ANTIBACTERIAL DEFENCE IN *ECHINUS ESCULENTUS* L

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Preface

I hereby declare that this thesis describes work carried out by myself unless otherwise cited or acknowledged and that it is of my own composition. The research was carried out within the period October 1979 to September 1982. This dissertation has not in whole or in part been previously presented for any other degree.

Matthew Service

Date 18/11/82

SUMMARY

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SUMMARY

Previous investigations at the University Marine Biological Station Millport had established that freshly drawn coelomic fluid from the common sea urchin Echinus esculentus had in vitro bactericidal activity against a heterotrophic marine bacterium designated Pseudomonas strain no. 111. The activity appeared to reside in the red spherule cells of the coelomic fluid but nothing was known about its biochemical basis. These observations provided the starting point for the present work.

The first objective of this study was to examine a variety of other marine bacteria for susceptibility to coelomic fluid bactericidal activity. For this purpose a panel of 23 different strains of heterotrophic bacteria was examined. Most of the strains were from the National Collection of Marine Bacteria but some were isolated by the author from the Clyde Sea Area but not identified. There were 11 Gram-negative and 12 Gram-positive strains altogether and the following genera were represented: Alteromonas, Beneckea, Brevibacterium, Cytophaga, Flexibacter, Micrococcus, Moraxella, Pediococcus, Photobacterium, Planococcus and Vibrio. All 23 strains were found to be susceptible in some degree to the in vitro bactericidal activity of coelomic fluid from E. esculentus at $8^{o}C$. Results of bactericidal tests were expressed at the survival index, defined as:

$$\text{Survival Index (SI)} = \frac{\text{viable count at time t}}{\text{viable count at time 0}} \times 100$$

There was much variation between the strains but highly sensitive and relatively resistant strains were found in both the Gram-negative and Gram-positive groups. There was little difference in average sensitivity of Gram-negative and Gram-positive strains when viewed collectively. This suggested that the target of the bactericidal system was probably not a component of the bacterial cell wall or envelope. None of the strains was able to grow in coelomic fluid and it was speculated that

if such a strain were discovered, it might be a pathogen for E. esculentus.

In trying to determine the basis of the susceptibility and resistance of the test strains, the possibility was considered that the growth rate in non bactericidal control fluid, which contained nutrient might be important. With those strains whose growth rate was measured there was a correlation between this variable and bactericidal susceptibility in coelomic fluid: the most rapidly growing strains were the most sensitive. This suggested that the bactericidal system was interfering with some metabolic function in the test organism.

The possibility was considered that coelomic fluid might contain more than one bactericidal system and that in different individual urchins the levels of these hypothetical systems might vary. Some support for this idea emerged from the observation of a lack of correlation in the extent of killing of a Gram-positive and a Gram-negative bacterium when mixtures of the two organisms were exposed to coelomic fluids from different individual urchins.

Because of the previous information on the involvement of red spherule cells in bactericidal activity and the fact that these cells contain echinochrome A, a naphthoquinone pigment, tests were made of the in vitro bactericidal activity of this substance on several strains of marine bacteria. Initial difficulties in dissolving the pigment in seawater were overcome by using various mammalian proteins as dispersants. For this purpose, bovine serum albumin (BSA), bovine gamma globulin (BGG), human serum albumin (HSA) and human gamma globulin (HGG) were compared. Few differences were noted except that BSA appeared to yield a slightly less bactericidal fluid when mixed with echinochrome A than when the other proteins were used. Routinely BGG at 2mg ml^{-1} in seawater was used as a dispersant for echinochrome A at concentrations of 50ug ml^{-1} or less. None of the proteins by themselves in seawater showed

bactericidal activity. The concentration of protein added to echinochrome A could be varied widely without noticeable effect on the bactericidal activity of the pigment protein solution.

The bactericidal activity of echinochrome A/BGG mixtures showed several similarities to that of whole coelomic fluid:

1) Killing of the test organism was not instantaneous but took place between 4 and 24h at $8^{o}C$.

2) The concentration of echinochrome A that was active in the artificial mixtures was within the physiological range of concentration (3-59ug ml^{-1}) found in coelomic fluid.

3) Both Gram-positive and Gram-negative bacteria were susceptible and the rank order of susceptibility of a panel of 7 test strains was similar to the susceptibilities in whole coelomic fluid under particular conditions (24h exposure time).

The final section of this investigation was concerned with exploring some of the environmental factors which might influence the bactericidal activity of coelomic fluid. The interest in this arose from the fact that although the majority of the several hundred specimens of E. esculentus tested in the courseof this work had bactericidally active coelomic fluid, there was considerable between-animal variation. Both seasonal and nutritional factors were considered worthy of study, and collections of E. esculentus were made in summer and winter from nutritionally rich and poor habitats. The animals were examined for several physiological parameters to see if any might correlate with the in vitro bactericidal activity of the coelomic fluid. Urchins from the rich environment yielded bactericidally more active coelomic fluid than urchins from the poor environment. Both echinochrome A concentration and gonad index were higher in urchins from the rich environment. As the gonads are the principle reservoir of stored energy in echinoids, the energy requirements for maintenance of the components of the

bactericidal system were considered as a possible reason for differences between animals from the two sites. There were no overall seasonal differences; nor was the haemagglutinin titre (HA) in the coelomic fluids correlated with nutritional or seasonal factors.

Preparatory to analysing the data from the above investigation, a study was made of the nature of the statistical distributions of the different variables since there was no previous information for guidance. Gonad indexes were shown to be approximately normally distributed; HA titre and EchA concentration were approximately log-normally distributed while SI did not follow any identified underlying distribution.

Because EchA is unique to echinoids, consideration was given to the question of its evolutionary origin and of what homologous substances might be present in animals on adjacent branches of the phylogenetic tree, particularily the vertebrates. The most attractive hypothesis was the idea that EchA and melanin might have shared a biosynthetic pathway which in echinoids eventually gave an end product with a defensive role against microbial invasion and in higher animals a pigment with diverse functions.

Contents

Index of Tables

Index of Figures

Abbreviations

ATP	adenosine triphosphate
BCFS	boiled coelomic fluid supernate
BGG	bovine gamma globulin
BSA	bovine serum albumin
CFU	colony forming units
EchA	echinochrome A
EGTA	ethyleneglycol-bis-n,n^{1}-tetra acetic acid
GI	gonad index
HA	haemagglutinin titre
HGG	human gamma globulin
HSA	human serum albumin
MBSW	marine broth seawater
NCMB	national collection of marine bacteria
Ps111	Pseudomonas strain 111
RBC	red blood cells
SI	survival index
SW	seawater

INTRODUCTION

General Features of the Marine Bacteria

There is a vast literature on the marine bacteria, and the reader is referred to Zobell (1946) and Sieburth (1979), for a general account of these organisms. What follows is a highly selective and brief account of those aspects most relevant to the present work.

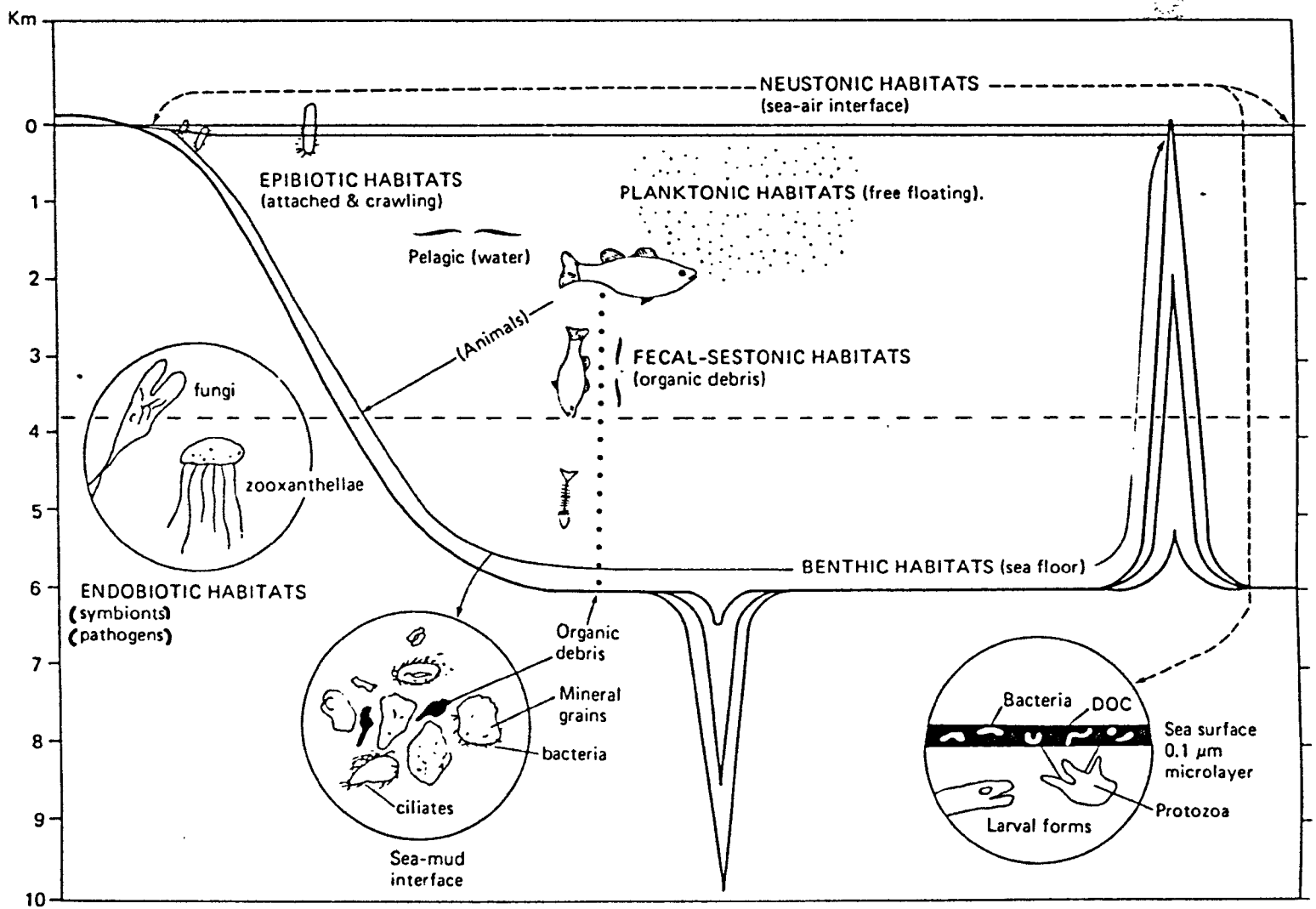
The majority of marine bacteria are Gram-negative asporogenous rods (Shewan, Hobbs and Hodgkiss, 1960) and some at least are characterised by a requirement for Na^+ for growth (Tyler, Bielling and Pratt, 1960; Macleod, 1965). Because of their thermosensitivity, most marine bacteria can be classified as facultative or obligate psychrophiles (Morita, 1974). Many marine bacteria can utilize nutrients present in minute concentrations (Rheinheimer, 1974) a prerequisite for growth in sea water which is normally poor in nutrients.

One of the main problems in understanding the ecology of marine bacteria is that of obtaining reliable estimates of population levels. Methods for enumerating marine bacteria include; direct counts by epifluorescence microscopy (Hoppe, 1976) and scanning electron microscopy (Krambeck, Krambeck and Overbeck, 1981), colony counts (Jannasch, 1958), micro-autoradiography (Hoppe, 1976), A.T.P. analysis (Hobbie et al., 1972) and muramic acid determination (Moriarty, 1975). These methods may provide widely varying estimates of bacterial populations from the same sample (Hoppe, 1976).

Sieburth (1979) has introduced a classification in which marine microbial habitats are divided into six groups (fig.1): planktonic, fecal-sestonic, neustonic, benthic, epibiotic and endobiotic.

Bacteria in the pelagic environment may exist as free living bacterioplankton or attached to other planktonic organisms or detritus (Taga and Matsuda, 1974). The same authors found that attached and free

FIGURE 1 Microbial habitats in the marine environment (from Sieburth, 1979)


Km
0
1
2
3
4
5
6
7
8
9
10
NEUSTONIC HABITATS
(sea-air interface)
EPIBIOTIC HABITATS
(attached & crawling)
PLANKTONIC HABITATS (free floating).
Pelagic (water)
(Animals)
FECAL-SESTONIC HABITATS
(organic debris)
fungi
zooxanthellae
ENDOBIOTIC HABITATS
(symbionts)
(pathogens)
BENTHIC HABITATS (sea floor)
Organic debris
Mineral grains
bacteria
ciliates
Sea-mud interface
Bacteria
DOC
Sea surface
0.1 μm
microlayer
Protozoa
Larval forms

living bacteria form two distinct populations. Murchelano and Brown (1970) demonstrated seasonal changes in abundance of bacteria coinciding with the seasonal change of the plankton in Long Island Sound, U.S.A. In the Humber Estuary of north-east England, Bent and Goulder (1981) showed that the density of attached bacteria was low in summer and high from autumn to spring. Furthermore these authors found that the density of attached bacteria was dependant on the concentration of suspended solids. However, not all planktonic organisms will support epiphytic growth of bacteria (Sieburth, 1968; Droop and Elson, 1966); many produce antibiotic compounds.

Free living bacterioplankton do not normally exist as individual organisms but as clumps or microcolonies found in conjunction with suspended detrital particles (Pearl, 1974 ; Jannasch, 1973). Bacterioplankton has been shown to be important as a source of food for many filter feeding invertebrates (Zobell and Feltham, 1938 ; Sorokin, Petuysa, Pavlova, 1970 ; Sorokin, 1974). Sorokin (1974) suggested that the formation of clumps of bacteria may increase the efficiency of filter feeding in some animals with feeding apparatus too coarse to capture individual bacterial cells.

Zobell (1946), who introduced the 2216E marine agar that is standard for the cultivation of heterotrophic marine bacteria, stated that the sediments of the sea floor contain the "most extensive and versatile bacterial flora". An earlier study by Lloyd (1930) in the Clyde Sea Area, Scotland showed that the highest numbers of bacteria are found in the top layers of sediment and that numbers decreased below this. Bacteria are often found attached to marine sand grains (Meadows and Anderson, 1968). In sediments, detritus feeders such as holothurians, gastropods and polychaetes ingest the sediment and digest the attached bacteria (Sorokin, 1974). Meiofaunal harpacticoid copepods have some

ability to feed preferentially on different species of bacteria (Rieper, 1982). Bacteria have been found in the deepest ocean sediments where the hydrostatic pressure is equivalent to 1150 atmospheres (Zobell and Morita, 1957).

From experiments following the sinking and recovery of the research submersible "Alvin", Jannasch et al., (1971) and Jannasch (1973) concluded that the rate of bacterial degradation of organic matter in the deep sea may be very slow. Schwartz (1976) proposed that the intestinal bacterial flora of deep sea invertebrates may be partly responsible for decompositional processes in the deep sea. More recently Jannasch and Wirsen (1981) have reported increased bacterial activity surrounding thermal springs at the Galapogos Rift and East Pacific Rise ocean spreading centres, at depths of 2500-2600 m. The chemolithotrophic bacteria found in these sites are, apparently the predominant food source for dense invertebrate populations in this environment.

Bacteria play an important part in the decomposition and recycling of organic detritus in the marine ecosystem and Fenchel and Jørgensen (1977) have reviewed the part played by bacteria in this process. Bacterial breakdown of polysaccharides such as a cellulose, agar and chitin (Wood, 1967) converts these substances into forms more easily assimilated by animals.

The Bacterial Flora of Marine Invertebrates (other than echinoderms)

The bacterial flora of marine invertebrates has been studied mainly from the standpoint of spoilage or contamination of the commercially important species of shellfish. However, surveys have been made on the natural flora of the bean clam (Beeson and Johnson, 1967) and the pacific

oyster (Colwell and Liston, 1962). These studies have reported a predominance of Pseudomonads, Flavobacterium and Achromobacter on the external surfaces, with Vibrio species dominating in the gut. Boyle and Mitchell (1981) found that Pseudomonas and Vibrio were the most common genera in the microflora of the marine wood-boring isopod Limnoria lignorum.

Bacterial Pathogens of Marine Invertebrates

The studies on bacterial pathogens of marine invertebrates have been limited mainly to commercially important species of crustaceans and molluscs. The most widely studied bacterial disease of invertebrates is probably Gaffkymia first described by Snieszko and Taylor (1947). This disease is a fatal infection of the lobster Homarus americanus and is caused by the micrococcus Aerococcus viridans var homari (= Gaffkya homari). A primary symptom is the reduced ability of the haemolymph to clot. A bacterium which reduces the clotting of the haemolymph has also been isolated from the blue crab Callinectes sapidus (Johnson, 1976).

A bacterial disease of Crustacea, known as "Shell Disease" and which accompanied significant mortalities of lobsters (Taylor, 1948) is thought to be caused by chitinoclastic Gram-negative bacilli (Hess, 1937). A similar disease in the blue crab C. sapidus was described by Rosen (1967).

A disease of the oyster Crassostrea gigas was shown to be due to the marine bacterium Pseudomonas enalia (Colwell and Sparks, 1967). Aeromonas and Vibrio sp. have been found which are pathogenic to larval bivalves (Tusbiash, Chanley and Leifson, 1965).

Studies on non-commercial species of invertebrates have produced a few scattered reports on infectious diseases. An early study by Inman (1927) reported amphipod crustaceans infected with Bacterium gardi, a

luminous bacterium. Bang (1956) observed the occurrence in the horseshoe crab Limulus polyphemus of a Gram-negative pathogen which produced pathological effects due to generalised intravascular clotting. The black line disease of brain coral is believed to be caused by bacteria (Garret and Ducklow, 1975).

Antibacterial Systems in Marine Invertebrates

Some marine invertebrates live in environments such as sediments which may contain large numbers of bacteria. There are many possible routes of infection of the animal and some of the bacteria may be potentially pathogenic. It might therefore be expected that marine invertebrates would possess effective defences against bacterial invasion.

Sindermann (1970) classified the defence mechanisms of marine invertebrates into cellular and humoral components. Cellular mechanisms include phagocytosis, thrombosis and encapsulation. Humoral defences are mediated by bactericidins, lysozymes or agglutinins in the body fluids.

Phagocytes

The process of phagocytosis involves the stages of chemotaxis, recognition, adhesion, injestion and breakdown (Bang, 1975). In marine invertebrates, phagocytosis has been extensively studied in the bivalve molluscs. Tripp (1960) demonstrated the rapid phagocytosis of bacteria injected into the oyster Crassostrea virginica. The phagocytes then migrated out through the gut epithelium or the inner and outer surfaces of the mantle. The most actively phagocytic cells in the giant clam Tridacna maxima were found to have a superficial resemblance to macrophages from other invertebrate species (Reade and Reade, 1972).

The lipase activity of the cells and sera of the clam Mya arenaria

increased when the animal was injected with bacteria (Cheng and Yoshino, 1976). Lipase is a constituent of lysosomes in mammals and Cheng and Yoshino (1976) have suggested that lipase in resting and phagocytic cells is responsible for the degradation of bacteria. Lysozyme activity has been demonstrated in the haemolyph of M. arenaria and may have been released from the cells of the haemolymph (Cheng and Roderick, 1974).

In the Crustacea, phagocytosis has been observed in the lobster Homarus americanus (Paterson, Stewart and Zwicker, 1976) and the shore crab Carcinus maenas (Smith and Ratcliffe, 1978). In C. maenas injected bacteria became deposited in the heart, hepatopancreas and gills (Smith and Ratcliffe, 1980).

Bactericidins

Humoral defence systems have been described from a wide variety of marine invertebrates and non-specific bactericidins have been found in representatives from most of the major marine phyla (table 1). Indeed the only major marine phylum from which there is no report of these substances is the Annelida. Although the majority of these bactericidins are relatively non-specific, the coelomic fluid of the sipunculid Dendrostomium zostericolum killed or strongly depressed the growth of marine Gram-negative bacteria but not of the Gram-positive Gaffkya homari or the terrestial Serratia marcescens (Johnson and Chapman, 1970b). Similarly in Limulus polyphemus there is a higher titre of bactericidin against marine Gram-negative bacilli (Furman and Pistole, 1976).

There have been few attempts to isolate or define the bactericidins from marine invertebrates. However, Krassner and Florey (1970) isolated a low molecular weight, heat-labile protein associated with the antibacterial activity of the sipunculid Golfingia gouldii. In the

TABLE 1 Phyla from which non-specific bactericidins have been reported

			Arthropoda		
Porifera	Coelenterata	Mollusca	Crustacea	Arachnida	Sipunculida
Jakowska and Nigrelli (1960)	Burkholder and Burkholder (1958)	Johnson and Chapman (1970c)	Acton, Weinheimer and Evans (1969)	Pistole and Furman (1976)	Johnson and Chapman (1970b
Berquist and Bedford (1978)		Prescott and Li (1960)		Furman and Pistole (1976)	Krassner and Florey (1970)
				Nachum et al (1979)	Evans et al (1969)

abalone, antibacterial action is due to substance(s) that are thermostable and non-dialysable (Prescott and Li, 1960). Bromolactones from tropical and temperate sponges have been found to have strong antibacterial activity (Sharma and Burkholder, 1967).

Lysozyme

The bacteriolytic enzyme lysozyme is present in annelids (Perin and Jollès, 1972) and bivalve molluscs (McHenery, Birkbeck and Allen, 1979 ; McDade and Tripp, 1967). Lysozyme may function as part of the antibacterial system in some invertebrates (McDade and Tripp, 1967) and has been shown to be released during phagocytosis (Cheng et al., 1975). Other evidence suggests that lysozyme may function as a digestive enzyme in some bivalves (McHenery et al., 1979).

Agglutinins and Haemagglutinins

Agglutinins have been implicated as an antibacterial defence mechanism in several marine invertebrates. Pauley, Krassner and Chapman (1971) found that body fluid from the sea hare Aplysia california agglutinated four species of marine bacteria but not the terrestial organism Serratia marcescens. The crayfish Procambrius clarkii also possesses a natural agglutinin that reacts with marine bacteria (Miller et al., 1972).

Haemagglutinins capable of reacting with vertebrate erythrocytes have been found in the body fluids of species from most of the major invertebrate groups (McKay, Jenkin and Rowley, 1969). Many invertebrate haemagglutinins show a specificity for certain blood types (McKay et al., 1969; Johnson, 1964). Although the function of invertebrate haemagglutinins is not known, several workers have suggested that they

act as opsonins, i.e. they enhance the rate of phagocytosis, and are functionally analagous to vertebrate immunoglobulins. Treatment of chicken red blood cells with normal Aplysia california serum enhanced their susceptibility to ingestion by phagocytes of A californica (Pauley et al., 1971). A similar phenomenon in the oyster was demonstrated by Tripp (1966). After pre-treatment of bacteria with haemolymph of the crayfish Parachaeraps bicarinatus, Tyson and Jenkin (1974) observed enhanced phagocytosis of the bacteria.

Immune Systems in the Invertebrates

There is little if any evidence that invertebrates possess an immune system of the types found in vertebrates i.e. there is no production of antibody or complement or cell-mediated immunity based on T-lymphocytes (Lafferty and Crichton, 1973). However, the induction of non-specific bactericidins by previous exposure to bacteria has been demonstrated in insects (Boman and Hultmark, 1981), crustaceans (Evans et al., 1968) and sipunculids (Evans et al., 1969). The best defined immune response in invertebrates appears to be in the specific recognition and rejection of allogenic tissue grafts. Second-set memory has been shown in sponges (Evans, Kerr and Curtiss, 1980), corals (Hildemann et al., 1977) and annelids (Cooper, 1976). However, in many phyla the necessity for colonial animals to preserve their integrity by preventing fusion with neighbouring colonies (Manning, 1979) may be the main physiological function for these systems.

Echinoderms

General features

The echinoderms are marine invertebrates with bilaterally symmetrical swimming larvae which are ultimately transformed through metamorphosis into organisms with five rays (Ubagh, 1969). The earliest echinoderms date from the early Cambrian (Fell and Pawson, 1966). Echinoderms are deuterostomes (Ubagh, 1969) that is, during development cleavage is radial and indeterminate, with the blastopore forming the anus. Adult echinoderms are characterised by having a calcareous skeleton made up of separate plates which bear spines or protroberances and a water-vascular system with podia on the exterior (Hyman, 1955). All present day echinoderms have a fluid-filled perivisceral coelom which has three additional subdivisions; the water vascular system, the perihaemal system and the haemal system.

All echinoderms are entirely marine animals, and very few species enter even brackish waters (Nichols, 1975). The echinoderms can be placed into four sub-phyla; the crinozoa, the asterozoa, the echinozoa and the holmazoa (Fell and Pawson, 1966). Of these, the holmazoa consists of completely extinct non-radiate forms. The remaining three sub-phyla contain the five living classes (fig. 2). The five classes are sometimes divided into the eleutherozoans, or motile forms, and the pelmatozoans, or attached forms (fig. 2). The origin of the echinoderms is still obscure (Nichols, 1969), however, figure 3 represents one possible phylogenetic tree showing their affinities. The echinoderms are often regarded as phylogenetically significant for vertebrate evolution in that the origins of echinoderms and chordates may lie close together (Russell-Hunter, 1969); figure 4 demonstrates a possible sequence of relationships between the echinoderms and other phyla.

FIGURE 2 The five classes of present day echinoderms (from Russell-Hunter, 1969)

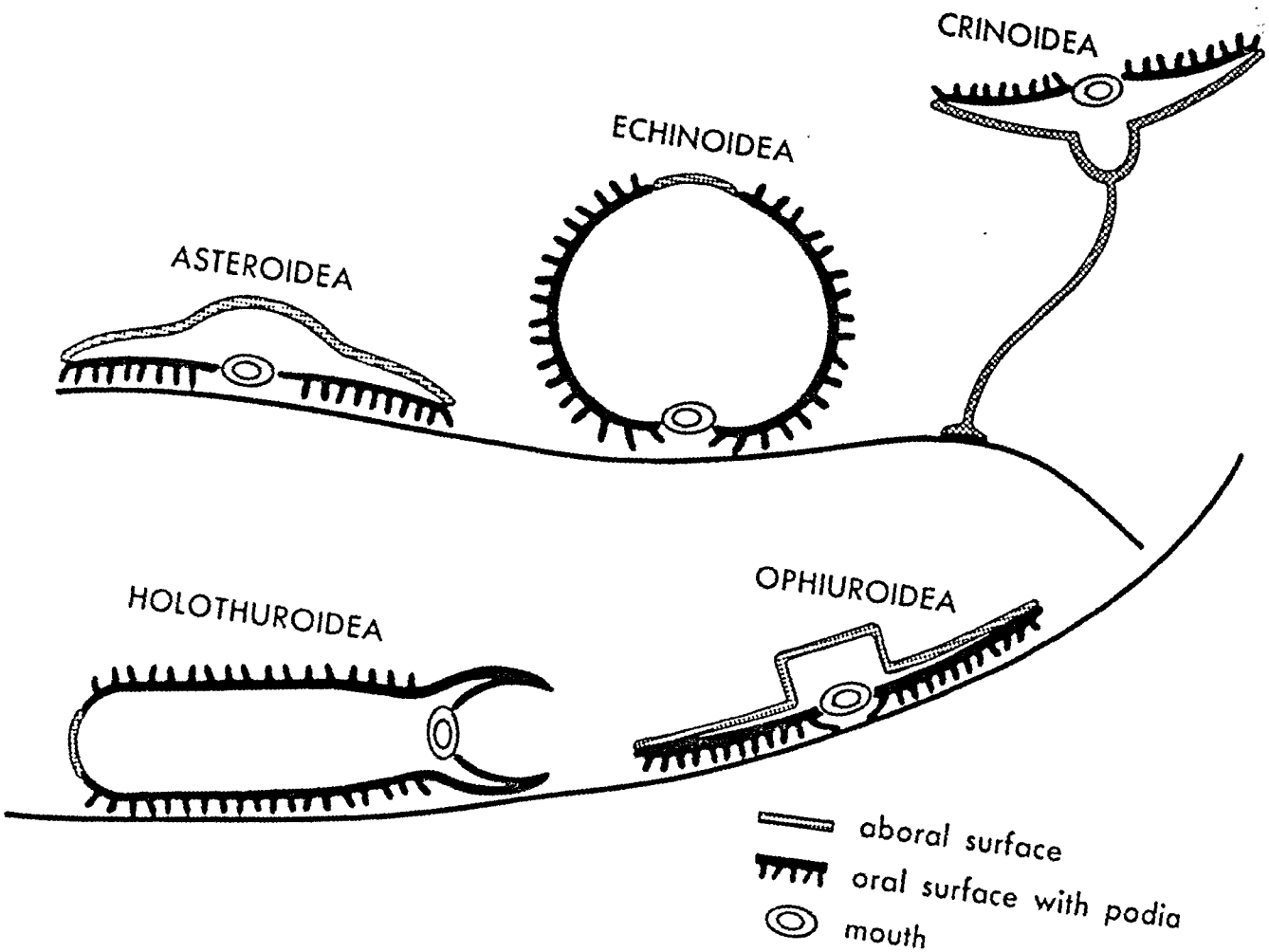

CRINOIDEA
ECHINOIDEA
ASTEROIDEA
HOLOTHUROIDEA
OPHIUROIDEA
aboral surface
oral surface with podia
mouth

FIGURE 3 Evolution of the echinoderms
(from Nichols, 1969)

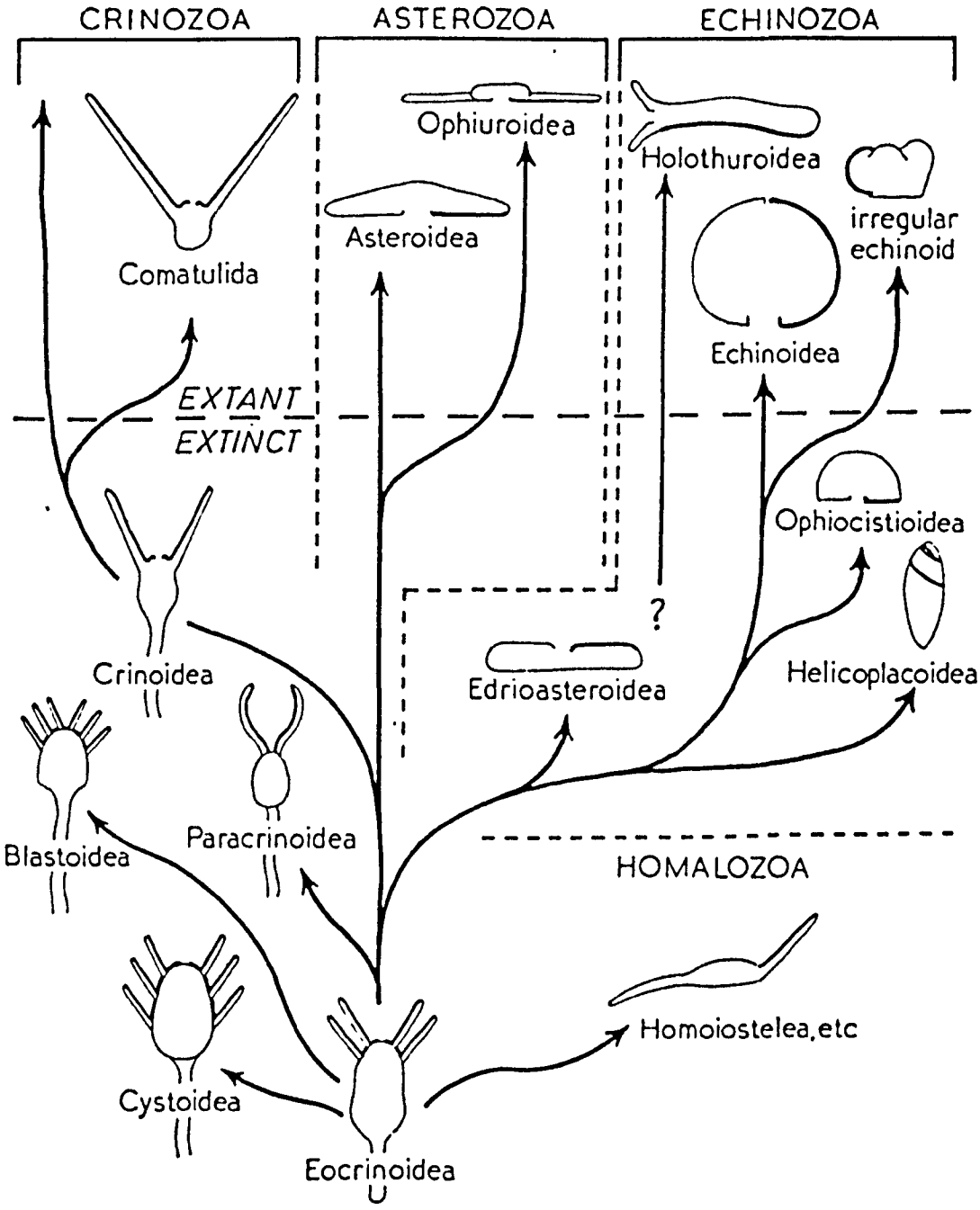
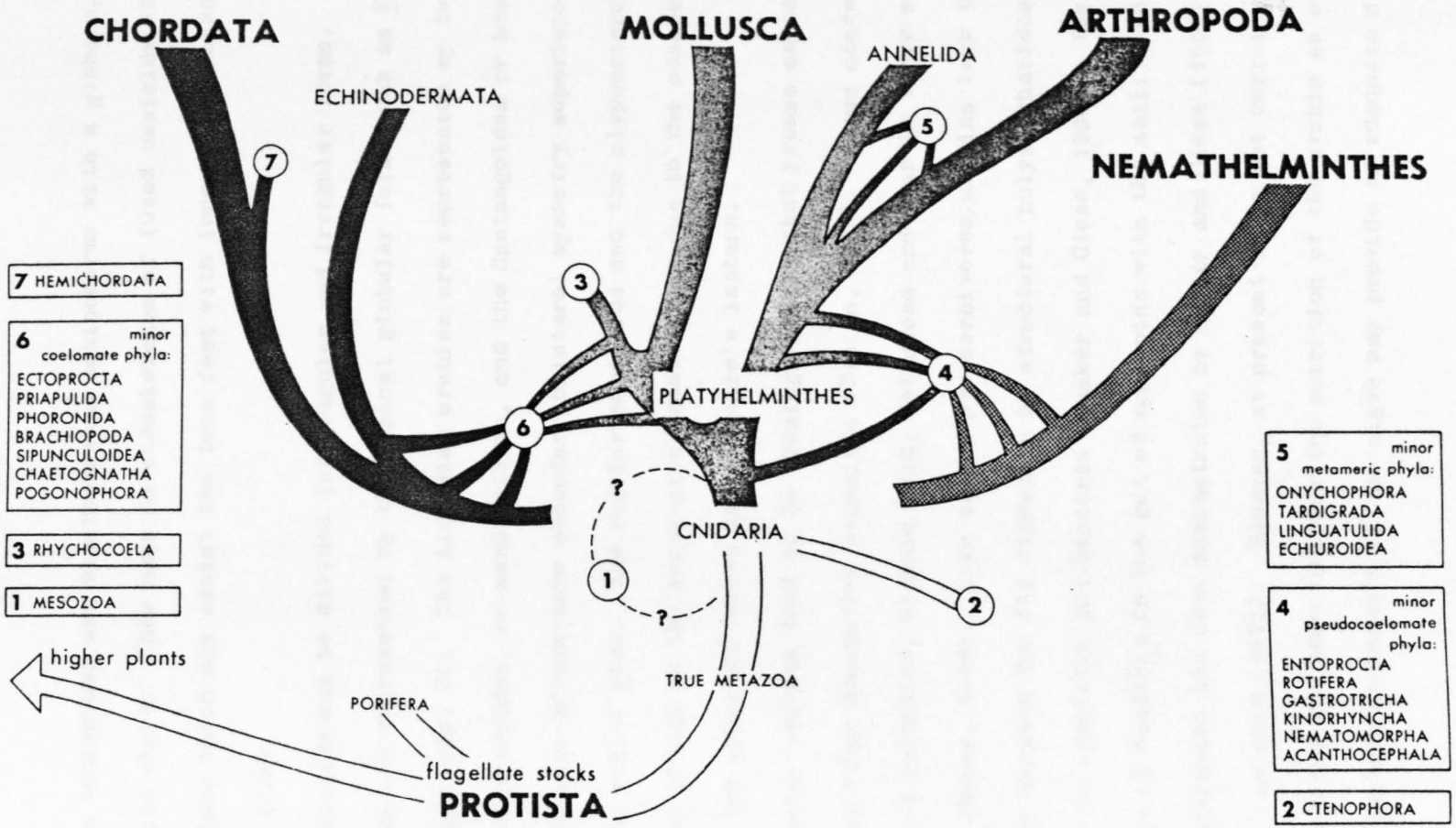

CRINOZOA
ASTEROZOA
ECHINOZOA
Ophiuroidea
Holothuroidea
irregular
echinoid
Asteroidea
Comatulida
Echinoidea
EXTANT
EXTINCT
Ophiocistioidea
?
Crinoidea
Edrioasteroidea
Helicoplacoidea
Blastoidea
Paracrinoidea
HOMALOZOA
Homoiostelea,etc
Cystoidea
Eocrinoidea

FIGURE 4 Relation of the Echinodermata to other phyla (from Russell-Hunter, 1969)


CHORDATA
ECHINODERMATA
MOLLUSCA
ANNELIDA
ARTHROPODA
NEMATHELMINTHES
PLATYHELMINTHES
CNIDARIA
TRUE METAZOA
PORIFERA
flagellate stocks
PROTISTA
higher plants
7 HEMICHORDATA
6 minor coelomate phyla:
ECTOPROCTA
PRIAPULIDA
PHORONIDA
BRACHIOPODA
SIPUNCULOIDEA
CHAETOGNATHA
POGONOPHORA
3 RHYCHOCOELA
1 MESOZOA
5 minor metameric phyla:
ONYCHOPHORA
TARDIGRADA
LINGUATULIDA
ECHIUROIDEA
4 minor pseudocoelomate phyla:
ENTOPROCTA
ROTIFERA
GASTROTRICHA
KINORHYNCHA
NEMATOMORPHA
ACANTHOCEPHALA
2 CTENOPHORA

Echinoids

The Echinoidea are eleutherozoan echinoderms with a globose, oval or discoid shape. They have an endoskeleton of fused ossicles which bear spines which may assist the tube feet with locomotion (Russell-Hunter, 1969).

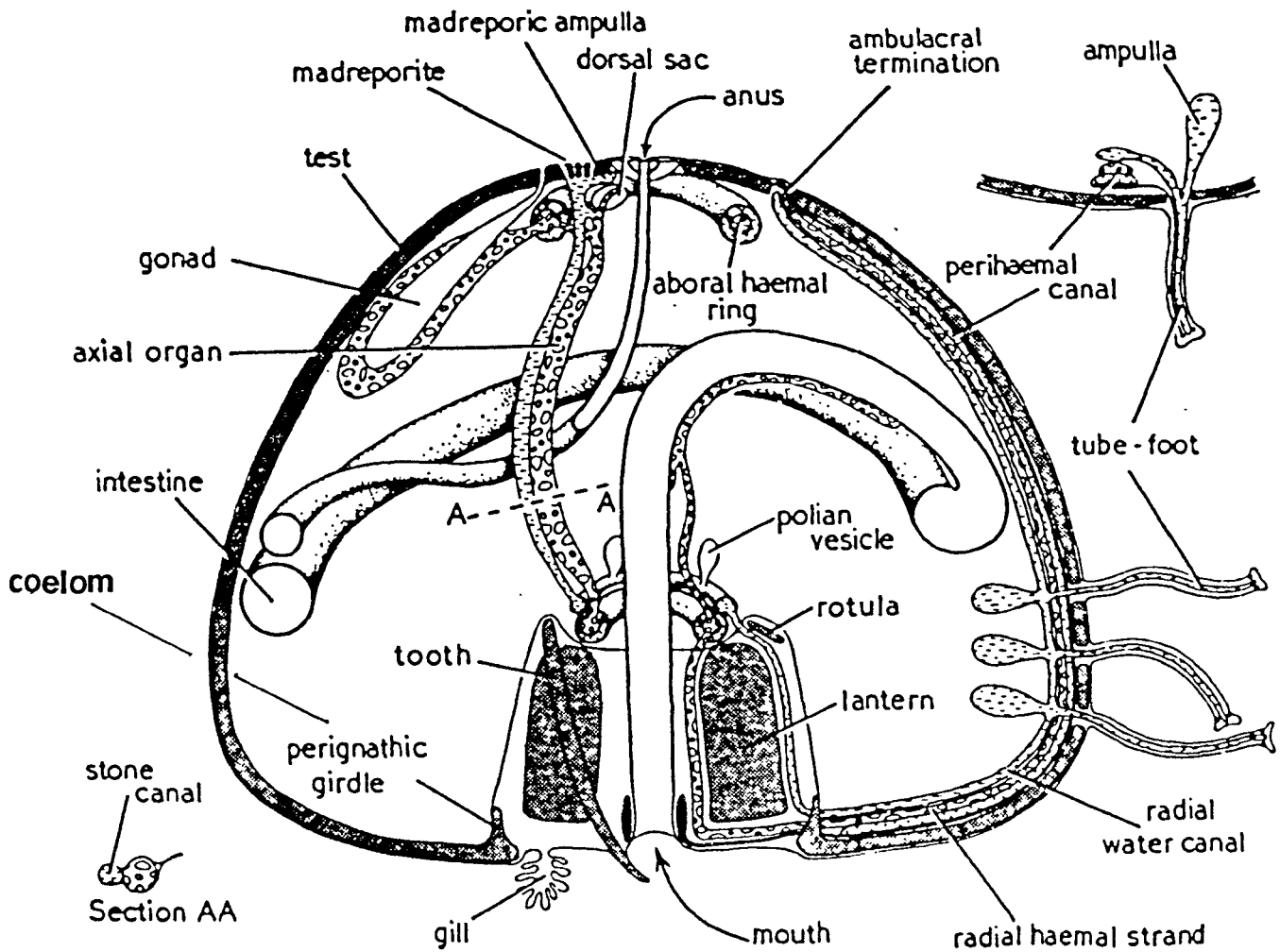
Echinoids can be divided into regular and irregular forms. Regular echinoids are represented by the typical globular forms such as Echinus esculentus (fig. 5). The irregular urchins are represented by two groups; the Clypeasteroidea, or sand dollars, and the Spatangoidea or heart urchins. Both irregular groups have secondary bilateral symmetry superimposed on the basic radial plan. The regular echinoids and the clypeasteroids are sometimes placed in the super-class gnathostoma due to the possession of a complex jaw apparatus known as Aristotle's lantern.

Regular urchins tend to be grazing animals that browse on marine algae and other encrusting organisms (Binyon, 1972). Algae contain large amounts of galactins, alginic acid, cellulose and agar in their structural make up (Boney, 1966). Like most other herbivores, urchins lack the necessary enzymes for the digestion of structural polysaccharides and may rely on symbiotic gut bacteria (Lasker and Giese, 1954). The existence of bacteria in the gut of echinoids with the ability to digest these substances has been demonstrated by Lasker and Giese (1954) and Prim and Lawrence (1975). However, at present it is not certain whether these bacteria are essential for the nutrition of the urchin as other less refractory components of the algae may provide an adequate diet.

Coelomocytes

The large fluid-filled perivisceral coelom of echinoids (fig. 5) contains a cell-rich coelomic fluid which has attracted several

FIGURE 5 Diagramatic transverse section of E. esculentus (after Nichols, 1969)


madreporic ampulla
madreporite
dorsal sac
anus
ambulacral termination
ampulla
test
gonad
aboral haemal ring
perihaemal canal
axial organ
tube-foot
intestine
A
A
polian vesicle
coelom
rotula
tooth
lantern
perignathic girdle
stone canal
radial water canal
Section AA
gill
mouth
radial haemal strand

investigators reviewed by Boolootian and Giese (1958) and Endean (1966). The ionic composition of coelomic fluid resembles that of seawater (Endean, 1966) with traces of protein also present (Holland, Giese and Phillips, 1967). There is some confusion in terminology of the types of cells recorded by different workers but part of this may be due to there being several developmental stages of particular cell types. Table 2 shows the cell types and nomenclature of various workers. A consistent feature is the basic division into phagocytes, spherule cells and vibratile cells (fig. 6). A more recent review by Smith (1981) confirms this division, but with the inclusion of progenitor cells as stem cells from which other coelomocytes are derived.

Soon after withdrawal from the animal, the coelomic fluid of echinoids clots by aggregation of the coelomocytes (fig. 7). Several workers have investigated the clotting mechanism (Kindred, 1921; Donellon, 1938; Boolootian and Giese, 1959; Abraham, 1964). The calcium chelator E.G.T.A. acted as an effective anticoagulant for E. esculentus coelomic fluid (Messer and Wardlaw, 1979) so it seems likely that the clotting is calcium-mediated. For the animal, rapid clotting is essential to prevent loss of coelomic fluid after wounding because the rigid body wall prevents closure by muscular contraction. The cells of the coelomic fluid also occur in the haemal channels, water vascular system and tissues of echinoids (Endean, 1966). However, these compartments are not easy to sample with a syringe and needle. The functions and interactions of the various coelomocytes are in many ways still unclear, but some may have multiple roles in defence and nutrition.

Using ^{14}C-labelled algae, Boolootian and Lasker (1964) showed that the red spherule coelomocytes of the purple sea urchin may be involved in transport of nutrients throughout the body. Other work by Pequignat (1966) has implicated the same cells in the digestion of material on the surface of the external epithelium.

TABLE 2 Coelomocytes of echinoids

Author and Method	Species	Phagocytes	Red Spherule Cells				Colourless Spherule Cells	Vibratile Cells
Kindred (1924) LM[a]	Arbacia sp.	Leucocytes	Coloured Amoebocytes	Brown Amoebocytes	Yellow Amoebocytes		Colourless Amoebocytes	Vibratile Cells
Bookhout and Greenburg (1948) LM	Mellita quinquiesperforata	Lobed Leucocytes	Granular Leucocytes	Brown Spherule Cells	Yellowish Brown Spherule Cells	Red Spherule Cells	Colourless Spherule Cells	
Liebman (1950) LM	Arbacia punctulata	Amoeboid Phagocytes	Petaloid Phagocytes	Fibroblasts	Green Trephocytes	Red Trephocytes	Colourless Trephocytes	Flagellated Phagocytes
Boolootian and Giese (1958) LM	Various sp.	Bladder Amoebocytes	Filiform Amoebocytes	Fusiform Corpuscles	Hyaline Haemocytes	Eleocytes	Colourless Spherule Amoebocytes	Vibratile Corpuscles
Burton (1966) LM	Diadema antillarum and Psammechinus miliaris	Phagocytes				Red Morula Cells	White Morula Cells	Vibratile Cells
Johnson (1969a) LM	Strongylocentrotus sp.	Phagocytic Leucocytes				Red Spherule Cells	Colourless Spherule Cells	Vibratile Cells
Vethamy and Fung (1972) EM[b]	Strongylocentrotus droebachiensis	Phagocytic Leucocytes	Lymphocytes	Granulocytes		Red Spherule Cells	Colourless Spherule Cells	Vibratile Cells
Bachman and Goldschmidt (1978) EM	Sphaerechinus granularis	Phagocytes	Leucocyte Type Cells			Full Morula Cells	Empty Morula Cells	Fibrocytes
Bertheussen and Seljelid (1978) DGS[c]	Strongylocentrotus droebachiensis	Phagocytes				Red Morula Cells	White Morula Cells	Vibratile Cells
Nesser and Yardlaw (1979) DGS	Echinus esculentus	Phagocytic Leucocytes				Red Spherule Cells	Colourless Spherule Cells	Vibratile Cells

[a] Light microscopy

[b] Electron microscopy

[c] Density gradient separation

FIGURE 6 Phase contrast photomicrographs of the coelomocyte types in the coelomic fluid of E. esculentus

a Vibratile cell x100

b Phagocyte x100

c Colourless spherule cell x50

d Red spherule cell x100

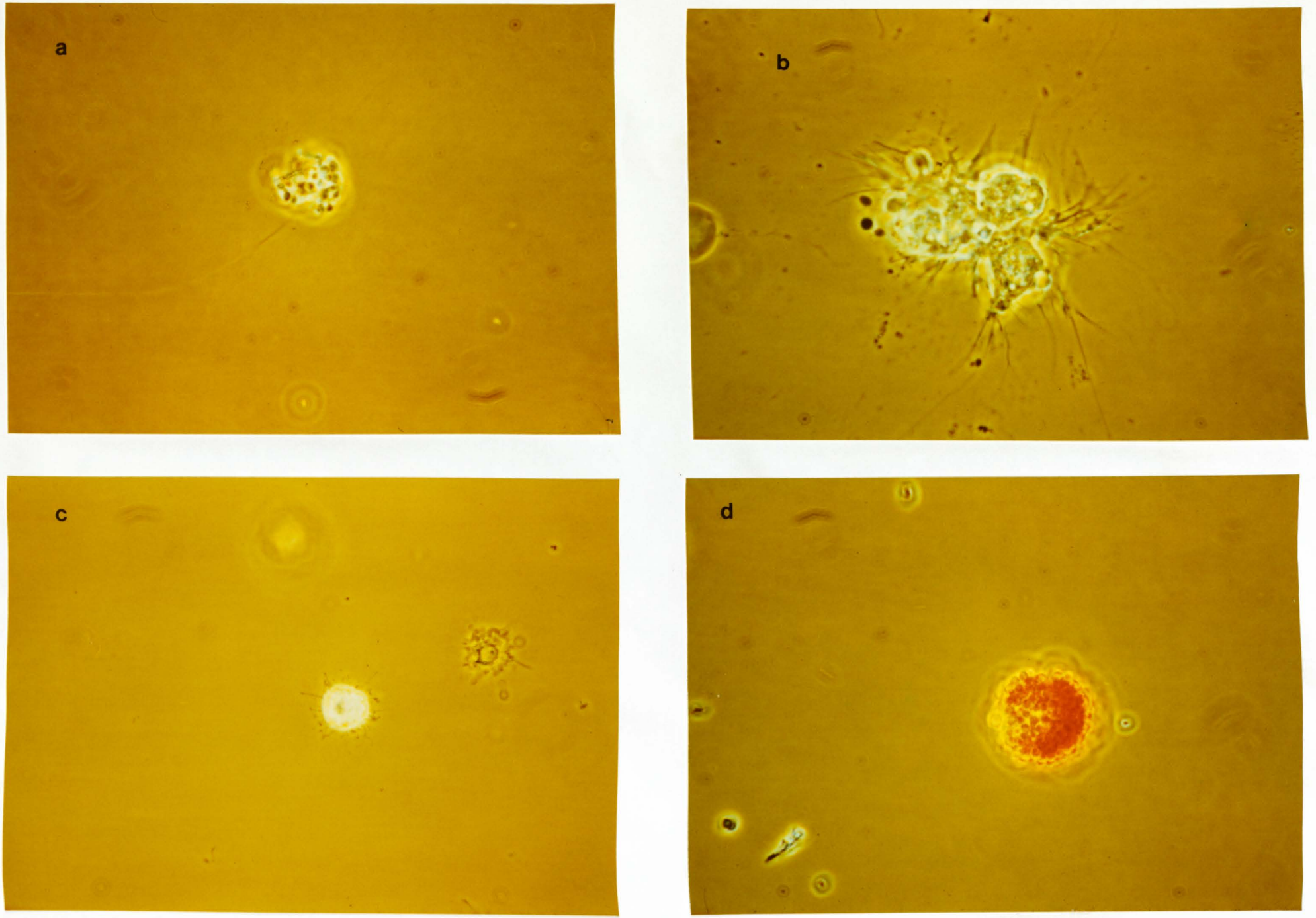
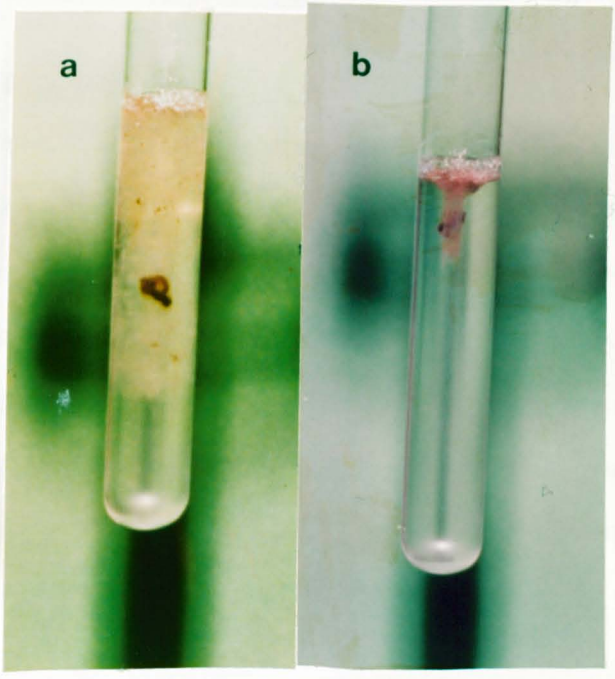

a
b
c
d

FIGURE 7 Clotted coelomic fluid of *E. esculentus*

a t = 0 after withdrawal

b t = 10 minutes after withdrawal


a
b

Naphthoquinone pigments

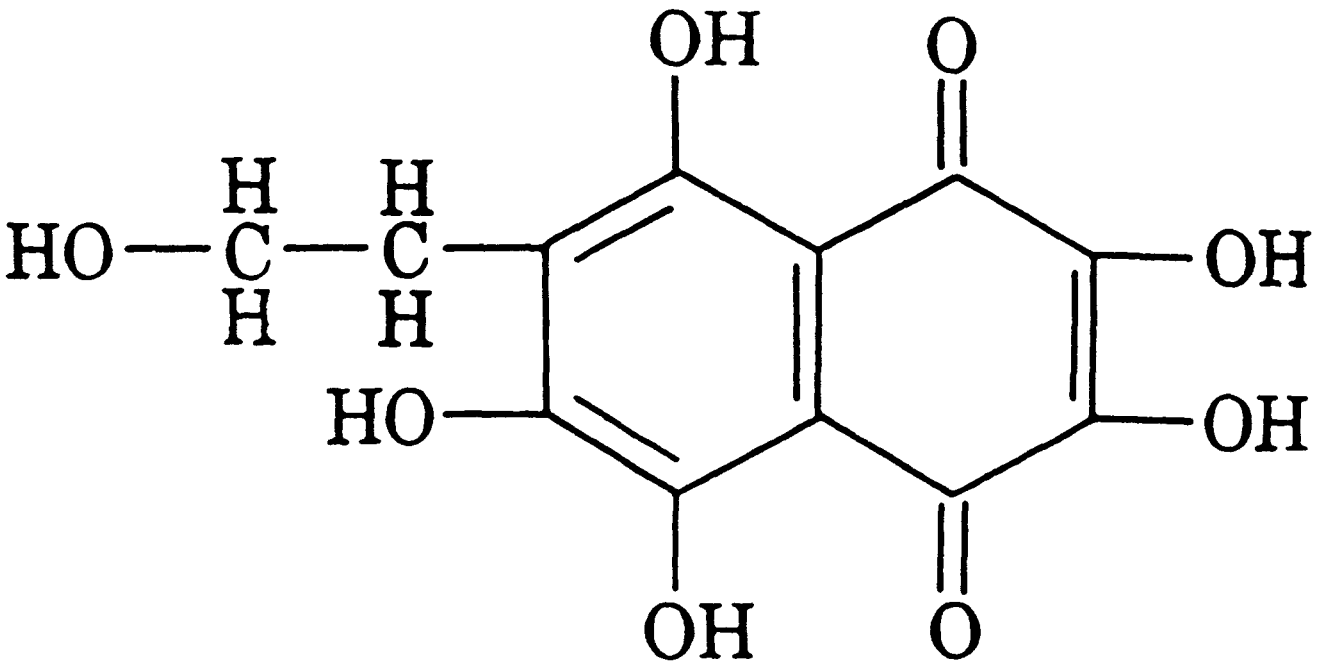
The calcareous test and the red spherule coelomocytes of echinoids contain naphthoquinone pigments. Echinoids are virtually unique in the animal kingdom in possessing substances of this class (Vevers, 1966). Naphthoquinones have also been shown to occur in bacteria (Ciegler et al., 1981) and cyanobacteria (Allen, Franke and Hirayama, 1967).

MacMunn (1885) first recorded the presence of the pigments, which he called echinochrome, in the red spherule coelomocytes of E. esculentus. Subsequently the pigment was isolated in crystalline form by McLendon (1912); later the chemical formula was established (Ball, 1936) and the structure established by Kuhn and Wallenfells (1940) (fig. 8). Confusion has arisen over the discovery of other naphthoquinone pigments in the spines of echinoids and which were termed spinochromes (Lederer, 1940). However, Goodwin and Srisukh (1950) and Anderson, Mathieson and Thompson (1969) have now clarified the nomenclature. It has been suggested that the naphthoquinone pigments function as algistats (Vevers, 1966). Experimental work by Johnson and Chapman (1970a) suggests that the pigment may act as a barrier to algal infection of the spines. In the red spherule coelomocytes, echinochrome A has been shown to occur in association with protein (Holland, Giese and Phillips, 1967; Johnson, 1970).

Antibacterial defence in echinoids

The coelomic fluid of the sea urchin E. esculentus is normally sterile (Unkles, 1977). A similar situation pertains in the asteroid Asterias forbesi (Bang and Lemma, 1962). The existence of a powerful bactericidin in E. esculentus coelomic fluid for a marine pseudomonad designated strain 111 (Ps111) was demonstrated by Wardlaw and Unkles (1978). The bactericidin which is heat stable and dialysable (Wardlaw and Unkles, 1978) resides in the red spherule coelomocytes (Messer and

FIGURE 8 Structure of echinochrome A



Echinochrome A

$C_{12}H_{10}O_8$

Wardlaw, 1979). In experiments using hanging drops of coelomic fluid of Strongylocentrotus sp., Johnson (1969b) found that the red spherule coelomocytes released their pigment in the presence of Gram-negative bacteria; Gram-positive strains were phagocytosed by the phagocytic cells. Earlier workers cited by Kindred (1921), also observed bacteria being phagocytosed. So far no observations have been reported on uptake of bacteria by the phagocytic cells in E. esculentus. As the bactericidal system in E. esculentus coelomic fluid does not require whole cells (Wardlaw and Unkles, 1978) their role in antibacterial defence is unclear. However, Bertheussen (1981) was able to observe uptake of Escherichia coli by phagocytes of Strongylocentrotus droebachiensis. Kaplan and Bertheussen (1977) found in S. droebachiensis receptors for the C3 component of the mammalian complement system on phagocytic coelomocytes with distribution similar to mouse peritoneal cells. Injection of bacteria into the coelom of the echinoid Arbacia punctulata produced gross structural changes in the animal's axial organ (Millot, 1966). This author suggested that the axial organ clears material ingested by the phagocytes of the coelomic fluid.

Immune Reactions in Echinoids

Coffaro and Hinegardner (1977) found an accelerated second set rejection of allografts in the sea urchin Lytechinus pictus similar to that in the holothurian Cucumaria tricolor (Hildemann and Dix, 1972). Drew (1911) described experiments which suggest E. esculentus coelomocytes can participate in the uptake of proteins; Hilgard, Hinds and Phillips (1967) reported that the coelomocytes are capable of discriminating between self and non-self proteins. Bertheussen (1979) concluded that the cytotoxicity of echinoid phagocytes to allogenic and xenogenic mixtures in vitro indicates that phagocytic coelomocytes are recognition

and effector cells in transplant reactions.

The coelomic fluid of some echinoids has been shown to possess powerful HA's and haemolysins against mammalian red blood cells (Ryoyama, 1973a and 1973b). The HA for rabbit erythrocytes is localized primarily in the colourless spherule coelomocytes of E. esculentus (Messer and Wardlaw, 1979). As it is thought that echinoderms lie close to the vertebrate line of evolution (fig. 4) attempts have been made to determine if the evolution of the vertebrate immune system can be traced through the echinoderms. The asteroid Asterias rubens has a haemagglutinin with an amino acid sequence which shows some correlation with the heavy chains of vertebrate immunoglobulins (Carton, 1974). This author, suggested that echinoderm HA is a possible candidate as an evolutionary precursor of the vertebrate immunoglobulins.

Smith (1978) induced pathological changes in the Polian vesicles of the holothurian Holothuria cinerascens with fish serum as an antigen, and proposed the Polian vesicle as an organ of immunologic responsiveness.

Echinus esculentus

Ecology

E. esculentus, the common or edible sea urchin, is one of the most conspicuous animals on hard substrates in the shallow sub-littoral waters of North Western Europe (fig. 9). Its range extends from North Norway to Portugal and westwards towards Greenland (Reid, 1935). In certain areas, E. esculentus may occur in extremely large numbers. In 1958, Forster (1959) estimated a 4 by $\frac{1}{2}$ mile strip of coast near Plymouth to have a population of 1.4×10^6 urchins, giving an average density of 4.7 m^{-2}. E. esculentus is a browsing animal and feeds on algae and encrusting organisms such as barnacles, bryozoans and tunicates. Recently

FIGURE 9 E. esculentus

Bonsdorffand Vahl (1982) have shown that it prefers to eat the alga Laminaria saccharina when the latter is encrusted with the bryozoan Membranipora membranacea.

Early work on the ecology of E. esculentus (Elmhirst, 1922 and Moore, 1935a) which suggested that larger urchins inhabit shallower water, is in contrast to recent studies using S.C.U.B.A. diving techniques. These latter studies (Larsson, 1968; Nichols, 1978) found larger urchins in deeper waters. Inshore populations of the animal showed a tendency to be thicker shelled and flatter (Thompson, 1942), a charge which has been attributed to the effects of wave action (Thompson, 1942). Moore (1935b , 1937c) detected seasonal changes in E. esculentus which accounted for changes in feeding habits, gonad composition and pigment deposition. Concentric pigmented rings in the test were shown to be annual and could therefore be used for estimating growth rates (Moore, 1937c). E. esculentus possesses a natural bacterial flora similar to that of other marine invertebrates (Unkles, 1977).

In recent years E. esculentus has come under increasing pressure from over-collection by divers (Nichols, 1978). The dried tests of large specimens are sold as souvenirs in certain areas of S.W. England and a drop in the number of large urchins inhabiting the depth range 5-15m in these areas has been observed. A more serious threat to E. esculentus populations is the possibility of commercial exploitation as food (Southward and Southward, 1975). In France the demand for sea urchin roes has exceeded the supply from local species and E. esculentus roes are being considered as a possible replacement.

Objects of the Research

Previous work from this department showed that the coelomic fluid of E. esculentus is bactericidal (Wardlaw and Unkles, 1978). This mechanism, which was tested against a black-colonied marine pseudomonad designated, as strain 111, is apparently based in the red spherule cells of the coelomic fluid (Messer and Wardlaw, 1979). The studies to be reported were designed to extend these initial observations.

a) by determining the susceptibility of a wide range of marine bacteria to killing by E. esculentus coelomic fluid.

b) to explore the possibility that the bactericidal activity of the red spherule cells might be due to the pigment echinochrome A.

A further section of the work focused on the question of whether certain identifiable environmental factors in the animal's habitat might influence the antibacterial defence mechanism.

MATERIALS AND METHODS

1. Sea Urchins

1.1. Collection

For general use, mature specimens of E. esculentus were collected from the sea bed near to Keppel Pier, Millport, Isle of Cumbrae, Scotland by the author wearing SCUBA diving equipment. Animals were also collected from environmentally different areas on and in the vicinity of Keppel Pier. In particular, collections were made from a) an area which was dominated by the brittle star Ophiocomina nigra and which was taken to be a site of poor food quality, and b) the pier piles of Keppel Pier which were heavily encrusted with barnacles and bryozoans and were grazed by E. esculentus; this was regarded as an environment with a rich food supply.

1.2. Maintenance of animals

Urchins were kept in concrete or plastic tanks with running seawater whose temperature ranged from 6^{o} in the winter to $12^{o}C$ in the summer. Before use in an experiment, animals were allowed to settle in the tanks for at least 24 h and up to 7 days. The animals used for environmental studies were processed without the settling period.

2. Bacteriological Culture Media

Marine agar 2216E and marine broth 2216E both from Difco Laboratories, Detroit, Michigan, U.S.A., were used. The media were dissolved in distilled water according to the manufacturer's instructions and sterilised by autoclaving for 15 minutes at 15 lbs in^{-2} pressure ($121^{o}C$).

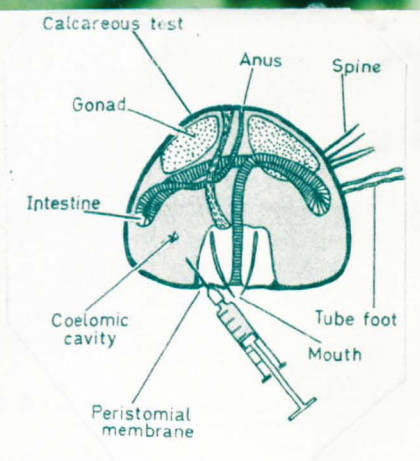
3. Bacterial Strains

Eighteen named bacterial strains were obtained from the National Collection of Marine Bacteria, Aberdeen. They were chosen by the curator in response to a request for strains that a) would grow on marine agar at 22°C, b) would survive and/or grow in seawater with a trace of added nutrient at 8°C, c) had coloured, or otherwise distinctive colony appearance and d) would contain as wide as possible a range of Gram-positive and Gram-negative representatives. In addition, the lobster pathogen Gaffkya homari (= Pediococcus cerevisiae, National Collection of Type Cultures 10331) was used. Also, five unnamed strains two Gram-negative and three Gram-positive distinctively coloured colonies with suitable growth characteristics were isolated from the sea near to Millport. These were included in the test strains. The black-colonied marine pseudomonad strain 111 (Wardlaw and Unkles, 1978) was also used. All strains were maintained on slopes of marine agar stored at 5°C.

4. Coelomic Fluid

To obtain coelomic fluid, an urchin was removed from the aquarium tank and water allowed to drain away from the peristomial membrane which was then swabbed with 95% ethanol. With the animal upright, the peristomial membrane was punctured with a 26 gauge half inch needle (fig. 10) to which was attached a 10 ml plastic, disposable syringe which had been chilled at 4°C. Fluid was withdrawn by moderate suction and was dispensed without delay into test tubes which already contained bacteria and which were held in an ice bath.

FIGURE 10 Technique for removing coelomic fluid from *E. esculentus*


Calcareous test
Anus
Spine
Gonad
Intestine
Coelomic cavity
Tube foot
Mouth
Peristomial membrane

5. Bactericidal Activity of Coelomic Fluid

Each test organism was grown on a marine agar slope, usually for three days at 22°C, and washed into suspension in sterile seawater. To standardize the concentration of bacteria, each suspension was adjusted, in a 16 x 150 mm tube to ten opacity units. This was done by diluting the suspension until its opacity, as determined visually, appeared to match that of the International Reference Preparation for Opacity (Perkins, et al., 1973), obtained from the National Institute for Biological Standardization and Control, London N.W.3. The ten opacity unit suspension was then diluted in sterile seawater by a factor (determined in a preliminary test) which ranged from 7×10^4 to 800×10^4 for different strains (table 3) so as to give a suspension with approximately 3500 colony forming units (CFU) per ml. This suspension was dispensed as 0.5 ml portions into 12 x 100 mm sterile capped glass tubes in an ice bath. To each tube was then added 1.5 ml freshly-drawn coelomic fluid or 1.5 ml control fluid (see below). This procedure differed for reasons of improved volumetric accuracy from that of Wardlaw and Unkles (1978) who added 1.8 ml of coelomic fluid to 0.2 ml of bacterial suspension. Immediately after mixing, a 0.1 ml zero-time sample was removed from the control tube and spread over the surface of a dried marine agar plate to obtain the initial viable count. An initial count of between 50 and 100 colonies per plate was desirable for ease of counting. To avoid disturbing the clotting process, the tubes containing coelomic fluid were not sampled at zero-time. However, samples from all the tubes were removed and plated out at 4, 24 and 48 h . The plates were incubated at 22°C for 2 to 4 days until colonies were large enough to count.

TABLE 3 The dilution factors required to reduce the concentration of each strain from 10 opacity units to 3.5×10^5 organisms/ml

Strain		Dilution Factor ($\times 10^4$)
Pseudomonas 111		30
NCMB strain no.		
	5	30
	8	20
	13	60
	19	30
	35	40
	308	15
	365	40
	570	50
	844	60
	1043	60
	1278	20
	1281	60
	1366	50
	1399	60
	1493	50
	1889	30
NCTC	10331	20
Clyde Isolate	ON1	150
	PP1	800
	YP1	7
	YP2	700
	YN1	80

6. Control Fluid for Bactericidal Tests

One type of control fluid, designated ECFS, consisted of pooled coelomic fluid from which coelomocytes had been removed by centrifugation for ten minutes at 2000x*g*. The supernate was heated for ten minutes at 100°C to kill possible bacterial contaminants. As an alternative control fluid, marine broth powder was reconstituted in SW(0.37 gl^{-1}) and the solution sterilised by autoclaving at 121°C for 15 minutes, this was referred to as 1% MBSW.

7. Clearance of Live Bacteria from the Coelomic Cavity of E. esculentus

A 1 ml suspension of Pseudomonas 111 containing 10^9 CFU was injected into the coelomic cavity of healthy urchins which were returned to separate cages in an aquarium tank. Subsequently at 4, 24 and 48 h , 1 ml of coelomic fluid was withdrawn from each urchin and 0.1 ml plated out onto marine agar. The plates were incubated for 24 h at 22°C before colonies were counted.

8. Proteins as Dispersants for Echinochrome A

Purified echinochrome A was obtained from Professor R.H. Thomson, Department of Chemistry, University of Aberdeen. The compound is only sparingly soluble in seawater but dissolves in the presence of various mammalian proteins (Johnson, 1970). To make a standard solution, a known weight of echinochrome A (typically 1 mg) was dissolved in a few drops of 0.1N NaOH and then treated with a 2 mg ml^{-1} solution of purified mammalian protein which had been dissolved in 1% MBSW. The mammalian

proteins used for this purpose were; bovine serum albumin (BSA), human serum albumin (HSA), bovine gamma globulin (BGG) and human gamma globulin (HGG) (Koch Light Laboratories Ltd., Colnbrook, England). The pH was adjusted to 8.0, although normally little adjustment was required. Following this, both the echinochrome A / protein solution and the stock protein solution, also at pH 8.0, were sterilised by passing through firstly, a 0.45 μm filter (Swinnex, Millipore S.A., Molsheim, France) and then a disposable 0.22 μm filter (Millex, Millipore S.A., Molsheim, France). The prefiltration by the 0.45 μm filter was precaution taken to remove the small amounts of protein that may have been denatured by the 0.1N NaOH in the previous step and to prevent blocking of the 0.22 μm filter.

9. Bactericidal Activity of Echinochrome A

The echinochrome A / protein solution was serially diluted in the corresponding plain protein solution to give 2 ml volumes of echinochrome A in solution at 50, 37, 25, 12 and 5 μg ml^{-1}. The 2 ml volumes were kept in 12 x 100 mm sterile, capped glass test tubes in an ice bath. To each tube was added 0.2 ml of bacterial suspension of 3500 CFU ml^{-1} prepared as previously described (5) and the tubes incubated at 8°C for 48 h . The smaller bacterial inoculum (0.2 ml) than used in studies with coelomic fluid (0.5) was to minimise the dilution of the echinochrome. Samples (0.1 ml) were spread onto marine agar plates at 0, 4, 24 and 48 h . The plates were then incubated at 22°C for up to three days and counted. Control tubes consisted of 2 mg ml^{-1} protein in 1% MBSW. On occasion, protein concentrations other than 2 mg ml^{-1} were used but without other alterations in the general procedure.

10. Physical and Biochemical Measurements on the Tissues of *E. esculentus*

10.1. External appearance of urchins

The external appearance of each urchin was noted after coelomic fluid had been withdrawn (below). Particular attention was paid to spine colour and the degree of algal infection in the spines.

10.2. Gonad index

After each animal had provided a sample of coelomic fluid, it was labelled with a numbered Dymo tape tag on an elastic band slipped around the ambitus of the animal which was then returned to the aquarium tank. Each urchin could subsequently be identified with a particular sample of coelomic fluid. When convenient, each urchin was weighed, measured and the external appearance noted. Following this, the gonads were dissected out and placed on tared dishes and weighed. The gonad weight as a percentage of body weight was expressed as the gonad index (GI) (Moore, 1935I):-

$$\text{Gonad index} = \frac{\text{wet weight gonads}}{\text{wet weight whole animal}} \times 100$$

10.3. Haemagglutinin titre

A 1 ml sample of coelomic fluid was dispensed into a chilled bijou bottle and the cells disrupted with five 30 second bursts from a ultrasonic probe (Konte, Vinland, N.J., U.S.A.). Using 0.02 ml volumes, serial two-fold dilutions in Dulbecco's phosphate buffered saline (PBS) (Dulbecco 'A', Oxoid Ltd., Basingstoke, England), were made in plastic microtitre diluters. To each row of dilutions, 0.02 ml of a 2% suspension of washed rabbit red blood cells in Bulbecco's PBS was added. After one hour, the trays were inspected and the haemagglutinin titre expressed as

the highest dilution of coelomic fluid which showed agglutination.

10.4. Echinochrome A concentration in coelomic fluid

To measure the concentration of echinochrome A, 5 ml of coelomic fluid was withdrawn and dispensed into a chilled test tube, centrifuged for ten minutes at 2000 g and the supernate discarded. Echinochrome A was extracted from the pelleted cells with 5 ml extraction mixture consisting 50% (V/V) acetone, 45% (V/V) ethanol and 5% (V/V) 0.1N HCl. After 30 minutes, the tube was again centrifuged for ten minutes, at 2000 g and the supernate scanned in a visible/U.V. spectrophotometer (CE.272, Cecil Instruments, Cambridge, England) against a blank consisting of the extraction solvent. The resulting spectrum, which matched that obtained with purified echinochrome A (fig. 11), allowed quantitation of the concentration of the pigment in the coelomic fluid. A wavelength of 475 nm was found most convenient.

11. Data Processing

11.1. Expression of bacterial counts

Colony counts at 4, 24 and 48 h in both coelomic and control fluids were expressed as the survival index (S.I.) (Wardlaw and Unkles, 1978):-

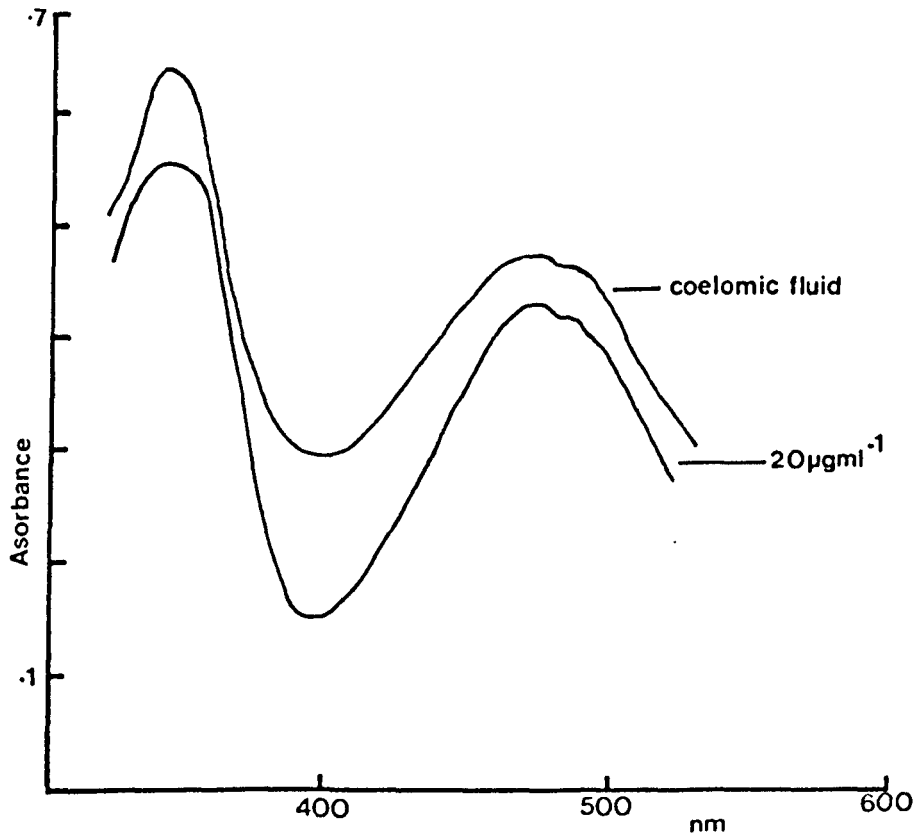
$$SI = \frac{\text{viable count at time T}}{\text{viable count at time 0}} \times 100$$

Use of this index facilitated comparison of bacterial suspensions whose initial counts were different.

11.2. Statistical analysis

A variety of standard statistical procedures was used to summarize and analyse the data gathered in this investigation.

FIGURE 11 The spectra of pure EchA compared with an acetone alcohol extract of coelomocytes


·7
·1
Asorbance
400
500
600
nm
coelomic fluid
20µgml-1

Histograms were drawn to provide visual illustration of frequency distributions of variables. Rankit and probit plots (Bliss, 1967), were employed to determine whether particular sets of data approximated to a normal or some other (e.g. lognormal) distribution.

Data which were normally distributed were summarized as the arithmetic mean and standard error of the mean, and differences between groups were analysed by Student's t-test. Lognormally distributed data were summarized as the geometric mean and the common logarithm of the data was used to analyse differences between groups with the Student's t-test. Data which appeared to depart markedly from both a normal and lognormal distribution were summarized as the median. Differences between such groups were investigated by the Mann Whitney U-test, and correlations were studied by the Kendall Rank-Correlation Coefficient.

RESULTS

1. Preliminary Observations

1.1. Clearance of injected live bacteria from the coelomic cavity of E. esculentus

Although Wardlaw and Unkles (1978) reported that E. esculentus could clear large doses of bacteria injected into the coelomic cavity, no detailed figures were presented. Figure 12 shows the results of an experiment aimed at quantitating this activity. In interpretating the data it is assumed that the initial innoculum of 10^9 Ps111ml^{-1} underwent an approximate hundred fold dilution in the coelomic fluid of the animal, to a final concentration of 10^7 organisms ml^{-1}. The viable count was rapidly reduced to 10 or fewer organisms ml^{-1}.

1.2. In vitro bactericidal activity of coelomic fluid on Ps111

In the course of this investigation a large number of observations on bactericidal activity of coelomic fluid from individual sea urchins on Ps111 were accumulated and were expressed as the survival indexes at 4, 24 and 48h. These data are presented as histograms in fig. 13. It is apparent that S.I. values are not distributed normally, and therefore it would be inappropriate to apply the arithmetic mean as the measure of central tendency. Instead, the median was employed. Thus the results from the 90 animals presented in fig.13 can be further summarized as the following median (M) survival indexes plus confidence limits (approx. 95%).

M

4h sampling time: $22 < 30 < 37$

24h sampling time: $12 < 18 < 50$

48h sampling time: $3 < 6 < 36$

FIGURE 12 Rate of clearance of injected live Pseudomonas 111 from the coelomic cavity of E. esculentus

△ Original Points

▲ Mean

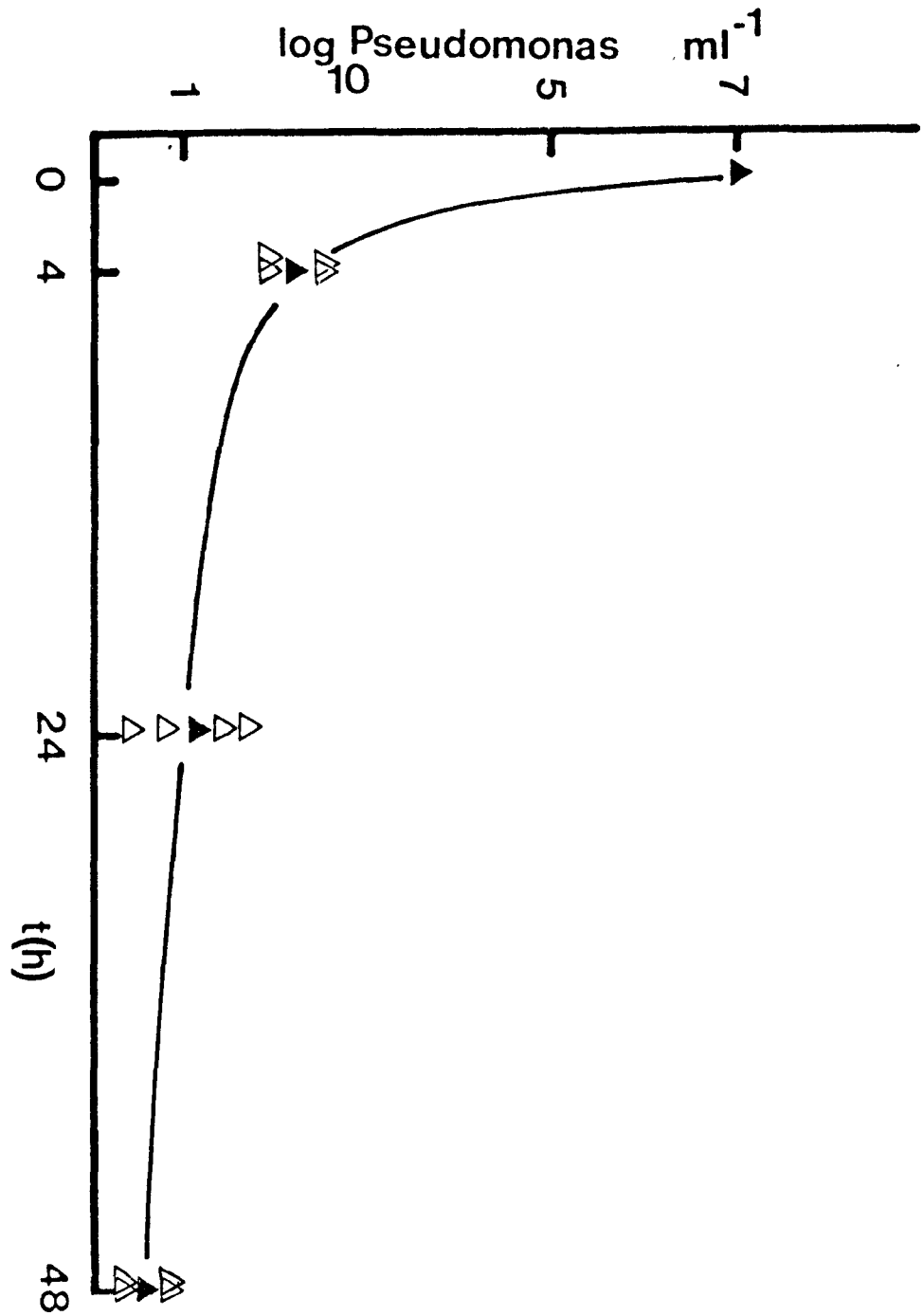
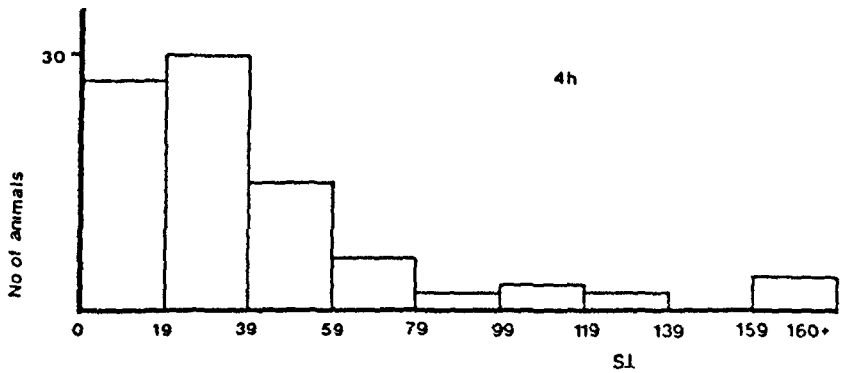
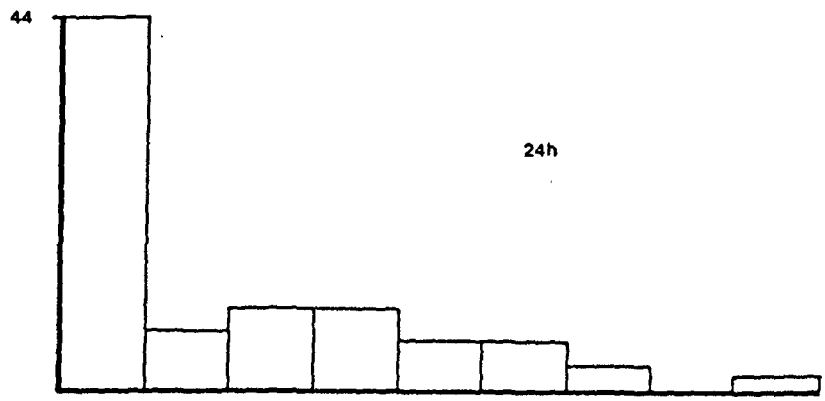
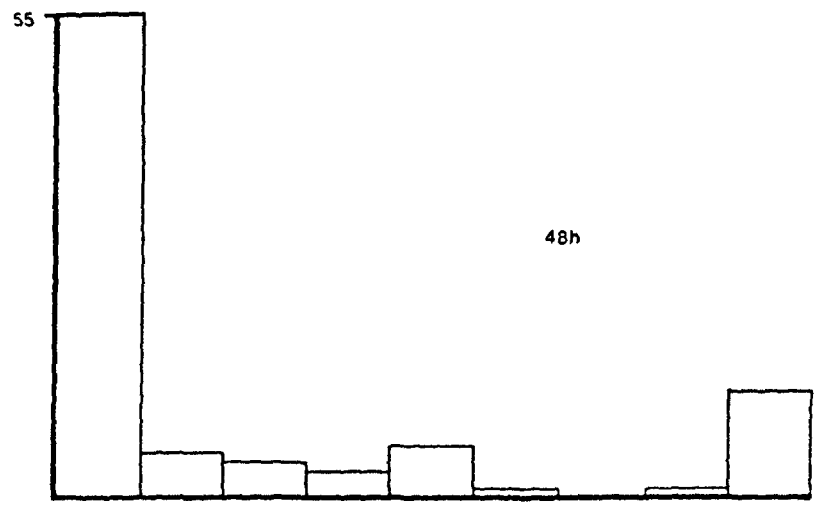

log Pseudomonas ml-1
10
1
5
7
0
4
24
48
t(h)

FIGURE 13 Histograms of survival indexes from *in vitro* bactericidal experiments on 90 different specimens of *E. esculentus* against *Ps111* as test organism


30
4h
No of animals
0
19
39
59
79
99
119
139
159
160+
SI


44
24h


55
48h

2. Characteristics of Test Bacteria

Before examining the various test bacteria for sensitivity to the bactericidal action of E. esculentus coelomic fluid in vitro, it was necessary to determine whether the bacteria would survive and/or grow at 8°C in seawater containing a trace of nutrient. This is because the coelomic fluid itself has the same ionic composition as seawater with a small amount of organic material (Ubagh, 1969). In the event two potential control fluids were examined: one was BCFS and the other 1% MBSW.

All 23 of the marine bacterial strains were able to survive or grow in one or both of the control fluids during the 48h of the test. These results are summarized in tables 4 and 5 together with information on the sources of the strains, Gram reaction, cell shape and colony appearance. Twelve of the strains were Gram-positive and eleven were Gram-negative and altogether they represented at least 11 genera (not all strains were identified to genus level).

Both control fluids proved to be equally effective in supporting the growth of the test strains; of the 23 strains 14 grew at 8°C and the rest survived without an increase in CFU. No difference was found between the growth of Gram-positive and Gram-negative strains in the control fluids (Mann-Whitney U-test; found value of U=50) (critical value of U=33, not sig. at $p \leqslant 0.05$). As a consequence of this survey, there was available a collection of 12 Gram-positive and 11 Gram-negative strains for tests for sensitivity to E. esculentus coelomic fluid.

TABLE 4 Cultural Characteristics of the Gram-negative test Bacteria

Strain No	NCMB Strain No	Organism	Morphology	Colonial appearance	Survival index after 48 h at 8°C in 1% MBSW[1]	Survival index after 48 h at 8°C in BCFS[1]
G.N.[3] 1	1904	Beneckea nigrapulchrituda	rod	grey/white	76	96
G.N. 2	1278	Vibrio fisheri	asporogenous rod	pale yellow, luminous colonies	103	98
G.N. 3	1281	V. fisheri	asporogenous rod	pale yellow, luminous colonies	122	129
G.N. 4	1889	Alteromonas citrea	rod	yellow	119	124
G.N. 5	844	Photobacterium phosphoreum	motile rod	luminescent/white	700	700
G.N. 6	308	Moraxella sp	coccus	white	ND[2]	700
G.N. 7	1366	Flexibacter litoralis	long filamentous rod	pink colonies	700	325
G.N. 8	19	Alteromonas haloplanktis	rod		700	700
G.N. 9	1399	Cytophaga latercula	short filamentous rod	orange/red agar-digesting colonies	282	255
G.N. 10	-	Clyde isolate YN1	rod	yellow	ND	700
G.N. 11	-	Clyde isolate ON1	coccus	orange	ND	219

1. B.C.F.S.: Boiled Coelomic Fluid Supernate; 1% MBSW = 1% Marine Broth in Seawater
2. Not Done
3. G.N. = Gram-negative
4. G.P. = Gram-positive

TABLE 5 Cultural Characteristics of the Gram-positive test Bacteria

Strain No	NCMB Strain No	Organism	Morphology	Colonial appearance	Survival index after 48 h at 8°C in 1% MBSW[1]	Survival index after 48 h at 8°C in BCFS[1]
G.P.[4] 1	-	Clyde isolate YP1	rod	yellow	ND[2]	90
G.P. 2	NCTC 10331	*Pediococcus cerevisiae*	coccus	white	107	94
G.P. 3	8	Cornyeform	rod	yellow	114	155
G.P. 4	570	*Micrococcus luteus*	coccus	yellow	87	91
G.P. 5	13	*Micrococcus sp*	small coccus	chalk white	99	129
G.P. 6	365	*Micrococcus sp*	small coccus	pink	130	111
G.P. 7	-	Clyde isolate PP1	coccus	pink	ND	138
G.P. 8	5	*Brevibacterium erythrogenes*	rod	yellow	258	223
G.P. 9	628	*Planococcus sp*	motile coccus	salmon pink	210	700
G.P. 10	1493	*Planococcus citreus*	motile coccus	orange/yellow	333	465
G.P. 11	35	Cornyeform	rod	orange	228	307
G.P. 12	-	Clyde isolate YP2	rod	yellow	ND	700

1. B.C.F.S.: Boiled Coelomic Fluid Supernate; 1% MBSW = 1% Marine Broth in Seawater
2. Not Done
3. G.N. = Gram-negative
4. G.P. = Gram-positive

3. Bactericidal Activity of Coelomic Fluid

3.1. On different bacteria

Previous work on the bactericidal effect of E. esculentus coelomic fluid (Wardlaw and Unkles, 1978; Messer and Wardlaw, 1979) were restricted to Ps111 as the test organism. The purpose of the present experiments was to extend the range of test organisms, with particular emphasis on strains from the marine environment.

The bactericidal effect of E. esculentus coelomic fluid on each strain is presented on figs. 14 and 15 as graphs of the S.I.'s plotted against time and compared with those in control fluid. In fig. 14 where the results from the 11 Gram-negative strains are illustrated, it is clear that all of these isolates were susceptible to the bactericidal effect of the coelomic fluid. This is shown by the progressive reduction in survival index from the initial value of 100. In this fig. the strains are arranged in approximate order of decreasing sensitivity to coelomic fluid, with no. 1 being completely killed after only 4h exposure and no. 11 showing a few surviving cells even after 48h. It will be noted that in the control fluid, strains 1-4 retained viability but showed no net growth (i.e. the survival index stayed around 100), whereas strains 5-11 all grew to give uncountable numbers of colonies during the period of exposure.

With the 12 Gram-positive strains (fig. 15) a similar pattern emerged, except that one strain G.P.12 (one of the isolates from the Clyde Estuary) was relatively insusceptable to killing by coelomic fluid. It showed slight net growth at 4 and 24h and a survival index of 83 at 48h. However, even with this strain, the coelomic fluid at 48h had been able to retard the growth of the organisms when compared with control fluid. Thus all 23 of the bacterial strains, 11 Gram-negative and Gram-positive, were sensitive in some degree to the

FIGURE 14 Bactericidal activity of E. esculentus coelomic fluid on 11 Gram-negative bacterial strains

●—● Whole coelomic fluid

O-O Control fluid (BCFS)

O--➤ Control fluid showing confluent growth

(minimum of 6 replicates per strain)

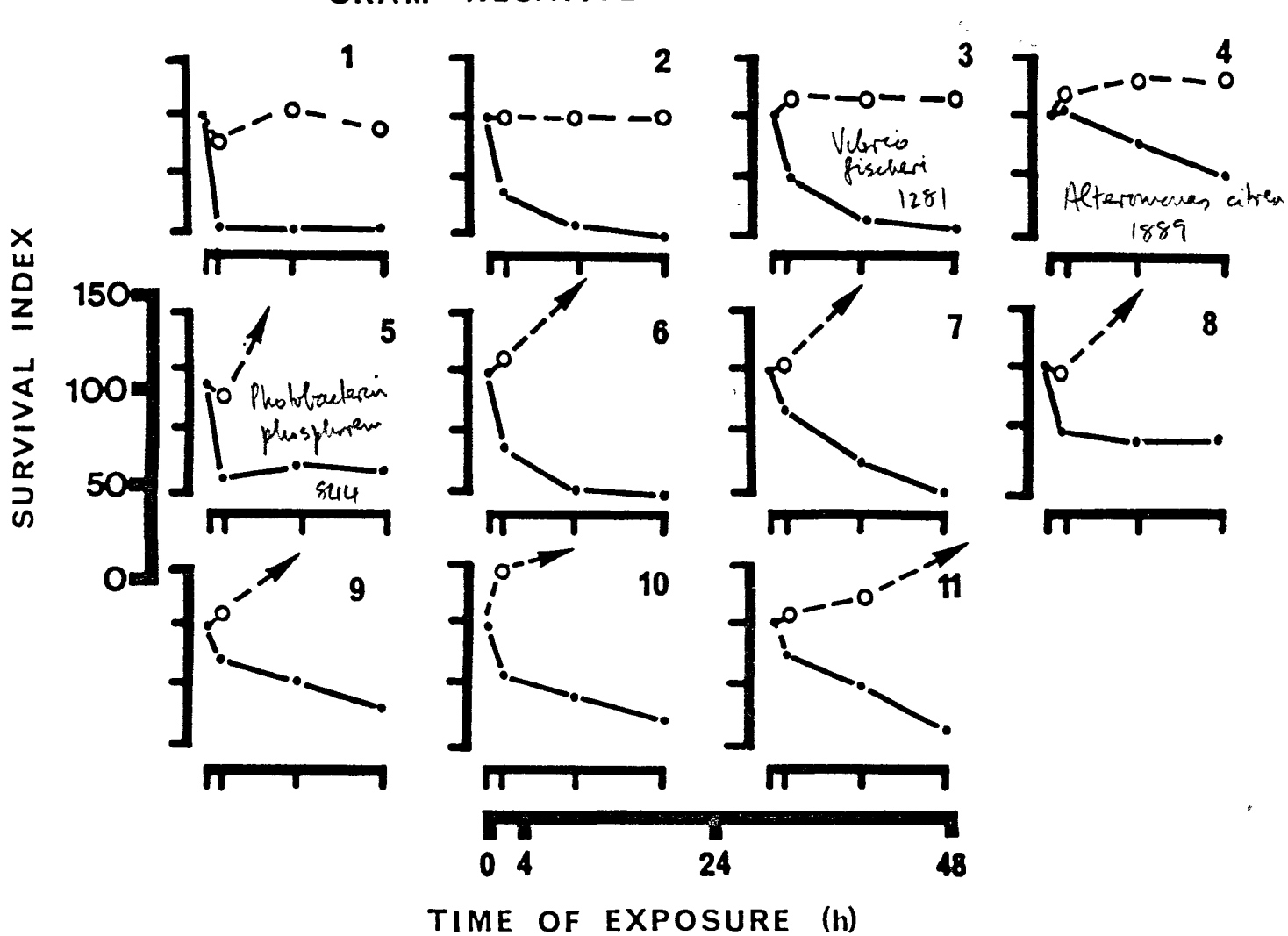

GRAM NEGATIVE STRAINS (GN)
SURVIVAL INDEX
150
100
50
0
1
2
3
4
Vibrio fischeri 1281
Alteromonas citrea 1889
5
6
7
8
Photobacterium phosphoreum
9
10
11
0 4 24 48
TIME OF EXPOSURE (h)

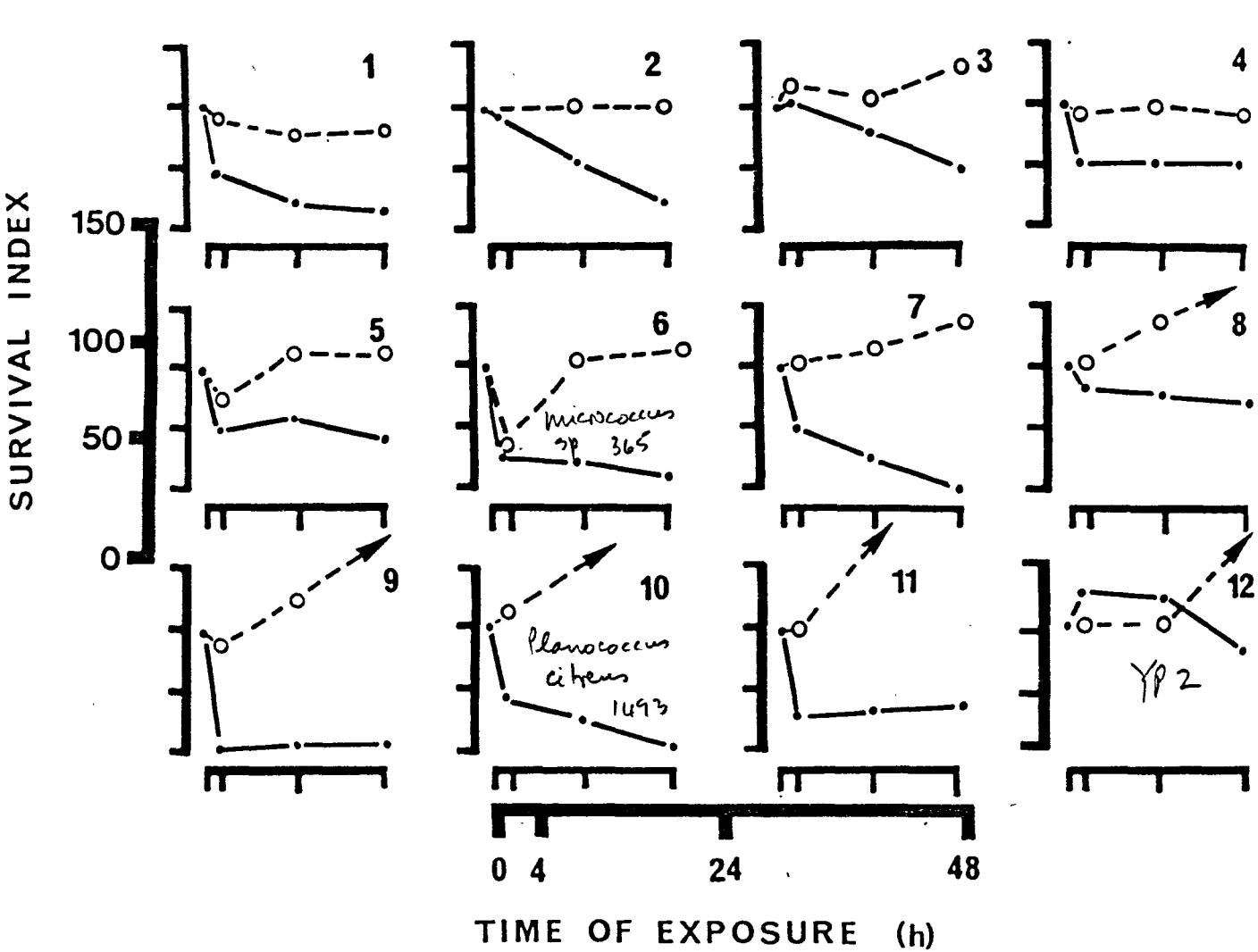
FIGURE 15 Bactericidal activity of E. esculentus coelomic fluid on 12 Gram-positive bacterial strains

●—● Whole coelomic fluid

O--O Control fluid (BCFS)

O--➤ Control fluid showing confluent growth

(minimum of 5 replicates per strain)


GRAM POSITIVE STRAINS (GP)
SURVIVAL INDEX
150
100
50
0
1
2
3
4
5
6
Micrococcus
sp 365
7
8
9
10
Planococcus
citreus
1493
11
12
YP 2
0 4 24 48
TIME OF EXPOSURE (h)

bactericidal effect of E. esculentus coelomic fluid. There was a slight trend for the Gram-negative strains to be somewhat more rapidly killed than the Gram-positives (*fig.* 16) but the difference was not statistically significant (Mann Whitney U-test, (Siegel,1956) U=39, critical value of U=33, not sig. at $p \leqslant 0.05$). The actual data is shown in appendix 2.

The marine pseudomonad strain no. 111 which was used in previous investigations (Wardlaw and Unkles, 1978; Messer and Wardlaw, 1980) and which is not included in *fig.* 14 gave a response very similar to that shown by G.N.6 or G.N.7 in fig. 14.

3.2. On mixtures of bacteria

Initially the possibility was considered that the bactericidal activity of coelomic fluid might be selective against particular strains and that this could be explored by inoculating the coelomic fluid with two distinctive strains simultaneously. A differential response to the two strains by coelomic fluid from different urchins may indicate the operation of two separate bactericidal mechanisms. In order to investigate this, a bactericidal experiment was set up according to the procedure described previously (materials and methods 5) using a mixture of two strains rather than one. Two tests were run in each case with the black-colonied Ps111 mixed with a Gram-positive strain either YP2 (yellow colonies) or PP1 (pink colonies) (table 6). No obvious departure in survival indexes from the values obtained when the strains were tested on their own (*fig.* 14) was apparent. The data obtained for the Ps111/YP2 mixture was used to produce the scatter diagram in fig. 17. On the basis of this diagram and a Kendall Rank Corellation Coefficient test ($S = -6$, $P = 0.117$) a differential response can be demonstrated i.e. the rank order of activity towards Ps111 does not correlate with the

FIGURE 16 Mean survival indexes of 11 Gram-negative and 12 Gram-positive bacterial strains

■ ■ Gram-negative strains

▲ ▲ Gram-positive strains

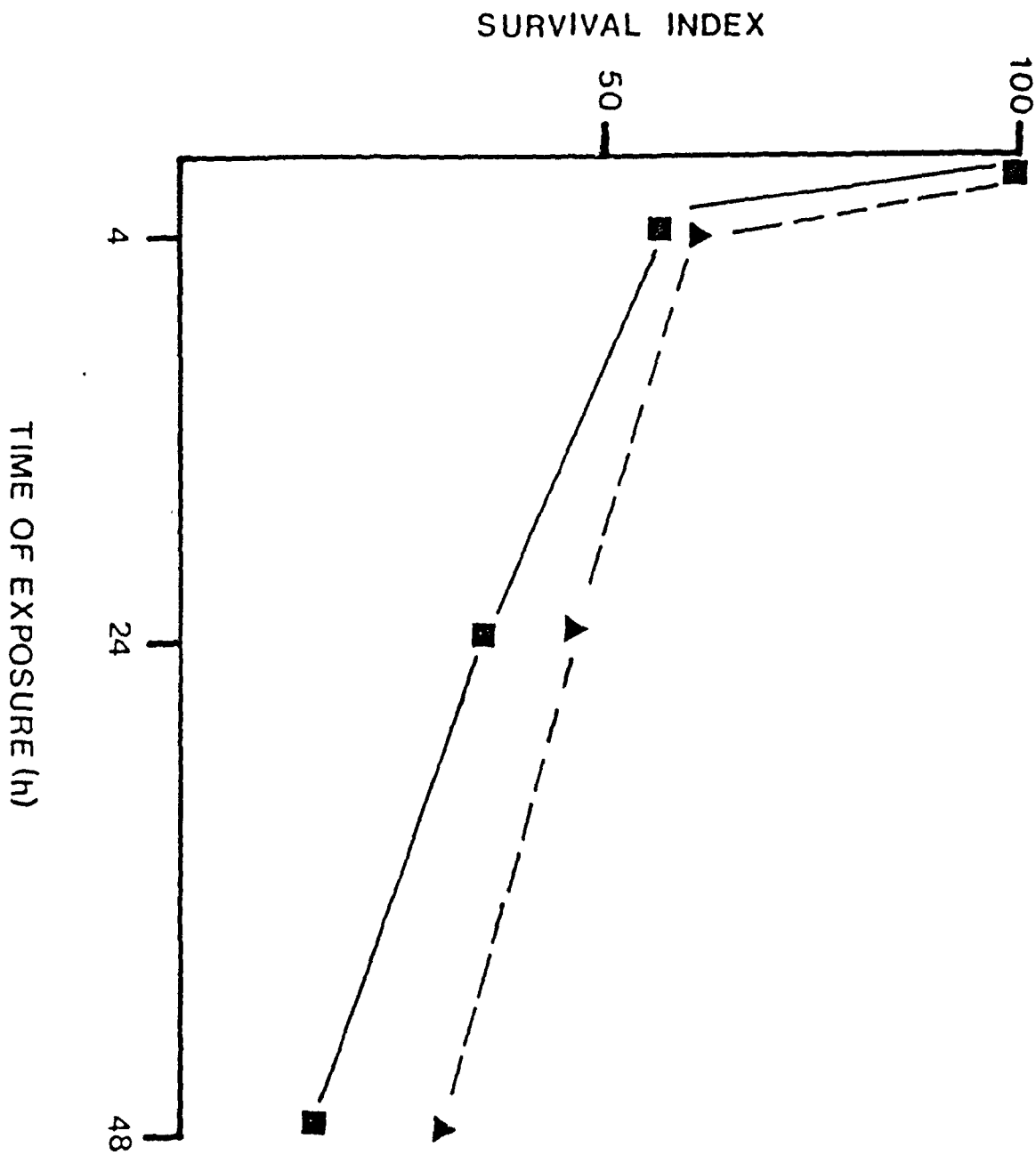

SURVIVAL INDEX
50
100
4
24
48
TIME OF EXPOSURE (h)

TABLE 6 Bactericidal activity of E. esculentus coelomic fluid on a mixture of Gram-negative and Gram-positive strains expressed as survival indexes

(a) Ps111/PP1

t(h)	C_1[a]		C_2		U_1[c]		U_2		U_3		U_4		U_5	
	Ps111	YP2[e]	Ps111	YP2	Ps111	YP2	Ps111	YP2	Ps111	YP2	Ps111	YP2	Ps111	YP2
4	79	94	85	65	59	105	85	97	62	93	5	101	72	68
24	495	80	392	79	10	82	0	71	3	97	0	63	8	86
48	700	[d]	700	[d]	0	55	0	101	0	93	0	72	0	51

(b) Ps111/YP2

t(h)	C[b]		U_1[c]		U_2		U_3		U_4		U_5	
	Ps111	PP1[f]	Ps111	PP1	Ps111	PP1	Ps111	PP1	Ps111	PP1	Ps111	PP1
24	400	72	48	39	9	17	4	19	4	14	0	6
48	700	[d]	9	8	0	8	0	11	0	14	0	3

[a] Control fluid (B.C.F.S.)

[b] Control fluid (1% M.B.S.W.)

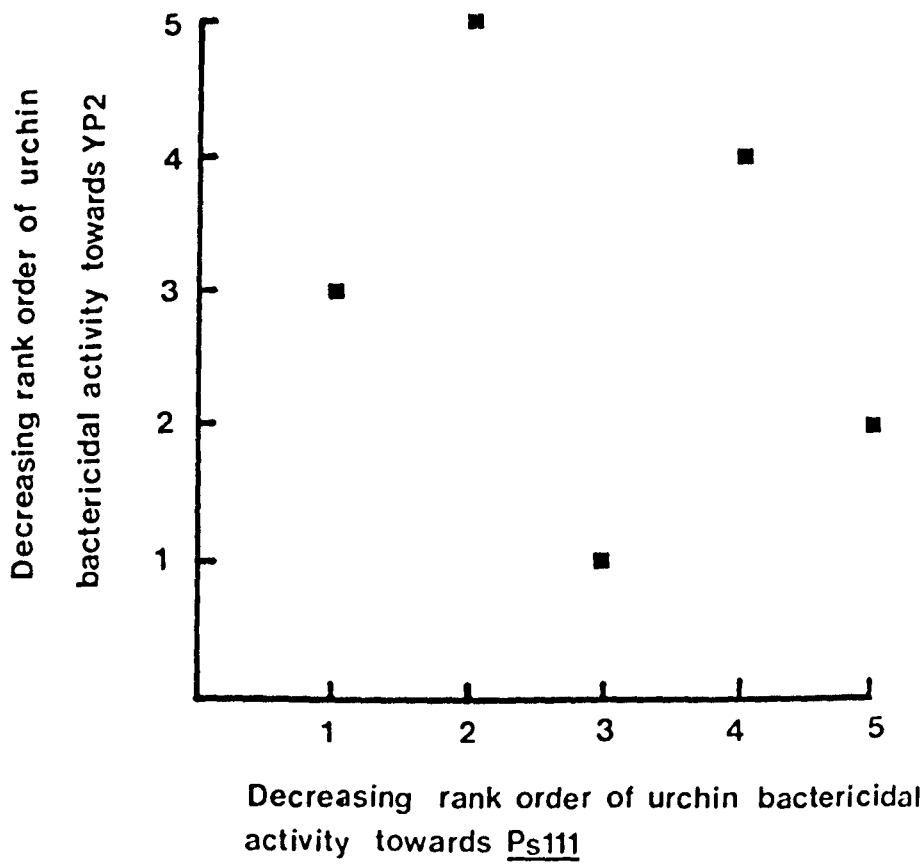
[c] Urchin no.

[d] Organism outgrown by Pseudomonas strain 111 in control fluid

[e] YP2 = G.P.12 } table5

[f] PP1 = G.P.7 }

FIGURE 17 Relationship between the survival indexes of bacterial strains Ps111 and YP2 in E. esculentus coelomic fluid


Decreasing rank order of urchin bactericidal activity towards YP2
5
4
3
2
1
1
2
3
4
5
Decreasing rank order of urchin bactericidal activity towards Ps111

rank order of activity towards YP2. This indicates the operation of at least two separate bactericidal mechanisms within E. esculentus coelomic fluid.

3.3. Growth of test bacteria and bactericidal action of coelomic fluid

The growth rate of the strains used to test the bactericidal activity of E. esculentus coelomic fluid showed considerable variation between strains. Figure 18 shows the relationship between the rank order of growth and the rank order of susceptibility to coelomic fluid for all the strains used (24 including Ps111). A Kendall Rank Correlation Coefficient test (Siegel,1956) indicated that no significant correlation existed ($Z = 0.802$, $P = 0.206$). However, when the strains that showed confluent growth at 48h were excluded the remaining 15 strains showed significant ($Z = 1.69$, $P = 0.04$) correlation between bactericidal sensitivity and growth rate. This indicates that the bactericidal effect of coelomic fluid may be related to bacterial metabolism.

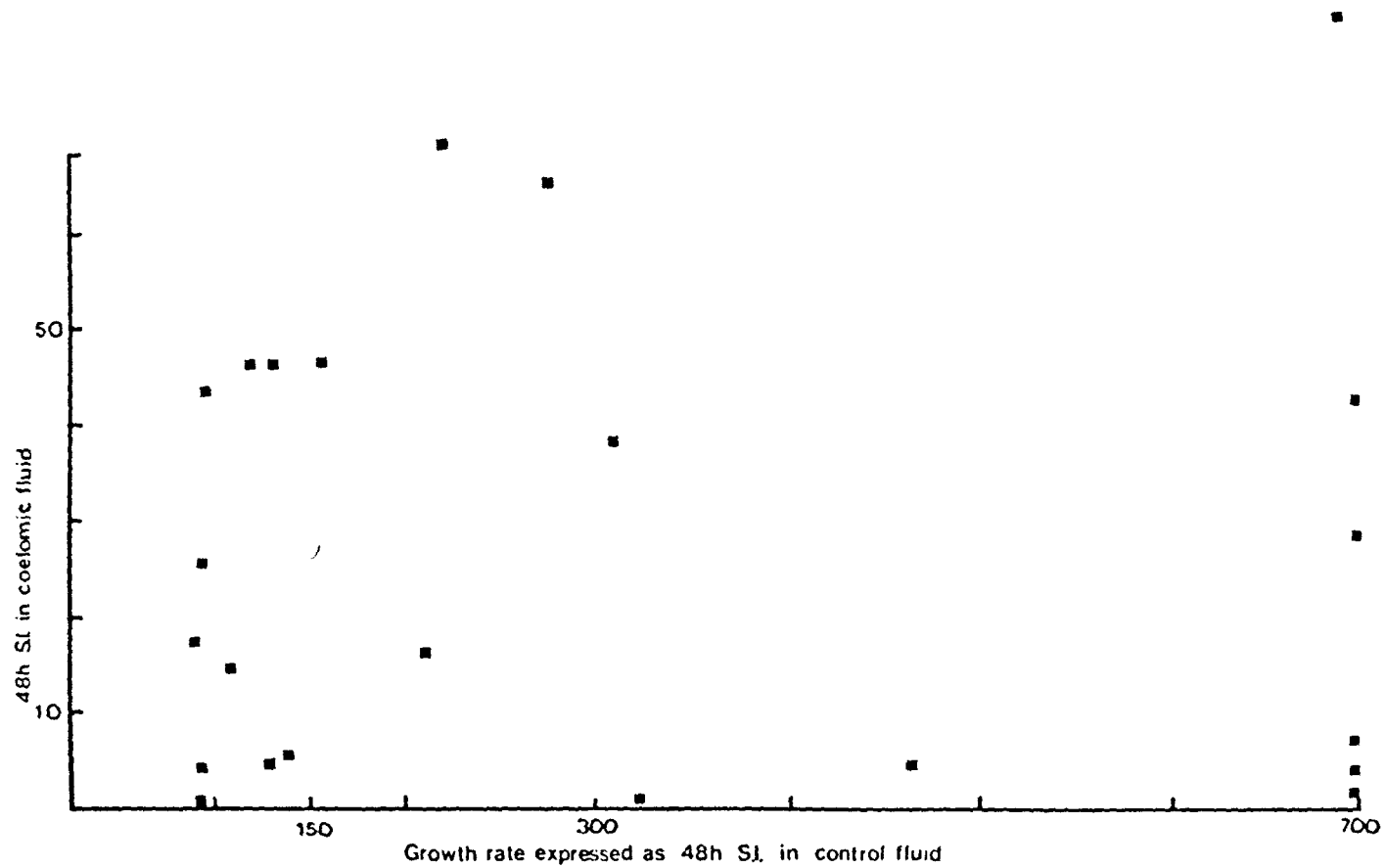
Bactericidal Activity of Echinochrome A

4.1. Preliminary observations

Before describing the experiments that demonstrate directly the bactericidal action of Echinochrome A, a number of incidental observations will be presented.

Animals which were held in outside tanks prior to processing showed a reddening of the test followed by a loss of most of the primary spines. This condition mimics the symptoms shown by an apparently diseased population of the sea urchin Strongylocentrotus franciscanus described by Pearse et al., (1977). In several individual E. esculentus this condition was followed by regrowth of the lost spines.

FIGURE 18 Correlation between the sensitivity of bacteria to killing by coelomic fluid and their ability to grow in coelomic fluid


50
10
48h SI. in coelomic fluid
150
300
700
Growth rate expressed as 48h SI. in control fluid

Animals suffering from an apparently similar condition were observed during dives at two separate locations; at Loch Riddon and Borradale, Loch Sunart both of which are sheltered sea lochs on the west coast of Scotland. The urchins at these sites all showed a general reddening of the test and spine loss. At Loch Sunart large numbers of dead animals could be observed.

Animals were often found during collecting dives with reddened patches on their test denuded of spines (fig. 19). This could possibly be due to damage by predators or micro-organisms. Similar reddening of the test was observed on animals which had been roughly handled during collection, or damaged by the net bags used for collecting the urchins.

The common factor in all these observations is the reddening found in damaged or diseased areas. As Echinochrome A bearing red spherule cells in E. esculentus coelomic fluid are known to possess bactericidal activity (Messer and Wardlaw, 1980) the reddening in these animals may be due to an accumulation of red spherule cells at the point of injury or disease. The possibility that Echinochrome A might be acting as an antimicrobial defence factor was therefore investigated directly.

4.2. Direct demonstration of bactericidal activity

It was not possible to test Echinochrome A on its own for bactericidal activity because the compound is too sparingly soluble in seawater to give solutions of the required concentration. However, note was taken of the report by Johnson (1970) that Echinochrome A could be dissolved in seawater containing added protein. Eventually four proteins were used as dispersants: BSA, HSA, BGG and HGG. A fifth protein, calf thymus histone, proved to be insoluble in seawater and was not tested further.

FIGURE 19 Damaged or diseased area on test of E. esculentus

Using Ps111 as a test organism, the bactericidal activity of Echinochrome A dissolved in solutions of four different mammalian proteins is shown in fig. 20a, b and c. All of the Echinochrome A / protein solutions possessed bactericidal activity with an effect being noted at the highest concentrations as early as 4h. At 24h almost total killing at concentrations higher than 25ug ml^{-1} Echinochrome A occurred in all 4 solutions. At 48h only the BGG solution remained effective at 25ug ml^{-1} Echinochrome A. In the solutions containing Echinochrome A in HSA, BSA, HGG there was regrowth at concentrations below 37ug ml^{-1} Echinochrome A. There was some indication that BSA/Echinochrome A solution was less effective bactericidally than the other three. This was most noticeable at 4 and 48h. The control fluids, which were the protein solutions without Echinochrome A, supported bacterial growth in all instances.

4.3. Effect of varying the protein concentrations on the bactericidal activity of Echinochrome A

The initial concentration of protein used as a dispersant for Echinochrome A was set arbitrarily at 2mg ml^{-1}. To investigate the possibility that protein concentration influences bactericidal activity, a repeat of the previous experiment using BGG was run with the protein concentration set at 4 different values. From the results in table 7 it is apparent that varying the protein concentration over the range used had no effect on bactericidal activity. Presumably, very low concentrations of protein would no longer act as effective dispersants of Echinochrome A but these were not explored.

4.4. Bactericidal activity of echinochrome A towards a range of different marine bacteria

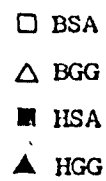
In addition to Ps111, it was decided that six of the strains which had previously been tested for their susceptibility to the bactericidal

TABLE 7 The effect of varying the BGG concentration on the bactericidal action of 30ug EchA ml^{-1} on Ps111

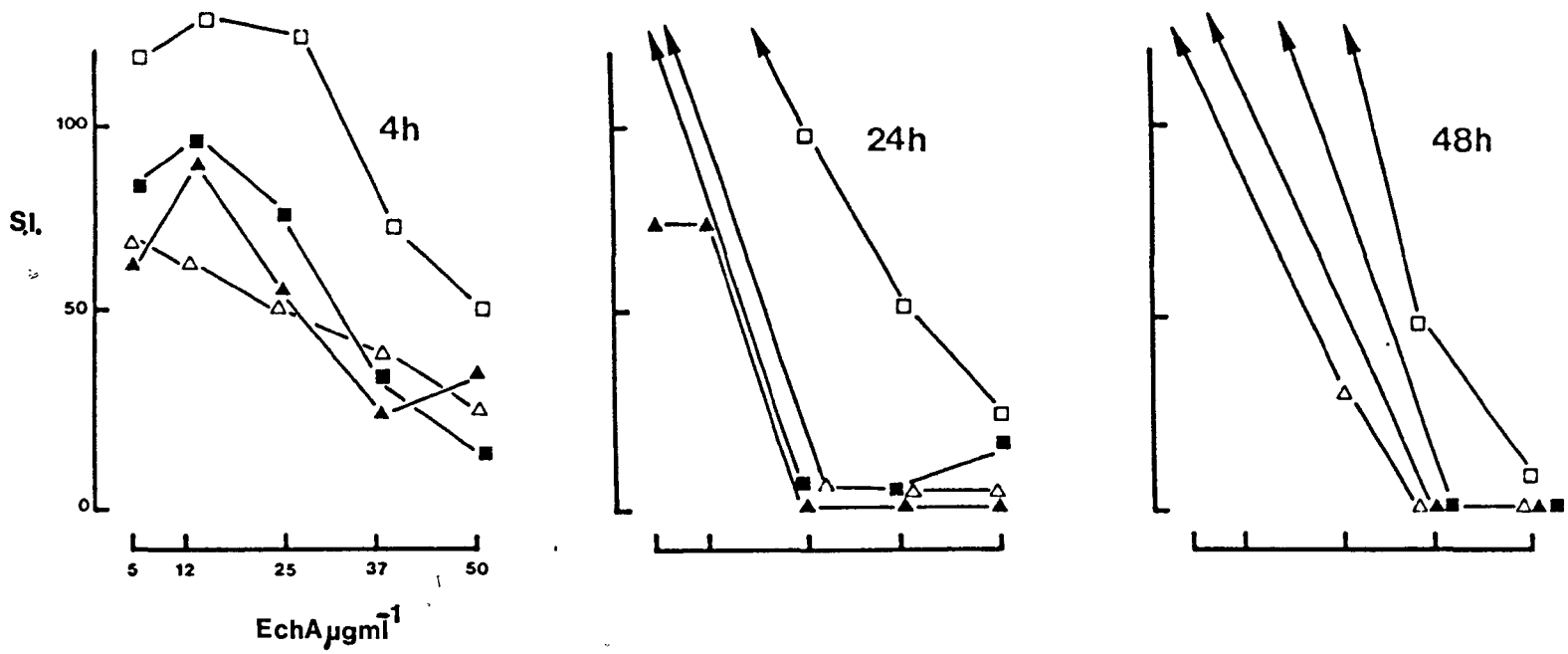
BGG(mg ml^{-1})	SI 4h	SI 24h	SI 48h
0.125	68(105)	14(155)	3(700)
0.5	60(109)	30(130)	0(700)
2	85(129)	27(94)	6(700)
8	92(81)	10(100)	3(700)

( ) control results with no EchA present

FIGURE 20 Bactericidal activity of EchA at a range of concentrations in association with 4 different proteins



(duplicate counts at each point)


4h
24h
48h
SI.
100
50
0
5
12
25
37
50
EchA µgml⁻¹

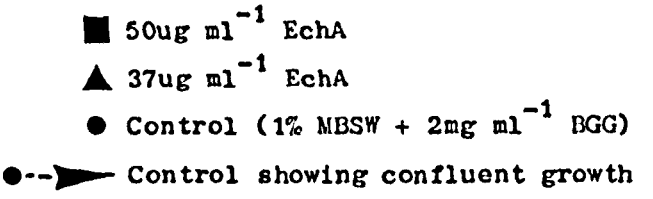
activity of whole coelomic fluid should be used as test organisms in the EchA system. This was to give information on whether the strains would show a similar order of susceptibility towards EchA as they did towards whole coelomic fluid. BGG was used as the dispersant protein for EchA in these tests.

The results for the Gram-negative strains, including Ps111, are shown in fig. 21 and for the Gram-positive strains in fig. 22. Altogether six of the seven strains were inhibited to some degree by EchA at concentrations of 50 and 37 $\mu g\ ml^{-1}$ when compared with growth in the control fluid. However, one strain, Alteromonas citrea, showed little difference between even the highest concentration of EchA used and the control fluid. Two of the remaining strains Ps111 and Vibrio fischeri were completely killed at the higher concentrations; a further two strains, YP2 and Plannococcus citreus were suppressed by comparison with the control fluid after 48h at both concentrations. Photobacterium phosphoreum showed only partial inhibition at 4h in the lower concentration of EchA and at 24h at the higher concentration, regrowing rapidly after 24h. The means of the survival indexes for Gram-positive and Gram-negative strains are shown in fig. 23. There is a degree of inhibition up to 24h followed by regrowth, with a tendency for a greater degree of killing of Gram-negative strains.

4.5. Relationships between the concentration of echinochrome A in coelomic fluid and bactericidal activity

Although EchA had bactericidal activity in association with various mammalian proteins, this does not necessarily imply that it is similarly active in coelomic fluid. Therefore two questions could be asked:
a) does the bactericidal activity of various coelomic fluids correlate with the concentration of EchA in the different coelomic fluids; and
b) do different bacterial strains show the same rank order of susceptibilities towards EchA as towards coelomic fluid? The concentrations of

FIGURE 21 The bactericidal action of two concentrations of EchA on Gram-negative bacteria



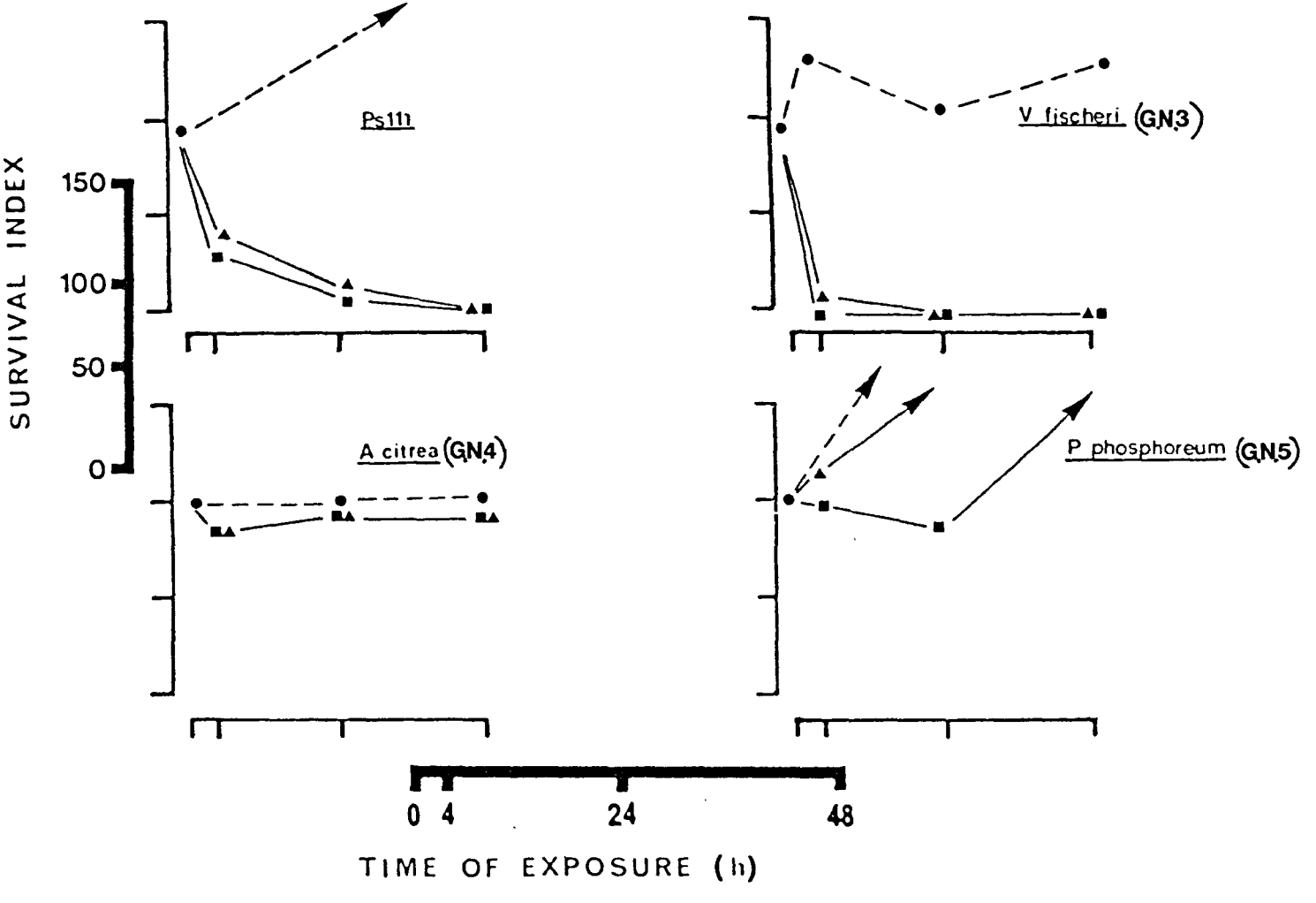
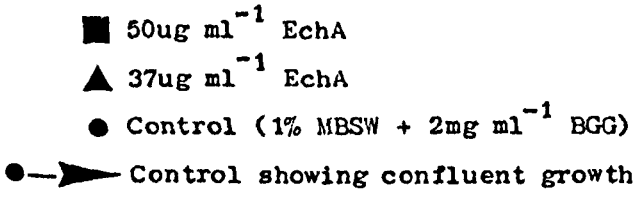

SURVIVAL INDEX
150
100
50
0
Ps111
V fischeri (GN3)
A citrea (GN4)
P phosphoreum (GN5)
0 4 24 48
TIME OF EXPOSURE (h)

FIGURE 22 Bactericidal action of two concentrations of EchA on Gram-positive bacteria



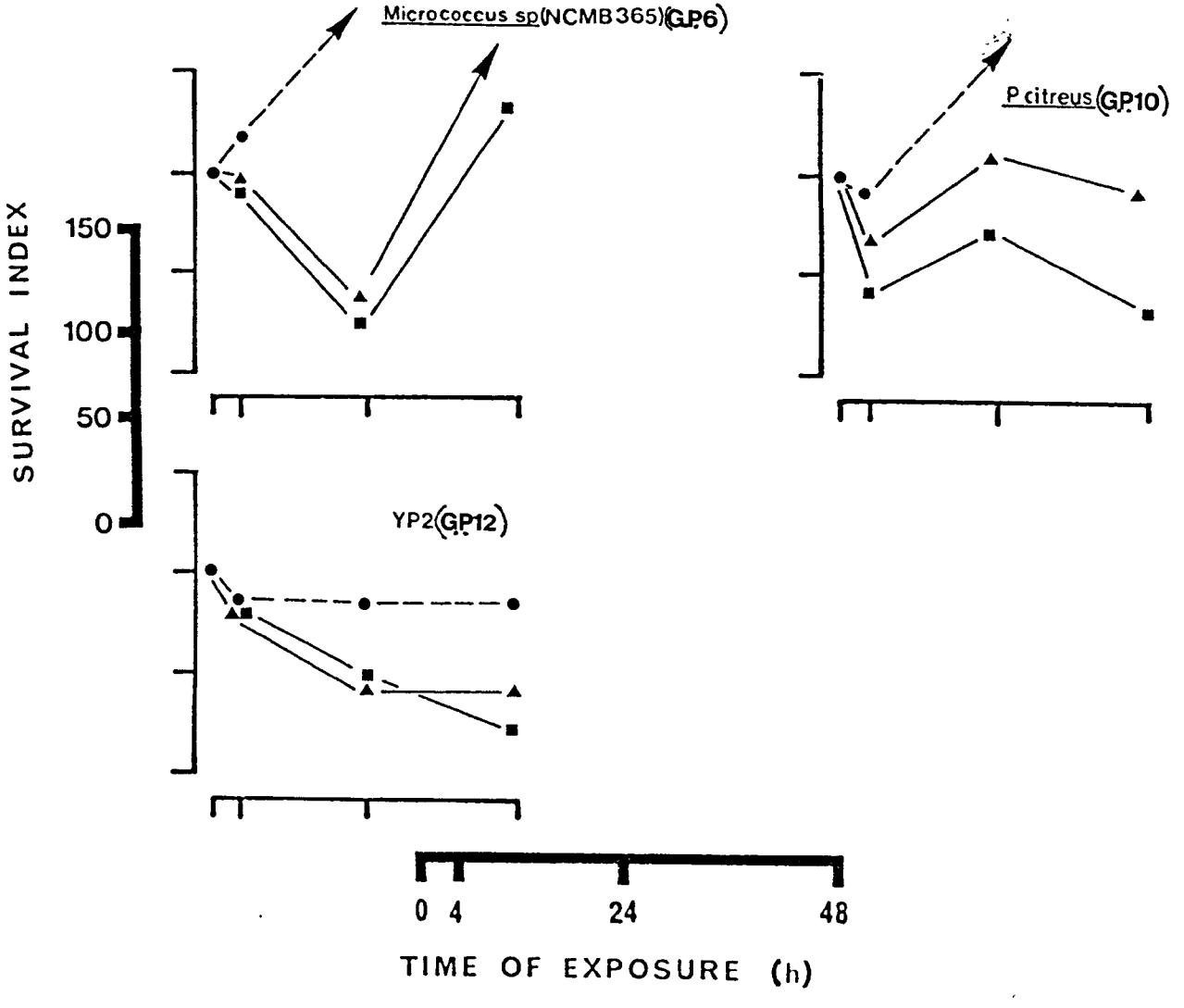

Micrococcus sp(NCMB365)(GP6)
P citreus (GP10)
YP2(GP12)
SURVIVAL INDEX
150
100
50
0
0 4 24 48
TIME OF EXPOSURE (h)

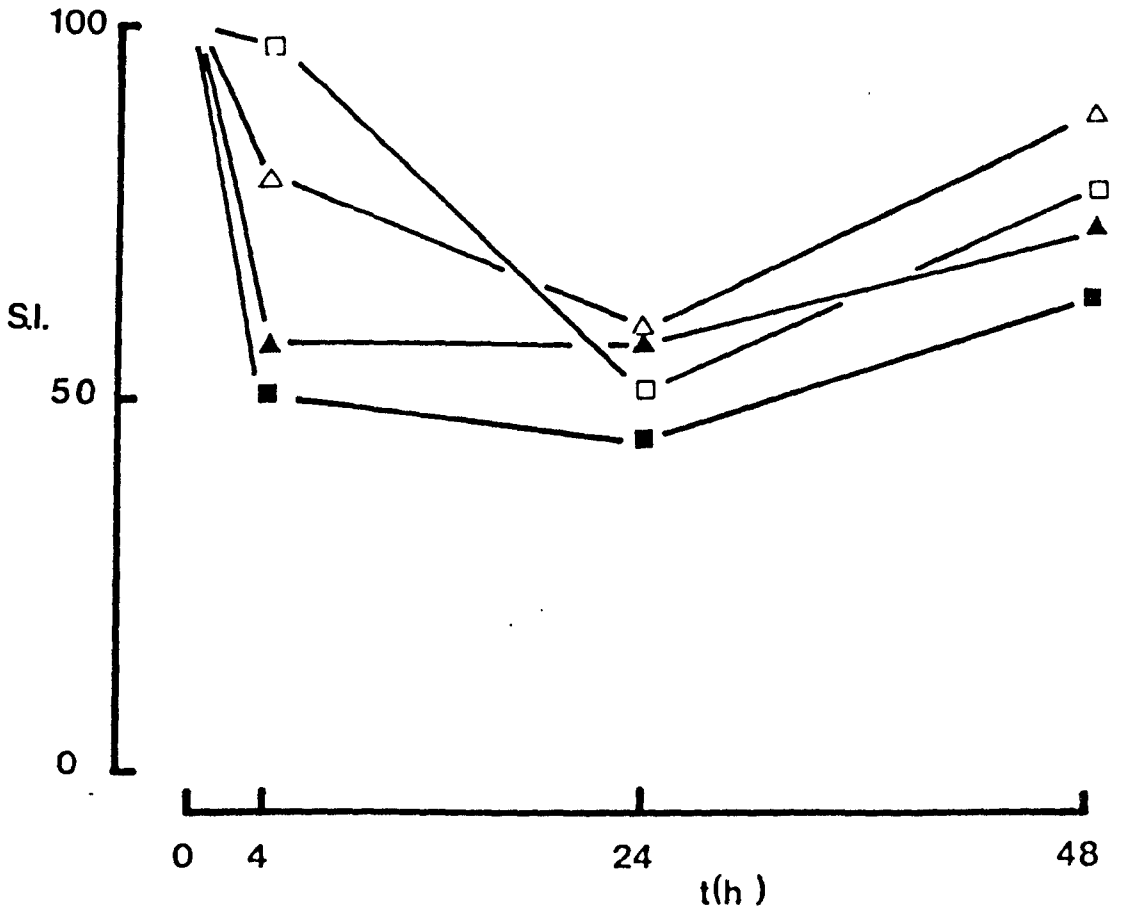
FIGURE 23 Mean survival indexes of Gram-negative and Gram-positive bacteria exposed to EchA/BGG

■ Gram-negative
▲ Gram-positive | 50 µg ml^{-1} EchA

□ Gram-negative
△ Gram-positive | 37 µg ml^{-1} EchA

(Gram-positive 3 replicates)

(Gram-negative 4 replicates)


100
S.I.
50
0
0
4
24
48
t(h)

EchA in coelomic fluid ranged from 59ug ml^{-1} to 3ug ml^{-1} with a geometric mean value of 14ug ml^{-1} (fig. 24). Probit analysis (fig. 25) indicated that the range in EchA concentrations followed a lognormal distribution. The concentrations at which EchA/protein solutions are effective lie within the natural range of concentrations found in the animal.

To answer the question: "does the bactericidal action of various coelomic fluids correlate with the concentration of EchAin the different fluids?" the 4, 24 and 48h SI values of Ps111 incubated in the coelomic fluid from 40 different urchins was compared with the EchA concentrations in the coelomic fluid from the same animals. Use of the Kendall Rank Correlation Coefficient test produced a value of $Z = 1.971$ indicating a significant positive correlation at $P \leqslant 0.05$ between the 24h survival index and EchA concentration. Earlier, the survival indexes in coelomic fluid for the seven bacterial strains used to test EchA were established. Comparison between the two was made using the Kendall Rank Correlation Coefficient test. A value of $S = 14$, indicating a significant positive correlation at $0.034 \leqslant P \leqslant 0.015$ was found between the survival indexes of the strains in coelomic fluid and their survival indexes in EchA at 24h.

5. Comparison Between Populations of E. esculentus From Different Sites and at Different Seasons

5.1. External appearance of urchins

Considerable variation was observed in spine colour between individual E. esculentus. Johnson and Chapman (1970d) speculated that the presence of absence of naphthoquinone pigments in the spines of a Pacific sea urchin Strongylocentrotus franciscanus influenced the degree of algal infection of the spines. Algal infection can be observed in the spines of many of the E. esculentus around Cumbrae. All of the animals

FIGURE 24 Concentrations of EchA in *E. esculentus* coelomic fluid

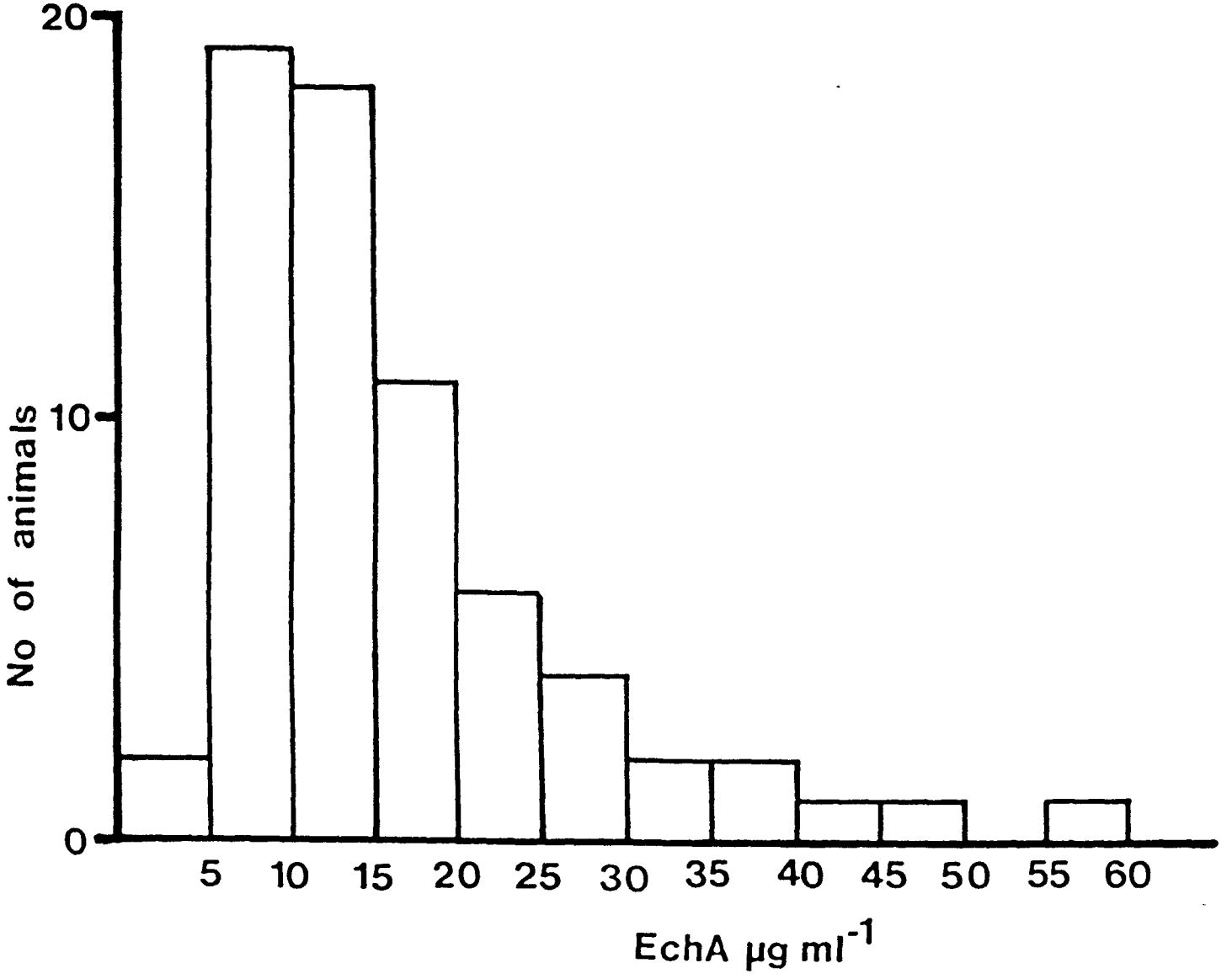
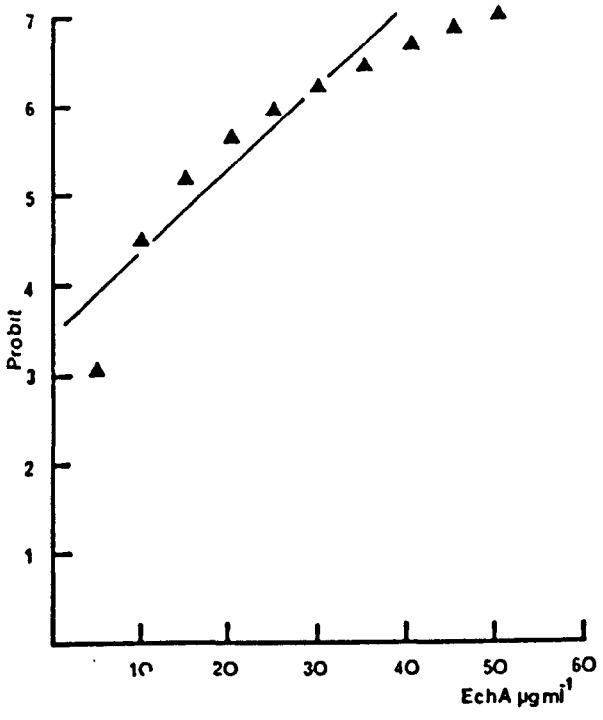

20
10
0
No of animals
5
10
15
20
25
30
35
40
45
50
55
60
EchA µg ml-1

FIGURE 25 Probit plot of EchA concentrations in *E. esculentus* coelomic fluid

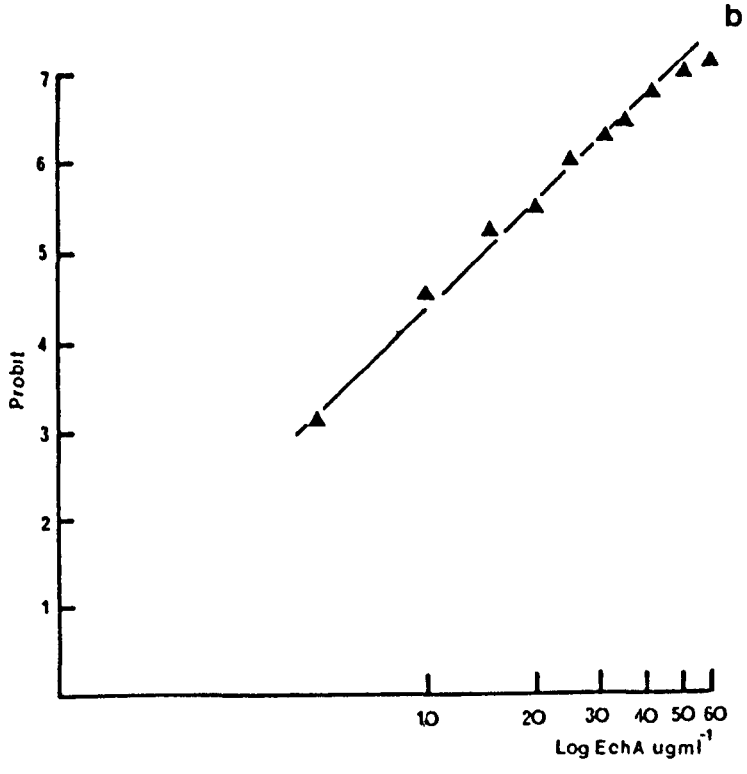
a Arithmetic

b Logarithmic

a


Probit
7
6
5
4
3
2
1
10
20
30
40
50
60
EchA µgml-1

b


Probit
7
6
5
4
3
2
1
10
20
30
40
50
60
Log EchA ugml-1

used in the environmental study were examined; the spine colour was noted and the animals were scored for the degree of algal infection (table 8). It can be seen that the degree of infection increases in the winter and this seems to be paralleled by an increase in the number of lighter coloured animals in the winter. This may support Johnson and Chapman's (1970) belief that lighter coloured animals have a greater tendency to suffer from algal infection and adds support for hypothesis of the antimicrobial function of naphthoquinone pigments.

5.2. Physical and biochemical observations

Three factors which it was thought could affect the bactericidal activity of coelomic fluid were chosen and measured from each animal sampled. Two areas (materials and methods, 10) were sampled in the summer and winter and sample groups were designated; summer rich, summer poor, winter rich and winter poor. Figures 26a, b and c shows the results for EchA concentration, GI and HA titre for each sample group. The actual data for each of the 4 samples is shown in appendix 1a and 1b.

5.3. Rankit plots

Rankit plots provide a relatively simple means for assessing whether or not data are normally distributed. This can be done by measuring the degree of scatter of plotted points around a fitted line. The choice of statistical test for analysing data was dependant on the result of the Rankit plot. Rankit plots for EchA concentration, HA titre and gonad index (GI) are shown on figures 25, 27 and 28, apparently both HA titre and EchA concentration follow a lognormal distribution (figs. 25, 27).

TABLE 8 The relationship between season and site of collection of E. esculentus and the spine colour and degree of algal infection of the animals

Season	Site	No. of animals with spine colour				No. of animals with infection level [a]			
		Purple	White/Purple	Red/White	White	-	+	++	+++
Summer	rich	1	3	2	2	5	0	1	2
	poor	3	2	1	3	6	2	1	0
Winter	rich	1	5	0	4	0	2	3	5
	poor	1	2	0	7	2	1	4	3

[a] the scoring system was:
- no algal infection
+ 10 spines infected
++ infected spines all around animal
+++ majority of spines infected

FIGURE 26 Histograms of physical and biochemical data from 4 different groups of *E. esculentus* taken in the environmental study

Sr Summer rich

Sp Summer poor

Wr Winter rich

Wp Winter poor

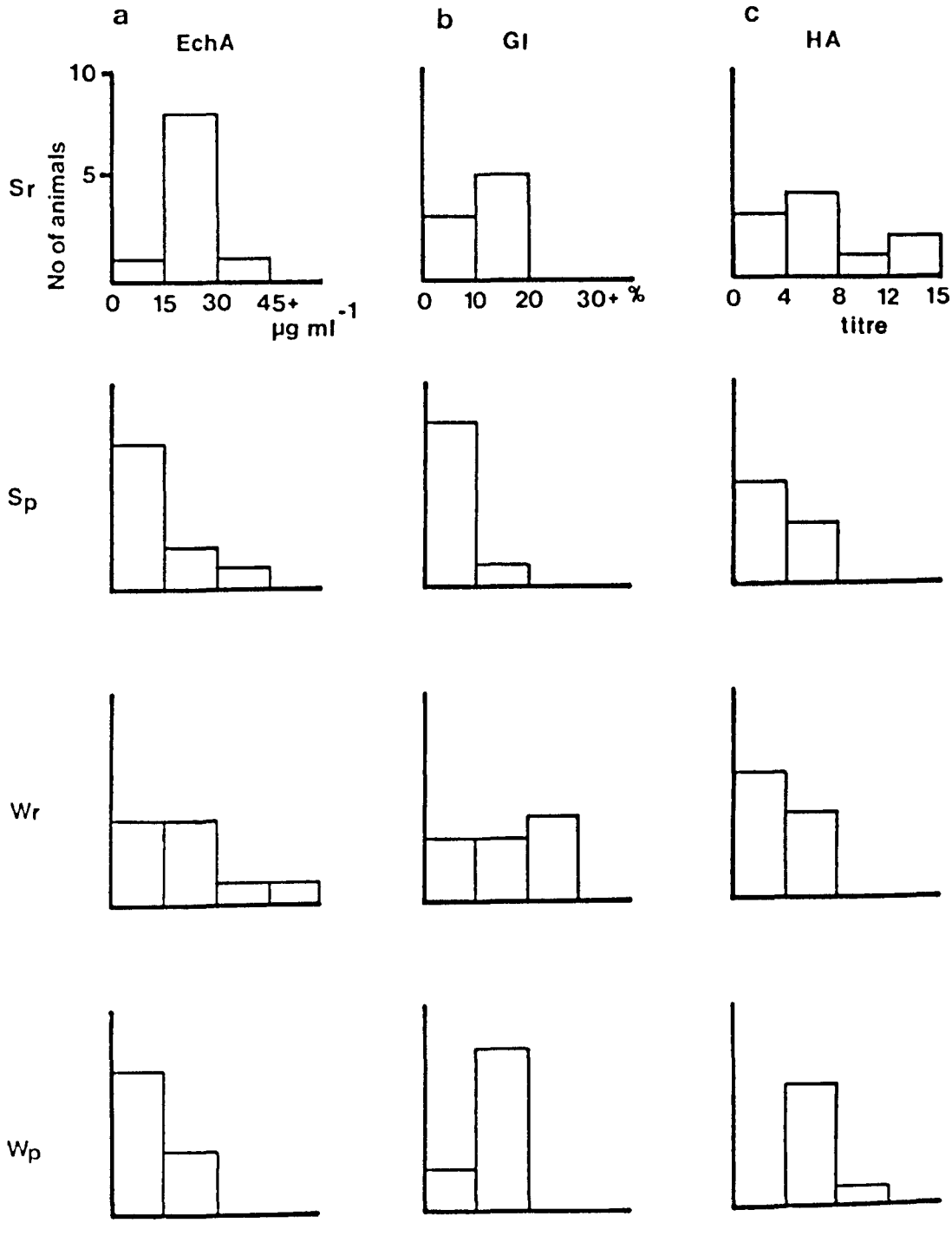
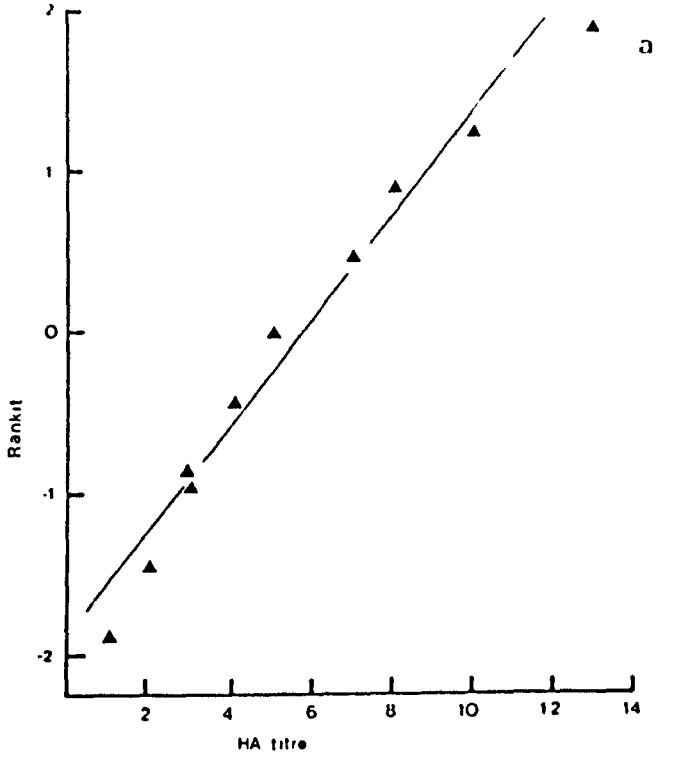

a
EchA
b
GI
c
HA
10
5
Sr
No of animals
0 15 30 45+
µg ml -1
0 10 20 30+ %
0 4 8 12 15
titre
Sp
Wr
Wp

FIGURE 27 Rankit plot of HA titres

a Arithmetic

b Logarithmic


a
Rankit
2
1
0
-1
-2
2
4
6
8
10
12
14
HA titre

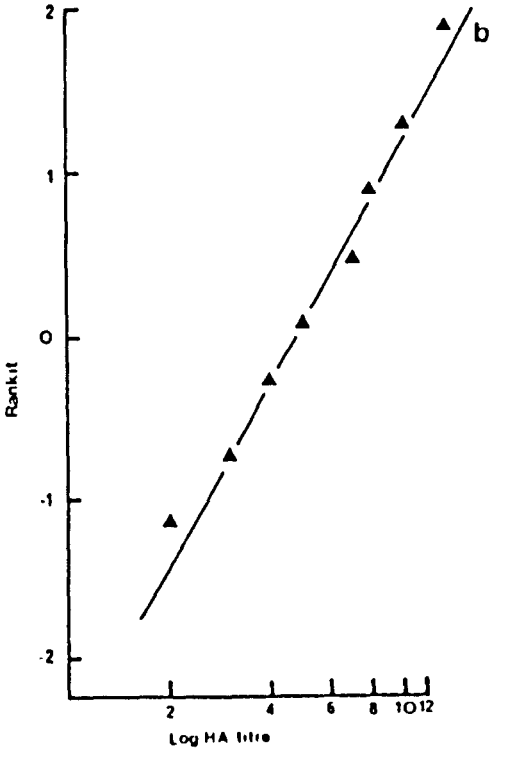
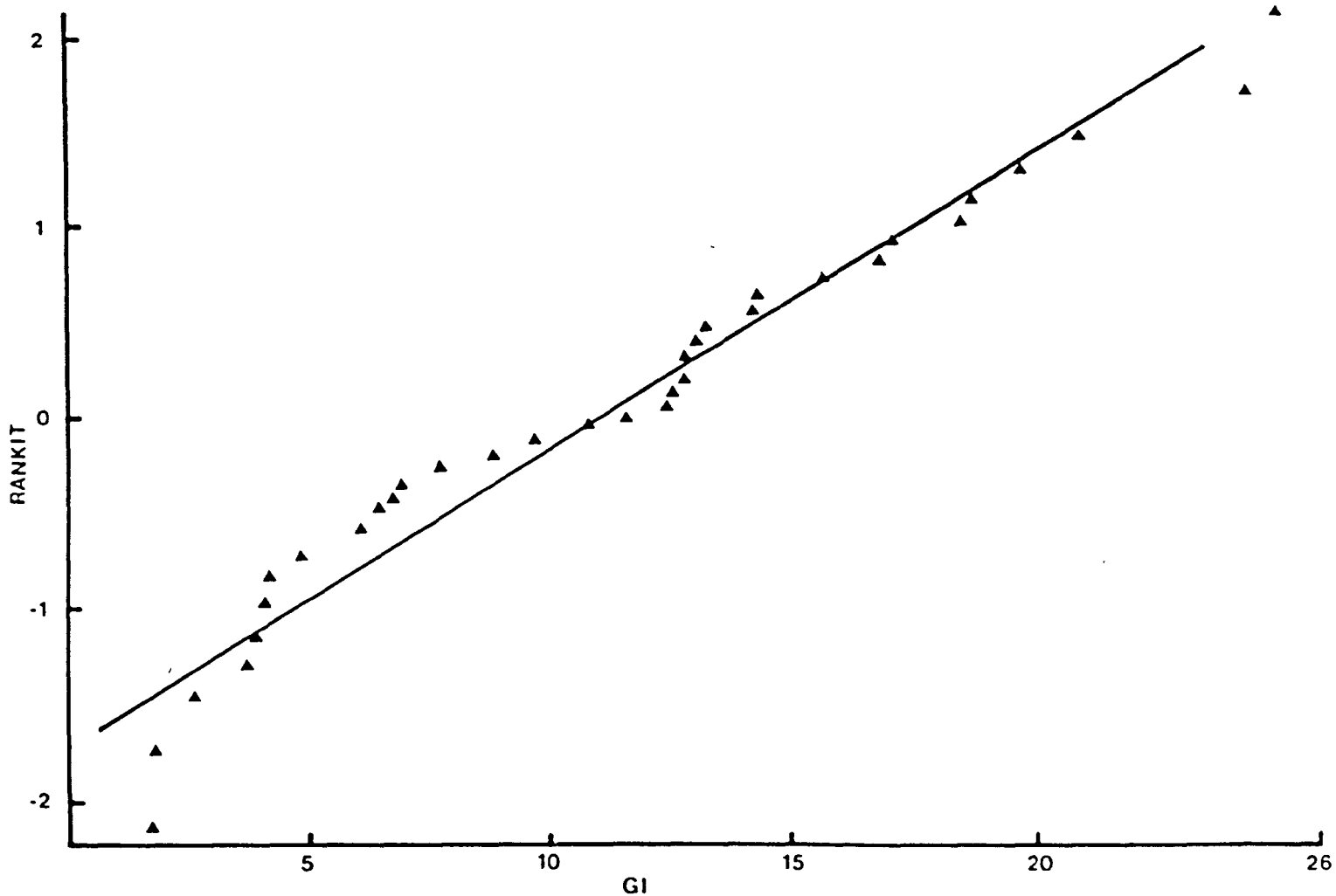

b
Rankit
2
1
0
-1
-2
2
4
6
8
10
12
Log HA titre

FIGURE 28 Rankit plot of gonad indexes


2
1
0
-1
-2
RANKIT
5
10
15
20
26
GI

5.4. Gonad indexes

In echinoids the gonads represent the main organ for the storage of nutrients (Moore, 1935). The proportion of gonad weight to body weight expressed as a percentage has commonly been adopted as a measure of this stored nutrient (Moore, 1935I). Differences were observed between sites indicating differences in food quality or perhaps different spawning times. The gonad indexes from the rich site in the summer were significantly higher than those from the poor site (Student's t-test, $t = 2.62$, sig. at $P \leq 0.05$). Similarily the gonad indexes from the poor site in the winter were significantly greater than the values obtained from the same site in the summer (Student's t-test, $t = 4.23$, sig. at $P \leq 0.05$).

Overall no significant differences were found between the rich and poor sites or between the winter and summer results (Student's t-test, $P > 0.05$).

5.5. Haemagglutinin titre

Although the function of the HA for rabbit r.b.c. found in the coelomic fluid is unknown,it may function as an opsonin. Therefore, any variation in HA titre may well have an effect on the rate at which bacteria are phagocytosed. As with EchA concentration, HA titre follows a lognormal distribution so the common logarithm of titres was used when applying the Student's t-test. There was an overall difference between the rich and poor sites, with a higher titre found at the rich site (Student's t-test, $t = 2.79$, critical value of $t = 2.01$, $P \leq 0.05$). Similarily the winter titre is higher than the summer value (Student's t-test, $t = 2.60$, critical value of $t = 2.03$, $P \leq 0.05$). Taking the two seasonal samples separately no significant differences occur between the two sites.

5.6. Echinochrome A concentration

As EchA may be a major contributory factor in E. esculentus antibacterial defence, any seasonal or environmental variation in its concentration might be expected to influence the bactericidal activity of coelomic fluid. As EchA concentration follows a lognormal distribution (fig. 25), the common logarithm of the concentration was used when applying the Student's t-test. No significant difference ($P > 0.05$) was found between the sites in either the winter or summer sample or between the seasons. However, taken overall the urchins from the rich site had a significantly higher EchA concentration (Student's t-test, $t = 2.746$, critical value of $t = 2.101$, $P \leq 0.05$).

5.7. Survival indexes for coelomic fluid at different seasons and sites

In conjunction with the physical and biochemical measurements already made of urchins from different sites and seasons, the coelomic fluid from the same animals was tested for bactericidal activity. Figure 28 shows the bactericidal activity of the coelomic fluid from the animals from each sample displayed as 4, 24 and 48h survival indexes. As has already been shown (results, 1.2.), survival indexes do not follow a normal or lognormal distribution and so the Mann Whitney U test was used for statistical analysis. From table 9 it can be seen that at all three time intervals the rich site shows a significantly lower survival index i.e. higher bactericidal activity and overall the "rich" survival index is consistantly lower. Whatever factor caused the survival indexes to differ in summer apparently does not affect the two populations in the winter.

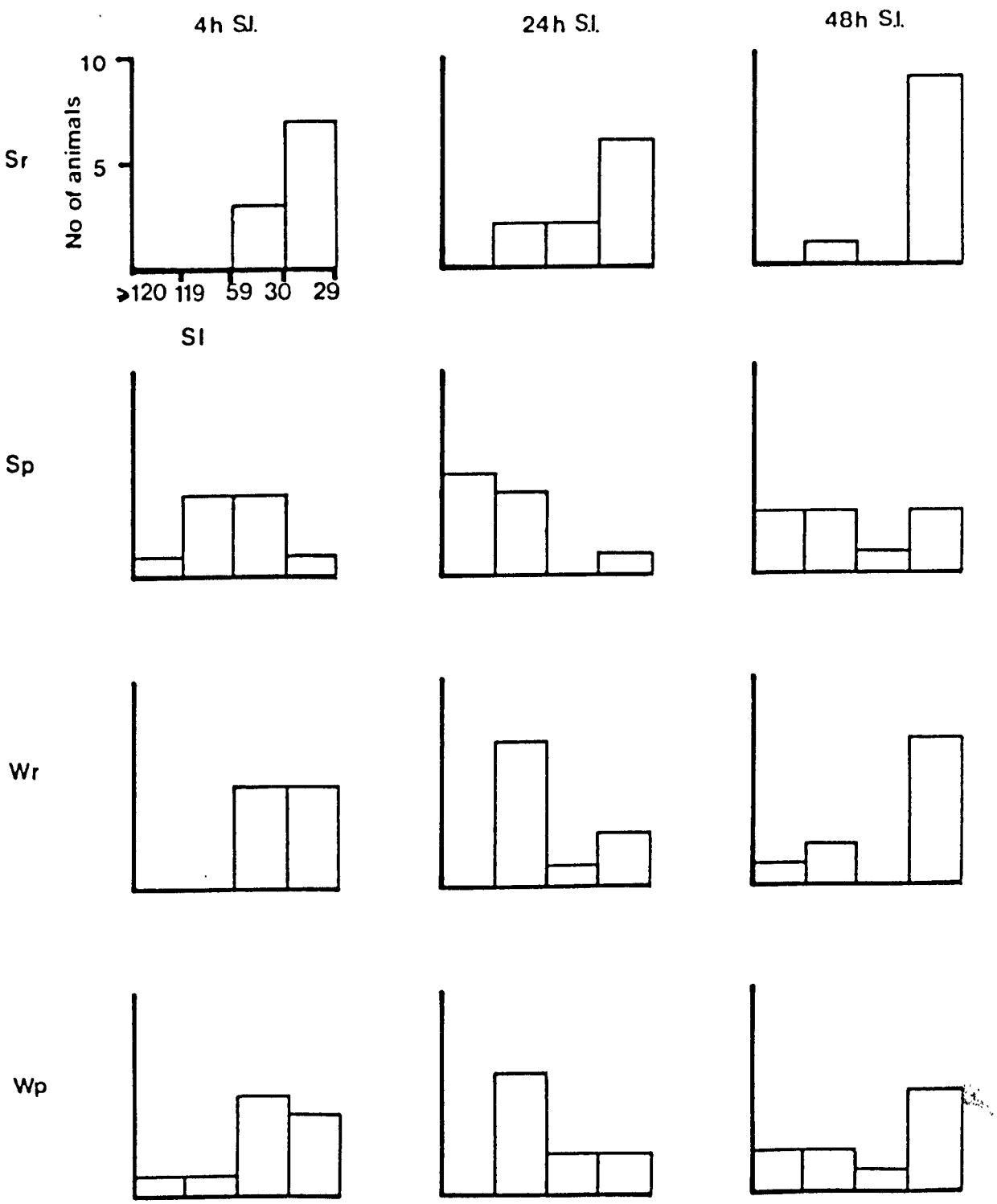
FIGURE 29 Histograms of the distributions of survival indexes from the 4 different groups of *E. esculentus* taken in the environmental study

Sr Summer rich

Sp Summer poor

Wr Winter rich

Wr Winter poor


4h S.I.
24h S.I.
48h S.I.
Sr
Sp
Wr
Wp
No of animals
10
5
>120
119
59
30
29
SI

5.8. Relationships between the physical and biochemical parameters and survival indexes

It can be established that certain physical and biochemical parameters as well as the bactericidal activity of individual *E. esculentus* vary according to habitat. In order to show whether or not there is any relation between the survival indexes and the other measurements a Kendall Rank Correlation Coefficient test was done comparing the 4, 24 and 48h survival indexes with each parameter (table10). From table 10 it is clear that whilst the gonad index has a constant relationship with all three survival indexes, both the HA titre and EchA concentration affect the survival index at different times. This is perhaps indicative that they play different but cumulative roles in the overall bactericidal mechanism.

TABLE 9 Comparison of survival indexes of coelomic fluid from E. esculentus sample at different sites and seasons

S.I.	summer rich v poor	winter rich v poor	overall rich v poor	overall winter v summer
4hr	S [a]	NS [b]	S	NS
24hr	S	NS	S	NS
48hr	S	NS	S	NS

[a] significant at $P \leqslant 0.05$

[b] not significant at $P \leqslant 0.05$

TABLE 10 Correlations between physical and biochemical factors and survival indexes (Kendall Rank Correlation Coefficient)

Parameter	4h S.I.	24h S.I.	48h S.I.
Gonad Index	S[a](2.28)[c]	S(1.70)	S(2.28)
Echinochrome A concentration	NS[b](0.536)	S(1.94)	NS(1.05)
HA titre	S(3.24)	NS(0.889)	S(2.29)

[a] significant positive correlation ($P < 0.05$)

[b] no significant correlation

[c] value of Z from Kendall Rank Correlation Coefficient

DISCUSSION

Spectrum of Bactericidal Activity of E. esculentus Coelomic Fluid

It was known at the start of this investigation and from tests on hundreds of sea urchins that the coelomic fluid of Echinus esculentus had the capacity to kill the Pseudomonas designated strain no. 111 which had been used in all previous work at Millport. However, the spectrum of bacterial strains which might be sensitive was entirely unknown. In order to explore this question it was desirable to obtain a widely diversified panel of test bacteria, the only provisos being that each strain would survive, and preferably grow, at 8°C in seawater supplemented with a low level of nutrient and have distinctive looking colonies on Zobell agar. From a panel of 21 strains provided by the curator of the National Collection of Marine Bacteria, 17 were found to meet these criteria and to these were added 6 coloured-colonied isolates from the Clyde Sea Area. This panel of 24 strains, 12 Gram-negative and 12 Gram-positive would appear to be the most extensive so far used by any investigator to characterize the spectrum of bactericidal activity in the body fluid of any marine invertebrate.

There was particular interest in determining whether broad differences might emerge in the sensitivities of the Gram-positive and Gram-negative strains, since this might reflect a response to endotoxin similar to that found in Limulus polyphemus (Levin and Bang, 1964). This, in turn, could lead to E. esculentus coelomic fluid providing the raw material for a laboratory reagent for endotoxin analagous to Limulus amoebocyte lysate.

In the event, the responses of the panels of Gram-positive and Gram-negative strains were very similar. Both categories contained strains with a range of sensitivities to the coelomic fluid. The most sensitive Gram-negative strain showed over 90% killing at 4h, the first

sampling time, while the most resistant Gram-negatives were killed to the extent of only 50% at 48h. For comparison, the most sensitive Gram-positive strain also showed about 90% killing at 4h and the most resistant about 10% killing at 48h. Overall, the average susceptibility of the Gram-negatives as a group were somewhat higher than the Gram-positives, but the difference was not marked. Thus while the various marine bacteria used in the foregoing varied considerably in susceptibility, the basis of this variation was not correlated with Gram-reaction and hence cell wall structure. Also the reported (Shewan *et al* 1960) predominance of Gram-negative bacteria in the marine environment is not reflected in the specificity of E. esculentus bactericidal action being preferentially directed at this category of organism.

Another possibility was considered: that susceptibility to the bactericidal activity of coelomic fluid might be related to the growth rate of the bacteria in the control fluid. However, when the whole panel of 24 strains was examined, no significant correlation between these two variables emerged. Nevertheless, when the strain that showed confluent growth from 48h samples from control media were excluded on the grounds of indeterminate count, the remaining 17 strains showed significant ($P \leqslant 0.05$) positive correlation between bactericidal sensitivity and growth rate. This suggests that rapidly growing (and therefore rapidly metabolizing) bacteria are more sensitive to the bactericidal system in coelomic fluid than cells that are less metabolically active.

Before leaving this topic, emphasis should be given to the observation that none of the panel of 24 strains was capable of actual growth in the coelomic fluid at 8°C. An organism with such a capability might well act as a pathogen of E. esculentus but it is of interest, that no such pathogen has so far been reported. Possibly an organism with this property could

be isolated by culturing the body fluid of animals found moribund in the natural environment, particularly in areas where mass mortalities of urchins appear to have occurred. Such events were noted by the author in Loch Riddon in 1981 and Loch Sunart in 1982 and could not be attributed, for example, to pollution. This remains a subject for further study.

E. esculentus Compared With Other Marine Invertebrates

As reviewed in the Introduction (Table 1), most of the major phyla of marine invertebrates contain species whose body fluids have been reported to possess *in vitro* bactericidal activity. However, none of the studies have been as extensive as the present work in terms of reported numbers of individual animals examined or range of test bacteria investigated. Johnson and Chapman (1970b,c; 1971) for example, studied five species of marine invertebrate but no one species was tested against more than six strains of bacteria. Furthermore the animals were not always examined at the place of collection and may have been exposed to the potential stress of shipment.

Echinochrome A

The foregoing discussion all relates to the whole coelomic fluid of *E. esculentus* without consideration of what might be the active component(s). The work of Messer and Wardlaw (1980) indicated that the red spherule cells were a principal locus of bactericidal activity and attention therefore fell on Echinochrome A, the red pigment which is concentrated in spherules within these cells.

Holland *et al* (1967) and Johnson (1970), both working with species of urchin of the genus *Strongylocentrotus*, showed that EchA migrated with particular coelomocyte proteins during electrophoresis. Johnson (1970) noting that EchA was insoluble in distilled water, employed three

different mammalian proteins (BGG, ovomucoid and calf thymus histone) as dispersants. However, the solutions were not examined for bactericidal activity. In the present studies, EchA was found to be insoluble in seawater but could be dissolved in seawater solutions of BGG, BSA, HGG and HSA. Typically 2000ug ml^{-1} of the mammalian protein was used to dissolve 50ug ml^{-1} of EchA. However, this ratio was not critical and concentrations of BGG ranging from 125 to 8000ug ml^{-1} seemed equally satisfactory for dissolving 30ug ml^{-1} EchA. It may be noted, for information, that the range of EchA concentrations in coelomic fluid is from 3 to 59ug ml^{-1} with a geometric mean of 14ug ml^{-1}. Two concentrations of 30 and 50ug ml^{-1} were chosen arbitrarily from within the physiological range.

However, the focus of this work was not to study EchA/protein interaction per se but to determine whether the pigment would be bactericidal towards some of the heterotrophic marine bacteria already examined. Initially, the tests were confined to Ps111 and with this strain a convincing demonstration of bactericidal activity was obtained with the pigment at concentration above 25ug ml^{-1}. This activity bore many similarities to that seen with whole coelomic fluid: it took place at 8°C, it was not instantaneous but required up to 24h for full effect, and there was no substantial difference in susceptibility of Gram-negative and Gram-positive bacteria.

Considerable effort was expended in comparing the 4 different mammalian proteins which were used as dispersants, but only minor quantitative differences emerged. On balance, BSA was marginally inferior (in terms of permitting the expression of bactericidal activity of EchA) to the other 3 proteins used. The underlying basis of this difference was not pursued. However, one might presume that the affinity of the pigment for the protein would be an important factor, and this could be investigated by, for example, equilibrium dialysis. Such a study

might also be valuable with the electrophoretically detected protein of the sea urchin with which the pigment is naturally associated. Meanwhile for convenience, only BGG was employed in the further studies.

It must be emphasised that in no instance did the protein solution in seawater act as other than a growth medium for Ps111. This observation is important from another standpoint, namely the fact that BGG (bovine gamma globulin) would be expected to contain a wide range of antibody specificities, some of which might fortuitously be directed against Ps111 and cause agglutination. Such an effect would mimic a bactericidal reaction by producing a fall in viable count due to clumping. However, this was not seen and therefore the fall in viable count in the BGG/EchA system can reasonably be attributed to the EchA.

In addition to Ps111, a further six marine heterotrophic bacteria; 3 Gram-positive and 3 Gram-negative were tested against EchA/BGG. These strains were chosen on the basis of exhibiting a wide range of susceptibilities to whole coelomic fluid. Thus they included the highly sensitive Planococcus citreus and Vibrio fischeri, the moderately sensitive Micrococcus sp. NCMB 365. Photobacterium phosphoreum and Alteromonas citrea, and the resistant Clyde isolate YP2. Six of the seven strains were susceptible in some degree to EchA/BGG but the 7th strain Alteromonas citrea neither grew nor died in either EchA/BGG or BGG control. In whole coelomic fluid, this strain was in the middle of the range of susceptibilities of the Gram-negative organisms examined.

The Kendall Rank Correlation Coeficient was used to test for any relationship between the susceptibilities of the seven strains to EchA and to whole coelomic fluid and a significant positive correlation was demonstrated at the 24h sampling time. Coupled with this is the separate observation that the amount of EchA in coelomic fluid of different urchins and the bactericidal activity of these coelomic fluids also showed a

positive correlation at 24h. It therefore seems possible that the role of EchA *in vivo* may be to restrict the growth of invading bacteria in the coelomic fluid and allow other bactericidal mechanisms such as phagocytosis, time to act. Therefore, EchA may act as a first line of defence against infection by becoming deposited in likely areas of microbial invasion. This is backed up by personal observations, reports by Johnson and Chapman (1970) and Pearse *et al* (1977). The former authors observed an apparent barrier of EchA-bearing red spherule cells which separated diatom-infected spine tips from healthy areas of the spines in *Strongylocentrotus franciscanus*. Pearse *et al* (1977) recorded that skeletal plates on apparently diseased individuals of *S. franciscanus* had a middle "red friable" layer, presumably due to EchA. Similar phenomena to the above were seen in *E. esculentus* (personal observation). Additional to the above is the report of Coffaro and Hinegardner (1977) that tissues of the sea urchin *Lytechinus pictus* which had been damaged during grafting experiments became reddened by an influx of red spherule cells. A similar response occurs in *E. esculentus* when the epithelium is abraded during collection (personal observation).

The only previous observation of the antimicrobial properties of sea urchin pigments was by Vevers (1966, and personal communication) who showed that pigments leached from the dried test of an unspecified echinoid placed on a culture plate of blue-green algae (cyanobacteria) inhibited their growth. Johnson (1969b) provided further circumstantial evidence for a possible antibacterial role of EchA when she observed release of the pigment by red spherule cells on contact with Gram-negative bacteria.

Although it appears that EchA functions as a disinfectant by killing marine bacteria, almost nothing is known of its biochemical mode of action.

The fact that EchA appears to take between 24 and 48h to achieve its maximum bactericidal effect suggests that it acts on bacterial metabolism rather than by disrupting the cell envelope. This harmonises with the suggestion made previously that the efficiency of bactericidal action of whole coelomic fluid is influenced by growth rate of the test bacteria and is consistent with the belief that EchA is involved in the bactericidal action of coelomic fluid. Whitton and MacArthur (1967) suggested that the action of 2,3-dichloro-1,4-naphthoquinone on blue-green algae was due to its interference with specific enzyme systems or its taking part in pseudocyclic photophosphorylation. However, the latter suggestion would not explain the effect on the heterotrophic non-photosynthetic bacteria used in the present study. Certain naphthoquinones form major components of the electron transport chain in bacteria (Dawes and Sutherland, 1976).

The nature of the proteins associated with EchA in coelomic fluid is unknown although one primary function may be to act as a carrier, serving to make the pigment soluble when coelomic fluid encounters foreign material. The readiness of EchA to associate with proteins may give some indication of its mode of attack on bacteria. Bacterial membrane proteins on Escherichia coli have been shown to bind penicillin (Franklin and Snow, 1981) and Lynn (1980) has stated that the action of chemical disinfectants "involves reactions with cellular constituents frequently proteins".

In addition to antimicrobial activity, a number of alternative functions for EchA have been proposed; respiratory pigment (MacMunn, 1885), cofactor for a digestive enzyme (Pequignat, 1966), antioxidative agent (Koltsova et al., 1981). Cannon (1927) questioned the respiratory function, although he stated that EchA could act as an "activator" for

oxygen transport in the coelomic fluid. Perry and Epel (1981) showed that EchA produced H_2O_2 during Ca^{2+} stimulated oxidation, a process which may help to harden the fertilisation membrane in embryos. They also suggested that Ca^{2+}- mediated EchA oxidation may also function in an antibacterial role. The notion that H_2O_2 is responsible for the antibacterial role would seem to be unlikely since several of the bacterial strains that proved most susceptible to both coelomic fluid and EchA in the present study (Ps111, Vibrio fischeri) were catalase-positive, implying resistance to the action of H_2O_2.

Although the present investigation has presented evidence for an antibacterial role for EchA, the possibility that the pigment has multiple functions should not be excluded. Indeed, as has already been mentioned in the introduction, the coelomocytes including the bactericidal red spherule cells have been shown to have multiple functions.

Antimicrobial Activity of Naphthoquinones

The basis of the antimicrobial activity of naphthoquinones in general has not been extensively studied and apart from this work, no direct investigation has been made of their role in echinoid antibacterial defence. There are, however, scattered reports of antimicrobial action by certain naphthoquinone compounds. Fitzgerald and Skoog (1954) showed that 2,3-dichloro-naphthoquinone was selectively toxic to blue-green algae in freshwater ponds, but had no harmful effect on eucaryotic green algae, higher plants, invertebrates and fish. Whitton and MacArthur (1967) were able to demonstrate that 2,3-dichloro-1,4-naphthoquinone was toxic to the blue-green alga Anacystis nidulans Blue-green algae have recently become classified as bacteria in the division Cyanobacteria (Buchanan and Gibbons 1974).

Although in the animal kingdom, naphthoquinones occur uniquely in the echinoids, they also are found as microbial products. Fleck et al (1980) isolated an antibiotic called granatomycin, a naphthoquinone, from Streptomyces lateritius. Granatomycin displayed antibacterial activity against Gram-negative and Gram-positive bacteria and antiviral activity against fowl-plague virus. Ciegler et al (1981) isolated two naphthoquinone pigments; xanthomegnin and viomellein from Penicillium viridicatum which possessed mycotoxic activity. Soderhall and Ajaxon (1982) demonstrated inhibition of a crayfish fungal parasite Aphanomyces astaci by synthetic 2-methyl-1,4-naphthoquinone.

Variation in Bacterial Activity Between Different Specimens of E. esculentus

Wardlaw and Unkles (1978) observed that the bactericidal ability of coelomic fluid from different specimens of E. esculentus varied considerably but found no evidence of seasonal effects. The results obtained in the survey part of this investigation are in accord with the earlier work but with the difference that habitat coupled with season was an influential variable. Moore (pers. comm.) has established that urchins taken from a high food quality environment, such as pier piles have higher respiration rates and higher gonad indexes than those from lower food quality sites such as the ophiuroid zone. In the summer sample of the present work, those animals taken from the pier piles showed significantly lower (i.e. higher bactericidal activity) survival indexes than the animals from the ophiuroid zone, however, these differences were not manifest in the winter samples. In an attempt to explain the between-site variation, various physical and biochemical features of the animals were compared from the standpoint of their relationship to the bactericidal activity of coelomic fluid and their

possible correlation with seasonal and environmental factors.

As a preliminary to the statistical analysis of the different physiological variables, it was necessary to determine the nature of the underlying frequency distributions. To the author's knowledge this has not hitherto been reported in respect of any physiological data from sea urchins. Using Rankits and Probits, it appeared that gonad indexes were approximately normally distributed. In contrast, EchA concentrations and Haemagglutinin titre were approximately lognormally distributed, while survival indexes were neither normal nor lognormal. Based on these findings, the appropriate parametric or non-parametric procedures were used for correlation analysis and comparisons of means.

The relationship between EchA concentration and bactericidal activity has already been discussed, the only positive correlation being with the 24h SI, suggesting the presence of other active mechanisms. This idea is supported by the observations described earlier (Results 3.2.) that coelomic fluid from different specimens of E. esculentus showed a differential response to two different strains of bacteria i.e. coelomic fluid from one urchin suppressed one strain at a greater rate than the other, while in another urchin the reverse might be true. A Kendall Rank Correlation Coefficient test showed that the bactericidal action of coelomic fluid from the different specimens of E. esculentus on the two strains was not correlated ($P > 0.05$); supporting the idea of two separate bactericidal mechanisms. It has been suggested that invertebrate haemagglutinins function as opsonins in enhancing the rate of phagocytosis (Anderson, 1975). Variation in the titre of rabbit r.b.c. HA in the coelomic fluid of E. esculentus may influence any role played by phagocytosis in antibacterial defence. Inverse correlations were found between HA titre and 4h and 48h survival indexes. This suggests that the HA may influence bactericidal activity as a separate

mechanism from EchA. However, the trend in the variation of HA titre from the different sites and seasons follows a different pattern from the survival indexes and EchA concentration. Although overall the titre from the rich site was higher than the poor site there was also a seasonal difference, with a higher titre present in the winter. Therefore, HA titre does not totally mirror the seasonal and environmental variation shown by EchA concentration or more importantly the survival indexes.

A positive correlation between gonad index and all three (4, 24, 48h) survival indexes was demonstrated and variation in gonad index between the 4 different samples paralleled the variation in survival indexes. This relationship may have two possible explanations which may be connected. Low gonad indexes may indicate recent spawning or the poor quality of the available food. In the first case, energy may be being channelled into reproduction and therefore not available for the maintenance of an antibacterial defence system. In the second, the energy may not be available or is channelled into the operation of essential body systems. Little or nothing is known about the energy requirements of antimicrobial systems in invertebrates in general. The varying levels of EchA in the coelomic fluid of E. esculentus may reflect the energy requirements for the synthesis of the pigment. A more extensive sampling programme in which animals would be collected throughout the year and the coelomic fluid tested for bactericidal activity and the gonad indexes measured should enable the relationship between reproduction and bactericidal activity to be clarified.

Perspectives

It is apparent from this investigation that much remains to be learned about the antibacterial defence mechanisms of E. esculentus. Although EchA would appear to function as a general disinfectant, nothing is known of its biosynthesis, modes of storage and release, mechanisms of bactericidal action and the role of its associated protein(s). To date no-one has demonstrated that phagocytosis occurs in the coelomic fluid of E. esculentus; however, evidence from other echinoid species suggests that it will almost certainly occur (Johnson, 1969band Bertheussen and Seljelid, 1978). Phagocytes may function by directly ingesting and killing live bacteria and also by clearing dead bacteria after action by other defence mechanisms. The role, chemical nature and specificity of the HA present in the colourless spherule cells awaits investigation. What other host defence mechanisms may be present is not known although the bacteriolytic enzyme lysozyme present in many invertebrates is not found in E. esculentus (McHenery, pers. comm.).

As the bactericidal system of E. esculentus is completely cellular (Wardlaw and Unkles, 1978), a profitable line for future work would be a study of changes in the cells of coelomic fluid during experimental infection. Also one may enquire, specifically what components of the bacterial cell wall are recognised by coelomocytes and how EchA is brought into contact with invading micro-organisms. Since it would seem that EchA must be released from the red spherule cells to be effective, an understanding of the mechanism of degranulation of red spherule cells is needed. Recent investigations by Bertheussen and Seljelid (1982) indicating the presence of receptors for the C_3 component of complement on echinoid phagocytes may prove important in understanding how echinoid coelomocytes recognise foreign particles.

Bertheussen and Seljelid (1982) have suggested that echinoids may possess a system similar to the alternative pathway of complement in vertebrates.

Reverting finally to Echinochrome A itself, we have already noted that this substance is unique to echinoids. However, certain chemically related substances are precursors of melanin which is common in diverse species in the animal kingdom. In this broader context therefore, the production of EchA by sea urchins can perhaps be regarded as a specialized adaptation of a widely distributed biosynthetic mechanism. Moreover melanin deposition may accompany host cellular defence responses (Smith and Ratcliffe, 1980) in some invertebrates such as Crustacea. Jacobson and Millot (1953) reported the presence of melanin and its precursors in the echinoid Diadema antillarum. Soderhall and Ajaxon (1982), have demonstrated mycotoxic activity by products arising from the generation of melanin and quinones from phenols. However, although the melanin precursor 5,6-dihydroxyindole (Dagly and Nicholson, 1970) and some phenols bear some structural resemblance to EchA, small changes in structure are known to completely remove the antibacterial activity of many compounds.

One may speculate that the evolutionary branch leading to sea urchins involved an ancestral creature which possessed biochemical pathways for precursors of either EchA or melanin. The branch leading to the chordates adopted the melanin pathway while that leading to the echinoderms took this pathway and also, in the echinoid sub-branch, the pathway leading to EchA.

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APPENDIX

APPENDIX 1a Data from summer collection of urchins

Measurement	Poor Site (Brittlestar bed)										Rich Site (pier piles)									
	U^{a}_{1}	U_2	U_3	U_4	U_5	U_6	U_7	U_8	U_9	U_{10}	U_{11}	U_{12}	U_{13}	U_{14}	U_{15}	U_{16}	U_{17}	U_{18}	U_{19}	U_{20}
Wet weight of urchin (g)	304	423	456	430	305	-	353	314	348	265	-	352	-	362	439	344	272	295	292	198
Wet weight of gonads (g)	13	18	59	26	15	-	13	21	13	7	-	22	-	62	8	45	24	47	32	27
Gonad index	4.2	4.1	13	6.1	4.8	-	3.7	6.9	4.1	2.6	-	6.1	-	17	1.8	12.9	8.9	15.8	10.8	13.4
HA titre	2^4	2^4	-	2^3	2^3	2^3	-	2^2	2^1	-	2^8	2^7	2^{13}	2^3	2^3	2^4	2^1	2^{13}	2^5	2^7
EchA (ug ml^{-1})	35	8	4	11	13	11	13	10	15	20	19	25	9	15	17	27	21	17	35	16
4h Survival index	69	60	22	91	52	62	53	198	48	41	28	12	14	49	48	17	38	16	9	5
24h Survival index	172	9	126	197	72	129	129	64	62	62	36	4	16	82	24	16	50	4	77	0
48h Survival index	700	5	0	700	86	45	700	85	17	95	5	4	0	2	14	0	2	0	91	0

[a] urchin number

APPENDIX 1b Data from winter collection of urchins

Measurement	Poor Site (Brittlestar bed)										Rich Site (pier piles)									
	U^{a}_{1}	U_2	U_3	U_4	U_5	U_6	U_7	U_8	U_9	U_{10}	U_{11}	U_{12}	U_{13}	U_{14}	U_{15}	U_{16}	U_{17}	U_{18}	U_{19}	U_{20}
Wet weight of urchin (g)	399	365	247	332	332	528	381	329	416	173	254	283	279	302	178	180	270	320	408	214
Wet weight of gonads (g)	53	68	43	23	32	34	48	47	49	23	36	54	35	60	14	44	5	81	16	45
Gonad index	13	19	17	7	10	7	13	15	12	13	14	19	13	20	8	25	2	25	4	21
HA titre	2^7	2^5	2^5	2^8	2^3	2^5	-	2^4	-	-	2^8	2^{10}	2^7	2^{10}	2^7	2^7	2^5	2^5	2^{10}	2^7
EchA (ug ml^{-1})	8	10	3	29	15	9	9	8	11	24	8	13	15	10	14	39	49	15	19	21
4h Survival index	41	37	15	102	26	57	35	28	28	35	30	20	50	28	19	32	48	37	7	13
24h Survival index	61	54	9	107	109	115	119	9	78	37	119	69	82	102	85	32	93	17	69	37
48h Survival index	4	63	4	700	159	24	9	83	0	30	15	0	13	89	61	11	700	4	0	13

[a] urchin number

APPENDIX 2 Sensitivity of test bacteria to the bactericidal activity of E esculentus coelomic fluid expressed as survival indexes(p37,50)

2(a) Gram-negative strains

Strain G.N.1.

t(h)	C[a]	U_1[b]	U_2	U_3	U_4	U_5	U_6
4	79	2	4	2	4	6	5
24	106	2	10	0	1	2	4
48	85	0	15	0	0	4	4

Strain G.N.2.

t(h)	C	U	U	U	U	U	U
4	102	65	22	12	16	106	15
24	97	25	1	1	0	24	0
48	101	0	0	0	0	0	0

Strain G.N.3.

t(h)	C	U	U	U	U	U	U
4	126	65	13	30	37	86	44
24	125	58	0	0	5	23	0
48	125	26	0	0	0	0	0

Strain G.N.4.

t(h)	C	U	U	U	U	U	U
4	112	105	99	95	123	92	114
24	125	77	89	55	77	92	40
48	122	59	62	46	52	34	24

Strain G.N.5.

t(h)	C	U	U	U	U	U	U
4	86	70	8	4	2	0	35
24	201	186	0	4	2	0	12
48	>700	>700	0	0	0	0	66

Strain G.N.6.

t(h)	C	U	U	U	U	U	U	U_7	U_8
4	112	41	73	36	52	67	15	37	30
24	207	13	15	4	4	2	4	11	8
48	>700	0	15	4	4	0	0	6	0

a Control Fluid (B.C.F.S.)

b Urchin No

Strain G.N.7.

t(h)	C	U_1	U_2	U_3	U_4	U_5	U_6
4	103	75	50	65	50	75	88
24	238	38	0	0	0	43	40
48	525	0	0	0	0	0	0

Strain G.N.8.

t(h)	C	U	U	U	U	U	U
4	86	58	26	29	69	58	14
24	180	113	0	40	61	4	0
48	>700	116	0	0	142	0	0

Strain G.N.9.

t(h)	C	U	U	U	U	U	U
4	162	228	137	119	139	123	102
24	230	235	105	63	124	77	98
48	272	172	84	42	16	51	26

Strain G.N.10.

t(h)	C	U	U	U	U	U	U	U_7	U_8
4	139	72	53	67	78	71	67	33	61
24	182	56	49	39	67	44	61	33	17
48	>700	1	7	8	31	27	11	33	0

Strain G.N.11.

t(h)	C	U	U	U	U	U	U	U	U
4	106	91	47	79	92	66	88	79	59
24	121	40	48	79	32	28	72	57	49
48	219	6	22	32	0	0	0	49	20

2(b) Gram-positive strains

Strain G.P.1.

t(h)	C	U	U	U	U	U
4	89	64	46	30	55	35
24	78	41	11	19	12	15
48	86	12	28	25	17	8

Strain G.P.2.

t(h)	C	U	U	U	U	U	U
4	95	94	79	126	96	115	54
24	103	45	40	40	42	53	3
48	100	26	25	31	29	31	4

Strain G.P.3.

t(h)	C	U_1	U_2	U_3	U_4	U_5	U_6
4	103	105	121	106	96	111	97
24	101	78	64	74	96	93	60
48	134	36	30	40	101	44	26

Strain G.P.4.

t(h)	C	U	U	U	U	U	U
4	90	52	47	52	62	54	32
24	95	50	26	47	75	63	39
48	90	41	17	50	51	47	41

Strain G.P.5.

t(h)	C	U	U	U	U	U	U
4	77	71	41	57	43	76	53
24	116	64	57	62	67	60	58
48	113	41	30	50	28	59	71

Strain G.P.6.

t(h)	C	U	U	U	U	U	U
4	40	29	15	32	26	29	38
24	107	6	9	38	12	49	26
48	116	6	23	9	6	26	12

Strain G.P.7.

t(h)	C	U	U	U	U	U	U	U_7	U_8
4	101	71	57	81	53	53	51	39	4
24	105	46	31	64	41	21	34	26	3
48	138	4	4	1	9	0	0	21	1

Strain G.P.8.

t(h)	C	U	U	U	U	U
4	100	85	76	106	72	67
24	136	83	88	59	56	96
48	240	80	88	35	32	112

Strain G.P.9.

t(h)	C	U	U	U	U	U	U
4	91	0	2	5	2	7	7
24	122	0	12	0	5	9	0
48	>700	0	14	0	21	2	0

Strain G.P.10.

t(h)	C	U_1	U_2	U_3	U_4	U_5
4	110	48	57	43	68	9
24	230	0	11	36	66	0
48	465	0	0	2	16	0

Strain G.P.11.

t(h)	C	U	U	U	U	U	U_6
4	100	49	41	27	13	38	13
24	158	35	0	32	65	41	22
48	270	35	16	32	65	19	62

Strain G.P.12.

t(h)	C	U	U	U	U	U	U	U_7	U_8
4	101	160	162	158	138	136	102	92	109
24	101	145	172	185	94	113	87	72	79
48	>700	81	123	126	121	106	36	0	68