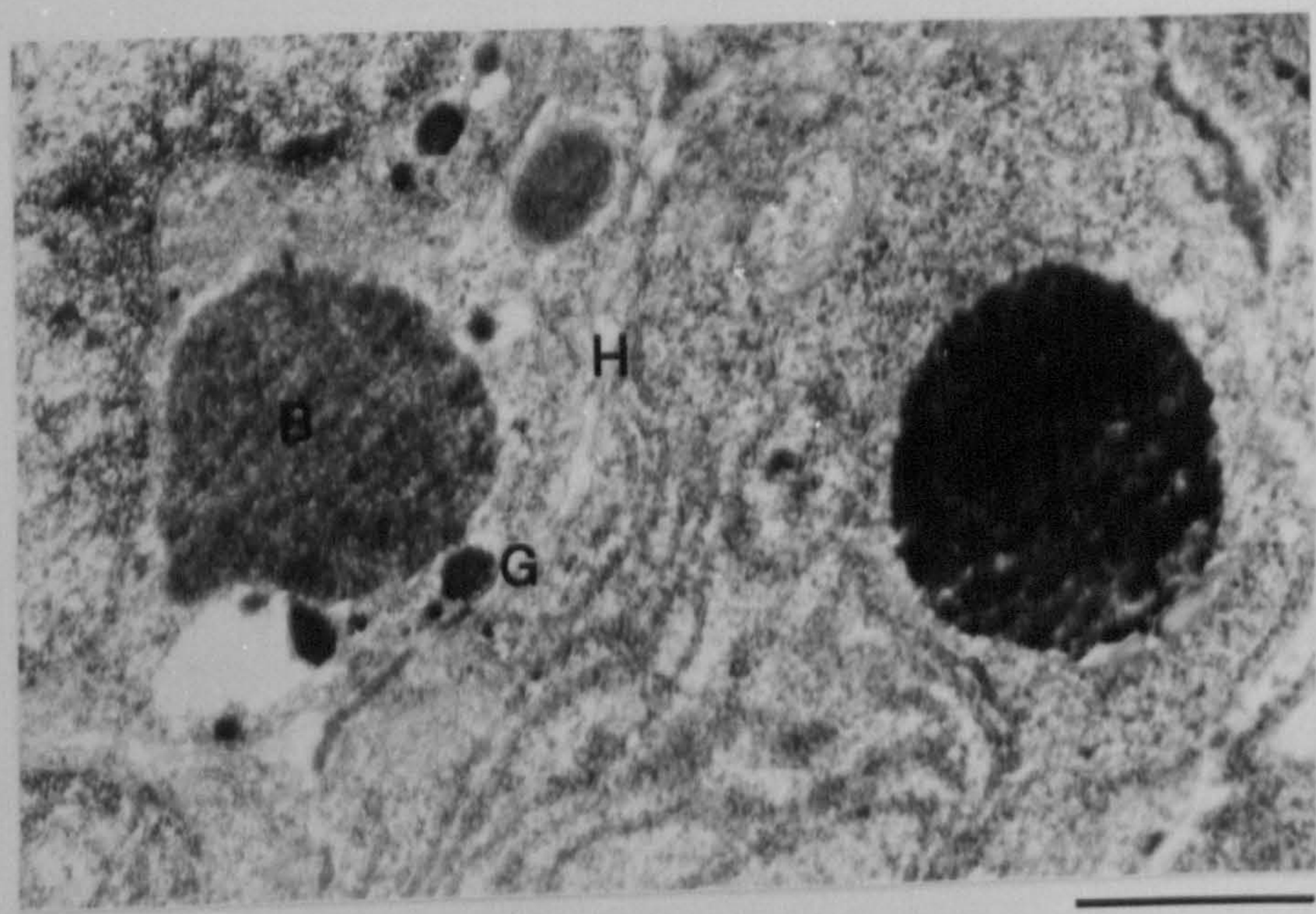
Fig. 9.26 0.9 μm

Figure 9. 27. TEM section of the white body 48 h after bacterial injection.

H = Haemocytes; B = Bacteria in the cytoplasm of the haemocytes; G = Granules in the cytoplasm of the haemocytes.

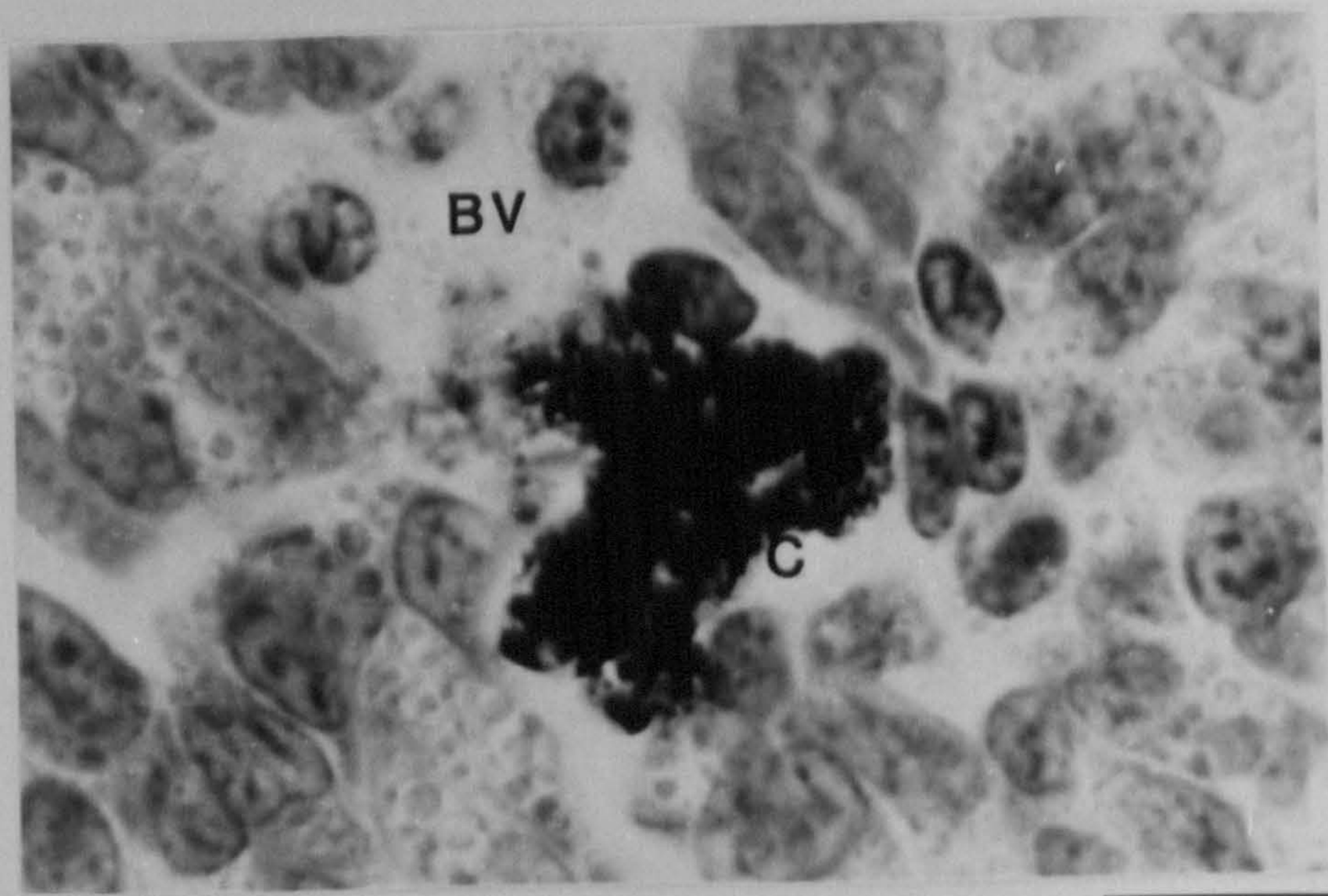
Figure 9. 28. H&E stained section of the white body 4 h after the injection of colloidal graphite.

BV = A blood vessel; C = Graphite contained within a delimited blood space.



0.8 μm

Fig. 9.27



6 μm

Fig. 9.28

undergoing degredation in the cytoplasm. Colloidal graphite (C) was located in a blood vessel (BV) of the white body (fig. 9.28) 4 h post-injection.

Haemocytes (Figures 9.29-9.34)

A TEM section of a haemocyte (H) sampled at 0 h from a control animal (fig. 9.29) showed the bi-lobed nucleus (N) and various electron-dense granules (G). The number of granules (G) present in the cytoplasm of the circulating haemocytes (H) increase in number, and larger granules are present, at sampling times of 4 h (fig. 9.31) and 24 h (fig. 9.32) compared to 0 h (fig. 9.30) from control animals. However, some haemocytes sampled 4 h (fig. 9.33) and 24 h (fig. 9.34) post-injection showed a decrease in the number of granules (G). Both at 4 h (fig. 9.33) and at 24 h (fig. 9.34) post-injection various particles which resemble bacteria (B), shown by dark stained material in non-stained matrices, and bacteria in various stages of breakdown were present in the cytoplasm of the haemocytes.

Figure 9. 29. TEM section of a haemocyte obtained from the 0 h sample.

H = Haemocyte; N = Nucleus of an haemocyte; G = Various electron-dense granules in the cytoplasm of the haemocyte.

Figure 9. 30. Polychrome stained section of haemocytes obtained from the 0 h sample.

H = Haemocyte; G = Cytoplasmic granules of the haemocyte.

Figure 9. 31. Polychrome stained section of haemocytes sampled from the control animals 4 h after the initial (0 h) sample.

H = Haemocytes; G = More and slightly larger cytoplasmic granules in the haemocytes.

Figure 9. 32. Polychrome stained section of haemocytes sampled from the control animals 24 h after the initial (0 h) sample.

H = Haemocytes; G = Granules in the cytoplasm of the haemocytes, more are present than at 0 h.

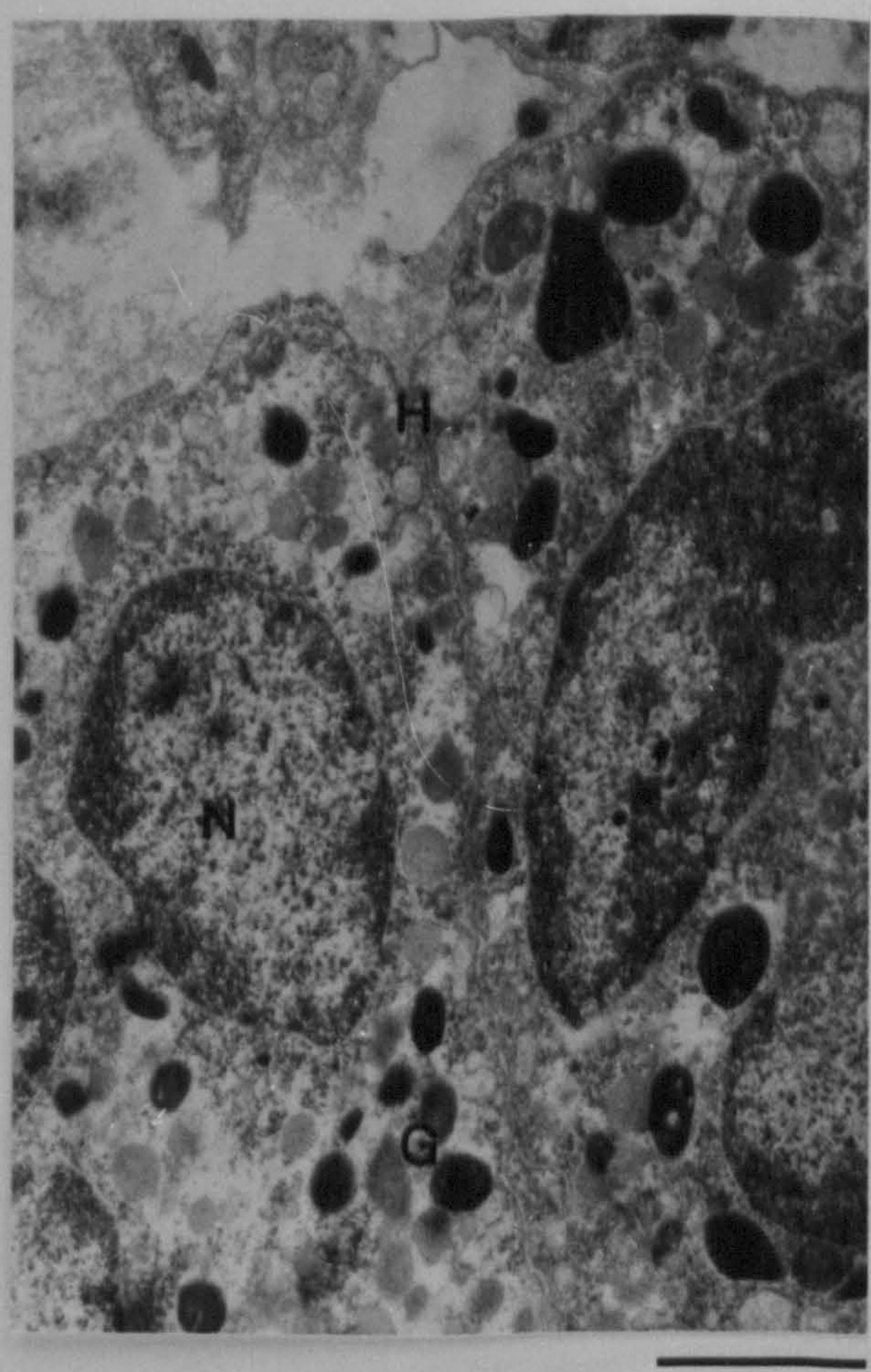


Fig. 9.29 5 μm

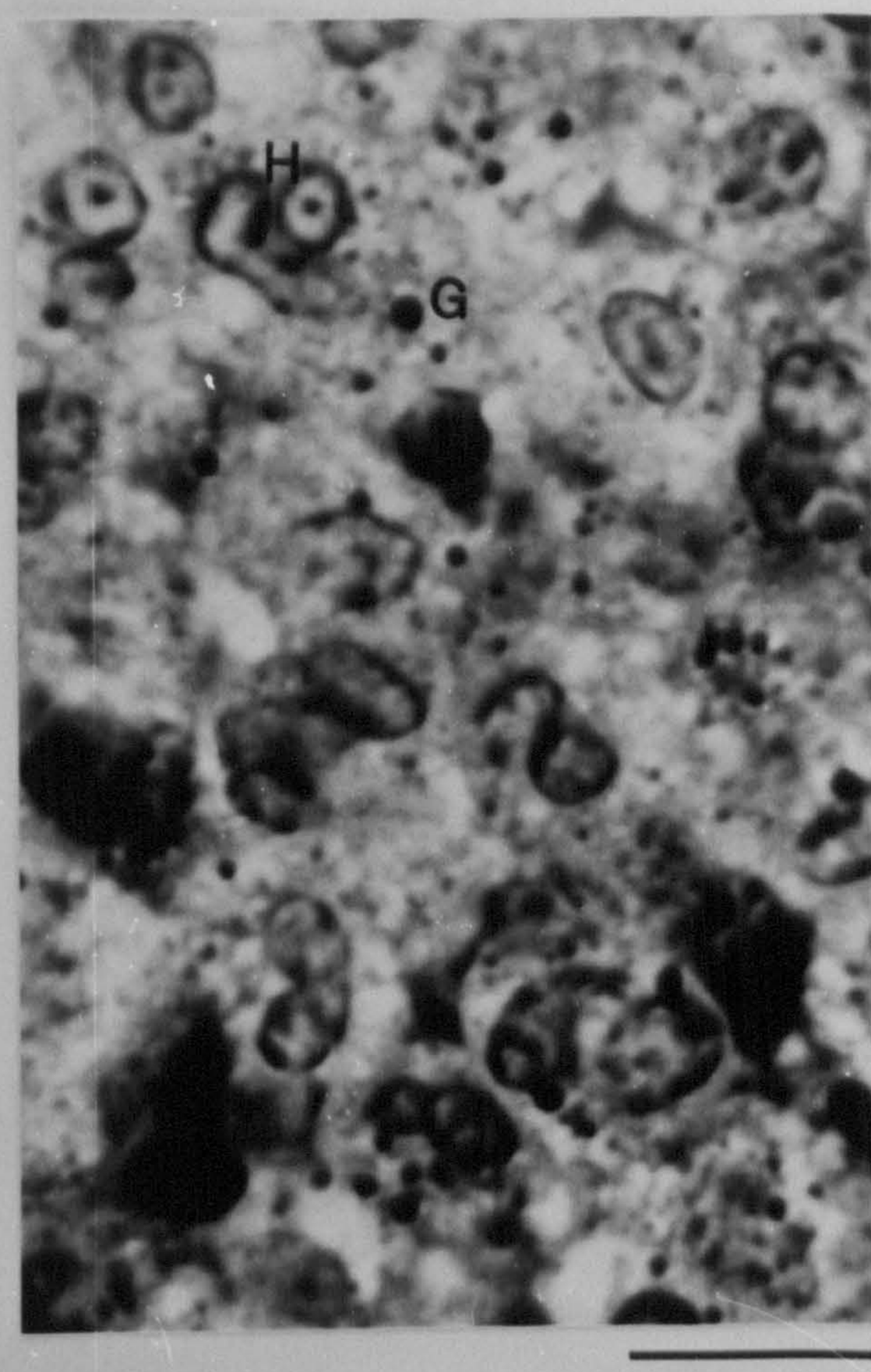


Fig. 9.30 2 μm

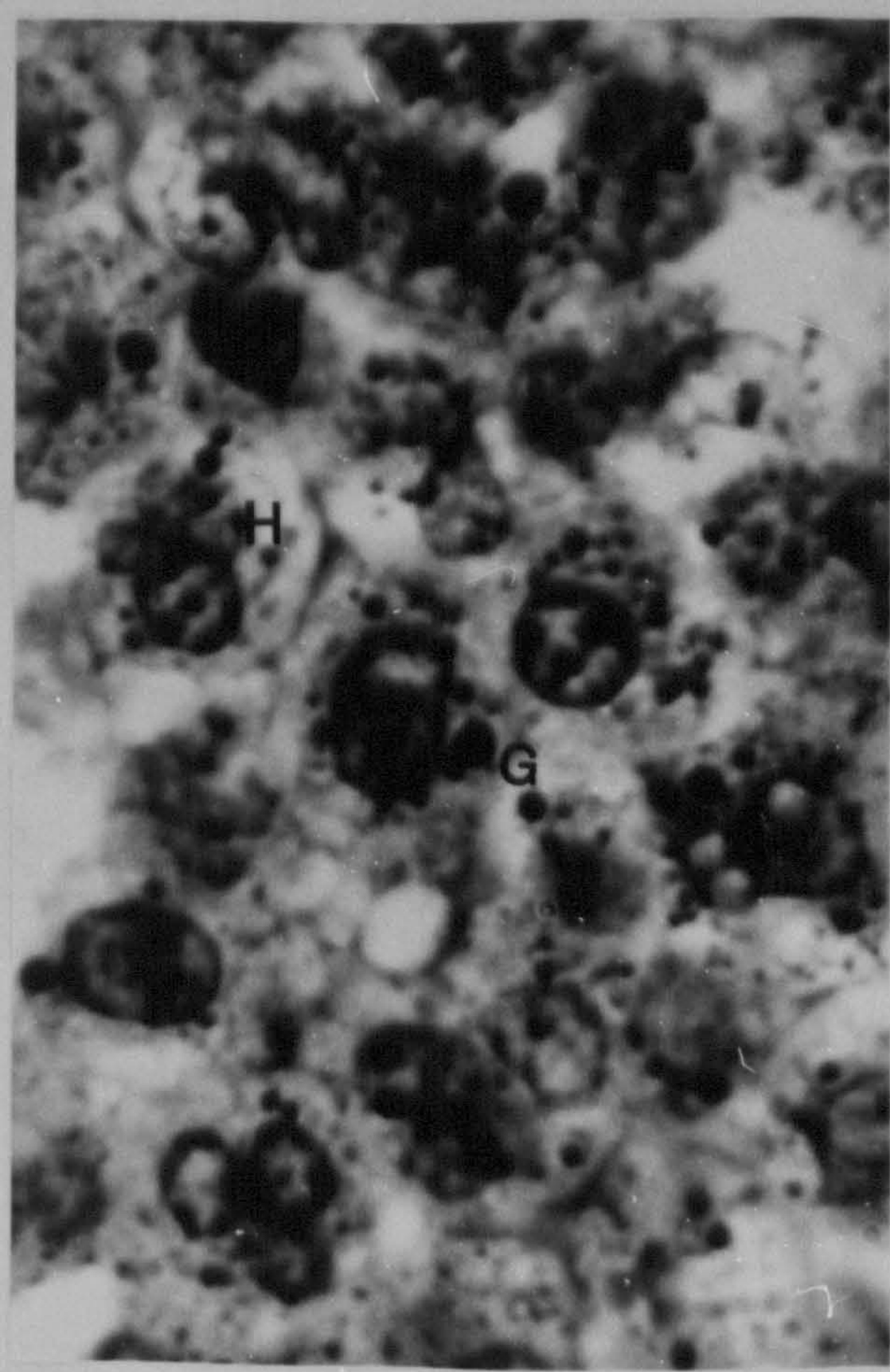


Fig. 9.31 5 μm

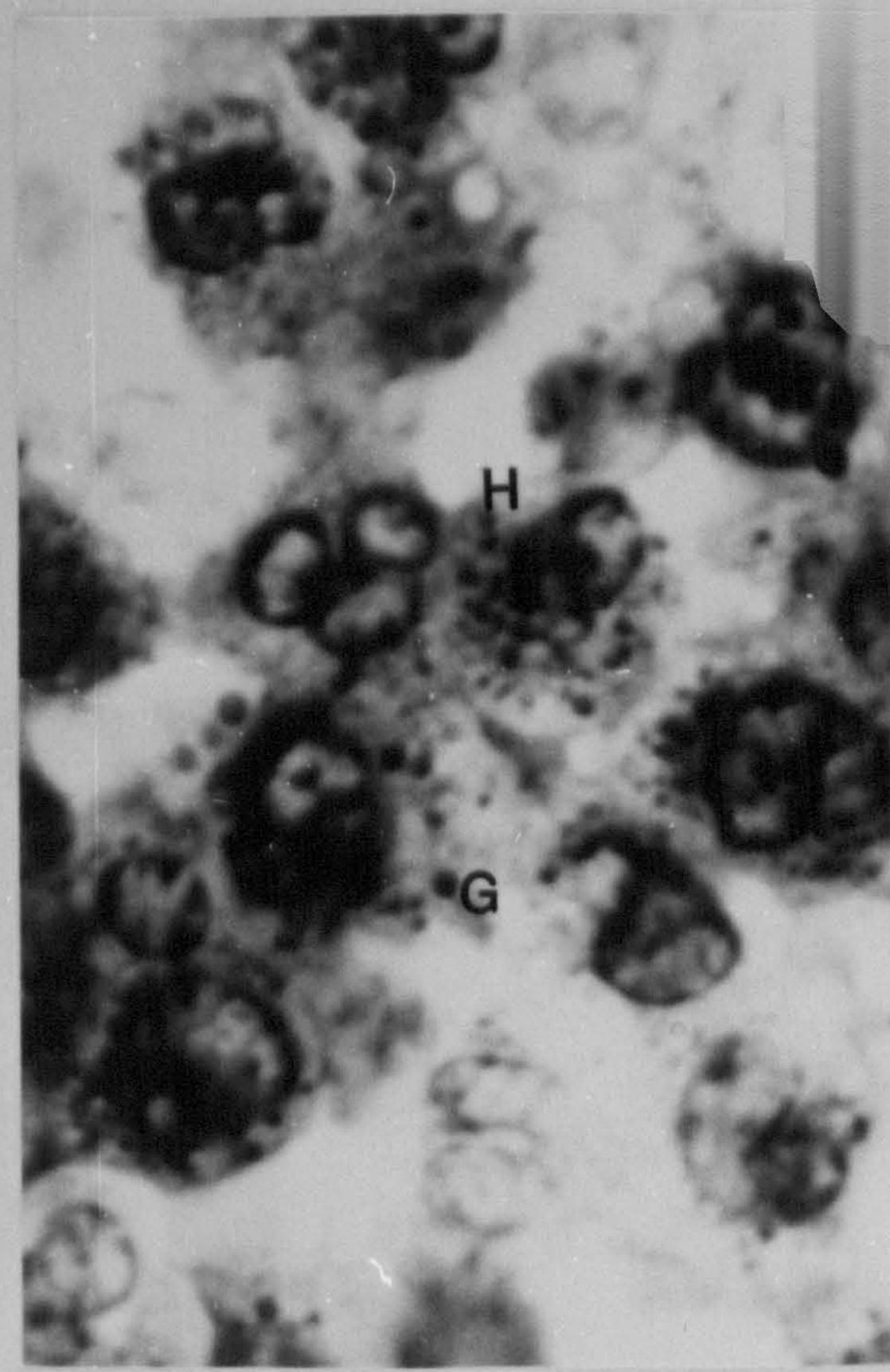


Fig. 9.32 5 μm

Figure 9. 33. Polychrome stained section of haemocytes sampled 4 h after the injection of bacteria.

H = Haemocytes; B = Bacteria in various stages of breakdown; G = Haemocyte cytoplasmic granules. Note less granules are present compared to the control 4 h sample.

Figure 9. 34. Polychrome stained section of haemocytes sampled 24 h after bacterial challenge.

H = Haemocytes; B = Bacteria in various stages of breakdown; G = Granules in the cytoplasm of the haemocytes. Note very few granules are present.

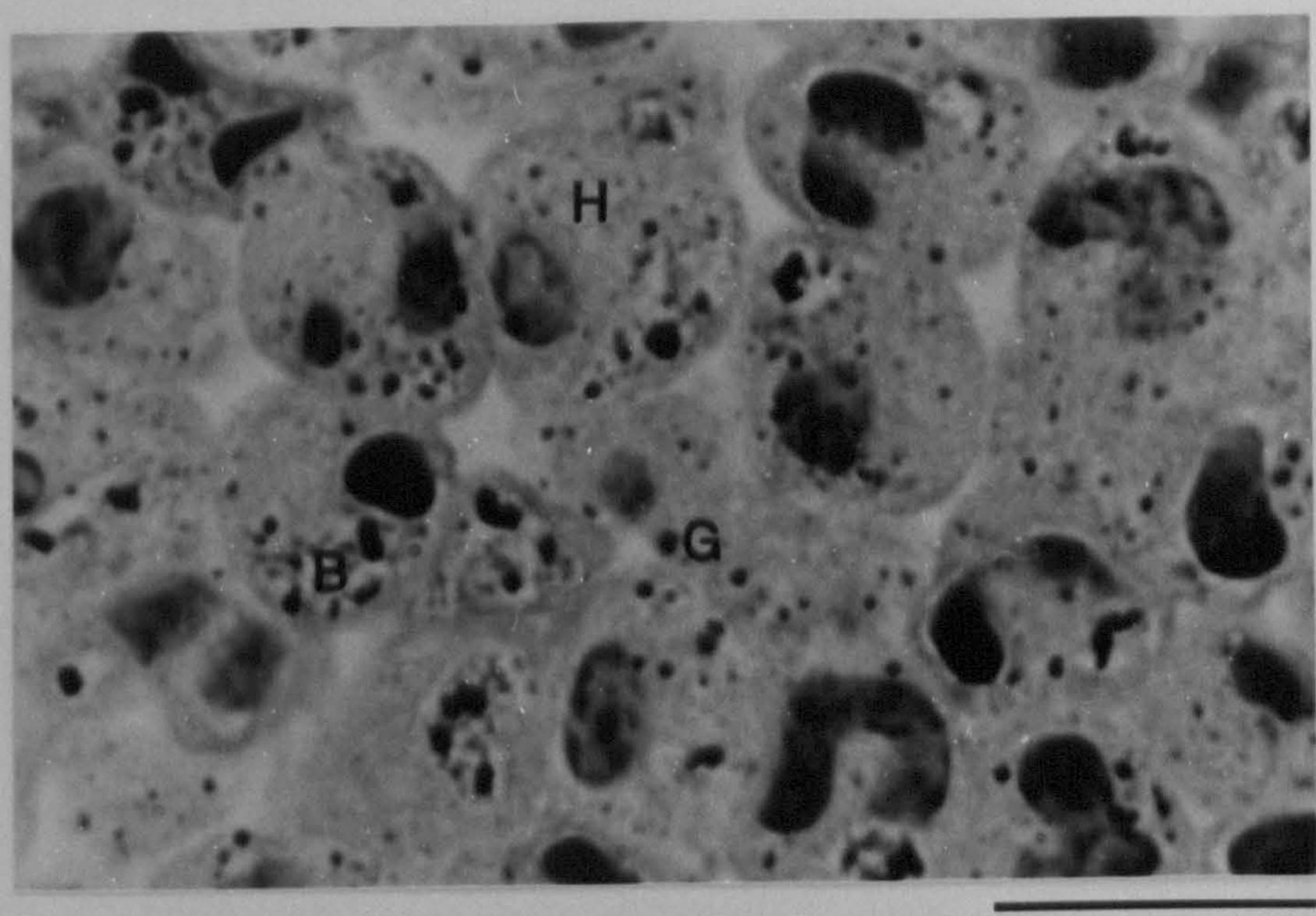


Fig. 9.33

10 μm

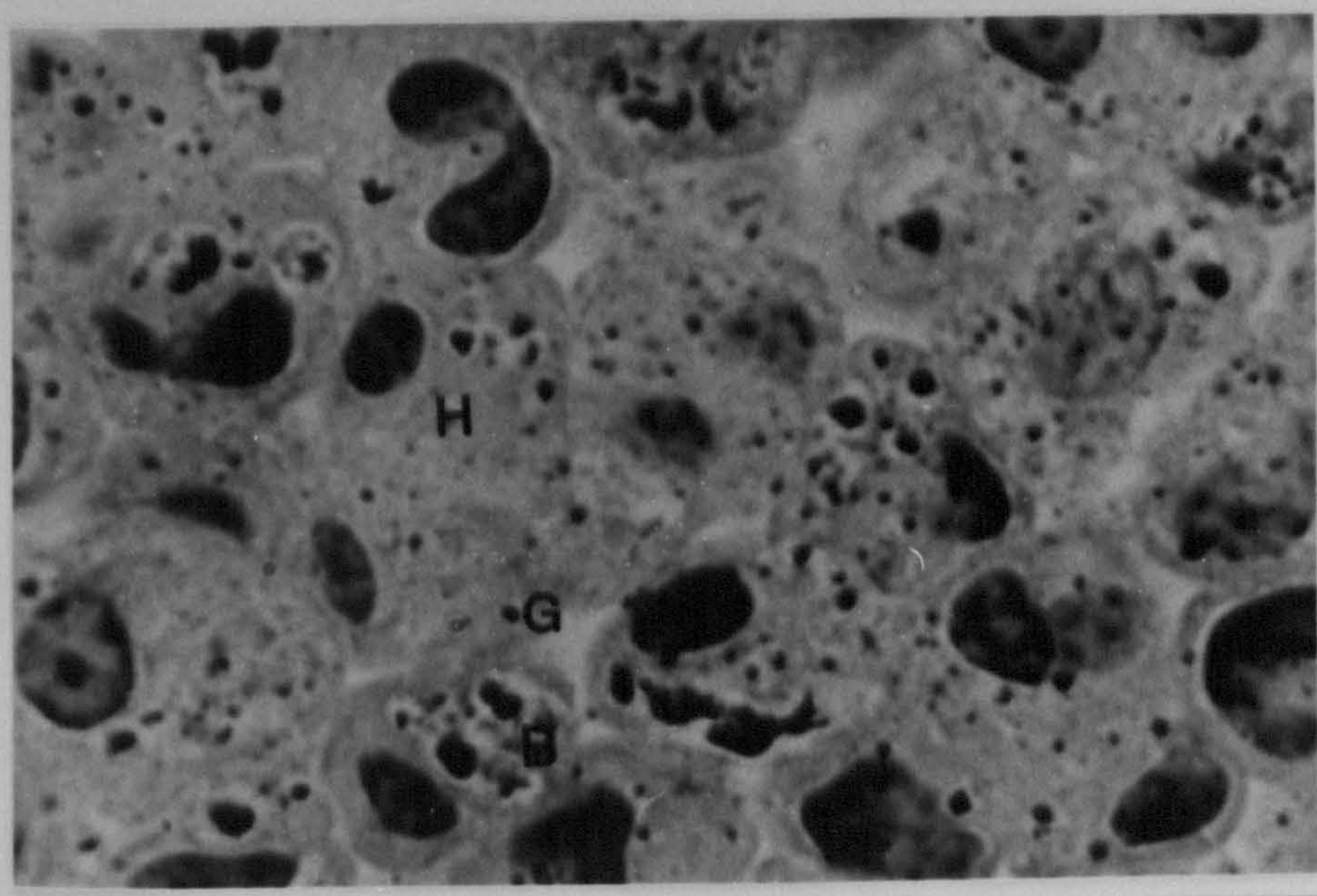


Fig. 9.34

5 μm

Discussion

The number of circulating haemocytes/ml in *Eledone cirrhosa* increase in response to sampling (Chapter 3) and after injection of phosphate buffered saline or live bacteria (Chapter 8). It has also been shown that live bacteria are either cleared from the circulation of *E. cirrhosa* or rendered non-viable in about 4 hours (Chapter 8). Opportunistic *Vibrio* (sp), including *V. anguillarum*, have been implicated in causing mantle lesions and even death in various species of cephalopods held in captivity (Hanlon & Forsythe, 1990).

Haemocytes have been shown in a variety of invertebrates to clear experimentally introduced particles (Ratcliffe *et al.*, 1985). In crustaceans for example, carmine (Smith & Ratcliffe, 1980) and live bacteria (White & Ratcliffe, 1982; White *et al.*, 1985) injected into *Carcinus meanas* were removed and killed in haemocyte clumps, which formed in the gills, as well as by fixed phagocytes in other organs (Johnson, 1987). Work with annelids has also demonstrated that in the earthworm *Eisenia foetida andrei* bacteria are initially degraded by humoral factors (Valembois *et al.*, 1992) before 'aggregation' by chloragocytes and the formation of brown bodies (Valembois *et al.*, 1993). Fitzgerald and Ratcliffe (1983) working with *Arenicola marina* showed 'coelomocyte aggregates' after live bacterial injection, followed by accumulation of bacteria in various organs where eventually brown bodies formed. Yui and Bayne (1983) also demonstrated the formation of 'cellular aggregrates' after live bacterial injection, in the sea urchin *Strongylocentrotus purpuratus*. Insect research has indicated that for example, phagocytosis occurs after the introduction of

low doses of bacteria into the Madeira cockroach *Leucophaea maderae*, whereas higher infection loads and larger particles are aggregated and encapsulated in nodules (Rahmet-Alla & Rowley, 1989).

Research into various molluscan species has also demonstrated that haemocytes play a role in clearance of introduced particles (Bayne, 1983). Stauber (1950) showed that injected Indian ink was eventually removed from the circulation of *Ostrea virginica* by mobile phagocytes and distributed throughout the organism. Haemocytes of the American oyster *Crassostrea virginica* are known to phagocytose and digest injected live bacteria (Feng, 1966; Tripp, 1960) and haemocytes from the clam, *Tridacna maxima* phagocytosed injected carbon particles (Reade & Reade, 1976; Sparks & Morado, 1988). Haemocytes and fixed phagocytes were however implicated in the clearance of particulate materials injected into the California Sea Hare *Aplysia californica* (Pauley & Krassner, 1972). Gastropod studies appear to indicate that circulating haemocytes are not the sole agents responsible for clearance of injected particles. Using *Helix pomatia* Bayne and Kime (1970) demonstrated an initial decrease in circulating haemocytes after bacterial injection. Further, Bayne (1973a) showed that experimentally introduced bacteria were cleared to particular tissues with the digestive gland being the most important. Also using *H. pomatia*, Renwrantz *et al.* (1981) found that circulating haemocytes were not involved in the initial stage of clearance of injected foreign cells, they first accumulated in the digestive gland, kidney and foot muscle of the snail. Haemocytes, which initially decreased in number in the circulation of *H. pomatia*, were apparently attracted by these organs containing

trapped foreign cells and later returned to the circulation containing the foreign particles. Interestingly, Renwrantz *et al.* (1981) indicated that the clearance and attachment of foreign cells was mediated by the level of opsonins in the haemolymph of *H. pomatia*.

It has also been demonstrated that octopods are able to clear their haemolymph of introduced foreign particles. Stuart (1968) injected *E. cirrhosa* with different amounts of colloidal carbon and sampled animals after certain time periods. The experiment showed that some organs, in particular the gills and posterior salivary glands, and to a lesser extent the white body and branchial heart, were able to remove carbon from the circulation of the octopod (Stuart, 1968). No carbon was detected in the circulating haemocytes of *E. cirrhosa* following the various experiments performed by Stuart (1968). Bayne (1973b) injected carbon and also live bacteria, *Serratia marcescens*, into *Octopus dofleini*. The bacterium was cleared rapidly leading Bayne (1973b) to suggest that fixed phagocytes rather than circulating haemocytes were responsible. This was also supported by the finding that the carbon injected into *O. dofleini* was found to have accumulated in the gills. The data presented in this paper are preliminary and only low doses of carbon and bacteria were injected. Also the carbon experiment and one of the bacteria experiments were only run for 4 h with the second bacterial experiment run for 48 h. However, the histological and TEM evidence does appear to suggest that within 4 h of live bacterial injection the circulating haemocytes are involved to some extent in phagocytosis of the bacteria, as shown by circulating haemocytes in the branchial gill, branchial heart, branchial heart appendage and white

body sections. The cells of the branchial heart, branchial heart appendage and maturing haemocytes in the white body were also involved in the removal of bacteria from the circulation. Though no evidence was observed for the involvement of the branchial gill in bacterial removal, this organ can not be ruled out and it is possible that other organs, e.g. the digestive gland and posterior salivary gland are also involved. Further experiments are currently underway to determine the involvement of other organs using different labelling techniques such as fluorescein iso-thiocyanate (FITC). The circulating haemocytes are possibly involved in the removal of graphite which aggregated in the blood vessels, as demonstrated by the 4 h histological sections of the branchial heart, branchial heart appendage, branchial gill and white body of *E. cirrhosa*. However it would be necessary to run this experiment for longer time periods and to investigate whether various other organs such as the posterior salivary gland, digestive gland and the circulating haemocytes are involved in clearance. The injection of bacteria into *E. cirrhosa* caused the branchial gill, branchial heart and heart appendage as well as a small portion of the kidney to turn green (pers. obs.) which was assumed to be similar to the inflammation reactions demonstrated in bivalves in particular (Sparks & Morado, 1988). Morphologically the branchial heart and white body changed after the injection of *V. anguillarum* and colloidal graphite into *E. cirrhosa*. The significance of the change in the large vacuole found in the branchial heart cells is unknown. It is assumed that the contents of the vacuole are involved in part of the immune defences of the animal and this needs to be further investigated. Further investigation is also required into the pleomorphic state of some haemocyte

nuclei seen 4 h after graphite injection and 48 h after bacterial injection. Of major interest was the response of the white body to the bacterial injection. It appears that large numbers of mature and maturing haemocytes are held in clusters in the white body (Cowden, 1972; Cowden & Curtis, 1974, 1981) and it is possible that there is fairly rapid release of haemocytes from this organ or other stores e.g. the posterior salivary gland (pers. obs.) into the circulation of the octopod after insult. Blood sampling increases the number of haemocytes present in the circulation of *E. cirrhosa* and the timing of sampling was also found to be important (Chapter 3), however bacterial challenge induces significantly higher numbers of haemocytes to be released compared to control (PBS) injections (Chapter 8). The newly released haemocytes also contain more cytoplasmic granules (Chapter 3) and haemocytes exhibit lysozyme and α_2-macroglobulin activity (Chapter 8) as well as acid phosphatase and peroxidase staining granules (Chapter 3). Observationally the haemocytes collected from the bacterial injected animals (4 & 24 h samples) appeared to contain fewer granules whereas those collected from control animals (4 & 24 h PBS injected samples) contained a greater percentage of granules than in 0 h controls.

Lie *et al.* (1975) showed that large numbers of amoebocytes were released from the amoebocyte producing organ of *Biomphalaria glabrata* after challenge with *Echinostoma* (sp). Exposure of *B. glabrata* to *Echinostoma* miracidia also results in morphological changes in the amoebocyte producing organ which when fully activated consisted of an increased number of amoebocytes arranged in zones of progressive maturation (Jeong *et al.*, 1983). Leucocytosis was demonstrated in the bivalves

Sunetta scripta and *Villorita cyprinoides* var. *cochinesis* after bacterial or sham injections (Suresh & Mohandas, 1990). The time period for leucocytosis in the bivalves was shown to depend on the nature of the particles/substances injected and also varied between the 2 bivalves.

In conclusion, preliminary observations indicated that the circulating haemocytes in *E. cirrhosa* aid in the removal of foreign particles as do the cells of the branchial heart, branchial heart appendage and the white body of the animal. Further research aims to determine whether other organs are involved in the clearance of injected particles and the nature of haemocyte involvement in phagocytosis *in vivo*.

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Chapter 10

General Discussion

Discussion

This study has established that the haemocytes, haemolymph and tissues of the lesser octopus *Eledone cirrhosa* (Lam.) exhibit both cellular and humoral immune reactions. Indications are that the cellular and humoral components interact to protect the octopod against potentially pathogenic bacteria if external barriers such as the skin and mucus are breached.

Haemocytes and Haemolymph of E. cirrhosa

In order to perform this study on the cellular and humoral components of the immune system of *E. cirrhosa* it was necessary to sample adequate quantities of blood, (repeated samples if needed). Further, it was also necessary to be able to separate the haemocytes (blood cells) from the haemolymph (plasma) and to culture the haemocytes *in vitro* under controlled conditions.

The blood sampling technique developed for *E. cirrhosa* is quick and guaranteed quantities can be obtained with 100% survival (Chapter 2). Improvements to the maintenance conditions for the animals (constant temperature, 10-12°C; abundant food; biological filters) has enabled repeated sampling of the same animals once every 5/6 days, without apparent ill effects, for periods of longer than 9 months. Further, the haemocytes can be separated from the sampled blood and cultured for up to 72 h without morulae formation.

The sampling and culture technique (Chapter 2) could be used in further studies of the immune system of *E. cirrhosa* or other cephalopods. Other applications could include

their use in assessing the health of *E. cirrhosa* in captivity; determination of reproductive development and the effects of pollutants. *In vitro* culture of haemocytes could also be used to determine whether subpopulations of haemocytes exist. Though there appears to be one main type of haemocyte in *E. cirrhosa*, subpopulations, or haemocytes at different maturity stages, could be present (Chapter 3).

Importantly however, sampling and repeated sampling does affect both the haemocytes and haemolymph of *E. cirrhosa* (Chapter 3). In particular, the haemocyte numbers/ml of blood and the percentage of haemocytes containing cytoplasmic granules increase while the amount of copper and initially the amount of protein in the haemolymph decrease.

Looking at the haemocyte response it appears that initially the increase in haemocyte numbers/ml due to sampling, is caused by loss of blood, i.e. a wounding response. Concomitant with this increase in the number of circulating haemocytes is a change in the staining pattern and as mentioned above an increase in the percentage of haemocytes containing cytoplasmic granules. Whether these 'new' haemocytes are released from the leucopoetic organ, or white body, (as suggested by preliminary data), or from other stores (such as the posterior salivary gland, the space around the white body or the branchial heart) or by proliferation of circulating haemocytes requires further investigation. Cowden and Curtis (1974, 1981) showed that the haemocytes matured in the white body of octopuses and upon release contained numerous cytoplasmic granules. Necco and Martin (1963) suggested that maturing haemocytes were held in the white body at telophase. If true then large numbers of newly mature

haemocytes could be quickly released if needed. With this in mind it would appear that loss of blood causes the release of haemocytes, which are mature or quickly complete maturation, and it is these cells which have the numerous cytoplasmic granules. Interestingly haemocyte numbers/ml and the percentage containing cytoplasmic granules increases over 3-5 days during repeat sampling whereupon the number of haemocytes/ml decreases before secondarily increasing again. However, the percentage of haemocytes containing cytoplasmic granules continually decreases from day 5. The timing of sampling is therefore important. If newly released haemocytes have cytoplasmic granules then these results suggest that only a certain number of 'new' haemocytes can be released over a particular time period. Since the samples obtained from new, previously unsampled animals shows only a small percentage of haemocytes containing cytoplasmic granules, it is assumed that the granules are either released by exocytosis or used during the life cycle of the circulating haemocyte. Only about 10% of the circulating haemocytes are normally newly released. The secondary increase in haemocytes/ml could be due to the release of haemocytes which are not fully mature (they have not yet developed their full complement of granules), to the proliferation of circulating haemocytes, or more likely to the mobilisation of older stored haemocytes. Stores of haemocytes in places, other than the white body, have been detected in the posterior salivary gland, which secretes proteases (Chapter 8) and the branchial heart, which contains pore cells with large vacuoles (Chapter 9) (pers. obs.). However, as will be seen later (Chapter 8), the haemocytes contain antiprotease activity (possibly within granules). Interestingly, haemocytes undergoing degradation

have been seen in the branchial heart which, as will be discussed later, has lysozyme activity (Chapter 8). So far there is no evidence to support the suggestion that there is proliferation of circulating haemocytes. In effect very little is known about the life cycle or replacement rate of the circulating haemocyte found in *E. cirrhosa* and further investigation into its activities and life span, in conjunction with studies on the white body, are required. How they are related to the tissue haemocytes is also unknown. With regard to the changes in the haemolymph parameters due to sampling, the decrease in the amount of copper is extremely important and could lead to a possibly fatal decrease in the oxygen carrying capacity of the blood of these aerobic animals. Though protein levels do decrease over a short sampling period they do return to original values (even if highly variable) over extended sampling times. Thus although the protein concentration is restored it is not in the form of the respiratory protein haemocyanin. Whether the replaced protein is in the form of apohaemocyanin remains to be determined. Interestingly, when sampled for 3 consecutive days over a 12 day sampling period, the copper content of the haemolymph does appear to remain stable. Preliminary data suggests that sampling once every 5/6 days allows the amount of copper in the haemolymph to return to original values. This effect however urgently needs further investigation and these experiments need to be repeated in conjunction with blood volume measurements.

Cellular Defence

As with other invertebrates, the haemocytes from *E. cirrhosa* will phagocytose bacteria (Chapter 4). *In vitro* phagocytosis occurs in the absence of haemolymph and is affected by temperature and the duration of haemocyte incubation with, in this case, formalised bacteria (*Vibrio anguillarum*). Additionally enhanced phagocytosis occurs if the bacteria are pre-incubated in haemolymph (10-100% concentrations), suggesting that opsonins are present. Stuart (1968) showed that erythrocytes were only phagocytosed by *E. cirrhosa* haemocytes after pre-incubation in haemolymph. It therefore appears that the opsonin acts as a recognition molecule (lectin) on the surface of particles either initiating or enhancing phagocytosis. This leads to the assumption that *in vivo* invading bacteria would be opsonized and quickly phagocytosed.

Opsonization of bacteria and therefore phagocytosis is however affected by a number of parameters. In particular, where bacteria are only opsonized for a short time (1-10 min) at low temperatures (5-10°C), the phagocytic rate decreases below that obtained for unopsonized bacteria. In effect, as suggested by Fryer and Bayne (1989), initial non-specific adsorption of a variety of plasma components (including opsonins) onto the surface of the bacterium could explain the inhibition. Longer exposure of the bacteria to the haemolymph at higher temperatures would allow more of the opsonin to bind, enabling faster recognition and therefore increased phagocytosis. Other factors which appear to affect enhanced phagocytosis by *E. cirrhosa* haemocytes include the presence in the buffer, used to dilute the haemolymph containing the opsonin, of the

divalent ions calcium and magnesium and the chelating agents ethylenediaminetetraacetic acid (EDTA) and ethylene glycol-bis (ß-aminoethyl ether) tetraacetic acid (EGTA). This appears to indicate that the attachment of the opsonin to the bacterium is sensitive to the presence of divalent cations. Further investigations into the nature of the opsonin is required and it would be interesting to determine the effect of the surface properties of particles, their size and any bound ions, on opsonization and subsequent phagocytosis by haemocytes.

The investigation of the recognition aspect of phagocytosis in *E. cirrhosa* has established that haemocytes will migrate towards certain preparations (Chapter 5). The fact that haemocytes migrate towards low concentrations of blood preparations is again suggestive of a wounding response. Molluscan blood does not clot, and it has been claimed that cephalopod haemocytes migrate into the wound and seal it (Polglase *et al.*, 1983; Wells, 1983; Féral, 1988; Ford, 1992). *In vivo* haemocytes presumably move towards an area of diluted blood, which would probably also contain lysed haemocytes, so plugging the wound and they would then phagocytose necrotic tissue. Blood preparations in which live bacteria had been incubated were also attractive to *E. cirrhosa* haemocytes suggesting that some of the bacterial secretions are chemoattractant(s). *In vivo*, wounding could also lead to the invasion of live bacteria which upon release of certain substances would be recognised by and attract the haemocytes. *In vitro* studies have also shown (Chapter 5) that haemocytes will migrate towards blood preparations which contain lipopolysaccharide (LPS). Since LPS is part of the cell wall of bacteria it appears that haemocytes will not only

recognise secreted bacterial products (from live bacteria) but will also recognise constituent molecules from bacterial walls. Interestingly, negative migration, compared to controls, was seen when blood preparations, haemolymph in particular, were used in which dead bacteria had been incubated. However, phagocytosis of dead bacteria does occur (Chapter 4). Investigations are however needed into the nature and effects of the chemoattractants secreted by live bacteria and whether other molecules such as other bacterial constituents also induce haemocyte migration. Further it would be phylogenetically interesting to investigate whether molecules which induce vertebrate macrophage migration such as tumor necrosis factors and interleukins also stimulate *E. cirrhosa* haemocytes.

The electron microscope results suggest that once the bacteria are internalised, the haemocyte appears able to kill and digest them (Chapter 4 & 9). The decrease in the number of live bacteria after incubation with haemocytes from *E. cirrhosa* is assumed to be due to phagocytosis (Chapter 6). Though it is possible that the haemocytes secrete bacteriostatic molecules this appears to be unlikely as regards the evidence from the NBT reduction experiments. The experiment, involving NBT reduction, showed that dead bacteria in particular, but also live bacteria and intriguingly LPS, caused intracellular reactive oxygen intermediate (ROI) production by *E. cirrhosa* haemocytes. The production of ROIs is in effect an indication that phagocytosis, or possibly pinocytosis with LPS, has taken place. Additionally, haemocytes contain lysosomal enzymes such as acid phosphatase (Chapter 8) as well as lysozyme and antiprotease activity, which could protect against bacterial proteases. Presumably,

other bactericidal molecules are present and it would be interesting to isolate and characterise these molecules as well as determining the exact nature of the action by haemocytes on the bacteria. Preliminary investigation with *E. cirrhosa* haemocytes was also carried out using phorbol myristate acetate (PMA) (a membrane stimulator), bacteria and LPS with ferricytochrome C which detects extracellular ROI production (unpub. data). However no extracellular ROI production was demonstrable for *E. cirrhosa* which could be due to either the buffers (containing EGTA) used to isolate and culture the haemocytes for the assays, or to the possibility that haemocytes do not produce extracellular ROIs. Additional experiments are necessary to determine whether ROIs are produced extracellularly.

Humoral Defence

E. cirrhosa haemolymph contains agglutinins for the bacteria *V. anguillarum, V. parahaemolyticus* and *Aeromonas salmonicida* (Chapter 7). Stuart (1968) also demonstrated the presence of agglutinins in *E. cirrhosa*. He also reported the presence of opsonins, which were also seen in this study (Chapter 4), and suggested that the respiratory pigment haemocyanin could be the opsonin. No evidence was obtained from this study for the involvement of haemocyanin in the humoral defence of *E. cirrhosa*, but it would be interesting to remove haemocyanin from the haemolymph and determine the effects on opsonization. Lectins have been demonstrated in another octopod, *Octopus vulgaris* (Rögener *et al.*, 1985, 1986, 1987). It appears therefore that invading particles are quickly cross linked in suspension (agglutination), marked

for recognition and opsonized for rapid phagocytosis by the haemocytes. Additionally the haemolymph of *E. cirrhosa* contains either a bacteriostatic or a bactericidal capacity, as well as lysozyme and possibly 'protective' antiprotease activities (Chapter 8). Experiments are required to determine whether the effect of the haemolymph on bacterial growth is due to bacteriostatic or bactericidal activity and what the causative agent is. It would also be interesting to determine the effect of the removal of haemocyanin on this activity of the haemolymph of *E. cirrhosa*.

In Vivo Clearance

Live bacteria injected into *E. cirrhosa* are cleared from the circulation in about 4 hours (Chapter 8). Additionally haemocyte and branchial heart lysozyme and haemocyte antiprotease activity were affected by bacterial injection, as were the number of circulating haemocytes/ml. Lysozyme activity was shown to increase in the branchial heart and decrease in the haemocytes possibly suggesting the production of lysozyme in the branchial heart and either its utilisation or release by the haemocytes. Lysozyme production in the branchial heart may be associated with the changes in the morphology of the large vacuole of the pore cells 4 hours after bacterial injection (Chapter 9). Additionally, haemolymph lysozyme activity did not change after bacterial injection (Chapter 8).

The decrease of antiprotease activity in the haemolymph suggests utilisation which could possibly protect the tissues of *E. cirrhosa* against possible bacterial proteases. Since blood was sampled from the animals before injection of bacteria, it is difficult to

determine whether the increase in haemocyte numbers/ml is due to the blood loss, or the injection, or both. However, more haemocytes/ml were detected in animals injected with bacteria, compared to the control, suggesting that the bacteria had a greater effect than sampling alone.

The final experiments (Chapter 9) showed that, as with other molluscs, injected bacteria were cleared by circulating haemocytes and also by certain tissues such as the branchial heart, the white body and the branchial heart appendage. Bacteria undergoing degradation were seen in these tissues and in the circulating haemocytes confirming that potentially pathogenic bacteria can be removed from, and destroyed by, the internal defences of the animal. This however does need further investigation and a better understanding of the clearance mechanisms could be obtained by using labelling techniques such as ^{14}C and fluorescein isothiocyanate(FITC) on the bacteria. Other factors which need investigating are the change in the large vacuole of the branchial heart cells and the changes in morphology of the haemocyte nucleus.

Injected colloidal graphite was detected as aggregates in blood vessels. This agrees with the findings of Stuart (1968).

In conclusion, *E. cirrhosa* is able to deal with invading potentially pathogenic bacteria by using combinations of both the humoral and cellular components of its immune system. Wounding, or bacterial invasion, causes an increase in circulating haemocytes, followed by agglutination, opsonization, and possible killing of the bacteria in the haemolymph. Haemocytes migrate towards, phagocytose and probably

kill any invader. If the invader escapes the haemocytes other tissues are also capable of removing and killing these foreign organisms.

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