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Characteristics of bacterial clearance were investigated in the purple sea urchin, Strongylocentrotus purpuratus (Echinodermata: Echinoidea). Primary clearance kinetics were determined for three bacteria, a marine Gram negative motile rod, a marine Gram positive non-motile rod, and a fresh water fish pathogen, Aeromonas salmonicida. Various doses of live bacteria were injected into the perivisceral coelom of urchins, and coelomic fluid samples were removed for viable counts. Clearance of the two marine bacteria occurred in two stages. Reduction in viable counts was rapid and exponential during the first 90 min, followed by a period of slower clearance. The two bacteria exhibited similar clearance rates during the first 90 min, however the Gram positive bacteria were subsequently cleared more slowly than the Gram negative. Clearance of A. salmonicida was linear and not in two stages, although the initial rate of clearance was much lower than that of the marine bacteria.

Attempts were made to determine whether a memory or inducible component exists in S. purpuratus' response to bacteria. After the kinetics of clearance of primary injections had been determined, the same urchins were challenged with a second injection of bacteria (9, 14, and 21 days after primary injections for the two marine bacteria, 19 days for A. salmonicida). The secondary clearance rates were not significantly different from the primary clearance rates for any of the three test bacteria.

The cell-free coelomic fluid of S. purpuratus was tested in vitro for bacterial agglutination and bactericidal activity against the two marine bacteria. Declines in viable counts of bacteria were observed with both bacteria in coelomic fluid, while viable counts in sea water controls did not change. Neither previous injection of urchins with bacteria nor prior heating of coelomic fluids (100°C, 10 min) significantly altered the viable counts. It is not clear from these experiments whether the declines in viable counts were due to bacteria killing or agglutination. However, when the coelomic fluid was tested, agglutination was not observed with either of the bacteria.

The cellular responses of the urchins were also investigated. After injection of Gram negative bacteria, total numbers of circulating coelomocytes declined by 93% by 90 min after injection, and decreased slowly thereafter. By 24 hr, cell counts had risen, but not to preinjection values. All four coelomocyte cell types contributed to the overall decline. The proportions of coelomocyte types also changed during the 6 hr after injection. A fluorochrome dye, acridine orange,

was used to observe phagocytosis and killing of bacteria. Clotting phagocytes trapped and engulfed bacteria. Within 2 hr after mixing bacteria and coelomocytes *in vitro*, dead bacteria were seen in some of the phagocytes.

Bacterial Clearance in the Sea Urchin, *Strongylocentrotus purpuratus*

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INTRODUCTION

All living species can recognize and respond to a myriad of substances and organisms which may invade their bodies. At the same time, these internal recognition and defense mechanisms generally do not act against the organism's own undamaged cells or certain normal symbionts - a diverse array of internal parasites, pathogens, mutuals and commensals. As Boyden (1963) pointed out, it is not likely that animals would have evolved beyond unicellular organisms had it not been for a discriminatory mechanism to distinguish between functional and damaged cells and foreign material. The basic question of how various invertebrate phyla discriminate and respond appropriately to non-self remains largely unknown and speculative.

The higher vertebrates (birds and mammals), on the other hand, have been well studied in this regard. They are known to respond to foreign invaders via a phagocytic reticuloendothelial system and complex and highly specific immune systems including antibody production, complement fixation, and cell cooperation, proliferation, and communication. The lower vertebrates (fishes, reptiles, and amphibians) also have been found to have many of the characteristics of the immune responses of the higher vertebrates (see Marchalonis, 1977 for a review of immunity in the classes of vertebrates). Therefore, the search for the origins of

many of the humoral and cellular mechanisms of vertebrate immunity requires an understanding of the immune systems of the invertebrates, especially the protochordates and echinoderms, which are deuterostomes and therefore considered to be the closest extant relatives of the vertebrates (Romer, 1962). Unfortunately, there is a paucity of data on the internal defenses of the invertebrates as a whole, and the protochordates and echinoderms in particular.

Two major experimental approaches have been used in the study of invertebrate immunity. One approach has been to adopt methods used in comparative vertebrate immunology such as complement fixation, amino acid sequencing in order to determine degrees of molecular homology, tissue transplantation, mitogenesis and others. In addition, some intriguing studies have found substances in invertebrates which actively modulate mammalian immune responses. The results of studies using these approaches are difficult to interpret in terms of internal defenses of the invertebrates.

The other major approach has been to study the in vivo and in vitro cellular and humoral defense responses of various invertebrates to non-self substances. Clearance of living and inert particles such as colloidal carbon or gold, allo- and xenogeneic cells, bacteria, viruses, and fungi has been observed in members of most phyla. In vitro studies have shown that humoral factors, hemagglutinins, hemolysins, bactericidins, bacterial agglutinins, and opsonins exist in the body fluids of many invertebrates (see Dales, 1979 for review). Cellular responses to parasites and transplants have also been observed in many phyla, especially the more economically important invertebrates, the

insects (Salt, 1970) and the molluscs (Bayne, 1982b, in press). Many different circulating cell types have been identified for various invertebrates, however, the terminology has been confusing and the roles of many cell types have yet to be elucidated (Ratcliffe and Rowley, 1979).

The primary difference noted so far between vertebrates and invertebrates is the lack of immunoglobulin (Ig) in the invertebrate body fluids. Hemagglutinins (multivalent lectins which bind carbohydrate on erythrocyte surfaces) have been found in microorganisms, plants and animals (Gold and Balding, 1975; Yeaton, 1981 for recent invertebrate review). Many of these molecules were purified, characterized and compared with Ig in hope of finding a possible precursor molecule to vertebrate Ig. These studies included hemagglutinins from the horseshoe crab, Limulus polyphemus (Marchalonis and Edelman, 1968; Finstad et al., 1972), the oyster, Crassostrea virginica (Acton et al., 1969), the land snail, Helix pomatia (Hammarström and Kabat, 1969; Hammarström, 1972), the mussel, Velesunio ambiguus (Jenkin and Rowley, 1970), and the sea star, Asterias forbesi (Finstad et al., 1972). Using data from Finstad et al. (1972), Carton (1974) found that hemagglutinins from Asterias and Limulus were similar in primary structure to hemagglutinins from Agnatha and Osteichthyes and the heavy and light chains of Ig. Acton and Weinheimer (1974) and Jenkin (1976), on the other hand, compared the above studies in terms of amino acid composition (not sequences), molecular weight, and number and size of subunits and concluded that none of the agglutinins were similar to Ig. Based on these characteristics they also suggested that

invertebrate hemagglutinins may be structurally related to one another.

Although they are probably not homologous with vertebrate Ig, the significance of lectins as recognition molecules in host-symbiont interactions has only recently been realized in invertebrates as well as plants and vertebrates. Hemagglutinins are responsible for the accelerated clearance of red blood cells (rbc) in the snail, Helix pomatia (Renwrantz and Mohr, 1978; Renwrantz et al., 1981) and for increasing rates of rbc phagocytosis and therefore clearance in the crayfish, Parachaeraps bicarcinatus (Jenkin, 1976). They may be involved also in invertebrate host-parasite interactions (Rhodnius - Trypanosoma, Pereira, Andrade and Ribeiro, 1981; Biomphalaria - Echinostoma, Jeong et al., 1981). Unfortunately, few hemagglutinins have been isolated and even fewer tested for possible biological roles in invertebrates.

One likely role for lectins in the natural internal defenses of invertebrates is bacterial agglutination. The lectins may act by enhancing the rate of phagocytosis by agglutinating the bacteria to one another, thereby concentrating them, and/or agglutinating bacteria to phagocytic cells. Bacterial agglutinins have been found in the body fluids of many invertebrates (see Dales, 1979 for review). Cross absorption studies between bacteria and rbc indicate that the recognition sites may be the same for both particles (Jenkin, 1976; Huang, Eble, and Hammen, 1981); this is not surprising because carbohydrates are present on the surfaces of both types of cell.

Because Ig is the basis of recognition specificity in most vertebrate immune responses, and Ig is evidently absent from invertebrates, Tripp (1974), Cunningham (1978), and others have suggested that invertebrates do not have sophisticated immune defenses and may depend on high rates of reproduction to replace diseased organisms. As Lackie (1980) pointed out however, invertebrate immunology is in the descriptive stage, and mechanisms are not understood in even the most studied invertebrate groups. Although lectins may be important recognition molecules in invertebrate defense responses, the diversity of lectins is unknown for any one species or group of invertebrates, and the level of recognition specificity obtainable with lectins is not yet clear. With such basic information lacking, generalizations about the phylogeny, complexity, and specificity of immune responses may be premature.

Echinoderm immunology

Despite the relative paucity of studies on echinoderm immune responses, echinoderms have been shown to possess sophisticated and sensitive internal surveillance systems sharing basic cellular and humoral characteristics with many other invertebrate phyla. In addition, the results of studies utilizing methods of comparative vertebrate immunology suggest that echinoderms may also have characteristics in common with vertebrate immune systems, and homology between the immune systems of the two groups has been suggested (Leclerc et al., 1980). However, it remains to be shown whether or not the functions or

mechanisms of these responses are similar in echinoderms and vertebrates.

The efficiency of their internal defense systems is evident from the aseptic nature of coelomic fluid in carefully collected and properly maintained echinoderms (Bang and Lemma, 1962; Unkles, 1977). To test their capacities to clear foreign material from the coelomic fluid, various substances have been injected into different echinoderms (carborundum and xenogeneic cells, Reinish and Bang, 1971; bacteria, Wardlaw and Unkles, 1978; Kaneshiro and Karp, 1980; proteins, Hilgard, Hinds and Phillips, 1967; Hilgard and Phillips, 1968; Hilgard, 1970; Hilgard, Wander and Hinds, 1974; bacteriophage T_4, Coffaro, 1978). Results indicate that (1) echinoderms are capable of removing rather high doses of proteins and particles from the perivisceral coelom and (2) there are differences in rates of clearance between different particles injected into the same species (Reinisch and Bang, 1971; Bertheussen, 1981). However, as with most invertebrates, we have little understanding of mechanisms of recognition and clearance, or even of the cells, tissues, and/or humoral factors involved.

Based on the few studies on specific recognition in echinoderms, it appears that echinoderm coelomocytes can discriminate between different injected proteins. Preinjection of the echinoid, Strongylocentrotus purpuratus, with bovine serum albumin (BSA) blocked coelomocyte binding of additional BSA but not bovine gamma globulin (Hilgard et al., 1967), indicating that different receptors are involved in the uptake of the two proteins. Similar results were obtained by adding BSA and chicken serum albumin to coelomocytes in vitro (Hilgard et al., 1974).

The coelomocytes did not appear to have separate receptors for BSA and human serum albumin (HSA) although receptor affinity was greater for BSA than HSA (Hilgard, 1970). The molecular basis of this discrimination has not been studied; it is unknown what portion(s) of the molecule is (are) recognized. Although Hilgard and Phillips (1968) were unable to elicit enhanced coelomocyte binding of BSA by preinjections, protein (BSA and whale myoglobin) injection resulted in an increase in numbers of specific protein-binding receptors in a subpopulation of Asterias rubens axial organ cells (Leclerc et al., 1980). However, this capability to recognize and respond specifically to foreign protein does not necessarily imply that this specificity is used defensively against foreign organisms.

Fourteen distinct coelomocyte types have been described in the five classes of echinoderms (Endean, 1966). Since my study focussed on the echinoid, Strongylocentrotus purpuratus, this discussion will be restricted to the cell types found in echinoids. Seven cell types were described for S. purpuratus (Boolootian and Giese, 1958). Subsequently, several of these types have been suggested to be the same. More recently, four major coelomocyte types have been described in Strongylocentrotus spp. (Johnson, 1969a; Chien et al., 1971; Bertheussen and Sejelid, 1978) and Echinus esculentus (Messer and Wardlaw, 1980). These cells include phagocytes, red spherules, colorless spherules, and vibratile cells. Each of these cell types has been implicated in some defensive role.

The phagocytes (leukocytes, bladder amebocytes) are the most abundant coelomocyte type and may be responsible for at least some of

the discrimination and clearance of particles from the coelomic fluid. Phagocytosis is a basic cellular response found in most uni- and multicellular animals, playing important roles in nutrition, developmental events, and defense. The defensive role of phagocytosis has been known in echinoderms since Metchnikoff's (1892) famous observation that phagocytes accumulated around a rose thorn placed in a sea star larva. Since that time, the phagocytes of several echinoderms have been shown to engulf particles such as India ink, carmine, ferritin, carborundum, red blood cells, bacteria, Sephadex and latex (Kindred, 1921; Bang and Lemma, 1962; Johnson, 1969b; Johnson, Chien and Chapman, 1971; Reinisch and Bang, 1971; Kaplan and Bertheussen, 1977; Kaneshiro and Karp, 1980).

Johnson (1969b), using hanging drop cultures of Strongylocentrotus spp. whole coelomic fluid, noted that bacteria were not all phagocytosed equally; some were immediately engulfed, others less consistently, and some not at all. Bertheussen (1981) determined rates of in vivo clearance and in vitro phagocytosis of various substances (treated and untreated rbc, carbon, bacteria, latex and yeast) and found distinct differences in the rates of responses to different particles. The hierarchies of rates of particle clearance and phagocytosis were similar, indicating that the phagocytes may be primary determinants of the clearance rate. In mixtures of allogeneic and xenogeneic phagocytes in vitro, 90% of xenogeneic and 70% of allogeneic mixtures resulted in cytotoxic responses (Bertheussen, 1979). None of the other coelomocyte cell types elicited this response, therefore, recognition and cytotoxicity may be attributable to these cells alone.

Phagocytes are the only cells capable of phagocytosis and cellular clotting (aggregation and syncytia formation). These cells accumulate in wound areas (Dermasterias, Karp and Hildemann, 1976; Sphaerechinus, Höbaus, 1980), and it is probable that they form clots which close off the wound area, and phagocytose damaged cells and contaminating foreign organisms. Cellular clotting by phagocytes apparently also plays a role in the clearance of some foreign substances. Bertheussen (1981) noted that clots formed in vivo in response to injections of bacteria but not other particles. Cellular "clumping" by the phagocytes was observed when Asterias were injected with Arbacia (echinoid) cells (Reinisch and Bang, 1971). Clots may mechanically trap bacteria allowing phagocytosis to proceed (Johnson, 1969). Clots probably do not cause release of bactericidal substances (Echinus, Wardlaw and Unkles, 1978), unlike the clots of horseshoe crabs (Limulus polyphemus, Furman and Pistole, 1976). Bactericidal activity was noted in purified populations of phagocytes (Messer and Wardlaw, 1980), although the activity was not as strong or consistent as that of the red spherules, and may have been due to contamination by those cells.

The ultimate fate of phagocytosed or trapped particles is not completely understood, although asteroid and echinoid phagocytes laden with foreign material most likely migrate out of the perivisceral coelom by diapedesis to the axial organ (Millot, 1967; Jangoux and Schaltin, 1977; Bachmann and Goldschmid, 1978) and/or Tiedemann bodies (Bachmann and Goldschmid, 1980; Kaneshiro and Karp, 1980), then to the water vascular system and out through the tube feet (Höbaus, 1979), dermal

papillae (Bang, 1970) and/or the stone canal and madreporite (Bachmann and Goldschmid, 1978).

Red spherules (eleocytes, red morulas) are distinct red cells with lobate pseudopodia and spherical cytoplasmic inclusions containing a napthaquinone pigment, echinochrome. These cells have also been implicated in defensive roles since they accumulate at wound sites (Sphaerechinus, Höbaus, 1980; Lytechinus, Coffaro and Hindegardner, 1977) and exhibit chemotaxis to certain bacteria in hanging drops of coelomic fluid (Strongylocentrotus sp., Johnson, 1969b). It is likely that the red spherules destroy invading organisms since purified populations of these cells have significant bactericidal effects on a marine pseudomonad (Messer and Wardlaw, 1980) and echinochrome may be an algastatic compound (Vevers, 1963). Since echinochrome is not found in all echinoderms, it is not likely to be of primary importance in general echinoderm defenses.

Colorless spherule cells look very similar to the red spherules but without the red pigment. Apparently, in Echinus, only these cells contain hemagglutinating activity for rabbit erythrocytes (Messer and Wardlaw, 1980).

Vibratile cells are spherical, motile cells, each with a single flagellum and granular cytoplasm. They can release a mucoid substance resulting in the "gelification" of areas of the coelomic fluid. This may immobilize bacteria facilitating phagocytosis of them (Johnson, 1969b). These cells apparently do not contain bactericidal compounds against the pseudomonad tested (Messer and Wardlaw, 1980).

The axial organ (AO), whose functions have long been enigmatic (Millot, 1966), appears to play some role in internal defense and wound repair (Millot, 1967, 1969: Jangoux and Schaltin, 1977; Bachmann and Goldschmid, 1978) and may be a possible early "lymphoid organ" (Leclerc, Brillouet and Luquet, 1981). The AO of the echinoid, Arbacia punctulata, became filled with cellular debris and phagocytosed cells after animals were injected with sperm, ciliates or other xenogeneic cells (Millot, 1967) or were injured (Millot, 1969). Injection of ferritin into the urchin Psammechinus miliaris resulted in migration of phagocytes laden with ferritin into the AO (Jangoux and Schaltin, 1977). At the ultrastructural level, cells of the AO appear to be identical to coelomocytes (Jangoux and Schaltin, 1977). Since, even without previous injection or wounding, phagocytes in the AO of Sphaerechinus granularis are packed with phagosomes, Bachmann and Goldschmid (1978) suggested a storage and degradative role for the phagocytes in the AO. Tiedemann bodies of the water vacular system may play a similar role (Bachmann and Goldschmid, 1980).

Leclerc and his colleagues have studied some characteristics of the AO cells of Asterias rubens using techniques adopted from vertebrate immunology. For example, athymic mice apparently accept and vascularize AO transplants (Leclerc, Panijel and Toyama, 1974). The AO cells survived in the mouse and some cells exhibited mitotic figures. Subcutaneous injections of AO cells into irradiated mice resulted in vascular reticulation (angiogenesis), and injections into neonatal chickens induced splenomegaly (Leclerc et al., 1977a); both responses are similar to those found after injection of vertebrate lymphoid

tissue. Digestive organ cells and oocytes did not cause these responses. Coelomocytes also failed to cause angiogenesis (Leclerc et al., 1977b), implying that the cell populations in the perivisceral coelom and the AO are different.

Subpopulations of AO cells have been separated by use of adherence properties (Leclerc et al., 1977b), lectin agglutinability (with wheat germ agglutinin [WGA] and soy bean agglutinin [SBA], Leclerc et al., 1980), and mitogenic responses to concanavalin A (Con A), pokeweed mitogen (PWM), and lipopolysaccharide (LPS) (Brillouet et al., 1980). The size of the SBA-agglutinating subpopulation increased, while the non-agglutinating subpopulation did not, after injections of either whale myoglobin or bovine serum albumin (Leclerc et al., 1980). It is unknown if the cells proliferated, migrated from elsewhere or were induced to transform from another cell population. From these studies, Leclerc et al. (1981) concluded that the sea star AO is likely to be an ancestral lymphoid organ retaining cell populations with characteristics which resemble those of vertebrate immunocytes. Although these studies are intriguing, the functions of these responding cells must be elucidated before phylogenetic comparisons will be meaningful. It is also unknown whether (1) echinoid AO cells serve the same functions and exhibit similar responses as asteroid AO cells, and (2) any other tissues, not likely to be involved in internal defenses, have similarly defined subpopulations of cells. In addition, it appears that responses to lectins and mitogens are common, even in phyla more distantly related to the vertebrates. Earthworm leukocytes respond mitogenically to Con A, phytohemagglutinin (PHA), and LPS (Roch and Valembois, 1977), and

even a cytotoxic ameba (Nuclearia) responded mitogenically to certain lectins, Con A, LPS, PHA, and Lentil lectin (Bayne, 1982a in press).

Humoral factors have not been very well characterized in echinoderm internal defenses. Hemagglutinins and hemolysins have been found in the coelomic fluid of several asteroids, echinoids and holothuroids (Finstad et al., 1972; Royoyama, 1973, 1974; Yokoyama et al., 1976; Parrinello et al., 1976, 1979). However, the biological roles of the hemagglutinins were not investigated. Bacteria agglutinins have not been reported in echinoderms despite the large numbers of hemagglutinins reported.

In vitro bactericidal activity in echinoderms has been found to be dependent upon the presence of coelomocytes in the sand dollar, Dendraster excentricus (Johnson and Chapman, 1970) and the urchin, Echinus esculentus (Wardlaw and Unkles, 1978). Neither of these experiments was designed, however, to determine whether coelomocytes released anti-bacterial factors during microbial infection. Lysozyme, an enzyme which hydrolyzes cell wall polysaccharide of certain bacteria, has been isolated and characterized from Asterias rubens (Jollès and Jollès, 1975), however, the location and function of the enzyme in these animals are unknown since whole animals were used.

Various echinoderm factors have been found to effect responses of vertebrate immune systems. Coelomocytes from Asterias forbesi contain a factor which can cause delayed inflammatory reactions and inhibition of macrophage migration in guinea pigs (Prendergast and Suzuki, 1969). This factor also activates macrophages and inhibits tumor cell proliferation (Prendergast and Liu, 1978), inhibits primary but not secondary immune responses to the T-cell dependent antigen, Listeria

monocytogenes (Willenborg and Prendergast, 1974), and inhibits precytotoxic thymus dependent cell differentiation in vitro (Prendergast, Gleiner, and Reinisch, 1976).

In addition, a factor isolated from sea star coelomic fluid has been shown to exhibit C3-like complement activity (Day et al., 1972). C5 deficient complement plus Ig-M can act as opsonin for the phagocytosis of red blood cells by echinoid cells (Bertheussen, 1981). Attachment of Ig-M-complement treated rbc occurred in the perinuclear region of the phagocytes, a location similar to that of the receptor sites for C3b in rat Kupffer cells and mouse peritoneal macrophages (Kaplan and Bertheussen, 1977). However, other invertebrate body fluids (even those of protostomes) also contain factors capable of activity similar to certain complement components (Anderson, 1975).

Two important characteristics of vertebrate immunology, memory and specificity, have been demonstrated in echinoderms by tissue transplantation. Sea cucumbers, Cucumaria tricolor and Protoreaster nodosus, and a sea star, Dermasterias imbricata, rejected second set body wall allografts more rapidly than first sets (Hildemann and Dix, 1972). Since accelerated rejection occurred more rapidly when third set grafts were from the same donor as the first graft than with a different donor, in 5 out of 5 D. imbricata, the response was specific (Karp and Hildemann, 1976). Similarly, the echinoid, Lytechinus pictus, rejected primary allografts in 30 d and secondary ones in 12 d (Coffaro and Hinegardner, 1977). Specificity was weak but present when secondary grafts from the same and different donors were compared (Coffaro, 1980), although specificity was not found in all animals. Based on these

rejection tests, Coffaro (1980) concluded that there is a high degree of histocompatibility polymorphism in L. pictus. A system of allogeneic polymorphism also appears to exist in the echinoid, Strongylocentrotus droebachiensis, as has been demonstrated with in vitro mixed phagocyte reactions (Bertheussen, 1979).

The relevance of these phenomena to the phylogeny of immune responsiveness is unknown, since members of many invertebrate phyla, including the sponges (Hildemann, Johnson, and Jokiel, 1979; Evans, Kerr, and Curtis, 1980), cnidarians (Hildemann et al., 1977), and annelids (Cooper, 1970) have also been found to respond to grafts with memory and specificity. The results of these studies may be equivocable since Parry (1978) was unable to verify the results with annelids, and memory and specificity were not found in graft rejection with several other species of sponges (Van de Vyver, 1980; Evans et al., 1980).

Allogeneic graft rejection and allogeneic polymorphism may be more meaningful in terms of (1) the internal organization of increasingly complex metazoans, (2) maintenance of individual integrity in space-limited colonial sessile organisms such as sponges, tunicates, corals, and anemones, and/or (3) reproduction and the prevention of self-fertilization, a means of maintaining genetic variability. The question remains to be answered whether the complex histocompatibility responses of many invertebrate phyla reflect a meaningful relationship to the animal's ability to respond to invasion by potential parasites and pathogens.

Hildemann, Bigger and Johnston (1979) suggest three criteria for the presence of immunological competence: (1) selective or specific recognition, (2) cytotoxicity or antagonistic reaction after sensitization, and (3) inducible memory or selectively altered reactivity on secondary contact. According to these criteria, it appears that the sponges, cnidarians, annelids and echinoderms are all immunocompetent based on tissue transplantation alone. In the vertebrates, however, these criteria also characterize clearance of foreign substances. Criterion (1), specific recognition, has been demonstrated in clearance of proteins in echinoderms (Hilgard et al., 1974) and other invertebrates, although the details of the specificity have not been determined. Cytotoxicity has been demonstrated in echinoderm phagocytes in vitro (Bertheussen, 1979). However, criterion (3), altered reactivity, has not been demonstrated in any echinoderm using test "antigens" such as particles or protein. The potential for altered reactivity in echinoderms has been demonstrated with the increase in numbers of specific receptors to injected proteins (Leclerc et al., 1980). Among the invertebrates, only the cockroach, Periplaneta, has been shown to be capable of inducible and specific immunologic memory in response to injections of foreign proteins (Karp and Rheins, 1980). Since the two toxins (honeybee toxin and a snake venom) are not biochemically related, the insect response may not be as specific as suggested by the authors. However, with respect to both speed and increased response, the secondary responses resembled vertebrate secondary responses to injected foreign substances.

The purpose of the research reported in this thesis was to determine whether an echinoderm, *Strongylocentrotus purpuratus*, exhibits memory or altered reactivity on secondary contact with biologically relevant antigens, namely bacteria. Since very little is known about the characteristics and mechanisms of bacterial clearance in echinoderms, I also studied the *in vivo* and *in vitro* primary responses of echinoderm coelomocytes and cell-free coelomic fluid to bacteria.

S. purpuratus was selected for this study as it is readily available in the rocky intertidal zone on the Oregon coast. The animals are relatively easy to maintain in laboratory conditions, are fairly large with large volumes of coelomic fluid, and much is already known about their coelomocytes and general biology. Also, in the harsh, wave-swept rocky intertidal, *S. purpuratus* might be subject to abrasion (due to wave action and sediments) and subsequent microbial invasion so that it might be expected to have very efficient antibacterial defenses.

METHODS

Collection and maintenance of animals

Strongylocentrotus purpuratus (80-150 g) were collected by hand from intertidal surge channels at Yaquina Head, Newport, Oregon where the species is abundant. Care was taken to avoid damaging the urchins during collection and transportation to the 22,700 liter (6,000 gallon) recirculating sea water system at Oregon State University in Corvallis. Water temperatures were held at 12-15°C, comparable with temperatures on the coast. Macroalgae (*Hedophyllum*, *Egregia*, *Ulva* in particular) were collected and fed to the urchins *ad libitum*.

Isolation and culture of bacteria

One Gram negative motile rod and one Gram positive non-motile rod were isolated from the coelomic fluid of sick sea urchins. Selection was based on growth characteristics (rate of growth, colony color and morphology). Marine broth 2216E (Difco Laboratories), enriched with peptone (5 g l^{-1}), and yeast extract (3 g l^{-1}) was used to grow all bacteria. Soft agar was made with nutrient agar (1%) in marine broth for pour plate viable counts. For each experiment, bacteria from a stationary phase culture (overnight) were inoculated at 15% in fresh, enriched broth in triple baffle side arm flasks and grown at room temperature (20-23°C) on a rotary table (100 rpm). The bacteria were harvested during log phase growth, generally at OD_{550} = 0.60 (see

Appendix I for standard plots). The suspension was then centrifuged at 1840 rpm (300 g) at room temperature, and resuspended in cold (10°C) sterile sea water to appropriate concentrations for injections.

Because the urchins may have previously encountered the marine bacteria, a freshwater bacterium, Aeromonas salmonicida, was also used. A. salmonicida is a salmonid fish pathogen, a Gram negative non-motile rod generally not present in the marine environment (although it has occasionally been found in marine salmonids, Fryer, personal communication). These bacteria were grown in tryptic soy broth using methods described for the other bacteria.

The three bacteria used were fairly distinct from one another and other, possibly contaminating, marine bacteria. White colonies of the marine Gram negative bacteria appeared approximately 48 hr after plating and incubation at room temperature. The marine Gram positive bacteria produced bright yellow colonies 72-96 hr after plating. A. salmonicida produced white colonies 24 hr after plating. The A. salmonicida colonies and surrounding agar turn brown after another 24 hr due to the production of a brown pigment on media containing tyrosine or phenylalanine (Fryer, personal communication). These characteristics were used to help ascertain that the bacteria grown from sampled coelomic fluid were the species injected.

Coelomic fluid volume estimates

A method for estimating the coelomic fluid volume was required for determining the quantity of bacteria to be injected for a specific

concentration in the perivisceral coelom. The weight, test diameter, test height, and peristomium diameter were measured for each of 28 urchins, in the general range of the animals used for the experiments. The coelomic fluid was then drained through a cut in the peristomium and the volume measured. Each external parameter was regressed on the coelomic fluid volume (Appendix II). Weight was found to be the best indicator of coelomic fluid volume ($R^2 = 0.96$). The following equation defined the relationship and was used in all experiments:

Coelomic fluid volume (ml) = 0.35 X weight (g) - 4.2.

Clearance experiments

Urchins were weighed, the coelomic fluid volume estimated, and the dose of bacteria calculated for a given final concentration of bacteria in the coelomic fluid. For ease in sampling, the urchins were placed into marked plastic berry containers (Figure 1). These containers allowed a certain amount of free movement of tube feet and proper orientation relative to gravity, while preventing the animals from attaching firmly to the substratum during the sampling period. Attachment would have necessitated damage due to torn tube feet when the animals were needed for sampling. With these containers, the peristomium was always accessible during sampling, the animals had good water circulation, and each individual was identified.

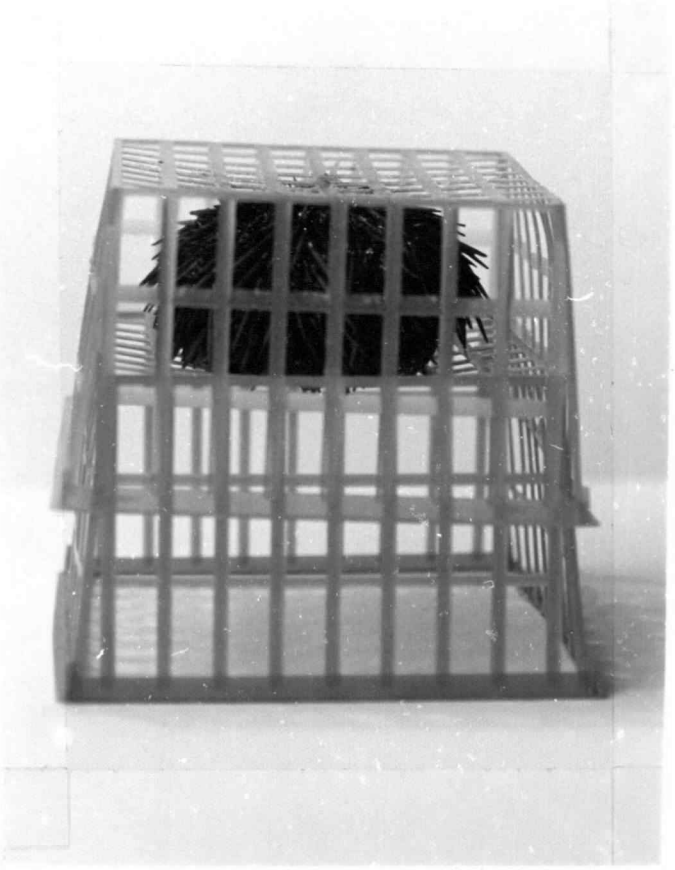

Figure 1: Photograph of containers used for the sea urchins during experiments.

The bacterial suspension (0.4 - 0.6 ml) was injected through the peristomial membrane with a 26 gauge, 0.5 inch needle and disposible 1 ml tuberculin syringe. Half of the inoculum was injected slowly, then the other half was injected 180° away from the initial injection site because of the time required (40 - 90 min) for the bacteria to disperse evenly 180° from the injection site (Appendix III). For sampling, the peristomium was washed several times with cold sterile sea water. Coelomic fluid samples (0.2 - 0.3 ml) were removed with a sterile 26 g, 0.5 inch needle and 1 ml syringe. The samples were either directly plated for sterility checks before the injection of bacteria or serially diluted in sterile sea water and plated by pour plate methods using soft agar at 47-49°C for viable counts. The bacterial suspensions used for injections were also serially diluted and plated to determine the actual initial inoculum because this varied somewhat between batches of bacteria. During the experiments, animals were placed in 20 liter plastic aquaria filled with sea water and aerated with air stones. All experiments were conducted in a 10°C cold room.

Urchins which were held for secondary clearance experiments were fed and maintained in individual containers in the circulating sea water system.

Humoral factors: Bactericidins

Coelomic fluid (5-10 ml) was removed with a 20g, 1.5 inch needle and plastic syringe or drained out through a cut in the peristomium. The coelomic fluid was immediately filtered through two Millipore prefilters

by slight positive pressure, into ice-cold beakers to remove cells without damage, then sterilized by passage through a 0.22 um Millipore filter. Two ml of cell-free coelomic fluid were placed in sterile 5 ml glass screw-cap vials. Sterile-filtered artificial sea water (Instant Ocean) or Hepes sea water medium (Bertheussen and Seljelid, 1978) was used as control fluid. Twenty ul aliquots of the bacterial suspension were added to each vial. All experiments were carried out at 10°C. Samples (10 and 100 ul) were removed at various times and plated directly by pour plate methods for viable counts.

To determine the effects of clotting on bactericidal activity, half of the coelomic fluid from one animal was allowed to clot for 30 min at 10°C before filtering out the cells; the other half was immediately filtered and held at 10°C. Because clotted coelomic fluid was found not to have a greater bactericidal activity than unclotted in the three urchins tested (Appendix IV), coelomic fluid was not clotted in subsequent experiments. To determine the heat stability of the bactericidal substance(s), half of the coelomic fluid from each urchin was heated to 100°C for 10 min, then cooled to 10°C, while the other half was kept at 10°C.

Humoral factors: Bacterial agglutinins

Urchins were bled through a cut in the peristomium into an ice-cold sterile beaker. The coelomic fluid (5-10 ml) was filtered as described for bactericidin tests. Cell-free coelomic fluid was added to the first well of a microtiter plate and serially diluted by one-half with 10°C

artificial sea water (Instant Ocean). The last well was a sea water-only control. Microtiter plates with either 6 rows of 10-100 ul wells, or 4 rows of 6-1 ml wells were used in different experiments. An equal volume of bacterial suspension in sea water was added to each well, then the plates were covered and placed on a rotary table (60 rpm) at 10°C. Plates were inspected for agglutination with a dissecting microscope and a 15-20 ul sample was removed for inspection under higher power magnification (400 X). The proportions of bacteria to coelomic fluid volumes and the times of inspection are given in Appendix V.

Total and differential coelomocyte cell counts

Sea urchins were injected with the marine Gram negative bacteria. Control urchins were injected with an equal volume of sterile sea water. Coelomic fluid was removed slowly with a 20 g, 1.5 inch needle, to prevent cell damage, into an equal volume of cold anticoagulant (30 mM EDTA in 0.3 M Hepes buffered sea water, after Bertheussen and Seljelid, 1978). Cells were dispensed onto a Brightline hemacytometer and two differential counts made and averaged. Phagocytes, vibratile cells, and red and colorless spherule cells were counted. Total cell counts were determined by adding the counts for the four cell types.

Phagocytosis and killing of bacteria

A unique property of the fluorochrome, acridine orange (AcO), is that microbes stained with a dilute solution of this dye will fluoresce

green if viable and red if dead, under ultraviolet light (Strugger, 1948). Pantazis and Kniker (1979) utilized this property to distinguish between viable and non-viable bacteria in human phagocytic leukocytes to measure the killing capacity of the cells. Their methods were modified for urchin phagocytes so that *in vivo* and *in vitro* phagocytosis and killing could be observed.

A $1:10^4$ solution of AcO was made by suspending 10 mg of AcO in 10 ml distilled water, then diluting by adding 0.1 ml AcO solution to 9.9 ml Hepes sea water medium (HSM).

In vitro tests: Twenty ul aliquots of bacterial suspension were added to acid rinsed (50% nitric acid, overnight, distilled water rinsed) glass coverslips. Whole coelomic fluid was removed with a 20g, 1.5 inch needle and 200 ul immediately dispensed onto each coverslip with bacteria. Coverslips were then placed in Petri dishes with moistened filter paper at 10°C. Coverslips were examined at various time intervals. The coverslip was gently rinsed with ice-cold sea water to remove the clot, dipped in AcO solution for one minute, rinsed with AcO-free HSM, blotted and sealed onto glass slides with nail polish. Cells were promptly observed with a Zeiss microscope equipped with an ultraviolet light source at 500 and 1250 X magnification.

In vivo tests: Bacteria were injected into urchins as previously described and samples removed at various times post injection. Cells were allowed to settle for 15 min before rinsing and dyeing as described above.

RESULTS

I. Primary Clearance

A. Gram negative bacteria

Injection of six urchins with 3.3×10^7 marine gram negative bacteria ml^{-1} of coelomic fluid (CF) resulted in rapid reduction in numbers of viable bacteria in the CF over the first 1-3 hr post-injection (p.i.), followed by a slower rate of clearance (Figure 2). Viable counts (v.c.), declined 95.7 ± 1.6% (mean ± standard error) by 1 hr p.i., 99.2 ± 0.3% by 3 hr p.i., and 99.86 ± 0.04% by 6 hr p.i. (Table 1). No further decline in v.c. was noted at 9 and 24 hr p.i. in this case. However, in later experiments when only two samples were taken from each animal, a further decline was observed of 99.40 ± 0.30% and 99.91 ± 0.06% at 6 and 24 hr p.i. respectively when 6×10^6 bacteria ml^{-1} of CF were injected (Figure 3).

From preliminary experiments with samples taken at shorter time intervals, the rate of bacterial clearance looked approximately exponential (i.e., $\log_{10}$ concentration of bacteria vs. time is linear) for the first 1.5 - 2.5 hr p.i. To verify this observation, four or five samples were removed from each of nine urchins injected with 10^5 - 10^7 bacteria ml^{-1} of CF. Lines were fitted to the log transformed data in two ways using linear regression: (1) calculating independent slopes for each urchin, obtaining a mean and standard error for those slopes ($R^2 = 0.963$), and (2) fitting nine parallel lines to the data

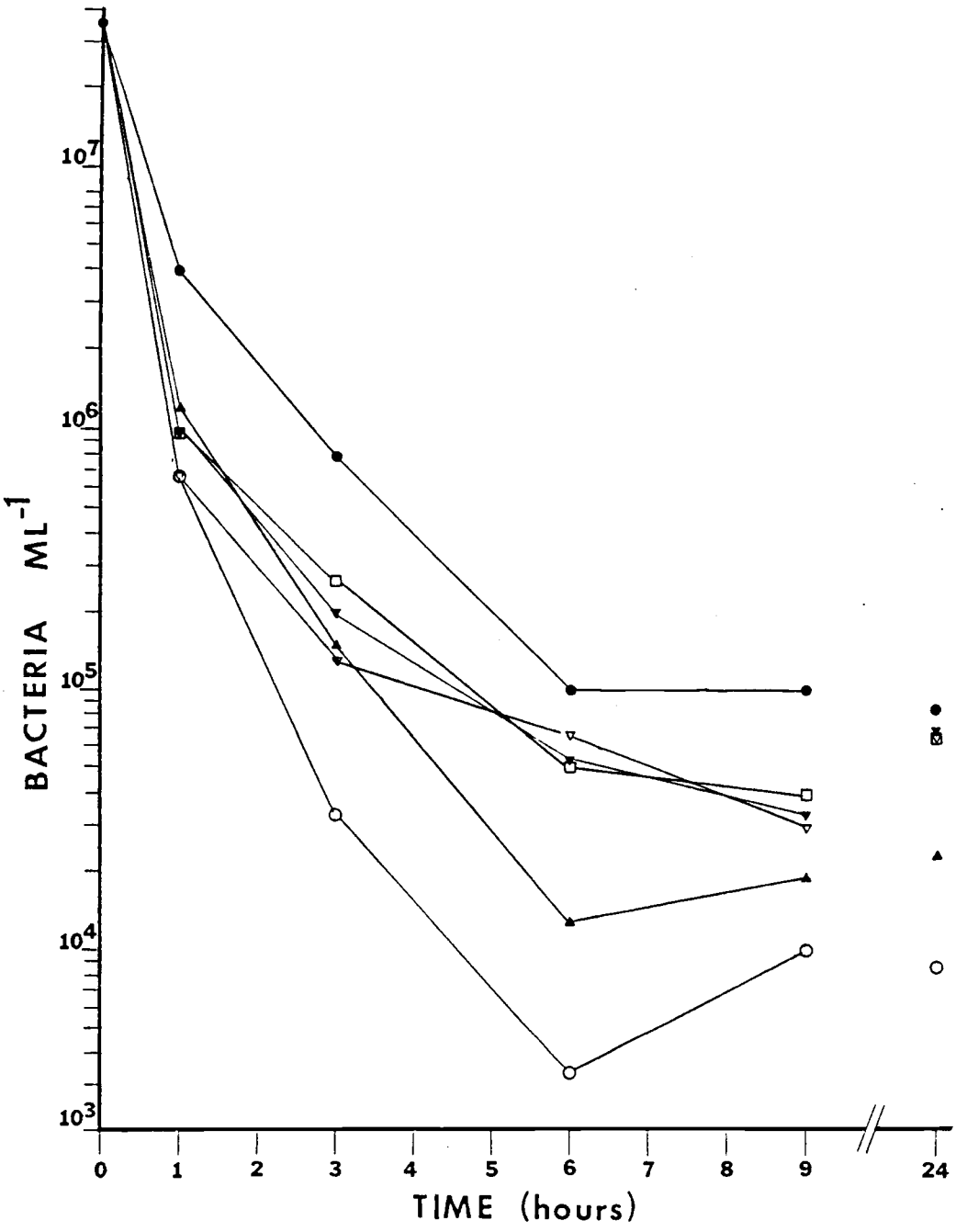

Figure 2: Primary clearance of the Gram negative bacteria; six urchins sampled 1, 3, 6, 9, and 24 hr after injection.

Table 1: Summary of primary clearance data (mean ± SE) for the three bacteria, Gram negative, Gram positive, and Aeromonas salmonicida. Number of urchins sampled in parentheses.

Bacteria	K (log_{10} bac min^{-1})	# bac ml^{-1} injected	% reduction in viable counts			
			1 hr p.i.	3 hr p.i.	6 hr p.i.	24 hr p.i.
Gram negative	-0.0179±0.0016 (9)	$3.3X10^7$ (6)	95.7±1.6	99.2±0.3	99.86±0.04	–
		$6.0X10^6$ (11)	–	–	99.40±0.30	99.91±0.058
Gram positive	-0.0195±0.0037 (3)	$1.5X10^7$ (2) $1.2X10^6$ (1)	97.7±0.5	90.9±3.3	95.14±2.07	–
		$1.7X10^6$ (12)	–	–	91.47±2.06	96.49±0.068
Aeromonas salmonicida	–	$3.3X10^7$ (3)	61.5±3.6	93.9±2.6	97.75±0.97	–
		$3.3X10^5$ (3)	75.4±13.9	99.3±0.5	99.91±0.03	–
		$1.7X10^7$ (6)	–	–	94.84±2.93	–
		$1.7X10^6$	–	–	99.67±1.02	–

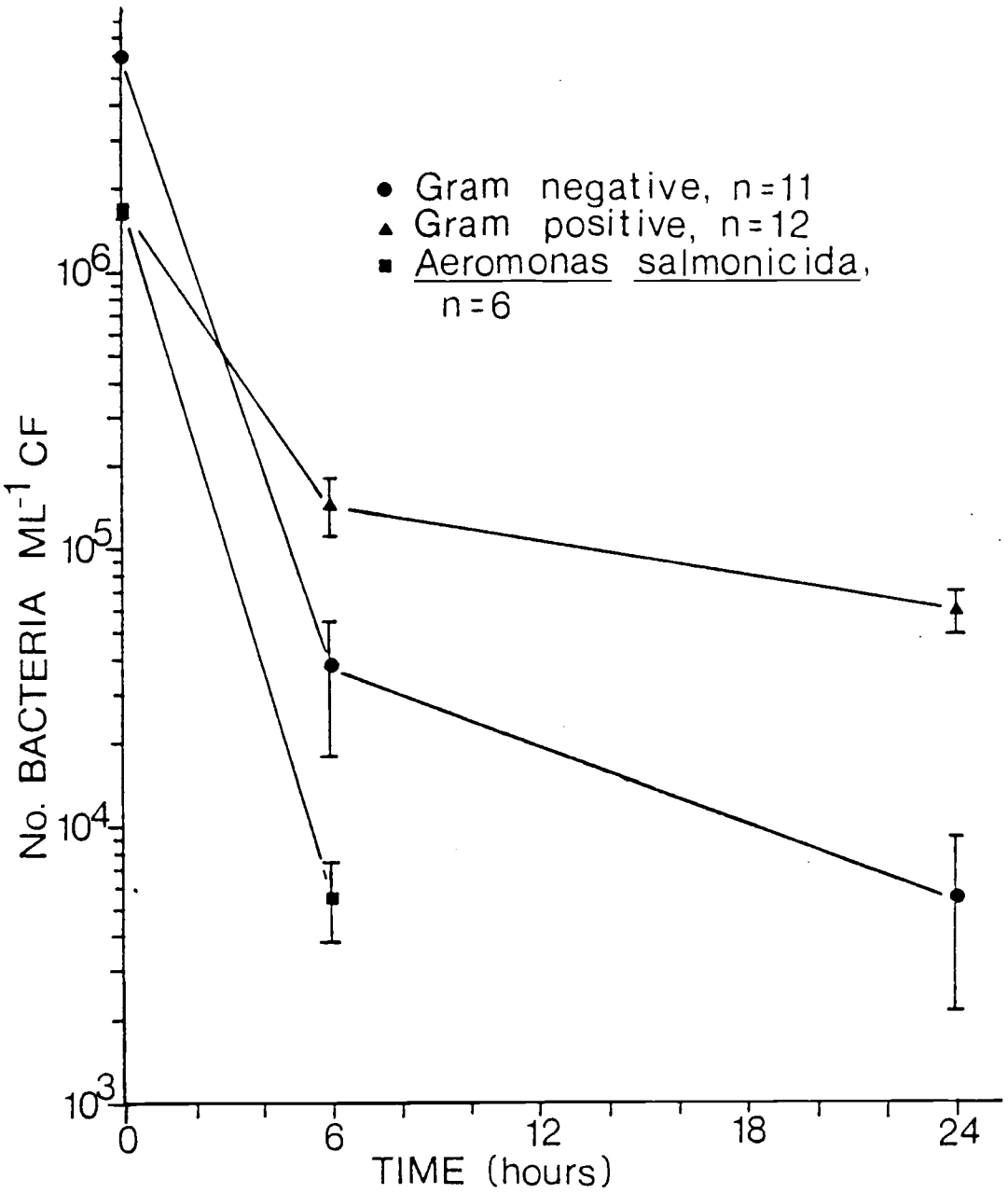

Figure 3: Primary clearance of the Gram negative and Gram positive bacteria, sampled at 6 and 24 hr after injection, and Aeromonas salmonicida, sampled at 6 hr (mean ± standard error.

(R^2 = 0.940). Quadratic equations were also fitted to the data to determine whether the data were better expressed by a curve than a line; however, the fit was not significantly improved (F = 0.349; p >0.5). The slopes of the lines fitted to the data were not different from one another using the two methods, being -0.0179 ± 0.0016 $\log_{10}$ bacteria min^{-1} using independent lines and -0.0181 using parallel lines. These slopes are equivalent to the "Phagocytic Index" or K value as defined by Biozzi, Benacerraf, and Halpern (1956) and Benacerraf, Sebastyen and Schlossman (1959) for the particle clearance kinetics in mammals, and Renwrantz and Mohr (1978) for particle clearance in the land snail, Helix. The slope does not appear to be strongly dose dependent since the K values for the two urchins injected with 2.6 x 10^5 bacteria ml^{-1} of CF fall into the range (although in the lower end) of the seven urchins injected with 2.6 - 3.9 x 10^7 bacteria ml^{-1} of CF (Appendix VI).

Based on the above experiments (and others with similar results), the following conclusions were made about primary clearance of the marine Gram negative bacteria in S. purpuratus:

(1) Clearance of 10^5 - 10^7 injected bacteria ml^{-1} is exponential for the first 1.5 - 2.5 hr p.i. After that time, clearance continues at a much slower rate over several days. When injected with approximately 10^7 bacteria ml^{-1} of CF, bacteria are generally undetectable by 4-8 d p.i.

(2) Although most urchins exhibit similar patterns of bacterial clearance as described in (1), there is high variability in the rates of clearance for individual urchins and this variability might mask changes

in secondary clearance. To eliminate some of the influence of individual differences and variations in initial concentrations of bacteria due to inaccurate estimates of CF volume, the same individuals were used for primary and secondary clearance determinations in memory experiments.

(3) Repeated sampling during the first 6 hr p.i. apparently reduced the ability of an animal to clear bacteria (Figures 2 and 3). To avoid this effect, fewer samples were taken from single individuals in later experiments. Because the mechanism of memory response (if it exists) is unknown and may require some time period for activation, 6 and 24 hr samples were generally taken, since values at these times are the cummulative result of all earlier responses.

(4) Injection doses of 10^6 - 10^7 bacteria ml^{-1} of CF were selected for further experiments because at those doses, bacteria were: (a) cleared without obvious trauma and no mortality, (b) detectable at high enough levels at 6 and 24 hr p.i. that a decline upon secondary injection would still be quantifiable even with small CF sample volumes, and (c) persistent in the coelomic fluid for a period of 4-8 d. This persistence of viable bacteria in the CF was considered advantageous because sensitization and induction of memory may require a long period of exposure (as seen in graft rejection). Booster injections were therefore also not required.

B. Gram positive bacteria

Primary clearance of the marine Gram positive bacteria was quite different from that of the Gram negative bacteria (Figure 4). The

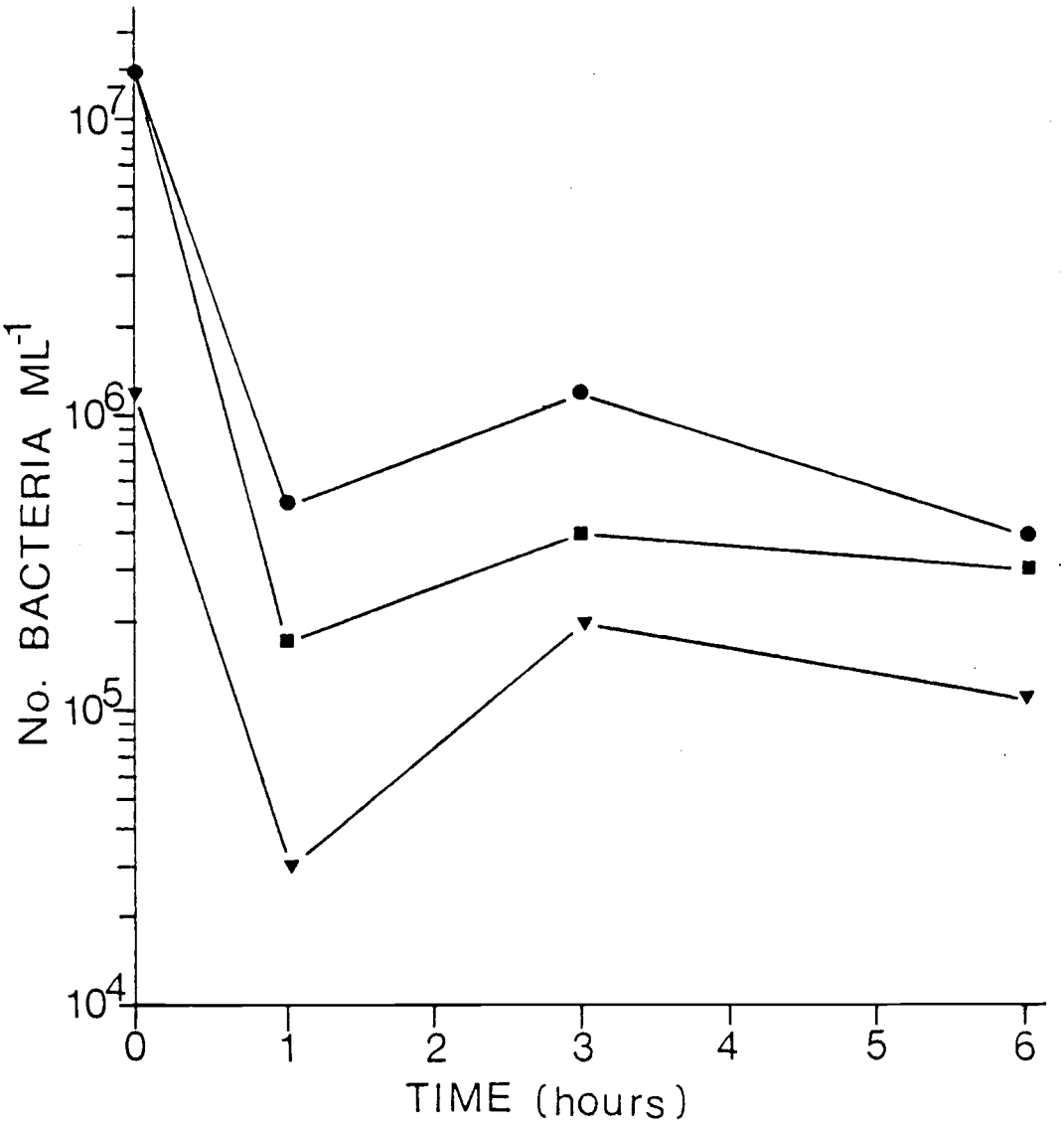

Figure 4: Primary clearance of the Gram positive bacteria; three urchins sampled at 1, 3, and 6 hr after injection.

initial drop in v.c. was similar to, or more accelerated than, that of the Gram negative, 97.7 ± 0.5% by 1 hr p.i. (Table 1). However, the v.c. had increased by 3 hr p.i. to a 90.9 ± 3.3% reduction and were back down to a 95.1 ± 2.1% reduction by 6 hr p.i. In later experiments, samples were taken at 6 and 24 hr p.i. (Figure 3). When 10^7 bacteria were injected, v.c. dropped 91.47 ± 2.06% by 6 hr and 96.49 ± 0.68% by 24 hr p.i. The differences in clearance between the two bacteria were significant at both 6 and 24 hr p.i. using a Student's t-test on the slopes ($p < 0.001$). Bacteria were generally undetectable by 8-12 d after injection with 10^6-10^7 bacteria ml^{-1} of CF. The K value (X ± SE of individual slopes) from the Gram positive bacteria was -0.0190 ± 0.0037 $\log_{10}$ bacteria min^{-1} for the first 90 min p.i. (n = 3), not different from K for Gram negative bacteria.

C. Aeromonas salmonicida

Clearance of 10^7 A. salmonicida ml^{-1} of CF differed from that of the two marine bacteria (Figure 5a). At 1 hr p.i., v.c. had dropped 61.5 ± 3.2%, a significantly lower rate of clearance than that of the marine bacteria ($p < 0.001$) (Table 1). By 3 and 6 hr p.i. however, clearance was 93.9 ± 2.6% and 97.75 ± 0.97% respectively, comparable to the percent decrease in the other bacteria. Declines continued to 99.997% by 48 hr. When sampled first at 6 hr, clearance was similar to that of the marine Gram negative, a 99.67 ± 1.02% reduction (Figure 3). Injection of 10^5 A. salmonicida ml^{-1} of CF resulted in a somewhat more rapid decline in v.c. than higher doses (Figure 5b), a 75.4 ± 13.9% decline by 1 hr p.i., 99.3 ± 0.5% by 3 hr p.i., and 99.91 ± 0.03% by 6 hr p.i. By 48 hr p.i., bacteria were no longer detectable in any of the

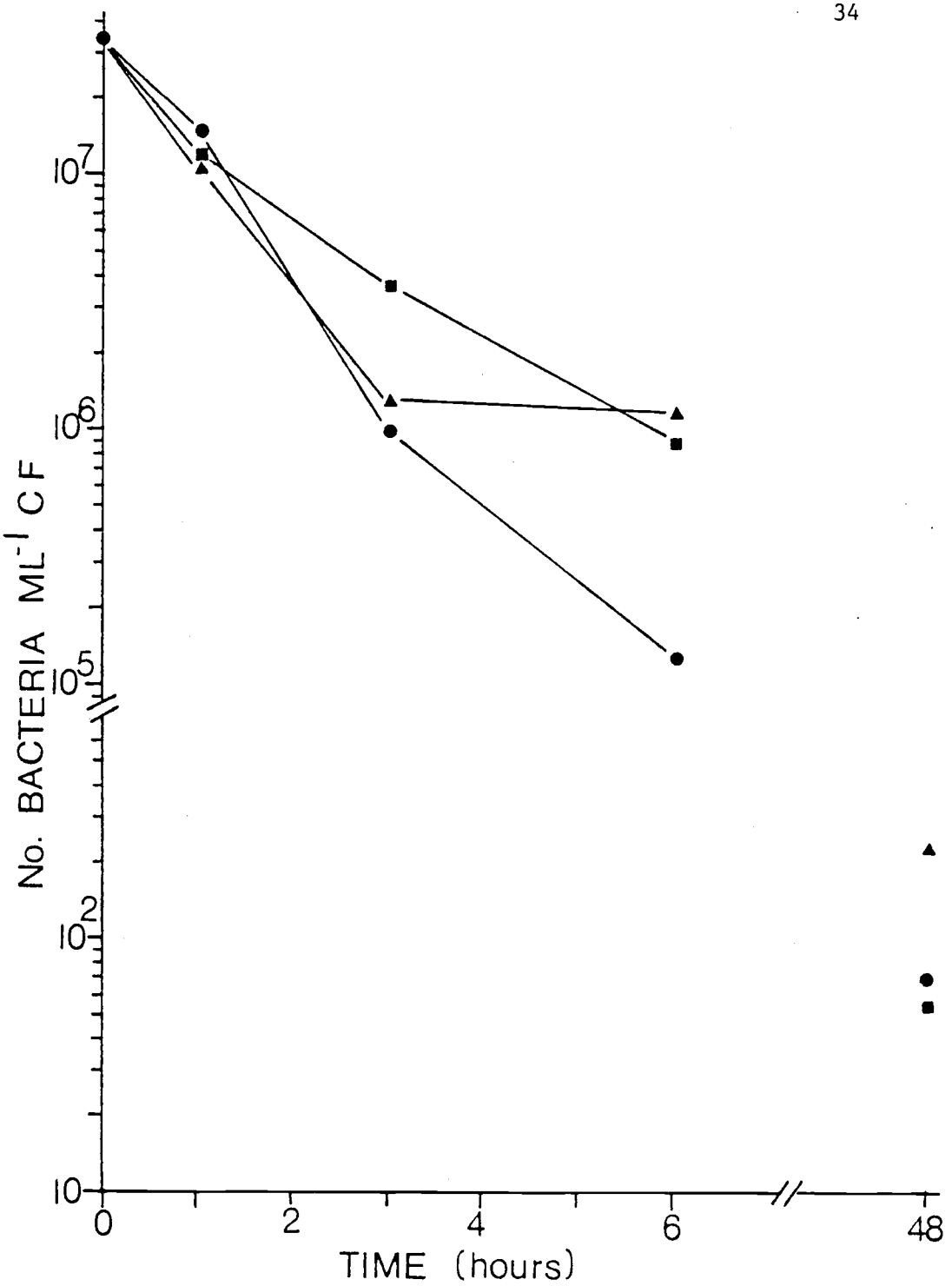

Figure 5a: Primary clearance of a high dose of Aeromonas salmonicida (3.3×10^7 bacteria ml^{-1} of coelomic fluid); three urchins sampled at 1, 3, 6 and 48 hr after injection.

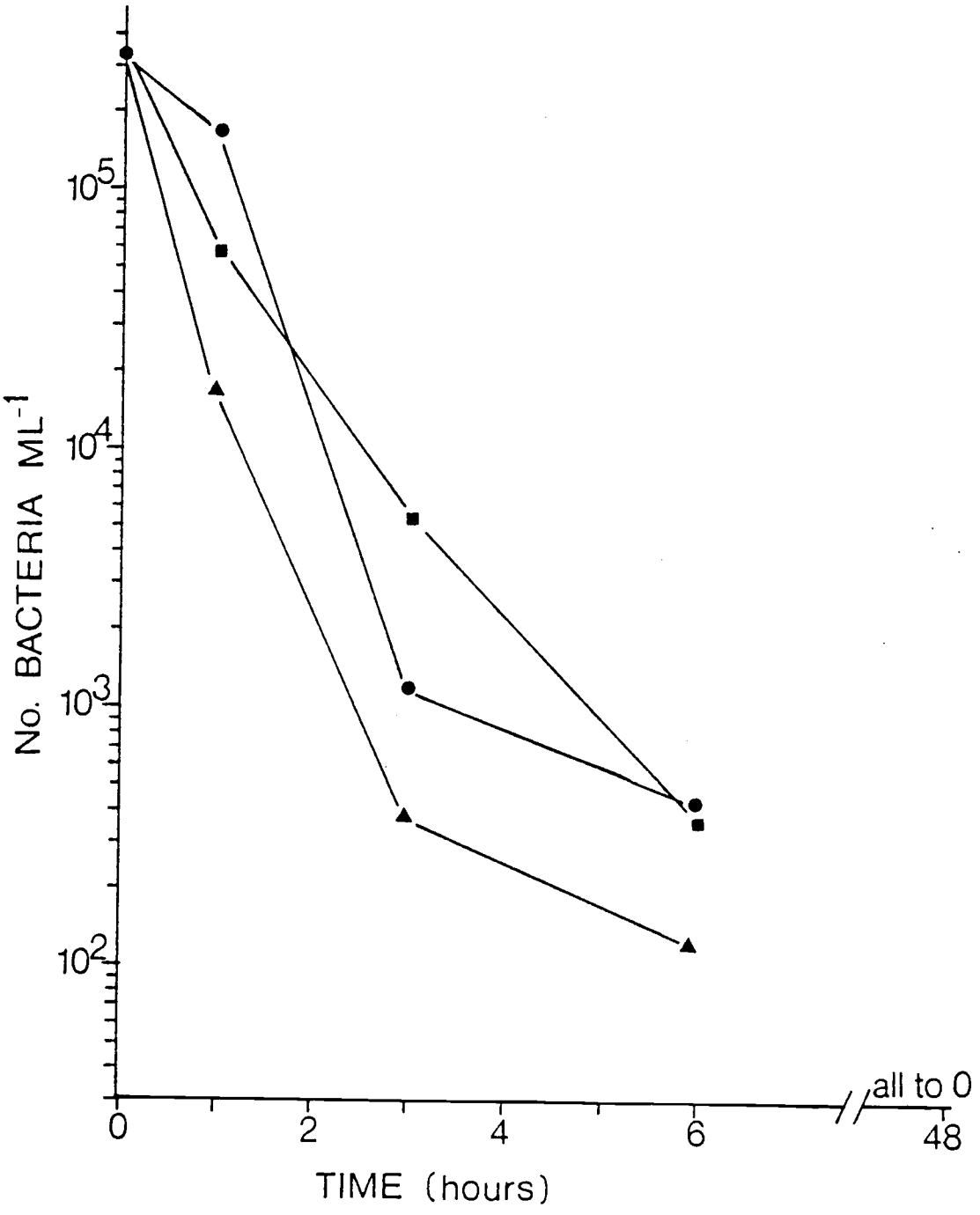

Figure 5b: Primary clearance of a low dose of Aeromonas salmonicida (3.3×10^5 bacteria ml^{-1} of coelomic fluid); three urchins sampled at 1, 3, 6 and 48 hr after injection.

three urchins. Samples were not taken during the first 90 min of clearance, so K was not determined.

II. Primary vs. Secondary Clearance

A. Gram negative bacteria

Primary and secondary clearance data for twelve urchins are given in Table 2. Since the first and second inoculation doses were never identical, the slopes of the lines were calculated from $\log_{10}$ transformed v.c. at time = 0 to 6 and 24 hr. The difference in slope between primary and secondary clearance for each urchin was calculated; a negative difference means secondary clearance was more rapid than primary clearance while a positive value indicates that secondary clearance was slower. The mean difference for the four urchins sampled at 6 and 24 hr p.i. was calculated and compared to 0, for injection intervals of 9, 14, and 21 d.

The mean values for the log transformed difference in slopes were not found to be significantly different from 0 when injection intervals were 9, 14, or 21 d, nor were they different when the data from all twelve urchins were pooled (Table 2). In a few cases (urchins 5, 7, and 9), the animals exhibited fairly low negative values, although only urchin 7 was consistently much lower at both 6 and 24 hr p.i.

B. Gram positive bacteria

When the experiment was repeated using the Gram positive bacteria, similar results were obtained (Table 3). Again, no significant difference was noted between the slopes of primary and secondary

Table 2: Clearance of primary (1^o) injections of the Gram negative bacteria and of secondary (2^o) injections given 9 and 14 days later.

1^o-2^o Interval (days)	Sample Time (hours)	Urchin #	# Bac ml^{-1} 1^o	# Bac ml^{-1} 2^o	Slope (log_{10}bac hr^{-1}) 1^o	Slope (log_{10}bac hr^{-1}) 2^o	Difference in slope (1^o-2^o)	$\bar{X} \pm SD$	p
	0	All	$6.0x10^6$	$1.3x10^7$					
	6	1	$6.7x10^3$	$9.7x10^4$	0.492	0.353	0.139		
		2	$4.0x10^4$	$5.7x10^5$	0.363	0.167	0.196	0.083 ± 0.106	>.2
9		3	$1.9x10^4$	$7.3x10^4$	0.417	0.375	0.042		
		4	$2.3x10^4$	$2.7x10^4$	0.403	0.447	-0.044		
	24	1	$2.7x10^3$	$2.0x10^3$	0.140	0.159	-0.019		
		2	$2.0x10^3$	$1.6x10^4$	0.145	0.121	0.024	0.0058 ± 0.0180	>.2
		3	$4.0x10^3$	$1.3x10^4$	0.133	0.125	0.008		
		4	$9.0x10^2$	$3.3x10^3$	0.160	0.150	0.010		
	0	All	$6.0x10^6$	$1.9x10^7$					
	6	5	$1.7x10^4$	$4.3x10^5$	0.425	0.275	0.150		
		6	$8.7x10^2$	$2.0x10^4$	0.640	0.497	0.143	0.038 ± 0.190	>.2
14		7	$6.3x10^4$	$6.7x10^3$	0.330	0.575	-0.245		
		8	$2.1x10^5$	$2.8x10^6$	0.243	0.138	0.105		
	24	5	$4.0x10^3$	$1.4x10^3$	0.133	0.172	-0.039		
		6	$4.0x10^4$	nd	0.090			-0.0303 ± 0.0515	>.2
		7	$5.3x10^5$	$2.4x10^4$	0.044	0.121	-0.077		
		8	$1.7x10^3$	$2.1x10^4$	0.148	0.123	0.025		

Table 2 (continued): Clearance of primary (1^o) injections of the Gram negative bacteria and of secondary (2^o) injections given 21 days later.

1^o-2^o Interval (days)	Sample Time (hours)	Urchin #	# bac ml^{-1} 1^o	# bac ml^{-1} 2^o	Slope (log_{10}bac hr^{-1}) 1^o	Slope (log_{10}bac hr^{-1}) 2^o	Difference in slope (1^o-2^o)	$\bar{X} \pm SD$	p
	0	All	$6.0x10^6$	$1.8x10^7$					
	6	9	$1.2x10^4$	$1.7x10^3$	0.450	0.672	-0.222		
		10	$9.7x10^3$	$1.7x10^5$	0.465	0.258	0.207	0.032 ± 0.182	>.2
21		11	$1.5x10^4$	$1.5x10^5$	0.433	0.338	0.095		
		12	$3.3x10^4$	$1.9x10^5$	0.377	0.330	0.047		
	24	9	$5.7x10^2$	$5.0x10^2$	0.168	0.190	-0.022		
		10	$4.7x10^2$	$5.0x10^3$	0.171	0.148	0.023	0.013 ± 0.024	>.2
		11	$3.7x10^2$	$4.0x10^3$	0.175	0.153	0.023		
		12	$5.0x10^3$	$7.0x10^4$	0.128	0.100	0.028		

Table 3: Clearance of primary (1^o) injections of the Gram positive bacteria and of secondary (2^o) injections given 9 and 14 days later.

1^o-2^o Interval (days)	Sample Time (hours)	Urchin #	# bac ml^{-1} 1^o	# bac ml^{-1} 2^o	Slope (log_{10}bac hr^{-1}) 1^o	Slope (log_{10}bac hr^{-1}) 2^o	Difference in slope (1^o-2^o)	$\bar{X} \pm SD$	p
	0	All	$1.7x10^6$	$4.4x10^6$					
	6	1	$3.3x10^5$	$2.7x10^5$	0.118	0.202	-0.082		
		2	$3.0x10^4$	$3.0x10^5$	0.292	0.195	0.097	-0.027 ± 0.108	$\geq .2$
9		3	$2.4x10^5$	$8.4x10^4$	0.142	0.287	-0.145		
		4	$7.0x10^4$	$2.5x10^5$	0.230	0.208	0.022		
	24	1	$9.0x10^4$	$3.2x10^5$	0.0533	0.0475	0.0058		
		2	$1.3x10^4$	$1.2x10^5$	0.0904	0.0650	0.0254	0.0107 ± 0.0115	$.1<p<.2$
		3	$3.2x10^4$	$1.7x10^5$	0.0721	0.0588	0.0133		
		4	$4.3x10^4$	$1.0x10^5$	0.0667	0.0683	0.0016		
	0	All	$1.7x10^6$	$2.2x10^6$					
	6	5	$1.0x10^5$	$5.0x10^5$	0.205	0.107	0.098		
		6	$1.3x10^5$	$1.0x10^5$	0.187	0.223	-0.036	0.027 ± 0.078	$\geq .2$
14		7	$1.3x10^5$	$9.0x10^4$	0.187	0.232	-0.045		
		8	$3.0x10^4$	$1.4x10^5$	0.292	0.200	0.092		
	24	5	$5.3x10^4$	$2.4x10^4$	0.0629	0.0817	-0.0188		
		6	$6.3x10^4$	$3.6x10^4$	0.0596	0.0745	-0.0149	-0.0084 ± 0.0160	$\geq .2$
		7	$1.1x10^5$	$6.0x10^4$	0.0496	0.0650	-0.0154		
		8	$2.2x10^4$	$6.7x10^4$	0.0788	0.0633	0.0155		

Table 3 (continued): Clearance of primary (1^o) injections of the Gram positive bacteria and of secondary (2^o) injections given 21 days later.

1^o-2^o Interval (days)	Sample time (hours)	Urchin #	# bac ml^{-1} 1^o	# bac ml^{-1} 2^o	Slope (log_{10}bac hr^{-1}) 1^o	Slope (log_{10}bac hr^{-1}) 2^o	Difference in slope (1^o-2^o)	$\bar{X} \pm SD$	p
	0	All	1.7×10^6	7.7×10^6					
	6	9	1.5×10^5	1.0×10^5	0.175	0.315	-0.140		
		10	2.7×10^4	1.4×10^5	0.300	0.291	0.009	-0.063 ± 0.061	$.1 < p < .2$
21		11	4.0×10^5	8.7×10^5	0.105	0.158	-0.053		
		12	1.0×10^5	1.8×10^5	0.205	0.272	-0.067		
	24	9	1.2×10^5_4	3.3×10^4_4	0.0480	0.0987	-0.0507		
		10	2.0×10^5_5	9.0×10^5	0.0804	0.0808	-0.0004	-0.0200 ± 0.0253	$> .2$
		11	1.2×10^4_4	6.0×10^5	0.0479	0.0463	0.0017		
		12	3.0×10^4	2.6×10^4	0.0729	0.1033	-0.0304		

clearance at 9, 14, and 21 d even when the data were combined from all 12 urchins.

C. Aeromonas salmonicida

Clearance was not accelerated with second exposure to A. salmonicida when the injection interval was 19 d, and no difference was evident when the data from both doses were combined (Table 4).

III. Humoral Factors: Bactericidins

A. Gram negative bacteria

Coelomic fluids from six urchins, three injected 3 d earlier and three uninjected, were tested for their effects on viability of the marine Gram negative bacteria. Sea water was also tested as a control (Figure 6). The results for each sampling time were compared using a one-sided Student's t-test. No significant differences were noted between any of the three treatments at 15 and 45 min post-inoculation. Although v.c. were lower in CF from preinjected than in uninjected urchins at 90 min or 5 hr, the difference was not significant ($0.2 < p < 0.3$). Viable counts were significantly lower in the CF from the six urchins than in sea water at 90 min ($p < 0.005$) and 5 hr ($p < 0.01$).

To determine whether the activity was lost after heating to 100°C for 10 min, coelomic fluids from four urchins were tested (Figure 7). In one case, 10^5 bacteria ml^{-1} of test fluid were added to the cell-free CF (heated and untreated) from a single urchin and to Hepes sea water medium (HSM) (Figure 7). V.c. in the untreated CF declined to

Table 4: Clearance of primary (1^o) injections of Aeromonas salmonicida and of secondary (2^o) injections given 19 days later.

Sample time (hours)	Urchin #	# bac ml^{-1} 1^o	# bac ml^{-1} 2^o	Slope (log_{10}bac hr^{-1}) 1^o	Slope (log_{10}bac hr^{-1}) 2^o	Difference in slope (1^o-2^o)	$\bar{X} \pm SD$	p
0	All	1.7×10^7	2.6×10^7					
6	1	1.4×10^5	6.7×10^4	0.347	0.431	-0.084		
	2	3.7×10^4	7.9×10^4	0.444	0.420	0.024	-0.011 ± 0.098	$\geq .2$
	3	1.7×10^4	1.2×10^5	0.500	0.389	0.111		
	4	1.7×10^5	7.0×10^4	0.333	0.428	-0.095		
0	All	1.7×10^6	2.6×10^6					
6	5	2.7×10^3	3.3×10^4	0.467	0.316	0.151		
	6	2.2×10^3	2.1×10^4	0.481	0.349	0.132	0.067 ± 0.090	$\geq .2$
	7	1.1×10^4	1.0×10^4	0.365	0.402	-0.037		
	8	1.1×10^4	2.3×10^4	0.365	0.342	0.023		

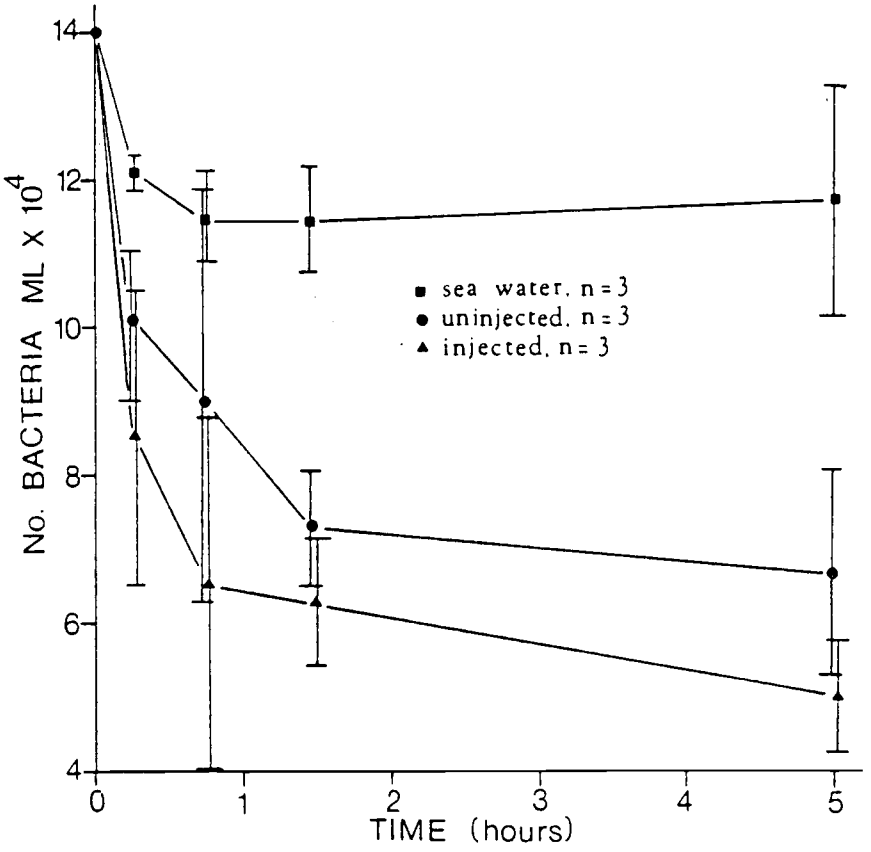

Figure 6: Viable counts (mean ± SE) for the Gram negative bacteria in sea water and in cell-free coelomic fluid from injected and uninjected urchins.

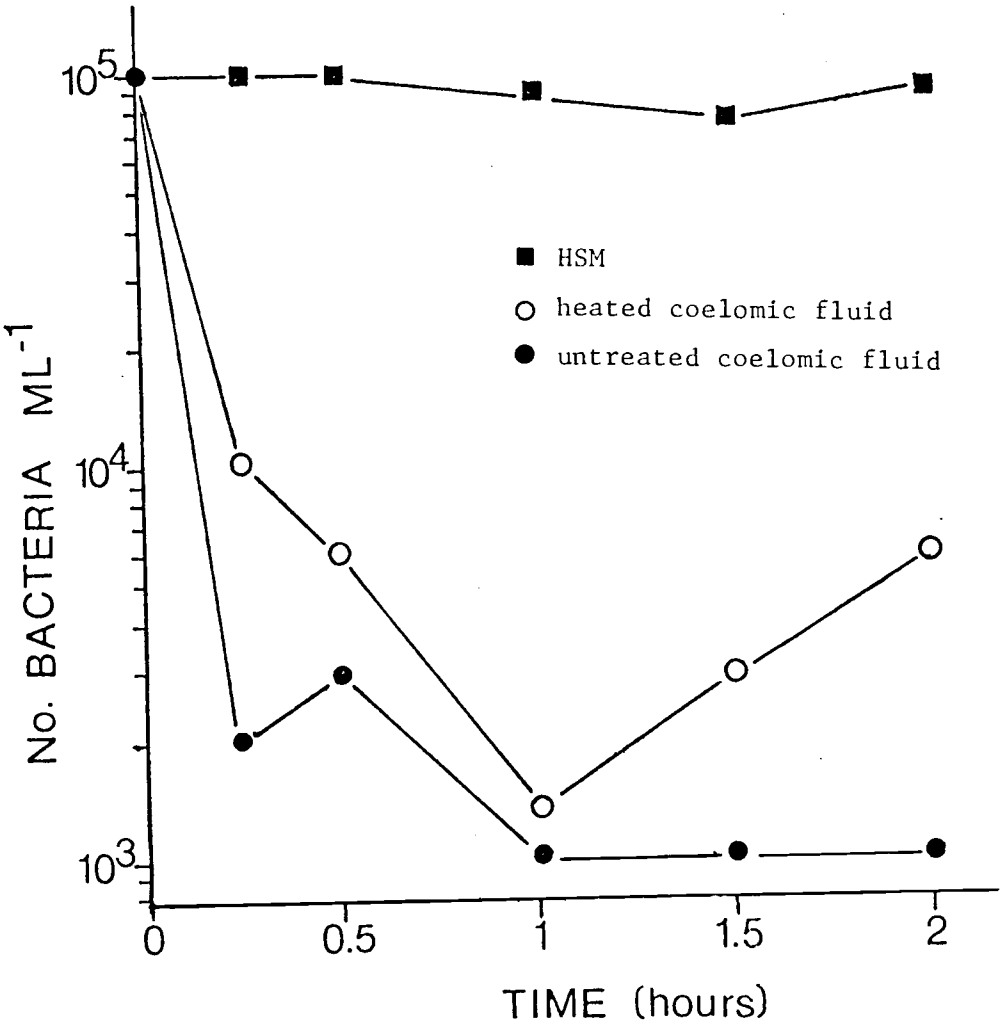

Figure 7a: Viable counts of Gram negative bacteria in Hepes sea water medium, heated and untreated cell-free coelomic fluid from a single urchin.

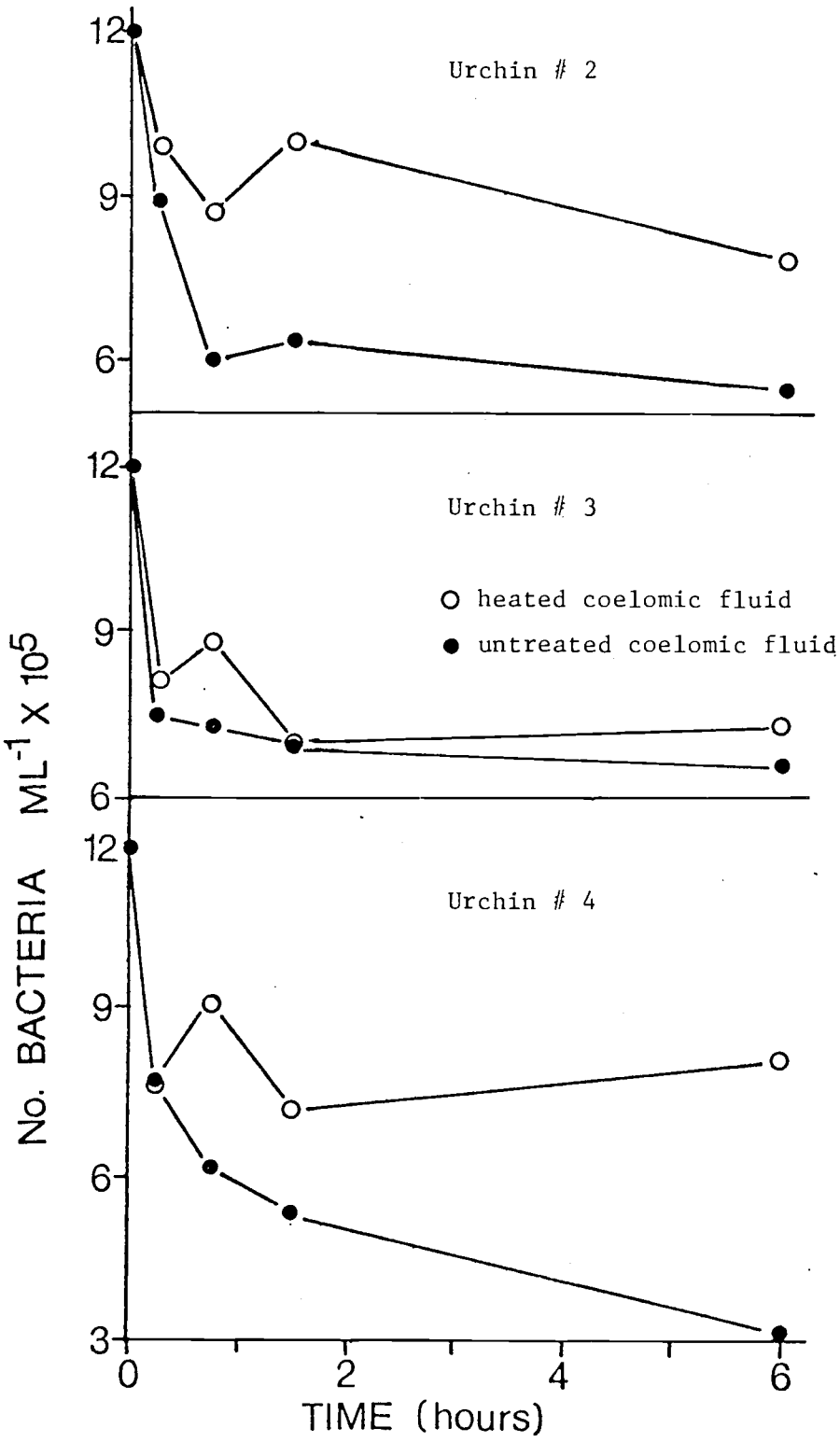

Figure 7b: Viable counts of Gram negative bacteria in heated and untreated cell-free coelomic fluid from three urchins.

approximately 10^3 v.c. (loss of 9.9×10^4 v.c. ml^{-1} or 99%) by 1 hr and to 5×10^3 (loss of 9.5×10^4 v.c. ml^{-1} or 95%) in the heated CF. V.c. in HSM did not change over the 2 hr sampling period. The lower values (approximately 10^3) for both CF samples were not very precise since only a few bacteria (1-6) were detectable at the lowest dilutions plated.

Inoculation of 12×10^5 bacteria ml^{-1} of test fluid in CF from three other urchins resulted in a decrease to $7-8 \times 10^5$ v.c. ml^{-1} (loss of $4-5 \times 10^5$ v.c. ml^{-1} or 33-42%) in 6 hr for heated CF samples and a decrease to $3-6 \times 10^5$ v.c. ml^{-1} (loss of $6-9 \times 10^5$ v.c. ml^{-1} or 50-75%) for untreated CF (Figure 7). The untreated CF v.c. were lower than the heated CF v.c. for all three urchins at 45, 90 min and 6 hr after inoculation. The difference was significant using a one-sided paired t-test at 45 min ($p < 0.025$), but not at 15 min ($0.100 < p < 0.200$), 90 min ($0.050 < p < 0.100$), and 6 hr ($0.050 < p < 0.100$).

B. Gram positive bacteria

The pattern of viable counts in seawater and CF with the Gram positive bacteria differed from that of the Gram negative (Figure 8). V.c. had declined 2.5 orders of magnitude below the initial inoculum and sea water control v.c. at 2 hr post-inoculation. All five test fluids from different urchins exhibited this large decline in v.c. at 2 hr while none of the three controls exhibited a similar response. This result cannot reflect bactericidal activity since counts returned to values not significantly different from sea water controls at 5 hr ($p > 0.200$). At 20 hr, v.c. in CF were lower than those in sea water ($p = 0.05$). The variability between v.c. for CF from different

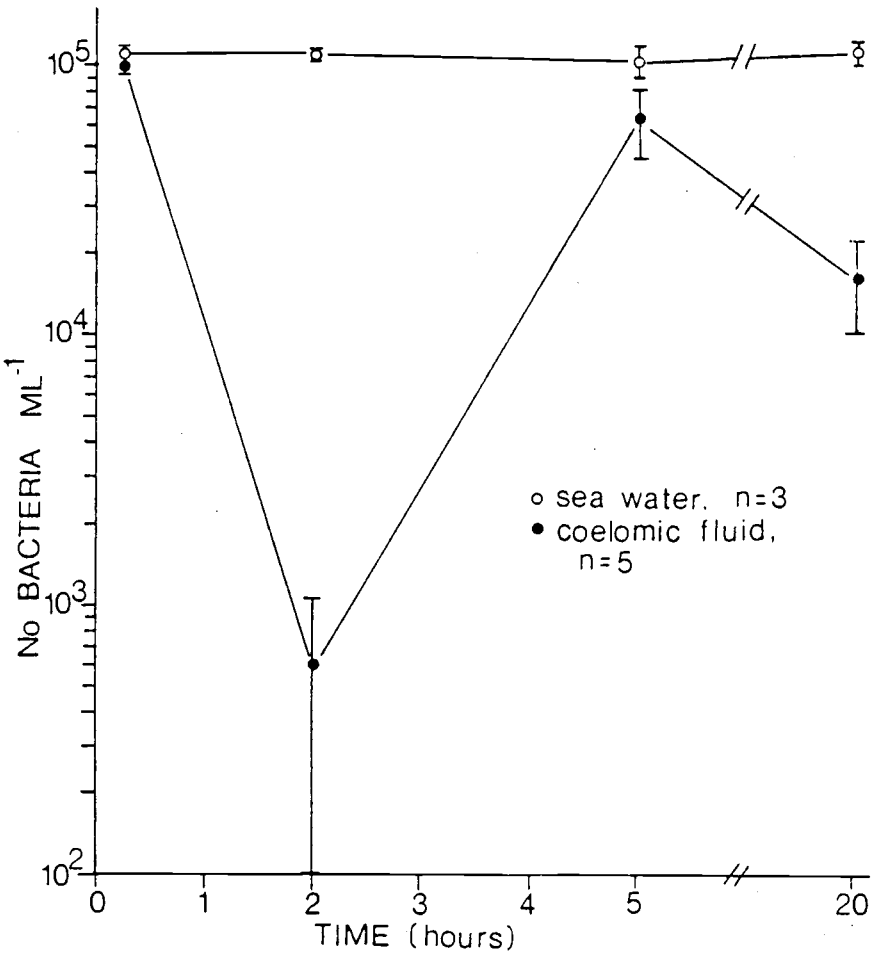

Figure 8: Viable counts (mean ± SE) for the Gram positive bacteria in sea water and in cell-free coelomic fluid.

individuals (10, 310, 1.6×10^3, 1.3×10^4, 3.1×10^4 v.c. ml^{-1} CF) was quite high compared to low variability in the controls (9×10^4, 1.2×10^5, and 1.1×10^5 v.c. ml^{-1} sea water), probably reflecting large differences in amount(s) of the humoral factor(s) present in the CF rather than differences due to sampling methods.

IV. Humoral Factors: Bacterial Agglutinins

Neither of the two marine bacteria were agglutinated in S. purpuratus coelomic fluid under the test conditions. CF samples from eight urchins were tested with the gram negative and four were tested with the Gram positive. A. salmonicida was not tested.

V. Total and Differential Counts of Coelomocytes

Results of total and differential cell counts obtained in August and September were quite similar to those obtained by Bertheussen and Seljelid (1978) for Strongylocentrotus droebachiensis (Table 4). The majority of the cells were phagocytes ($\bar{x}$ ± SD: 67.8 ± 4.4%), followed by vibratile cells (16.8 ± 3.8%), red spherules (10.5 ± 3.1%), and colorless spherules (5.0 ± 1.3%). The total number of cells averaged 10^7 ml^{-1}. In October and November, however, cell counts were somewhat depressed and more variable. The relative proportion of phagocytes decreased (none were found to be in the range of those sampled in August and September) while the proportion of vibratiles increased. The percentages of red and colorless spherule cells did not change significantly. October and November were the months in which gonadal

Table 5: The total number of coelomocytes ml^{-1} of S. purpuratus coelomic fluid and the percentages of each coelomocyte type (mean, SD, and range), comparing urchins sampled in August-September and October-November (Student's t-test, p values given). Ranges from Bertheussen and Seljelid (1978) with S. droebachiensis are also given for comparison.

Months	n		% Phagocytes	% Red Spherules	% Vibratiles	% Colorless Spherules	Total # cells ml^{-1}
Aug-Sept	6	$\bar{X}$	67.8	10.5	16.8	5.0	$10.4X10^6$
		SD	4.4	3.1	3.8	1.3	$1.9X10^6$
		range	60-72	7-15	13-22	2-7	$7.5\text{-}13X10^6$
Oct-Nov	17	$\bar{X}$	44.4	14.9	35.4	4.9	$7.2X10^6$
		SD	10.6	7.1	7.3	2.4	$2.9X10^6$
		range	17-56	6-35	25-50	1-11	$3.2\text{-}14X10^6$
		p value	<0.001	>0.200	<0.001	>0.200	<0.025
from Bertheussen and Seljelid (1978):							
		range	64-70	6-10	15-22	4-9	$5\text{-}10X10^6$

tissue was found in coelomic fluid samples, and animals that were dissected had ripe gonads. In these months gonad weight is maximal in S. purpuratus populations on the Oregon coast (Gonor, 1973). It is possible that the noted variation was due to grazing the ripe gonads with the needle, although care was taken to avoid this.

Differential counts of the four cell types were made up to 6 hr after injection of 10^7 gram negative bacteria ml^{-1} of CF (Figure 9). The sea water-injected control urchin exhibited a sharp but brief decline in cell numbers during the first hour followed by rapid recovery to pre-injection values. Total cell numbers dropped even more sharply in the urchins injected with bacteria. The sharpest decline was seen during the first 1-5 hr p.i., and continued more slowly to about 5 hr. The overall drop in total cell numbers was from 8.8×10^6 to 6×10^5 ml^{-1}, a 93% decline. The 24 hr samples shown on the figure were from the only urchin sampled at that time and are included to indicate that by 24 hr all cell types were returning to, but not quite at, pre-injection values.

All cell types contributed to the decline (Figure 9), however, the relative proportions of each cell type changed (Figure 10). The percentages of phagocytes and red spherule cells declined disproportionately while the percentages of vibratiles increased during that time. The percentage of colorless spherule cells did not seem to change although the values were at such low levels as not to be countable with great accuracy. In the one animal for which cell counts were obtained at 24 hr p.i., the proportions of cells were approaching pre-injection values.

Figure 9: Total and differential coelomocyte counts (mean $\pm$ SE) after injection with 10^8 Gram negative bacteria ml^{-1} of coelomic fluid (n=3), and total coelomocyte counts for the sea water injected control (n=1). The 24 hr sample was from only one of the three bacteria-injected urchins.

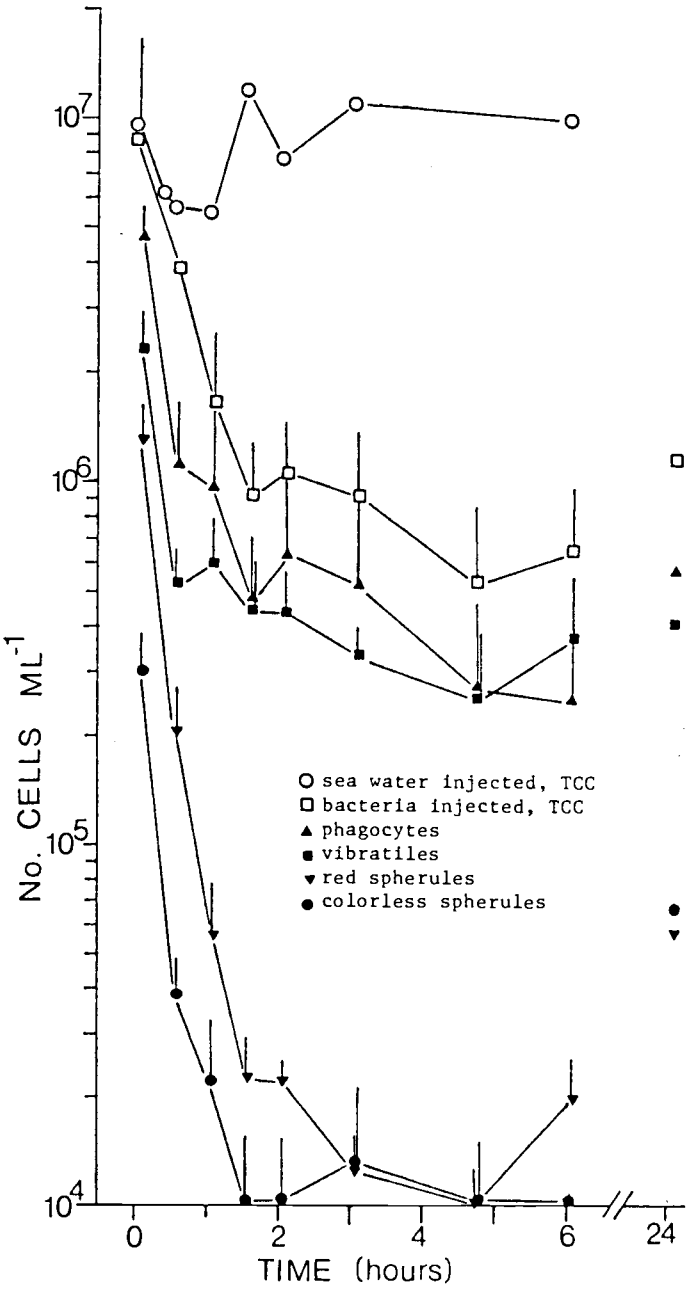
No. CELLS ML-1
10^7
10^6
10^5
10^4
0
2
4
6
24
TIME (hours)
O sea water injected, TCC
□ bacteria injected, TCC
▲ phagocytes
■ vibratiles
▼ red spherules
● colorless spherules

Figure 9

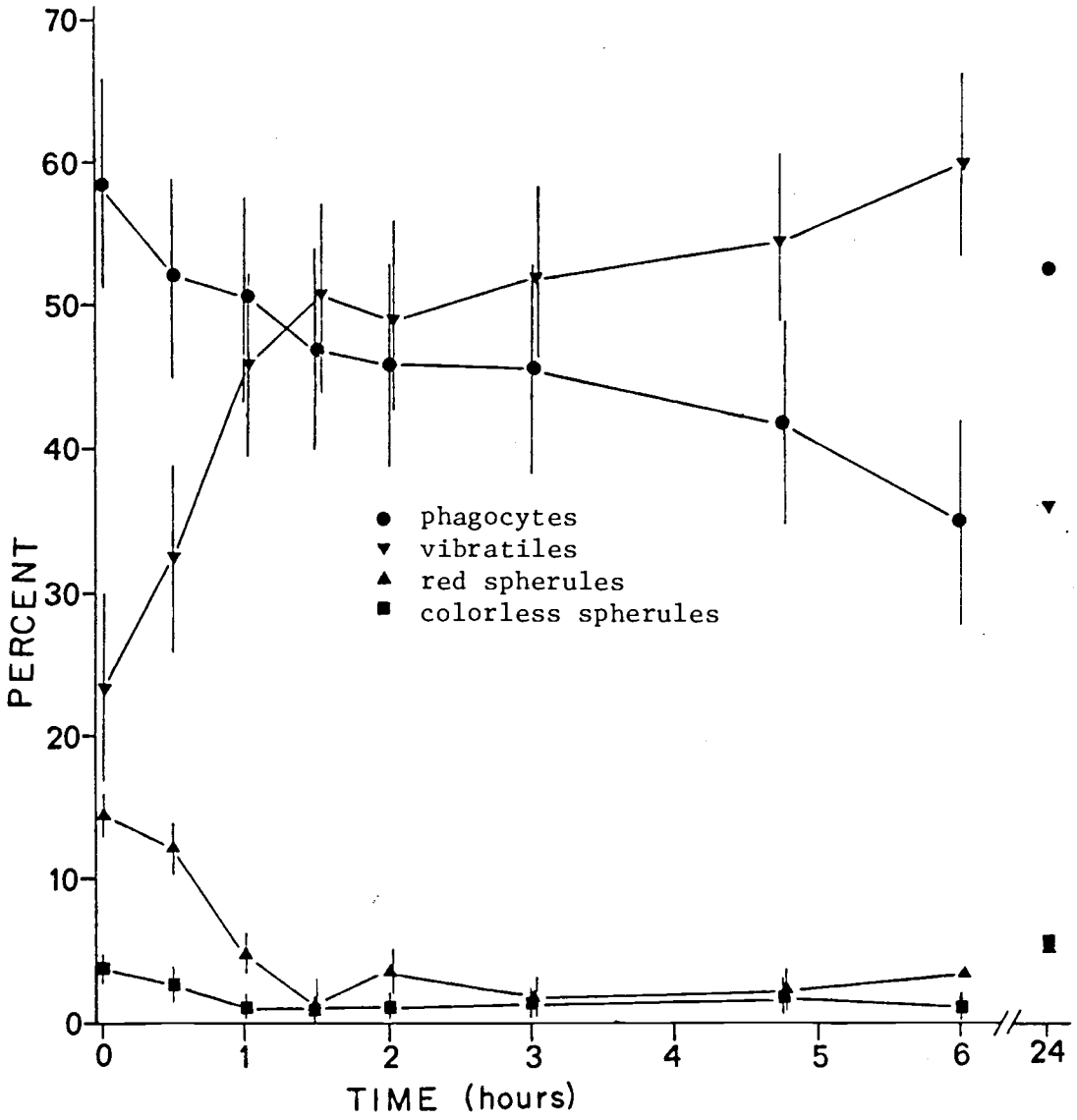

Figure 10: Percentages (mean ± SE) of the four coelomocyte types after injection of Gram negative bacteria (10^8 bacteria ml^{-1} of coelomic fluid)(n=3). The 24 hr sample was from only one of the three urchins.

VI. Phagocytosis and Killing

Both marine bacteria, when live, fluoresced green with acridine orange under uv light; heat-killed bacteria fluoresced red. Coelomocytes from uninjected urchins were dyed and observed for uptake of the acridine orange. Red spherules did not fluoresce while colorless spherules fluoresced green uniformly. Vibratile cells were generally removed with rinsing, and were quite distinct from other cells. Phagocyte nuclei fluoresced green but their sizes and shapes were distinct from those of bacteria. The cytoplasm fluoresced light green, so that the veils of the cells were apparent. Occasionally small red dots appeared in the cytoplasm, but they were generally distinct from intact bacteria. None of the coelomocytes autofluoresced.

Phagocytosis was difficult to quantify in vitro and in vivo because of aggregation of the phagocytes on the glass coverslip. Bacteria were seen in association with these cell aggregations but it was difficult to distinguish bacteria on the surfaces of cells from phagocytosed bacteria. When individual phagocytes were found, internalized bacteria were more apparent as they were usually lined up in groups in vacuoles.

In the in vitro study, no dead (red) Gram negative bacteria were observed at 30 and 75 min although some live (green) bacteria were obviously internalized. At 2, 3, and 3.5 hr, most internal bacteria were live but a few were dead.

When live Gram negative bacteria (10^8 ml^{-1} of CF) were injected into an urchin, most phagocytes had at least one bacterium associated with

it, but only a few were internalized at 10 min. The samples taken at 1.5, 2.5, and 8 hr p.i. had many green, orange (probably alive but dying), and red bacteria associated with the phagocytes. Phagocytes with any internal bacteria usually contained several (up to 6). Generally, internalized bacteria were the same color. By 20 hr p.i. no bacteria were seen associated with phagocytes.

DISCUSSION

I. Primary Clearance

Strongylocentrotus purpuratus efficiently cleared all three bacteria from its coelomic fluid. Viable counts were reduced 90-99% in 3-6 hr after injection of doses of 10^6 - 10^7 bacteria ml^{-1} of CF. Clearance appeared to take place in at least two stages, with the rate of clearance of one of the bacteria different from that of the others at each stage. Clearance data comparing the three bacteria are given in Table 1.

Clearance was approximately exponential for the first 1.5 - 2.5 hr p.i. for the Gram negative bacteria. Both marine bacteria exhibited quite similar clearance rates over the first 90 min as determined by K values and percent reduction in v.c. at 1 hr p.i. A value for K was not determined for the first 90 min of Aeromonas salmonicida clearance, but the percent reduction in v.c. at 1 hr p.i. clearly indicated a slower rate of clearance than those of marine bacteria ($p < 0.001$).

After the first 1.5 - 2.5 hr, clearance was generally slower than the initial exponential decline. Based on the percent reduction in v.c. at 6 hr p.i., A. salmonicida had been cleared as well as the marine Gram negative bacteria despite the slower initial rate, suggesting a higher rate of clearance after 1 hr compared to the other bacteria. The marine Gram positive, on the other hand, had a significantly lower reduction in v.c. at 6 hr p.i. compared with the marine Gram negative bacteria and A. salmonicida. At 24 hr the percent reduction of Gram positive bacteria

was still lower than that of the Gram negative. A. salmonicida was not sampled at 24 hr p.i.

The results of this study are similar to those of others working with other echinoderms. Wardlaw and Unkles (1978) injected about 10^7 Pseudomonas sp. into Echinus esculentus (of unknown coelomic fluid volume) and found that the coelomic fluid was sterile again by 24 hr p.i. Strongylocentrotus droebachiensis cleared 90% and 50% of 2 x 10^9 injected Escherichia coli and Vibrio anguillarum, respectively, by 2 hr p.i. (Bertheussen, 1981). The urchins (6-7 cm in diameter) also cleared 90-100% of 5 x 10^8 injected sheep red blood cells (untreated and tannin- and aldehyde-treated) in 2 hr, 70% of the same quantity of yeast cells, and 80% of 5.7 um latex beads. When 2.6 x 10^7 Arbacia cells were injected into Asterias vulgaris, the urchin cells were completely cleared by 1 hr (Reinisch and Bang, 1971). On the other hand, 1.1 x 10^7 carborundum particles were not completely cleared from the coelomic fluid until an average of 47 hr p.i. in A. vulgaris. A four day lag period was noted before Lytechinus pictus started to clear 2 x 10^9 bacteriophage T_4 (Coffaro, 1978), and by 10 d p.i., 10^2 particles ml^{-1} were still remaining. Hilgard and Phillips (1968), using injected radioactively labeled bovine and human serum albumin, found that over 85% of both proteins was removed in 22 hr.

None of the previous studies on echinoderms have quantitatively followed in vivo bacterial clearance. In other invertebrates, however, results have been quite similar to those of this study. The molluscs, Helix (Bayne and Kime, 1970), Lymnaea (van der Knaap et al., 1981), Octopus (Bayne, 1973), and Aplysia (Pauley et al., 1971) and the

crustacean, Carcinus (Smith and Ratcliffe, 1980), cleared 90-99% of the bacteria (10^6 - 10^9 bacteria per animal) in the first 2-3 hr p.i., with slower subsequent declines after 3 hr. Unlike S. purpuratus, the crab, Carcinus, cleared the Gram positive Bacillus cereus and the Gram negative Moraxella sp. equally well (Smith and Ratliffe, 1980). On the other hand, the snail, Lymnaea, cleared the Gram positive Staphylococcus saprophyticus more effectively than the Gram negative Escherichia coli (van der Knaap et al., 1981); the difference was especially obvious after 8 hr p.i.

The results of this study are also similar to those obtained in mammalian bacterial clearance studies (Rogers, 1960 for review). The first phase of clearance in mammals is rapid and exponential, with 90-99.9% reductions in circulating bacteria. This phase is apparently relatively independent of the nature of the microbe, the animal under study, and the subsequent outcome of infection. The second phase is either the persistance of the microbe at lower concentrations or gradual removal at slower rates over several hours or days. This phase differs considerably with different bacteria. Unlike echinoderms, it is known that the reticuloendothelial system, especially in the liver and spleen, is responsible for the removal of most of the injected bacteria in mammals.

Total and differential cell counts taken during primary clearance of Gram negative bacteria indicated a massive (93%) reduction in numbers of all four coelomocyte types accompanying bacterial clearance. Similar declines in circulating hemocyte numbers have also been observed in crustaceans (Cornick and Stewart, 1968; Smith and Ratcliffe, 1980),

insects (Wittig, 1965; Gagen and Ratcliffe, 1976), molluscs (Bayne and Kime, 1970; van der Knaap et al., 1981; Renwrantz et al., 1981), and in leukocyte numbers in mammals (Rogers, 1960).

Clotting, due to aggregation of phagocytes, no doubt contributes to some of the observed decline in numbers of cells and bacteria. Cellular clots were often observed when coelomic fluid samples were removed after injection of bacteria. Few urchins were injected with sea water, so it is unknown if sea water alone can elicit clot formation, although Bertheussen (1981) noted clot formation in S. droebachiensis only after injection of bacteria and not after injection of other particles (red blood cells, yeast, latex). This aggregation may serve to enhance phagocytosis, since closely packed vertebrate leukocytes may use one another's surfaces for trapping bacteria (Wood, 1960). The observed decline in phagocyte numbers may also be due to attachment of phagocytes to the coelomic epithelium, for phagocytosis, since vertebrate leukocytes trap and phagocytose encapsulated bacteria more avidly when attached to tissue or moistened filter paper (Wood, 1960).

Clotting may explain the observation that red spherule cells declined proportionately with phagocytes while vibratile cells increase proportionately. Red spherules are relatively non-motile (they depend on ameboid movement), therefore they may be passively caught in with aggregating phagocytes as observed in hanging drops (Johnson, 1969a). Although the percentage of non-motile colorless spherules did not appear to change, actual counts were very low so changes were difficult to detect. Vibratiles, being highly motile, may extricate themselves from

the clots or may be recruited more rapidly from elsewhere, causing the observed increase in relative abundances.

In vivo and in vitro phagocytosis of the marine Gram negative was observed by fluorescence with acridine orange under ultraviolet light. Although insufficient experiments were done to document rates of phagocytosis and killing, some phagocytes were observed, in vivo and in vitro, to contain live (green) and/or dead (red and swollen) bacteria. Generally, if a phagocyte contained bacteria there were usually more than one, all either alive or dead; not all phagocytes contained bacteria however. It is possible that the dead bacteria in the phagocytes were phagocytosed after death rather than being killed by the urchin cells.

Studies on in vitro effects of cell-free coelomic fluid indicate that there is (are) some humoral factor(s) active against both Gram negative and Gram positive bacteria. Other authors (Wardlaw and Unkles, 1978; Johnson and Chapman, 1971), to the contrary, found that coelomocytes were required for bactericidal activity. Since the quantity of humoral factor(s) would be limited and not renewable in a cell-free in vitro system, the number of bacteria per given volume of coelomic fluid would be critical in the detection of bactericidal activity. This may explain the previous failure of other authors to find humoral activity against bacteria. In addition, observable activity may depend upon the species of bacteria used and the echinoderms from which test fluids are derived.

Declines of 6×10^4 to 9×10^5 v.c. ml^{-1} of CF from 6 urchins were observed in vitro by 2 hr after inoculation with Gram negative bacteria,

enough to account for at least some of the decline observed *in vivo*. It is possible that the decrease in v.c. was due to agglutination rather than bacterial killing, although no agglutination was detected in this study.

Results of the bactericidal tests using the Gram positive bacteria are difficult to explain since agglutinins were not found. Massive declines in v.c. were noted in the 2 hr samples from the CF from all 5 urchins tested. These declines could not be explained by bacterial killing because v.c. returned to control levels by 5 hr p.i. and these bacteria do not grow that quickly at 10°C. These results, however, do correspond with the lower v.c. noted at 1 hr than at 3 hr in *in vivo* clearance of the Gram positive. Although v.c. in CF *in vitro* were not significantly lower than in sea water at 5 hr, the 20 hr counts were 8-11 x 10^4 v.c. ml^{-1} lower than the sea water controls and the initial inoculation dose. In addition, since the decline in Gram positive v.c. was much greater than that of the Gram negative, it appears that two different factors may have been involved.

The active factor(s) was (were) not enhanced by allowing clotting to occur before the removal of cells nor was activity completely destroyed by heating at 100°C for 10 min. Heated CF had higher v.c. than untreated CF in fluids from all 4 urchins tested, although some differences were small. V.c. for heated CF were all lower than initial inoculation doses and, in the one case tested, lower than counts in the HSM control. Wardlaw and Unkles (1978) also found that clotting did not affect bactericidal activity and that heat-treated CF (100°C for 10 min)

lost some, but not all of its activity in Echinus, although it was cell-dependent activity rather than humoral.

Although bacterial agglutination was not observed, these experiments do not rule out the possibility that agglutinins function in clearance of bacteria. In fact, the results of the bactericidal test using the Gram positive suggest that agglutination may occur temporarily at least at 2 hr p.i. Relative proportions of bacteria to concentration of active molecules in the CF may have been inappropriate for the observation of agglutination. Since hemagglutinins have been found in many echinoderms, and rbc and bacteria share some surface determinants, it is likely that these molecules function in the agglutination of certain bacteria as well.

Differences in the rates of primary clearance of the three bacteria tested may depend on (1) differences in specific or non-specific cellular recognition and response, (2) the quantity and effectiveness of bactericidal substances, agglutinins, and/or opsonins, and (3) the rate of bacterial reproduction. Based on these studies of in vivo and in vitro primary clearance, it appears that both cellular and humoral factors are involved in bacterial clearance. Clotting and the overall decline of coelomocytes paralleled bacterial clearance. Bacteria were observed to be caught in the cellular clots, as well as being phagocytosed by some phagocytes. In vitro studies on cell-free CF indicate that some factor(s), bactericidins and/or agglutinins, exist in the CF. Different quantities of the factor(s) or number of responding cells may cause the large individual variation observed between urchins.

The two stages of clearance in S. purpuratus are probably the result of a decline in active cells and/or molecules in the first few hr p.i., with further clearance being dependent on the level of cell recruitment, or activation and/or release of active molecules.

II. Primary vs. Secondary Clearance

Accelerated secondary clearance of bacteria was not observed using three different bacteria and 32 urchins. The few other attempts to induce elevated responses in echinoderms to foreign materials, other than grafts, have also failed. Coffaro (1978) was unable to elicit accelerated clearance of the bacteriophage T_4 in Lytechinus pictus after injection of 10^9 particles into each of ten urchins. An increase in the rate of foreign protein uptake was not detected in S. purpuratus after injection of four doses of protein at 18 hr, 3 d, 7 d, and 2 months after primary injection (Hilgard and Phillips, 1968). Asterias vulgaris was inoculated with 5×10^5 Arbacia cells six times but a change in the rate of cell disappearance was not observed (Reinisch and Bang, 1971).

Secondary responses to bacteria have been elevated in other phyla, including both deuterostomes (e.g., vertebrates) and protostomes, in which molluscs (see Bayne, Sminia, and van der Knaap, 1980 for review; Bayne, 1980), spiunculids (Evans et al., 1969), crustaceans (Acton et al., 1969; Stewart and Zwicker, 1974), and insects (Boman et al., 1972; Faye et al., 1975) have yielded positive results. However, in the invertebrate examples, either enhancement has been non-specific or the extent of specificity has not been thoroughly tested.

The lack of accelerated secondary clearance in this study can be explained in the following ways:

(1) There is no memory component to bacterial clearance.

(2) Memory does exist but was undetected due to:

(a) previous exposure to the same or similar bacteria, or

(b) inadequate protocols.

Immunological memory with some specificity has been demonstrated and confirmed in echinoderms in graft rejection experiments. However, there is no evidence that memory is a factor in bacterial clearance in S. purpuratus. The defense phenomena in any animal, including mammals, include both specific and non-specific components (Rowley, 1960). If only specific components may be enhanced, and if most bacteria are recognized primarily on non-specific properties (e.g., hydrophobicity, Van Oss and Gillman, 1972; overall charge or charge configurations, Lackie, 1980), a response with memory would not be expected against these bacteria. Absence of memory in anti-bacterial defenses of echinoderms does not preclude the possibility that memory may exist in responses to parasites, fungi, algae, or other invading organisms.

It is possible that memory does exist in anti-bacterial defenses but most of the S. purpuratus tested have encountered the bacteria before. In this case, the primary clearance rates obtained were actually secondary responses which could not be further enhanced. It is not likely that A. salmonicida was previously encountered by these urchins since it is a freshwater species. It is possible that the urchins have been "sensitized" by other species of bacteria sharing certain common surface characteristics critical for recognition and response, like the

cross-reactivity of antigens in vertebrate immune responses. This explanation might account for the few urchins in which secondary responses did appear to be accelerated over the primary responses.

As with all experiments testing for immunological memory, it is difficult to select the protocol that will show, with greatest sensitivity, enhancement of secondary responses. Parameters such as environmental temperature, the interval between primary and secondary injections, duration of exposure to antigen, timing of booster injections, the dose and quality of the "antigen" are probably critical for optimally eliciting enhanced secondary responses. Under any set of circumstances variations observed between individual urchins, and in the same animal over time, would tend to obscure alterations in secondary responsiveness.

Despite such considerations, there is a need to conduct experiments to identify the conditions under which echinoderms will respond optimally to immunologic challenge. Without such studies, the mechanisms of echinoderm internal defenses and the evolutionary history of immune responsiveness will remain obscure.

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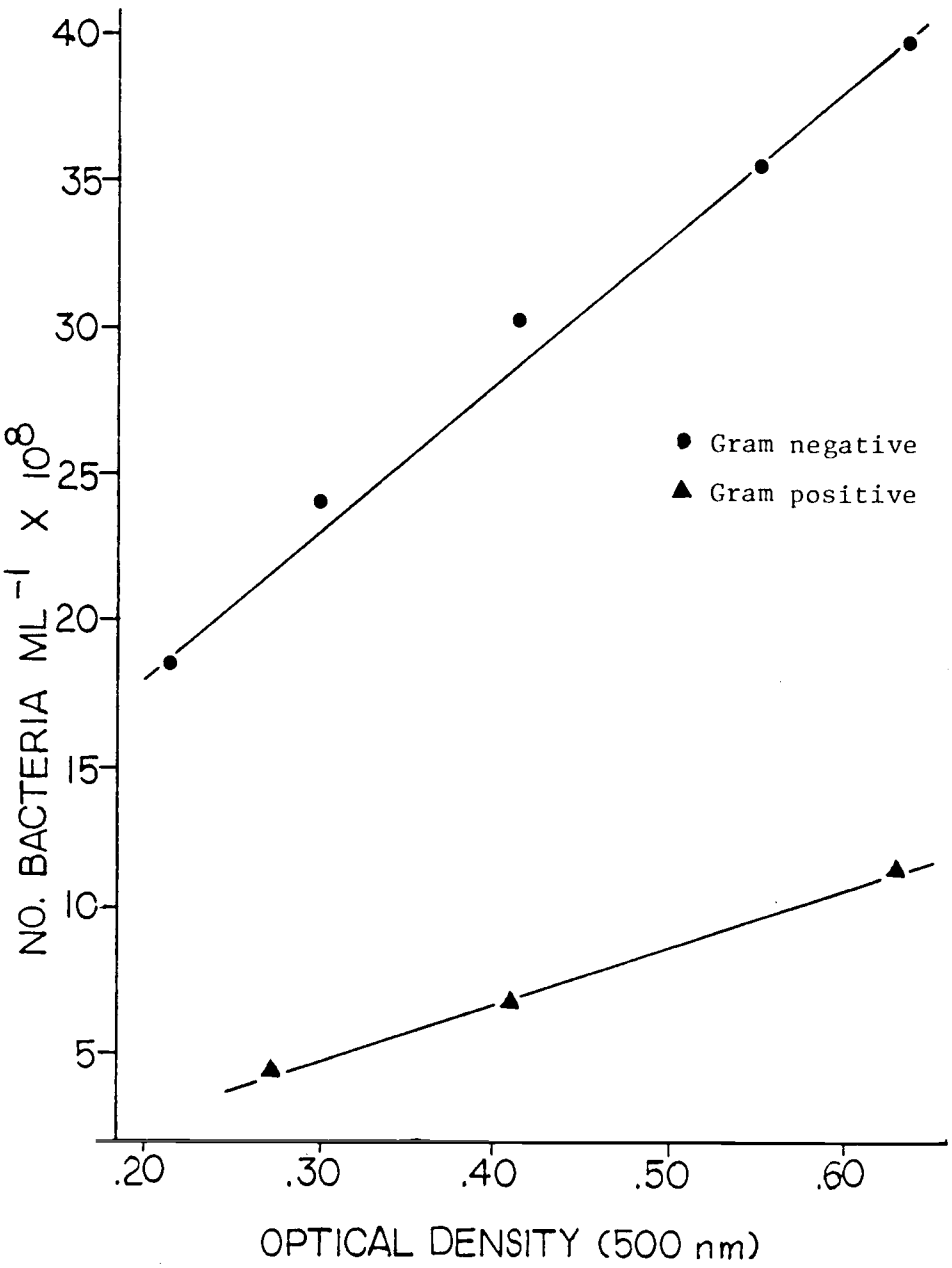
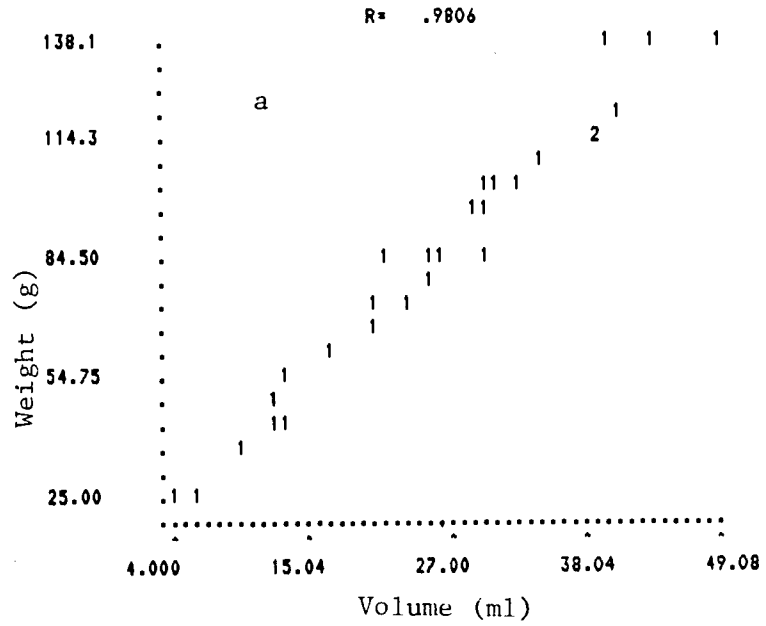
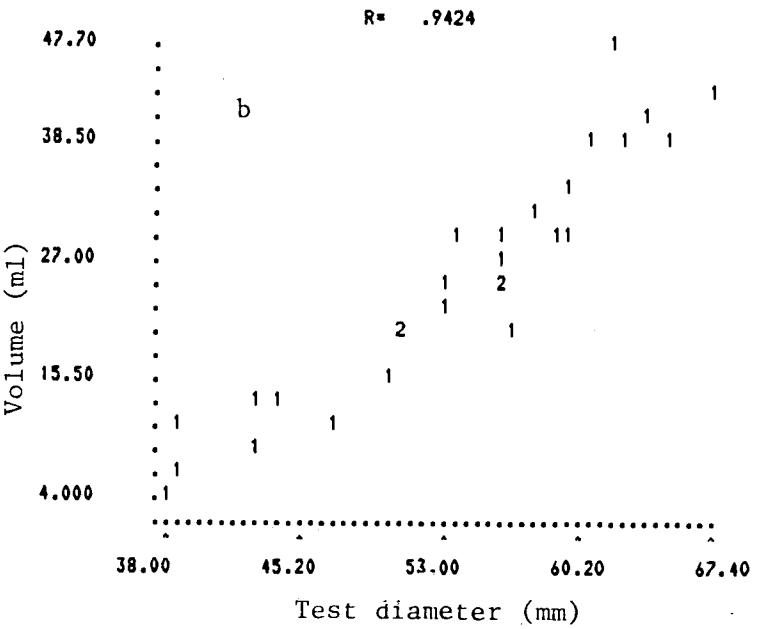
APPENDICES

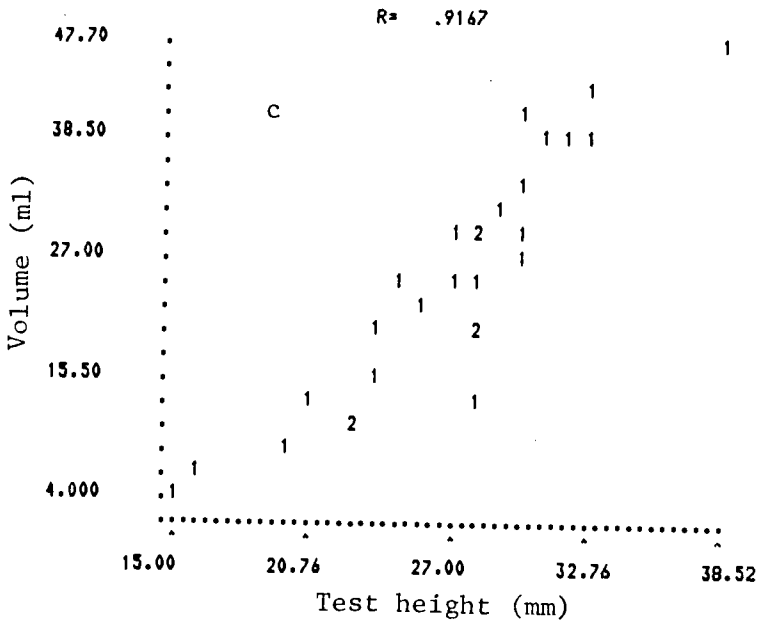


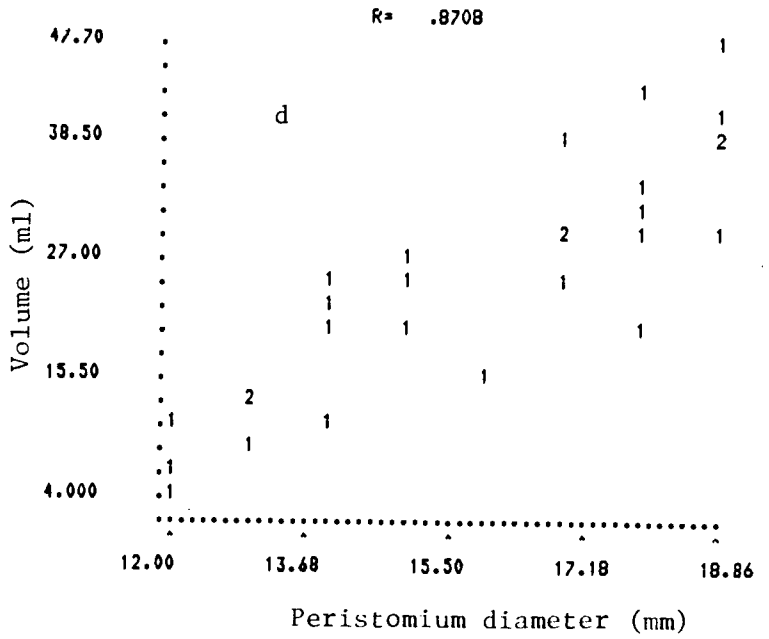
Appendix I: Relationship between the optical density at 550 nm and the viable count (number of bacteria ml^{-1} X 10^8) for the Gram negative and Gram positive bacteria grown in enriched marine broth at room temperature (23°C) and 100 rpm.





Appendix II: Regression of sea urchin weight and test diameter on coelomic fluid volume, n=28.





Appendix II (continued): Regression of sea urchin test height and peristomium diameter on coelomic fluid volume, n=28.

Appendix III: Dispersal of the Gram negative bacteria through the coelomic fluid, viable counts measured at the point of injection, 0^{o}, and 180^{o} (opposite) from the point of injection (n=1).

Time (min)	viable counts # bac ml^{-1} CF 0^{o}	viable counts # bac ml^{-1} CF 180^{o}	Difference (0^{o}-180^{o})
15	$13X10^{4}$	$3.6X10^{4}$	$9.4X10^{4}$
40	$6.2X10^{4}$	$3.6X10^{4}$	$2.6X10^{4}$
90	$2.7X10^{4}$	$2.4X10^{4}$	$0.3X10^{4}$

Appendix IV: Effect of coelomic fluid clotting on viable counts of the Gram negative bacteria (n=3).

Time (min)	# bacteria ml^{-1} X 10^4 unclotted CF urchin # 1	2	3	clotted CF urchin # 1	2	3	sea water sample # 1	2	3
15	1.5	1.1	1.3	1.9	1.3	1.2	1.4	1.3	1.6
45	1.5	0.1	1.1	1.6	1.2	1.2	1.4	1.7	1.4
90	1.3	0.5	0.9	1.5	1.3	1.0	1.6	1.6	1.5
360	1.5	0.5	0.5	1.9	1.1	0.5	1.4	1.0	1.3
change*	0	-	-	0	0	-	0	0	0

* general trends over the 6 hr period: 0 = no change
- = decline in bacteria numbers
+ = increase in bacteria numbers

Appendix V: Protocols for bacterial agglutination experiments.

Bacteria	Well volume	# bacteria added per well	# urchins tested	times checked (hours)
Gram negative	100 ul	$5X10^7$	2	1,2
	100 ul	$5X10^5$ $5X10^4$	2	2
	100 ul	$5X10^5$ $5X10^4$	2	1,2,3
	1 ml	$5X10^5$ $5X10^4$	2	0.5,2
Gram positive	1 ml	$5X10^7$	2	20
	1 ml	$5X10^5$ $5X10^4$	2	0.5,2

Appendix VI: The slopes (K) of the lines derived from the linear regression of 4-5 samples from each urchin taken during the first 90 min after injection of marine Gram negative bacteria.

Urchin #	# of bacteria injected ml^{-1} CF	K (log_{10}bac min^{-1})
1	$3.9X10^7$	-0.0117
2	$3.9X10^7$	-0.0228
3	$3.1X10^7$	-0.0180
4	$2.9X10^7$	-0.0141
5	$2.9X10^7$	-0.0197
6	$2.9X10^7$	-0.0256
7	$2.6X10^7$	-0.0208
8	$2.6X10^5$	-0.0168
9	$2.6X10^5$	-0.0120
		$\bar{X}$= -0.0179
		SE= 0.0016