ANTIBACTERIAL ACTIVITY IN THE SEA URCHIN

ECHINUS ESCULENTUS

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SUMMARY

The studies described in this thesis fall into two parts:

1. A survey of the normal bacterial flora of the common British sea urchin, Echinus esculentus, in comparison with the bacterial flora of seawater and sand from the same locality.

2. An investigation of the antibacterial activity in E. esculentus. This formed the main part of the work.

In part 1, the normal bacterial flora of the sea urchin was examined with isolates from the coelomic fluid, the peristomial membrane and the gut. Aerobic heterotrophic organisms from these sites were identified by a scheme based on that of Shewan, Hobbs and Hodgkiss (1960). The main genera identified were Pseudomonas, Vibrio, Aeromonas, Flavobacterium, Acinetobacter and Moraxella. A few Gram-positive bacteria were also isolated. Of 188 urchins examined, two-thirds had sterile coelomic fluid and it is likely that organisms found there had been introduced by damage to the animal and do not form a permanent indigenous flora.

In part 2, initial experiments showed that urchins were capable of clearing, within 24 h, large doses of marine bacteria which had been injected into the coelomic cavity. This indicated that sea urchins possess an efficient antibacterial mechanism.

A procedure was developed to examine in vitro the coelomic fluid of sea urchins for antibacterial activity. As test bacterium

in these experiments a marine pseudomonad, strain 111, was chosen because it produced characteristic black, agar-digesting colonies on marine 2216E agar which were not readily confused with contaminating bacteria. A non-bactericidal control fluid was included in all tests. This consisted of the boiled supernatant of coelomic fluid which was considered to be nutritionally and ionically equivalent to coelomic fluid, and which allowed growth of the test bacterium. Strain 111 incubated in coelomic fluid for 48 h was usually reduced to less than 5% of its initial viable count, whereas in the control fluid the bacteria multiplied.

Coelomocytes clot almost immediately when coelomic fluid is withdrawn from urchins but this appeared to have no effect on in vitro antibacterial activity. In vitro the fluid from all 188 urchins studied showed antibacterial activity. The activity was temperature-dependent (optimum $4^{o}C$) but was independent of the sampling date, over a 6 month period, and of the initial bacterial count.

By comparing the antibacterial activities of coelomic fluid and the cell-free supernatant of coelomic fluid it was shown that the activity was associated with the coelomocytes and that the supernatant acted as a bacterial growth medium.

Antibacterial activity was not dependent on intact coelomocytes but could be obtained in a clear, cell-free extract prepared by sonication of coelomocytes followed by centrifugation. Freezing

had little effect on the antibacterial activity of the extract.
Dialysis greatly reduced the activity of the extract but boiling
for 30 min caused only a slight decrease.

The nature and mechanism of action of the antibacterial
substance(s) was not determined. However, the fact that the
supernatant of sonically-disrupted coelomocytes was still active
indicates that phagocytosis of the bacteria is not essential for
the antibacterial effect. The drop in viable count was not due
either to agglutination or lysis of the bacteria since apparently
intact but non-viable bacteria were seen in test mixtures at 48 h.
The results of the experiments on heat-treatment and on dialysis
suggest that activity may involve a relatively low molecular
weight substance which may be released by coelomocytes during
in vitro incubation and which inhibits bacterial metabolism.

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LIST OF ABBREVIATIONS

a.c.	=	almost confluent
c.	=	confluent
0/129	=	2:4-diamino-6:7-di-isopropyl-pteridine phosphate
o.u.	=	opacity unit
S_0	=	supernatant of coelomic fluid
S_0/B	=	boiled supernatant of coelomic fluid
S_1	=	unconcentrated extract of coelomocytes
S_2	=	concentrated extract of coelomocytes (first fraction)
S_3	=	concentrated extract of coelomocytes (second fraction)
w/v	=	weight for volume

INTRODUCTION

The sea contains many species of vertebrates and invertebrates living in a medium in which large numbers of bacteria may be suspended. Whereas vertebrates in general are known to have the capacity for making immune responses to foreign cells or macromolecules, invertebrates either lack this capacity or possess it only in a rudimentary form. Some of the economically-important categories of marine invertebrates, notably members of the mollusca and crustacea, have been studied from the standpoint of susceptibility to infectious diseases and antibacterial defence mechanisms. The echinoderms, however, have been relatively neglected, despite their close phylogenetic relationship to the vertebrates. This thesis is concerned with determining whether the common sea urchin, Echinus esculentus, has any readily demonstrable antibacterial defence mechanism. This animal was chosen because of its abundance along local coasts and the large quantity of body fluid which can be obtained from it for in vitro studies.

To introduce the subject, let us review briefly

a) The diversity of marine invertebrates, showing the phylogenetic position and features of the Echinoderms.

b) The marine bacteria.

c) The antibacterial defence mechanisms of marine invertebrates in general and echinoderms in particular.

BRIEF SURVEY OF THE MARINE INVERTEBRATES

Invertebrates, by sheer force of numbers and wide distribution, dominate the environments of the world. Of the 23

phyla of the animal kingdom recognised by Smith (1971), 5 contain between 10^3 to 10^6 species.   The Arthropoda is by far the largest.   It consists of almost one million species (80% of the species of the entire animal kingdom) and includes insects, spiders and crustacea.   The phylum Mollusca, next in size, contains some 10^5 living species.   Marine in origin and still predominantly so, the Mollusca contains many varieties of shell fish, gastropods, lamellibranchs and cephalopods.   The other 3 major phyla are the Aschelminthes (mainly class Nematoda), the Protista (unicellular organisms) and the Chordata.   The phylum Chordata is composed mainly of vertebrates but includes the sub-phyla Cephalochordata, Hemichordata and Tunicata, marine animals which represent the first pre-fish essays in chordate design and link, far back in time, with the early ancestors of the echinoderms. The remaining phyla contain from 10^3 down to 10, or fewer, species. The largest of these are the Cnidaria or Coelenterata, marine polyps and medusae, the Platyhelminthes, worm-like animals including flukes, tapeworms and marine representatives, the Annelida, segmented worms including the marine class, Polychaeta; the Echinodermata, the star fish, sea urchins, sea cucumbers etc.; the Porifera, sponges, and the Ectoprocta, sea-mosses and sea-mats.

The remaining phyla contain from hundreds down to tens of species and represent less than 0.2% of the species of living animals.   These are the Nemertina and the Sipunculida, marine worms;   the Brachiopoda, lamp shells;   the Acanthocephala, worm-like parasites of vertebrates;   the Poganophora, marine worm-like animals;

the Ctenophora, medusa-like animals; the Chaetognatha, marine

arrow worms; the Entoprocta, marine stalked animals; the

Phoronida, horseshoe worms, and the Mesozoa, parasites of marine

invertebrates.

Much of the evolution of the invertebrate phyla appears

to have taken place in the Pre-Cambrian period which represents

four-fifths of the total history of life on earth, and for which

there are few fossil remains.   There is, however, some evidence

to show that members of the phyla Porifera, Annelida, Coelenterata,

Arthropoda and Echinodermata existed at this time.   In Cambrian

rocks there is a sudden increase in the range and numbers of

invertebrate fossils.   Unfortunately, no "transition forms",

namely animals linking phyla, have been found, suggesting that

evolution from primitive ancestors took place in the Pre-Cambrian

era.

The Paleozoic era, when the chordates originated, was a

period of high evolutionary diversification with concomitant

increases in ecological possibilities as plants colonised the land.

However, at the end of this era many species became extinct, but

the forms which survived to the Mesozoic era apparently made full

use of the ecological opportunities left by the extinct fauna and

produced a wide variety of new forms.   This diversification

continued until at the end of the Mesozoic, there was a marked fall

in sea level causing the extinction of many groups.   At the

beginning of the Cenozoic era therefore, the way was clear for those

invertebrates left to evolve into the species, most of which are

still alive today.

The relationships of the different phyla are difficult to trace because of the paucity of Pre-Cambrian fossils. The phylogeny of the invertebrates is therefore based mainly on the living representatives of the groups and from the fossils which have been obtained after the evolution of the major groups. The phylogenetic tree (Figure 1) is therefore only one possible way of interpreting the evolution of invertebrate phyla (Nichols, 1971).

THE ECHINODERMS

The present-day phylum Echinodermata is represented by about 5000 species of world-wide distribution divided into 5 classes within 3 subphyla. The subphylum Crinozoa contains only one living class, the Crinoidea, sea-lilies and feather-stars. It is the most primitive class, its adult members being mostly sessile on the seabed. The subphylum Asterozoa is comprised of the Asteroidea, starfishes, and the Ophiuroidea, the brittle-stars and basket-stars. These 2 classes are sometimes combined as the class Stelleroidea. The other living subphylum is the Echinozoa which includes the Echinoidea, the sea urchins, sand dollars and heart urchins, and the Holothuroidea, the sea-cucumbers. The last 4 classes are all free-living.

The features which distinguish the echinoderms from other invertebrates are that the adult members possess a pentameric symmetry, i.e. structures are present in fives. They also have tube-feet which are used in attachment, locomotion and respiration, and a calcite skeleton, referred to as the test. Some of today's

Figure 1.   Summary scheme of one possible way in which the main

invertebrate phyla can be related.   (after Nichols, 1971)

Chordata

Hemichordata

Echinodermata

Brachiopoda
Ectoprocta   Chaetognatha
Phoronida
                                    Pogonophora

Sipunculoida

Arthropoda

Annelida

Nemertina   Mollusca

Aschelminthes

Platyhelminthes

Coelenterata

Planuloid Ancestor

Porifera

Protozoa
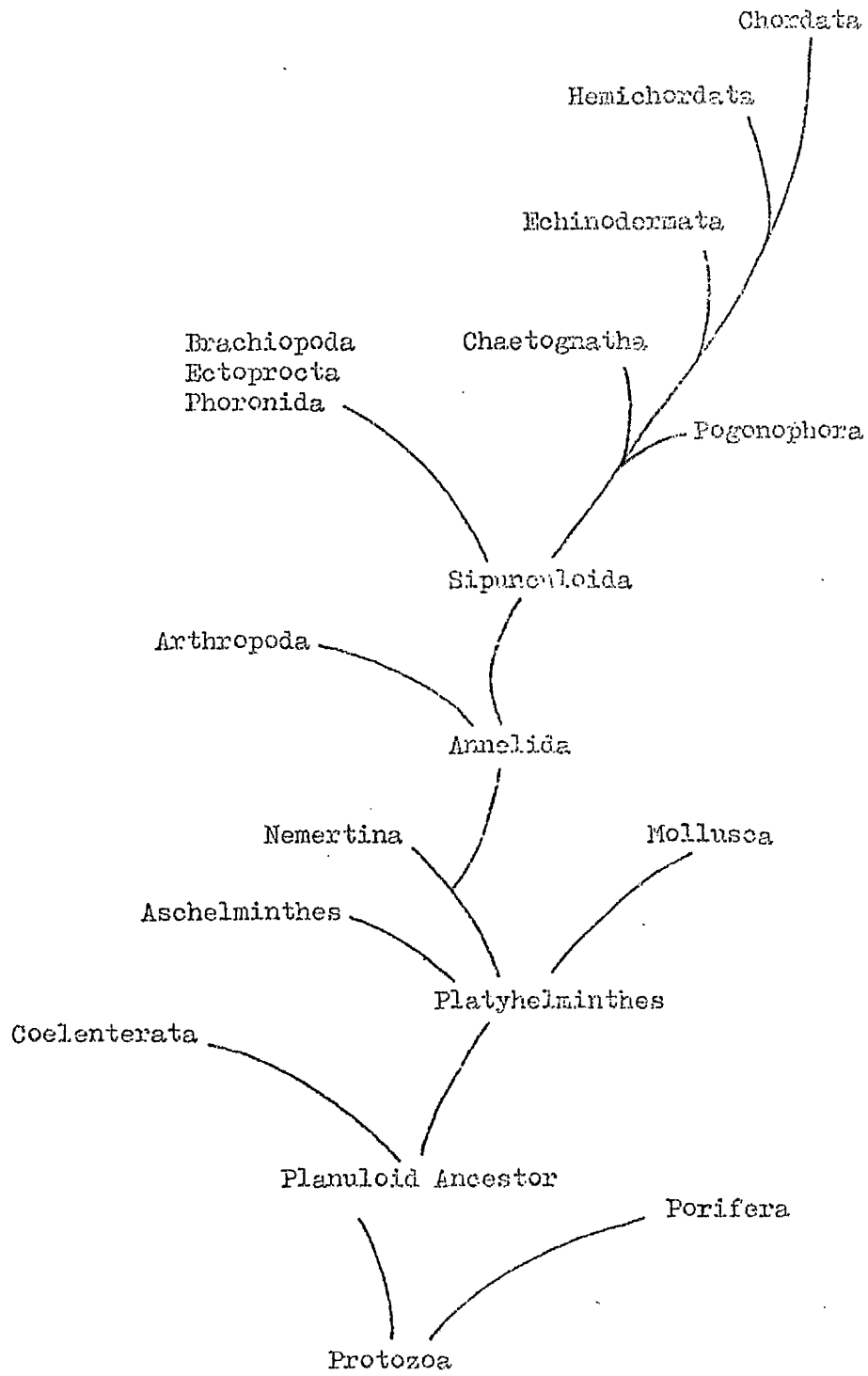
species show departures from pentameric symmetry with a secondary bilateral symmetry, as in the sea cucumbers and irregular urchins, but the basic structures are laid down in fives.

The original echinoderm was probably a sessile creature stationed on, or in, the sea bed and feeding on the rain of detritus falling from the waters above. It may have had a cup-shaped body with arms or brachioles supported by a skeleton, and was possibly a descendant of the Sipunculoida. The living class which most closely resembles this ancestor is the Crinoidea. The earliest fossil remains of echinoderms are found in the Cambrian period. They are representatives of the classes Crinoidea, Cystoidea, Eocrinoidea, Edrioasteroidea and Helicoplacoidea, all of which became extinct by the Permian period with the exception of one order of Crinoidea. The most primitive of these classes is not known but it is possible that it is from the Eocrinoidea that the other classes evolved (Figure 2). The eochrinoids were pentamerous and probably evolved into the non-pentamerous and unsuccessful subphylum Homalozoa.

The Ordovician period brought innovations in echinoderm physiology. Until this time, the echinoderms had been attached to the sea bottom and relied for food on the rain of detritus from above. Although the crinoids continued to use and exploit this way of life, the first free-living forms are seen with the mouth facing downwards, feeding off the sea floor. First came the Asteroidea and from them evolved the Ophiuroidea. At this time also the first echinoids and holothurians are found. There

Figure 2. The radiation of the echinoderm classes. (after Nichols,

1969)

CRINOZOA   ASTEROZOA   ECHINOZOA

Ophiuroidea

Crinoidea

Holothuroidea

irregular echinoid

LIVING

Asteroidea

Comatulida

Echinoidea

EXTINCT

Ophiocistioidea

Crinoidea

Helicoplacoidea

?

Edrioasteroidea

Paracrinoidea

Blastoidea

Homoiostelea, etc.

Cystoidea

Eocrinoidea
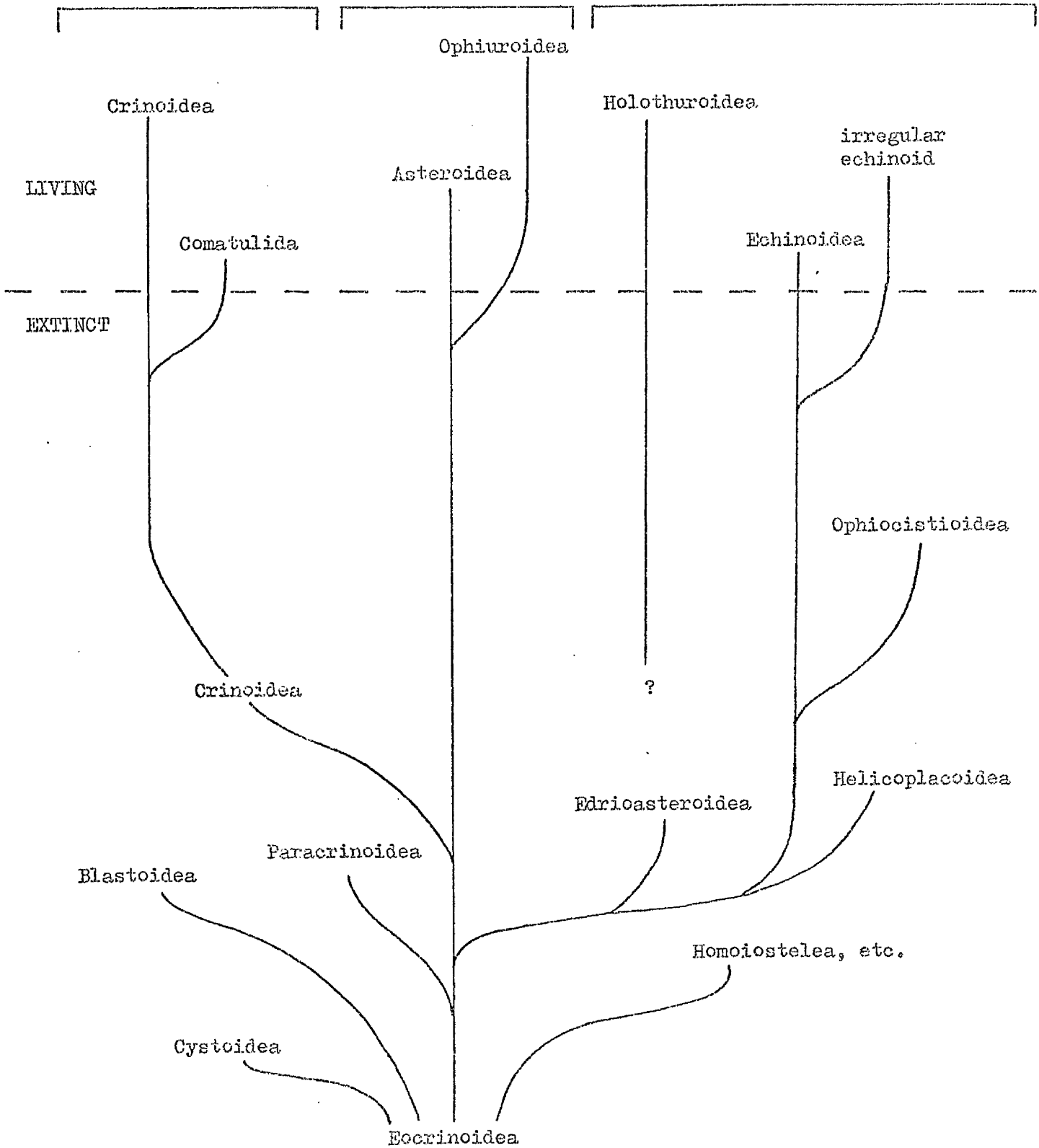
continued a period of evolution and diversification until the Permian period, the last of the Paleozoic era, when widespread extinction of invertebrate groups resulted in the loss of all but a few genera of echinoderms. The Triassic period saw the evolution of those that survived, including the irregular echinoids. From these species have evolved the present-day echinoderms.

An important aspect of the phylum Echinodermata is its relation to the vertebrates (Nichols, 1969), the basis of which comes from observations of larval forms. The early dipleurula larva has a band of cilia underlain by a nerve tract. It is thought that due to increase in size, this form developed a notochord, for support of the nerve tube, and gills.

Since this larva was well suited to exploit the plankton of the sea, a process of neotony, in which the animal becomes sexually mature while retaining the larval form, was favourable. Further increase in size meant that the notochord became inadequate and a bony structure was incorporated around it. Hence, a stage was reached where the animal was definitely fish-like.

Echinus esculentus

The sea urchin, E. esculentus, belongs to the class Echinoidea in the subphylum Echinozoa of the phylum Echinodermata. It is a regular urchin, having pentameric symmetry and is common along the coast of the British Isles. Sea urchins are essentially

browsers and scavengers, the chief food being encrusting algae and seaweeds.

Figure 3 shows a diagrammatic transverse section through the body of Echinus. It has a rigid test through which protrude the tube-feet. The test is covered with spines which are used in locomotion. Inside the mouth is the Aristotle's lantern, a complex masticatory apparatus consisting of 40 skeletal pieces intricately interbound with muscles and connective tissue. It carries 5 strong teeth which are used to rasp encrusting organisms, such as algae, from the sea-floor. The oesophagus leads from the mouth to the intestine which runs $1\frac{1}{2}$ times round the inside of the test before ascending to the anus. The coelomic fluid fills the body of the urchin and is more extensive in echinoids than in the other classes of echinoderms. The ionic composition and osmotic pressure of Echinus coelomic fluid, as of other echinoderms, are very close to those of seawater (Table 1). The coelomic fluid also contains a variety of cells which will be discussed later.

Echinoderms are often highly pigmented. E. esculentus is usually pinkish or purplish in colour. This has been shown by Goodwin and Srisukh (1950) and Anderson, Mathieson and Thomson (1969) to be due to the pigments spinochrome A and B which are present in the spines and test. These are naphthaquinone pigments, the relative amounts of which are thought to produce the subtle variations in colour. Another pigment, echinochrome, is found in Echinus but only in the coelomic fluid and its cells.

Figure 3.   <u>Diagrammatic transverse section through the body of</u>
<u>Echinus</u>   (after Nichols, 1969), showing position for
insertion of hypodermic needle for withdrawal of
coelomic fluid.
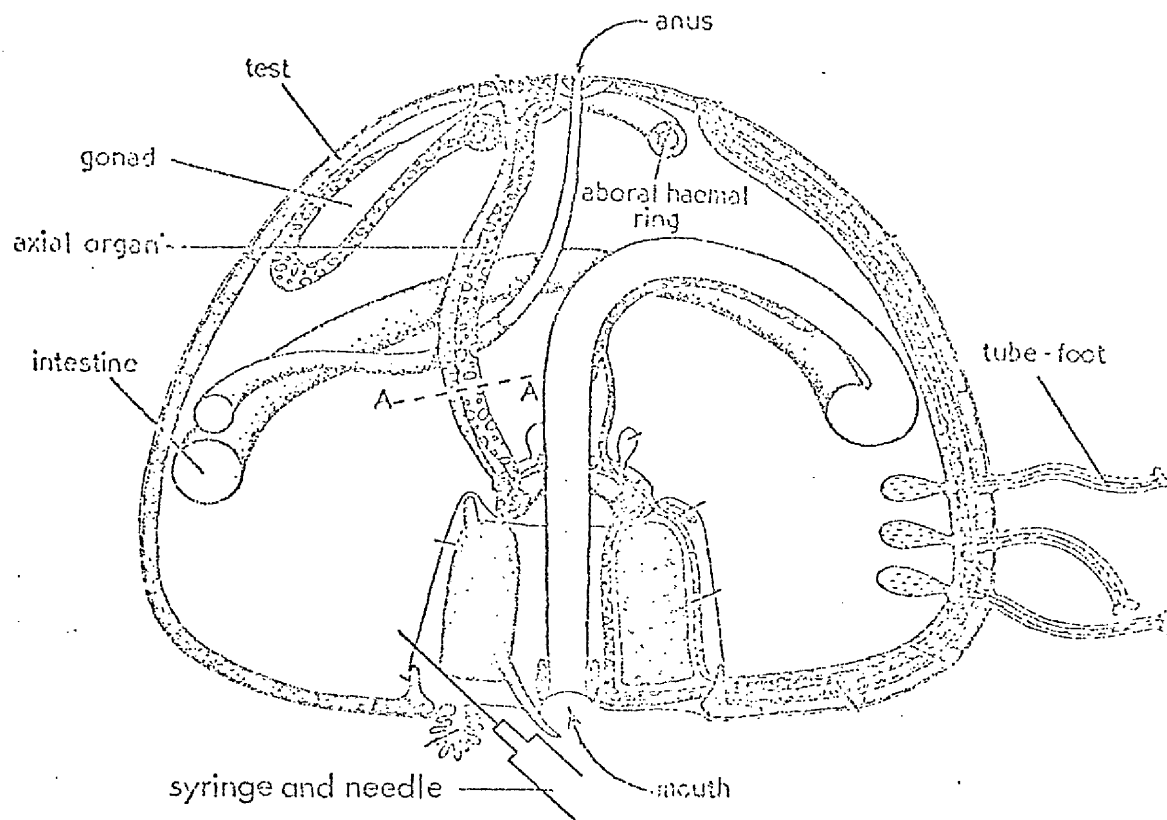
Table 1.   Ionic composition and osmotic pressure of echinoderm

body fluid and seawater.   (after Binyon, 1972)

Species	Na	K	Ca	Mg	Cl	SO_4	CO_2	pH	Osmotic Pressure Δ (°C)
			millimoles per litre						
Echinoidea									
Echinus esculentus									
C.F.	530	13.4	12.8	50.6	546	–	–	–	-1.86
S.W.	487	13.6	10.5	–	525	–	–	–	-1.86
Paracentrolus lindus									
C.F.	–	12.7	12.7	51.8	621	32.1	–	–	–
S.W.	–	12.3	12.7	61.7	627	31.2	–	–	–
Strongylocentrotus droebachiensis									
C.F.	420	9.7	9.6	48.6	488	29.0	6.00	7.2	-1.77
S.W.	458	8.5	9.7	33.8	492	25.4	2.15	8.1	-1.75
Holothuroidea									
Holothuria tubulosa									
C.F.	–	13.5	13.7	59.7	649	31.5	–	–	–
S.W.	–	12.3	12.7	61.7	627	31.2	–	–	–
Cucumaria fronulosa									
C.F.	456	7.4	8.9	33.2	501	25.5	2.14	7.8	-1.750
S.W.	458	8.5	9.7	33.8	492	25.4	2.15	8.1	-1.759
Asteroidea									
Asterias rubens									
C.F.	428	9.5	11.7	49.2	487	26.7	2.82	7.2	-1.86
S.W.	429	9.5	10.8	49.0	494	25.4	2.52	7.8	-1.90

MARINE BACTERIA

Gram-negative rods comprise 80-90% of marine bacteria, although Gram-positive rods and cocci can be found, sometimes showing considerable pleomorphism. Many bacteria isolated from the sea have a requirement for seawater or sodium ions in the growth medium (MacLeod, 1965). As a rule, marine bacteria tend to be proteolytic rather than saccharolytic but have the capacity to utilise a wide range of complex organic substrates such as agar, chitin and starch (ZoBell, 1942; Bianchi, 1971).

Descriptions of the genera and species of bacteria found in the sea are given by ZoBell and Upham (1944), Kriss (1963), Leifson et al. (1964), Pfister and Burkholder (1965) and Baumann et al. (1972). The principle genera found are, in order of numbers, Pseudomonas/Alteromonas, Vibrio, Flavobacterium, Moraxella, Acinetobacter and Gram-positive cocci. The groups Moraxella and Acinetobacter were formerly classed as Achromobacteriaceae (Baumann, Doudoroff and Stanier, 1968a,b). Figure 4 shows a scheme, based on that of Shewan, Hobbs and Hodgkiss (1960), by which Gram-negative rods can be allocated to the main genera.

When considering the distribution of marine heterotrophic bacteria it is simplest to divide their environment into 3 parts - the oceans, the sediments, and the shores and estuaries.

The seas cover 70% of the Earth's surface but, surprisingly, literature on the bacteriology of these waters is scarce. This environment is comparatively constant, for although

Figure 4.   Scheme of identification of marine Gram-negative

rod-shaped bacteria   (simplified from Shewan et al.,

1960)

Gram-negative rod

motile; oxidase-positive
- oxidative attack on glucose or no change; O/129 resistant → **Pseudomonas**
- fermentative attack on glucose
  - O/129-resistant; penicillin-resistant → **Aeromonas**
  - O/129-sensitive; penicillin-resistant → **Vibrio**

non-motile
- pigmented, oxidase-variable; variable attack on glucose → **Flavobacterium**
- non-pigmented; oxidative attack on glucose or no change
  - Oxidase-negative; penicillin-resistant → **Acinetobacter**
  - oxidase-positive; penicillin-sensitive → **Moraxella**

there are differences in temperature, salinity and pressure, these differences occur over large distances and long periods of time. Most bacteria in the ocean exist as clumps (Seki, 1971) or attached to detritus particles or plankton (Waksman et al., 1933). It is difficult, therefore, to assess their numbers accurately by ordinary viable counting methods, but there seem to be between 10 and 10^3 heterotrophic bacteria per ml of seawater. This figure increases when an increase in plankton numbers occurs, suggesting a relationship between the 2 populations.

The number of bacteria appears to be greatest in the surface layer of water, the euphotic zone, where light can penetrate and photosynthesis may occur to a depth of up to 80 m. In the aphotic zone below this, where photosynthesis does not occur and the temperature drops due to lack of penetration of solar radiation, the bacterial count decreases markedly (Lloyd, 1930; ZoBell, 1942, 1946; Kriss, 1963).

Although the sediments are partly derived from material from the sea and are in contact with it, they form a different environment. Microbial populations are much higher in sediments due to the concentration of nutrients. Rittenberg, Emery and Orr (1955), in a study of the bacterial activity in sediments, suggest that the bacteria are responsible for the chemical equilibrium between the sediment and water and that the bacteria restore the nutrients to the water.

Most studies on the bacteriology of sediments have been concerned with shallow, coastal waters (Lloyd, 1930; Waksman et al., 1933; Bianchi, 1971). Bacterial counts in the surface layers of the sediments are between 10^4 and 10^6 organisms per gram. However, the numbers of bacteria decrease with depth in the sediment. ZoBell and Morita (1957) were the first to show that bacteria exist in the sediments of the deepest parts of the world's oceans. These organisms are barophilic and many will not grow at atmospheric pressure.

The estuarine environment differs from that of the open ocean in that it is much more variable. Salinity varies because of dilution by rivers and depends mainly on the tides and rainfall. The temperature is also more variable than in the ocean. Due to drainage from the land, terrestrial and fresh-water bacteria as well as those of human and animal origin may be found, along with the normal marine flora.

The importance of bacteria directly and indirectly in the food chain has been recognised. At one time it was believed that the major role of bacteria in the oceanic environment was the conversion of organic material to inorganic, liberating inorganic compounds which could be used by other bacteria and phytoplankton. Although it is known that bacteria do produce more easily assimilable substances, this activity now seems to be of secondary importance. It may be that bacteria are more important in the gut of marine animals where they act on the

otherwise indigestible residues thus releasing more nutrients. Bacteria are, however, important in the breakdown of complex organic substances to simpler ones. Such substances include organic polysaccharides such as cellulose, agar, chitin and alginates as well as proteins, nucleic acids and other substances from dead animals and plants (Wood, 1967). These breakdown products are often used by plants without the need for mineral-ization to inorganic compounds.

A major activity of bacteria in the sea appears to be the assimilation of dissolved organic matter into the bacteria themselves. The bacteria then become available as food for zooplankton and other animals and are thus directly associated with the food chain. Newell (1965) has shown that two filter-feeders, the prosobranch snail, Hydrobia ulvae, and the clam Macoma balthica, obtain their food by digestion of bacteria adhering to fine silt particles. Vacelet (1975) and Reiswig (1975) showed that sponges ingest bacteria, and ZoBell and Feltham (1938) that marine worms and oysters could grow on a bacterial diet.

Although much effort has been expended in studying human enteric bacterial pathogens in shellfish and crustacea, little systematic work has been done on the pathogenicity of marine bacteria to marine invertebrates. The interest lies mainly with those invertebrates used by man as food and therefore commercially valuable.

Hess (1937) found a chitinovorous bacterium attacking the shell of live lobsters, in one case causing an epizootic. Snieszko and Taylor (1947) showed that the organism, Gaffkya homari (recently renamed Aerococcus viridans var. homari) is the cause of widespread mortality of the American lobster, Homarus americanus. In later experiments involving deliberate infection, Rabin (1965) found that the organism was able to overcome the host defence mechanisms and produce bacteraemia, associated with loss of coagulation of haemolymph, followed by death. Rabin and Hughes (1968) showed that bacteraemia could be produced in other marine invertebrates but the organism was usually cleared from the haemolymph after a week.

Colwell and Sparks (1967) have described a marine bacterium, Pseudomonas enalia, which can kill oysters. The organism infects and causes extensive breakdown of the tissues. In the case of G. homari and Ps. enalia, it is possible that the bacteria come from the commensal flora and are only pathogenic when the animal is weakened by injury or environmental changes.

Among the few other infectious diseases known in marine invertebrates, mention may be made of the progressive bacterial disease of the horse-shoe crab, Limulus polyphemus, described by Bang (1955). He noted that the endotoxin from the infecting Vibrio spp. caused amoebocytopenia, extensive intravascular coagulation, subsequent incoagulability of the blood and death. It was also shown that endotoxin alone could cause death in other marine invertebrates. The shore crab, Carcinus maenus, is

susceptible to infection with a Gram-negative bacillus (Cantacuzène, 1925) and infection is again associated with loss of coagulability of the blood and also loss of the blue colour of the plasma which is normally present as the respiratory pigment.

The normal bacterial flora of marine invertebrates has been almost totally neglected. Although some early work gives indications of the types of bacteria to be found in marine invertebrates - Symons (1921) and Rice (1929) on Mya arenaria, the soft-shelled clam, and Harrison and Hood (1923) on lobsters - most reports are concerned with the incidence of potential human pathogens and the transmission of human disease, such as typhoid, by molluscs in sewage-polluted waters. Also, incidental in the study of the spoilage of edible molluscs and crustacea has been the identification of certain genera of indigenous commensal bacteria i.e. Achromobacter, Pseudomonas, Flavobacterium and Micrococcus which are responsible for such spoilage (Hunter, 1920; Novak, Fieger and Stolzle, 1960).

One of the few systematic studies of the normal bacterial flora of marine invertebrates is that of Colwell and Liston (1960, 1962) on the Pacific oyster, Crassostrea gigas, and other marine invertebrates. They concluded that there was a "very characteristic bacteriological flora consistently associated" with the invertebrates studied, with only very minor differences due to extrinsic factors such as temperature, salinity, etc. The maintainance of the normal flora seems to depend therefore on the physical environment within

the animals. The predominant genera they found were
Pseudomonas, Achromobacter, Flavobacterium, Micrococcus and
Vibrio.

Further work on the bean clam, Donax gouldi, by Beeson
and Johnson (1967), on the oyster, Crassostrea gigas, by
Vasconcelos and Lee (1972) and on the blue crab, Callinectes
sapidus, by Sizemore et al. (1975) have substantiated these
conclusions.

An early part of the present investigation was to
examine the normal bacterial flora associated with E. esculentus.

ANTIBACTERIAL MECHANISMS IN MARINE INVERTEBRATES,
OTHER THAN ECHINODERMS

As outlined above, the sea is rich in bacteria and other
microorganisms, especially near coasts where counts may reach tens
of thousands per gram of sediment. It would be surprising,
therefore, if marine invertebrates, over the millenia, had not
evolved defence mechanisms to cope with potentially pathogenic
microorganisms which they often ingest and which surround them in
the sea. Table 2 summarises some of the features of marine
invertebrates (except echinoderms, which will be discussed later)
which might be relevant to antibacterial defence.

Phagocytosis appears to be the primary defence mechanism
against invading particles, although encapsulation may occur with
particles which are too large to be phagocytosed. Extensive use

Table 2.   Features of antibacterial defence in marine invertebrates,

excluding echinoderms.

Invertebrate phylum	Phagocytosis	Clotting and Coagulation	Bactericidins	Agglutinins and Haemolysins	Lysozyme	Transplantation
Mollusca	Tripp, 1960; Stauber, 1950; Tripp, 1958; Feng, 1967; Feng and Feng, 1974; Cheng and Cali, 1974; Foley and Cheng, 1975	Bang, 1961; Levin, 1967	Weinheimer, Acton and Evans, 1969b; Li, 1960; Johnson and Chapman, 1970b; Schmeer, 1966	Pauley, Krassner and Chapman, 1971; Tripp, 1966; McDade and Tripp, 1967; Tripp, 1970a,b; 1974	McDade and Tripp, 1967; Feng, 1974; Cheng and Rodrick, 1974; Cheng et al., 1975	Cheng, 1970; Tripp, 1970b; Cheng and Galloway, 1970; DesVoigne and Sparks, 1969
Tunicata	Anderson, 1971; Wright, 1974		Johnson and Chapman, 1970a.	Anderson and Good, 1975		Freeman, 1970
Porifera	Cheng and Yee, 1968a.		Jakowska and Nigrelli, 1960			Cheng et al., 1968b
Sipunculoida	Cushing, MacNeely and Tripp, 1969		Bang, 1967; Cantacuzène, 1922; Krassner and Flory, 1970; Evans et al., 1969	Weinheimer et al., 1970; Cushing et al., 1969; Fuke and Sugai, 1972		Anderson, 1971
Annelida	Dehorne, 1922; Fretter, 1953; Dales, 1961; Marsden, 1966				Jollès and Zuili, 1960; Perin and Jollès, 1972	Cooper, 1970
Arthropoda	Cornick and Stewart, 1968; Paterson, Stewart and Zwicker, 1976	Levin, 1967; Bang, 1955; Shirodkar, Warwick and Bang, 1960; Bang, 1970	Evans et al., 1968; Stewart and Zwicker, 1972	Pauley, 1974a, b; Cohen, Rozenberg and Massaro, 1974; Marchalonis and Edelman, 1968; Weinheimer et al., 1969a; Finstad, Good and Litman, 1974		
Coelenterata			Burkholder and Burkholder, 1958			Hildemann, Dix and Collins, 1974

has been made of molluscs in the study of phagocytosis because
of their importance as food and as reservoirs of human pathogens
(Feng, 1967; Tripp, 1970a,b).

The process of phagocytosis involves 3 steps; first,
the particles stick to the phagocytes, secondly, the particles
are ingested and thirdly, there is intracellular digestion or
elimination of the particles to the exterior of the animal. The
reaction is generally non-specific although it has been noted in
the oyster that certain bacteria are not phagocytosed (Bang,
1961), and in a tunicate there was a difference in reaction when
different types of erythrocyte were used (Wright, 1974). Since
marine invertebrates are poikilothermic, temperature has an effect
on phagocytosis and in molluscs, for example, phagocytosis is
depressed at low temperatures of 4-9°C (Feng and Feng, 1974;
Foley and Cheng, 1975).

Cell-clotting and subsequent coagulation of body fluid
seems to be an important reaction to invading microorganisms
especially in arthropods. Clotting of cells occurs on withdrawal
of fluid or when an animal is injured. The clots serve to close
wounds (Bang, 1961, 1970). Extracellular coagulation occurs
after cell clotting, presumably due to the release of cell factors,
in response to bacteria and their endotoxins (Levin, 1967) and the
coagulation of amoebocyte lysate of Limulus provides a laboratory
test for endotoxin (Jorgensen and Smith, 1974). Coagulation may
immobilise invading organisms and thereby prevent spread throughout
the body. Although coagulation has been studied mainly in

arthropods, Bang (1961) described extracellular coagulation
in vitro in the oyster.

Neither specific induceable antibodies nor any
convincing equivalent have been demonstrated in marine
invertebrates, even those which are ancestrally close to
vertebrates, i.e. protochordates and echinoderms. Non-specific
antibacterial substances, which seem to be widespread throughout
the marine environment have, however, been described in a number
of invertebrates, although few have been purified and described
in detail. Among those animals whose fluids have been found to
be antibacterial are sponges (Jakowska and Nigrelli, 1960),
corals (Burkholder and Burkholder, 1958), molluscs, including
abalones (Prescott and Li, 1960), oysters (Limasset, 1962),
lobsters (Weinheimer et al., 1969a) and sipunculid worms (Rabin
and Bang, 1964; Krassner and Flory, 1970). The 2 substances
most extensively studied are the "paolins" from abalones
(Prescott and Li, 1960) and "retine" or mercene (Schmeer, 1966),
an antitumour agent, from clams. Both are proteins which have
an inhibitory effect on a number of organisms. The other
bactericidins studied also appear to be proteins and show some
specificity for marine bacteria (Johnson and Chapman, 1970a). In
some cases, notably the spiny lobster (Evans et al., 1968) and the
American lobster (Stewart and Zwicker, 1972), the bactericidin
can be increased by immunisation with bacteria. However,
Weinheimer et al. (1969b) were unable to stimulate a bactericidin
of the oyster.

Marine invertebrates contain a variety of agglutinins against bacteria and vertebrate erythrocytes (Tripp, 1966, 1974; Acton and Weinheimer, 1974; Cohen et al., 1974; Anderson and Good, 1975; Pauley, 1974a). Most of these are proteinaceous (Pauley, 1974b; Acton and Weinheimer, 1974), although the haemagglutinin of an ascidian has been described as a large polysaccharide (Fuke and Sugai, 1972). Agglutinins are generally high molecular weight agents composed of several subunits, and some require calcium ions for maintenance of structure or activity (Tripp, 1966; Hall and Rowlands, 1974).

Haemolysins also occur in certain marine invertebrates and are generally non-specific. Although agglutinins and lysins which are active against bacteria are an obvious advantage to the animal, the role of haemagglutinins and haemolysins is not clear. Haemagglutinins may, however, act as opsonins and enhance phagocytosis (Tripp, 1966; McKay, Jenkin and Rowley, 1969; Pauley, 1974b).

The bacteriolytic enzyme, lysozyme, has been reported in some marine invertebrates. Those most extensively studied are the marine polychaete, Nephthys hombergi (Jollès and Zuili, 1960; Perin and Jollès, 1972) and the oyster, Crassostrea virginica (McDade and Tripp, 1967; Feng, 1974; Cheng and Rodrick, 1975). Lysozyme has also been found in the soft-shelled clam, Mya arenaria (Cheng and Rodrick, 1974) and the quahaug clam, Mercenaria mercenaria (Cheng and Rodrick, 1975). Cheng et al. (1975) have found that the lysozyme of the quahaug clam is released from the

haemolymph cells during phagocytosis. The biological role of lysozyme in the serum of marine animals is not, however, clear. It is possible that the enzyme kills and lyses susceptible bacteria which have not been phagocytosed.

A recent method of determining immunocompetence in invertebrates is tissue transplantation. The work in this field is reviewed by Hildemann et al. (1974) and Hildemann (1974). Despite the difficult surgical techniques involved, many species of invertebrates have now been studied for immune response to, and specific memory of, grafted tissue. Results have generally been indecisive although there is some evidence for recognition and rejection of certain grafts even at the level of the corals. Annelid worms have been shown to develop short-lived but specific memory, although studies with other marine invertebrates have been unsuccessful, possibly due to failures of technique rather than lack of immunocompetence.

ANTIBACTERIAL MECHANISMS IN ECHINODERMS

Before discussing the defence mechanisms, it is necessary to describe the cell types found in echinoderm coelomic fluid. This however is difficult as investigators have used different terminologies. The cells of Pacific echinoids have been observed in detail by Johnson (1969a,b,c). She has also correlated other studies of urchin coelomocytes and so it is her nomenclature which I use. Table 3 shows the different types of coelomocytes found in echinoids.

Table 3.   The coelomocytes of echinoid coelomic fluid

Cell type	Primary [1] Morphology	Secondary [1] Morphology	Activity
Leucocyte (Bladder amoebocyte)	Petaloid ecto-plasmic extensions. Small granules in endoplasm. 20-30 μ diameter.	Flattened cells with numerous pointed ecto-plasmic processes.	Phagocytosis and cell aggregation.
Vibratile cell	Finely granular cytoplasm, spherical 7-14 μ diameter. Motile by single long flagellum.	–	Possibly localisation of invading material by release of mucopoly-saccharide.
Red spherule cell	Round 10-15 μ diameter when floating in fluid. Large cytoplasmic inclusions containing the pigment ectino-chrome.	Flattened cells with lobate pseudopodia when in contact with glass or other cells.	Possibly release of algistatic and bacterio-static echino-chrome-protein complex.
Colourless spherule cell	Similar to red spherule but pigment absent.	Flattened cells with lobate pseudopodia when in contact with glass or other cells.	?

1   Primary morphology is seen in freshly drawn hanging drops of coelomic
     fluid.   In contact with a surface such as glass, or in cell aggregates
     of older drops, secondary morphology is evident.

As indicated previously, marine invertebrates appear
to possess 3 main mechanisms of defence against bacteria:
clotting mechanisms (agglutination of cells and coagulation of
body fluid), phagocytosis and bactericidal substances.

Extracellular coagulation of body fluid is widespread
among the marine invertebrates but there is no clear evidence
of such a reaction in echinoderms. Johnson (1969b,c) however
reported that when the gut of the echinoid, Strongylocentrotus,
was punctured during the removal of fluid and the yellowish
liquid from the gut flowed into the coelomic cavity, vibratile
cells which came in contact with this liquid rapidly decreased
in size. The coelomic fluid which was removed appeared viscous
or like a semi-gel but this disappeared during 12-24 h in vitro.
Vibratile cells have been shown to be rich in an acid muco-
polysaccharide (Johnson, 1969a) which, when released, could form
this semi-gel. Although in vitro the semi-gel is only
temporary, in vivo it could be constantly replenished by more
vibratile cells until the infected fluid, containing debris,
bacteria, protozoa, etc. was removed by phagocytosis.

The cells of the fluid of all marine invertebrates
studied appear to possess, to varying degrees, the ability to clot.
Clotting in echinoderms seems to involve only cell-aggregation.
The reaction appears to be initiated by the leukocytic cells
which extend long ectoplasmic processes, forming a network in
which are trapped the other cells of the coelomic fluid. In some
cases, however, the leukocytes may fuse together to form a

plasmodium. Clot formation seems to be primarily a reaction to injury as shown by Henri (1906) and Donnellon (1938) who found that clots served to close wounds in the gut and integument of sea urchins, and by Bang and Lemma (1962) who found that the degree of trauma was consistent with the degree of clumping of cells in the star fish, Asterias. The reaction may, however, be involved in the prevention of spread of microorganisms and toxic substances throughout the coelomic fluid (Davidson, 1953; Johnson, 1969c).

Clotting in echinoderms was first observed by Geddes (1880), Cuénot (1891) and Schaefer (1882) when they withdrew fluid from these animals. Since this early work, several researchers have suggested mechanisms for clotting. Schaefer suggested that a mucin provided the clotting matrix; Théel (1921) thought a fibrin was involved; Donnellon (1938) stated that the breakdown of the red spherule cells in Arbacia liberated a substance involved in clotting. Kindred (1921) and Bookhout and Greensburg (1940), working with the echinoids Arbacia and Mellita respectively, have suggested that the leukocytes in the coelomic fluid secrete a sticky substance which causes adhesion of other coelomocytes.

These hypotheses were made from direct observation of clot formation by microscopy. Boolootian and Giese (1959), however, investigated the mechanism of clot formation of several species of echinoderms by use of various anticoagulants. They observed 3 types of clot by phase-contrast microscopy; the first

in which cells aggregated and maintained their identity; the second where the cells agglutinated and gradually lost their identity to form a plasmodium; and the third where the cells agglutinated and formed a meshwork of fibres in which all types of cells were trapped. Using calcium-removing substances, e.g. sodium citrate or EDTA, they found that only the first type of clot was calcium-dependent. This was seen with an echinoid (the sand dollar), a holothuroid, an ophiuroid and a crinoid. The 2 remaining types of clot were typical of asteroids and the other echinoids studied. Clot formation was also inhibited by reducing agents and by substances with a specificity for sulfhydryl groups. It seemed therefore that disulphide bonds were necessary for cell aggregation and that these bonds were formed directly between cells or with the aid of an enzyme "bridge". These clots were found to be only temporary, but Johnson (1969c) has pointed out that this may be due to the anticoagulants used and to poor maintainance of the cells, leading to cell degradation and clot-disintegration.

Johnson (1969c) observed the reaction of Pacific urchin coelomocytes in hanging drops containing Gram-negative bacteria. The most common reaction of the cells was the formation of a wall-like clot which limited the bacteria to the site of inoculation. The leukocytes were the only active agents in the wall formation, other cells being incorporated passively. Cells in contact with the bacteria eventually formed a plasmodium. Chemotaxis of red spherule cells occurred to a varying extent

and the cells sometimes changed colour as did the fluid of the bacterial area, presumably due to release of the pigment echinochrome. Lysis of bacterial cells took place on occasions when the coelomocytes acted quickly to overcome the bacteria. The migration of sea urchin spherule cells to the outer surface of clots has been reported before -- Abraham (1964) showed colourless spherule cells at the outer edges of Paracentrotus lividus clots and red spherule cells on the surfaces of Arbacia lixula clots.

Although clotting has been implicated as a mechanism of defence in echinoderms, the primary line of defence to foreign material, as in all invertebrates, seems to be phagocytosis. Phagocytosis as a defence mechanism was first observed by Metchnikov (1882), cited by Bang (1975). This was the classical observation that a splinter introduced into the body of a sea star larva was soon surrounded by "mobile cells". It is now clear that these mobile cells were the phagocytic leukocytes of the body fluid.

Much work on phagocytosis in marine invertebrates has been done with molluscs (Tripp, 1960; Bang, 1961; Feng, 1967; Foley and Cheng, 1975). Phagocytosis has been studied in echinoderms but less fully. Phagocytic cells have been reported by Liebman (1950) in Arbacia and by Boolootian and Giese (1958) who studied the coelomocytes of 15 species of echinoderms representing all living classes. Kindred (1921) found that when carmine or India ink were injected into the urchin, Arbacia,

phagocytic leukocytes (bladder amoebocytes) contained dye
particles after 30 min. Bang and Lemma (1962) working with the
sea star, Asterias forbesi, showed convincingly that India ink
and carmine were phagocytosed by leukocytes and observed
leukocytes filled with dye in the sea water surrounding the
injected stars. Johnson and Beeson (1966) have also described
phagocytosis of carmine by coelomocytes of the starfish, Patiria.

Johnson (1969c), using hanging drops, investigated
in vitro phagocytosis of bacteria by coelomocytes of Pacific sea
urchins. She observed that only Gram-positive bacteria were
phagocytosed and suggested that this is the primary defence
against Gram-positive organisms (compare the reaction to Gram-
negative bacteria discussed previously). The reaction was often
intense -- after 5 days no bacteria were visible in the fluid of
the drop. However the bacteria could be seen within the
phagocytes even in cell aggregates or plasmodia. The phagocytic
cells were neither attracted nor repelled by the bacterial
species she used but merely consumed the bacteria in much the
same way as inert dye particles. No Gram-negative bacteria were
seen to be phagocytosed although they were seen sticking to
leukocytes which is a phenomenon which precedes phagocytosis by
oyster leukocytes (Bang, 1961). There does seem therefore to be
a certain specificity of reaction of urchin coelomocytes towards
Gram-positive bacteria. Using electron microscopy however,
Johnson, Chien and Chapman (1970) detected phagocytosis of one of
their Gram-negative bacterial strains.

Turning now to a consideration of soluble anti-
bacterial and similar substances in echinoderms, we find only
scanty reports in the literature. Johnson and Chapman (1971)
found that the whole fluids and also sera (cell-free filtered
fluid) of the sea cucumber, Stichopus tremulus, and the sand
dollar fluid) of the sea cucumber, Stichopus tremulus, and the sand
and dollar, Dendraster excentricus, inhibited a variety of marine
and terrestrial bacteria but without killing them. The whole
fluid was consistently more inhibitory than the serum and they
concluded that the bacteriostatic system mainly involved the cells.

In echinoids, it has been suggested by Vevers (1963)
that echinochrome, which is a naphthaquinone pigment belonging
to a group of chemicals which are algistatic agents, acts in this
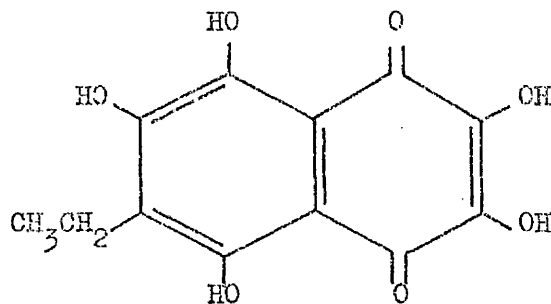
Echinochrome

capacity in the sea urchin, Echinus. It has been shown by
Holland, Giese and Phillips (1967) and Johnson (1970) that sea
urchin echinochrome is in complex with one or more proteins and
may be released from red spherule cells as an algistatic and
possible antibacterial agent.

Haemolysins have also been detected in a number of
echinoderms. Alender, Fiegen and Tomita (1965) showed that a
crude extract from homogenates of the globiferous pedicellariae

of the sea urchins Tripneustes gratilla, E. esculentus, Paracentrotus lividus and Psammechinus miliaris contained a non-dialysable, thermo-labile protein which lysed the erythrocytes of guinea pig and sheep. Saponin has been found in the Holothuroidea and the Asteroidea (Nigrelli et al., 1967) but not as yet in the Echinoidea. Ryoyama (1973) described a haemolytic substance in cell-free coelomic fluid of 3 echinoids. This is a heat-labile protein, or protein-like substance, with activity against rabbit, mouse and human erythrocytes. The biological role of these substances in defence mechanisms is unknown.

Parker, in an undergraduate research project in this Department (1974), found a haemagglutinin in E. esculentus. It had a high molecular weight, was probably a protein and showed a specificity for rabbit, rat and human group B erythrocytes. These conclusions were concordant with the work of Ryoyama (1974) on two other urchin haemagglutinins, and Finstad et al. (1972) on starfish haemagglutinin. Ryoyama also described a haemagglutinin in another urchin, Hemicentrotus pulcherrimus, which did not appear to be a protein, but was probably a large molecular weight carbohydrate similar to that of the ascidian (Fuke and Sugai, 1972). Carton (1974) has found that the haemagglutinin of Asterias shows similarities in amino acid composition to vertebrate immunoglobulins and suggests that certain parts of echinoderm haemagglutinin may have been conserved during the evolution of immunoglobulins.

Since marine invertebrates will not, under normal circumstances, encounter mammalian erythrocytes, the question arises as to the biological role of these haemagglutinins. It is thought that they may act, at least in some cases, as opsonins, promoting phagocytosis of foreign particles by an adhesive effect (Tripp, 1966; Tripp and Kent, 1967). They may also act directly by immobilising parasites (Cushing et al., 1969).

The enzyme lysozyme has been found in the starfish Asterias by Jollès and Jollès (1975) but it is not known whether it is found in cell-free coelomic fluid or whether it has any biological activity other than as a lysosomal enzyme during phagocytosis.

Although not necessarily connected with antibacterial mechanisms, tissue transplantation studies have shown that echinoderms can recognise foreign material. Graft recognition and rejection in starfish (Ghiradella, 1965) and specific memory in a sea cucumber and a star fish (Hildemann and Dix, 1972) have been observed. Injection of sea stars with cells from sea urchins resulted in clumping of recipient coelomocytes and rapid clearance of injected cells, but sequential challenge gave no indication of memory (Reinisch and Bang, 1971). Hilgard and Phillips (1968) showed that coelomocytes of a Pacific urchin responded selectively in the removal from the coelomic fluid of ^{14}C-labelled foreign macromolecules and that foreign molecules were removed more rapidly than labelled autologous molecules.

OBJECT OF THE RESEARCH

There is an extensive body of literature on the
associations and interactions of bacteria with man and other
vertebrate animals.   In contrast the information available
on the antibacterial mechanisms and the normal bacterial flora
of the numerous species of marine invertebrates which might be
studied, is limited.   Although few species have been studied
it has been shown that marine invertebrates do appear to have
a characteristic bacterial flora and one aim of this investigation
was to define the normal bacterial flora of the sea urchin, and
discover if the flora corresponded to that which has been
previously described in other marine invertebrates.

No evidence of the complex specific humoral and
cellular immune reactions of the vertebrates has been shown to
occur in the marine invertebrates.   However, reactions against
bacteria such as phagocytosis, cell clotting, coagulation of
body fluids and the production of bactericidal substances have
been shown in some species.   These reactions appear to exhibit
little specificity and it has been found that all marine
invertebrates do not necessarily possess all these antibacterial
activities.   The major objective of this investigation was
therefore to discover if E. esculentus possessed an antibacterial
activity and if so, to investigate some factors affecting it.

MATERIALS AND METHODS

URCHINS

Collection and maintenance

Urchins were collected off Great Cumbrae Island by scuba divers or from the shore at low tide. They were hand-picked, care being taken to avoid damage such as ripping off the tube feet and breaking the spines. Large urchins, greater than 6 cm in diameter, were chosen because of the large volume of fluid which could be obtained from them. Urchins were kept in the aquarium for at least 2 days before use.

Initially, the urchins were kept in a wire mesh cage on the sea bed and in a tank in the Marine Station, Millport. The cage was 1 m^2 x 0.75 m, the base raised 30 cm off the sea bed in water of depth 3.5 m at high tide. Inside was put the gravelly mud of the sea bed and a few rocks with seaweed growing from them. The cage held 10 urchins, which were taken by divers as required.

Latterly, only an indoor aquarium tank was used. It was suitable for keeping 6 urchins at a time with constantly-running fresh sea water. The tank was divided into 6 sections by a wooden frame and plastic mesh and an urchin placed in each compartment. Seaweed was given initially as food but this was found to be unnecessary for survival of the urchins. The tank was cleaned only when an urchin had died.

Sampling coelomic fluid

Since the sea urchin has an encasing shell, the test,
the best way to withdraw fluid is by puncturing the peristomial
membrane surrounding the mouth (Figure 3). For small amounts,
it was found that a syringe with a 26g½ in. needle was best
since a longer needle was liable to puncture the gut or gonads.
If the needle was inserted at an angle away from the mouth, the
likelihood of puncturing any of the internal organs was small.
Fluid was withdrawn slowly to avoid damage of its cells. For
multiple withdrawals, the needle was left in place while the
syringe was emptied.

When large amounts of fluid were needed, the
peristomial membrane was washed first with sterile seawater and
cut with a sterile scalpel. The urchin was inverted and the
fluid allowed to run into a sterile collecting vessel.

BACTERIOLOGICAL METHODS

Estimation of numbers of aerobic heterotrophic bacteria

The numbers of bacteria colonizing the peristomial
membrane, the coelomic fluid and the gut of the urchin were
estimated.

For estimation of numbers from the peristomial
membrane, the area was scrubbed with a toothbrush which had
previously been sterilised in 70% alcohol and rinsed repeatedly
in sterile distilled water. The end of the toothbrush, after

scrubbing the peristomial membrane, was then rotated in 5 ml of

sterile seawater in a universal container. Dilutions (10-fold)

of this were made in sterile Marine 2216 broth (Difco) and 0.1 ml

of dilutions 10^{-1} to 10^{-4} spread on the surface of a Marine 2216E

agar plate (Difco) previously well-dried at 37°C. Plate 1 shows

the appearance of a mixed population of bacteria from the

peristomial membrane.

The coelomic fluid, obtained as described above, was

dispensed in 0.1 ml portions directly from the syringe and spread

on dried 2216E agar.

Sections of gut were obtained by cutting the urchin

open with scissors, initially avoiding cutting through the gut.

The gut was then cut to give approximately 3 cm pieces and put in

10 ml sterile 3.2% saline (w/v). The gut contents were included

where possible. The mixture was then shaken vigorously for

20 - 30 sec. Serial dilutions (10-fold) were made in sterile

3.2% saline and 0.1 ml of dilutions 10^{-2} to 10^{-5} were spread on

dried 2216E agar.

All agar plates were incubated for at least 7 days at

20 - 22°C before counting.

Isolation of bacteria and maintenance of pure cultures

Marine 2216E agar medium was used throughout for the

isolation and purification of bacteria. Plates with not more

than 30 well-separated colonies were chosen from those inoculated

Plate 1.   Organisms from the peristomial membrane

after 3 days incubation at 22°C on 2216E

agar.
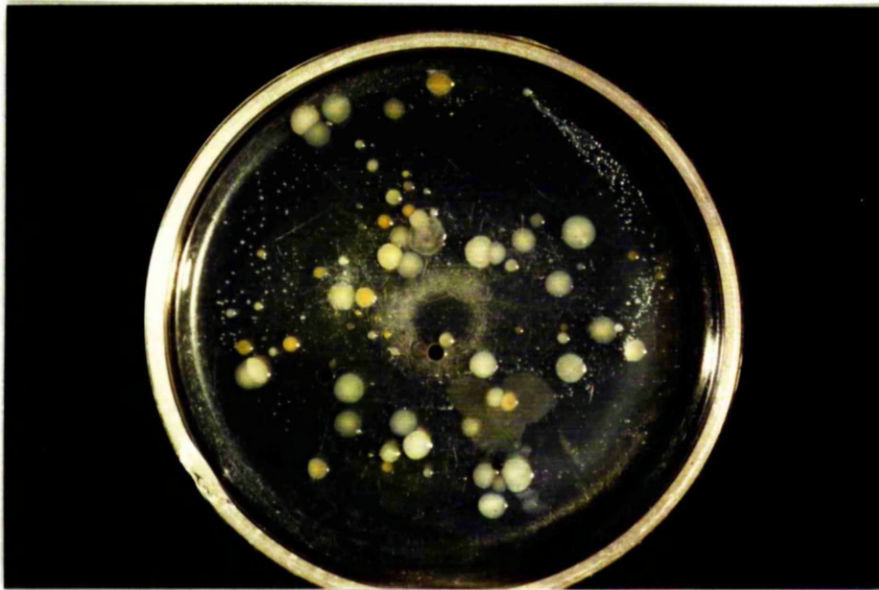
in the preceding section. Representative colonies of different
appearance were selected and sometimes every colony from a single
plate was taken. Pure cultures were obtained by plating out and
incubating at 20 - 22°C for 2 - 3 days or until growth appeared.
The cultures were numbered according to the date on which they
were isolated and the sequence in which they were selected.

From a single isolated colony of a pure culture, a
subculture was made on a 2216E agar slope in a universal container.
The stock cultures were incubated at 20 - 22°C until good growth
was apparent and stored at 4°C. These cultures were transferred
every 3 - 4 months.

Identification of cultures

Colony appearance and Gram reaction were determined
after growth of the organisms on 2216E agar for 24 - 48 h at
20 - 22°C depending on the growth rate of the organism. Phase-
contrast microscopy was used to observe motility and morphology
of 24 - 48 h cultures grown in 2216 broth. To detect pigment
formation, 2216E agar incorporating 30% skim milk was used (Shewan
et al., 1960). This was streak-inoculated and incubated for 48 h.

The oxidase test was carried out by the method of
Anderson (1962). A small strip of Whatman No. 1 filter paper was
impregnated with a 1% solution (w/v) of Kovac's oxidase reagent
(BDH) and laid on the surface of a colony for a few seconds. The
paper was then removed and according to the nature of the colony,
it either adhered to the paper or remained on the agar. A positive

reaction was recorded if the colony turned deep purple within 15 sec.

Oxidative or fermentative attack on glucose was determined using the Marine Oxidation Fermentation (MOF) medium of Leifson (1963) (Difco). The strains were stab-inoculated into duplicate 16 x 150 mm test tubes containing 5 ml of medium. One set of cultures was incubated aerobically and one anaerobically in a McIntosh and Fildes' jar. All cultures were incubated at room temperature for 3 days before examination. If only the aerobic culture produced acid, this was designated an oxidative attack, while, if both the aerobic and anaerobic cultures produced acid, this was designated a fermentative attack. If neither culture produced acid but growth was seen, it was recorded that the strain produced no change.

Sensitivity to penicillin, 2 units, was examined by first heavily inoculating a 2216E agar plate. This was done by flooding the plate with the bacterial suspension, removing the excess liquid and allowing the plate to dry. A penicillin disc, 2 u., was placed on the agar and the plate incubated for 24 - 48 h. Sensitivity was recorded if there was a zone of inhibition around the disc.

Sensitivity to the vibriostatic compound 2:4-diamino-6:7 di-isopropyl-pteridine phosphate (EDH), referred to as 0/129, (Shewan, Hodgkiss and Liston, 1954) was determined by inoculating a 2216E agar plate as above. A Whatman antibiotic disc was

impregnated with a 0.14% solution (w/v) of O/129, dried, sterilised by autoclaving at 15 lb/in^2 for 15 min and placed on the plate.   After incubating for 24 - 48 h a positive result was recorded if a zone of inhibition was seen around the disc.

ANTIBACTERIAL TESTS WITH COELOMIC FLUID IN VITRO

Test organism

The test organism, designated strain 111 and isolated from the sand of Kames Bay, Millport, was chosen because it grew as a black, agar-digesting colony and so was easily distinguished from contaminating organisms.   It was a Gram-negative, motile, oxidase positive, pleomorphic rod having an oxidative attack on glucose and resistance to penicillin and compound O/129.   The organism was therefore placed in the genus Pseudomonas.   Plate 2 shows the appearance of strain 111 on 2216E agar and for comparison, Plate 3 shows the typical appearance of organisms from the coelomic fluid.

In order to obtain a uniform inoculum for the anti-bacterial tests, a 9 opacity unit (o.u.) plastic rod Opacity Standard was used (Perkins et al., 1973).   This consisted of a 16 x 150 mm test tube containing an opalescent plastic rod of an opacity nominally equivalent to a suspension of about 10^9 bacteria per ml.   The bacterial suspensions were assumed to match the standard when a printed sheet looked the same viewed through the standard and the suspension held side by side.   A suspension of the test bacterium in 3.2% saline was made from a 2-day 2216E

42

Plate 2.   Appearance of strain no. 111 after 2 days incubation
           at 22°C on 2216E agar.

Plate 3.   Organisms from the coelomic fluid after 2 days
           incubation at 22°C on 2216E agar.
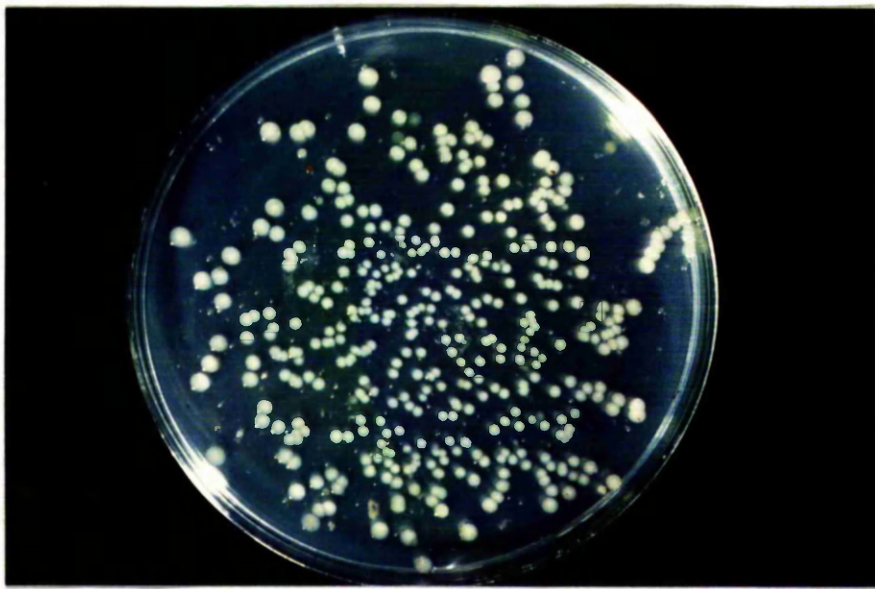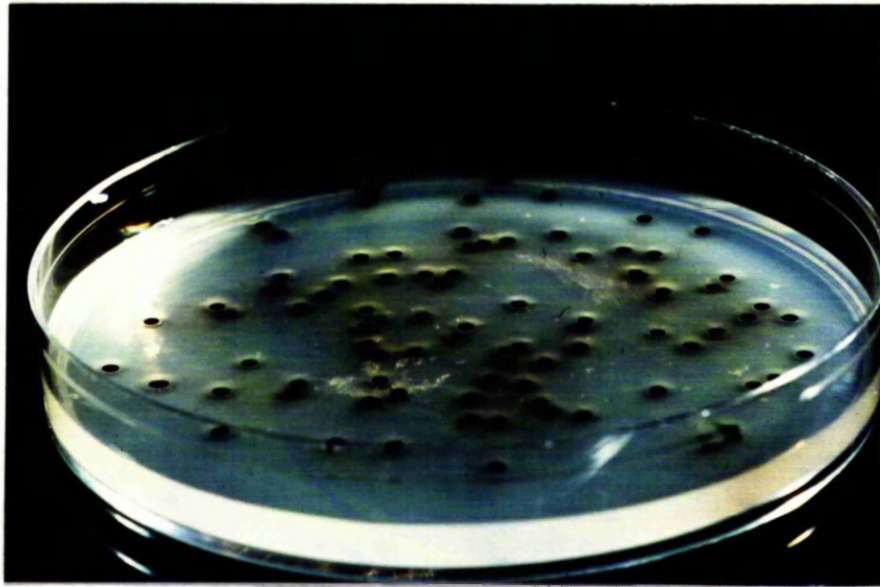
agar slope culture equal in opacity to the standard. The bacterial suspension was serially diluted as shown in Figure 5 in sterile 3.2% saline 1/100 to a concentration of 10^5 bacteria per ml and finally 1/20, giving a concentration of between 5 and 10×10^3 bacteria per ml. This dilution provided the inoculum for the antibacterial tests.

Procedure

Coelomic fluid was taken from urchins in 2 ml amounts or more as previously described. Urchins which seemed sluggish and lacked rigidity of the spines were discarded since these features suggested that the animal might be unhealthy. Fluid (1.8 ml) was then immediately dispensed into sterile 16 x 150 mm test tubes which had previously been placed either in water at the incubation temperature or on ice, and 0.2 ml of bacterial test dilution was added, giving a final bacterial concentration of 500 bacteria (colony-forming units) per ml.

To test the effect of pre-clotting of the coelomocytes on the antibacterial activity, the coelomic fluid was left in the tank water, $8^{\circ}C - 14^{\circ}C$, after being dispensed, for 2 h before addition of the bacterial test dilution.

The non-bactericidal control fluid which was found to be most satisfactory was boiled supernatant of coelomic fluid referred to as S_0/B although sterile seawater or a 1/20 dilution of 2216E agar were used early in the work. To prepare S_C/B, coelomic fluid was centrifuged at 4000 rpm for 15 min in an MSE

Figure 5   Scheme of dilution of bacterial strain no. 111

in 3.2% saline
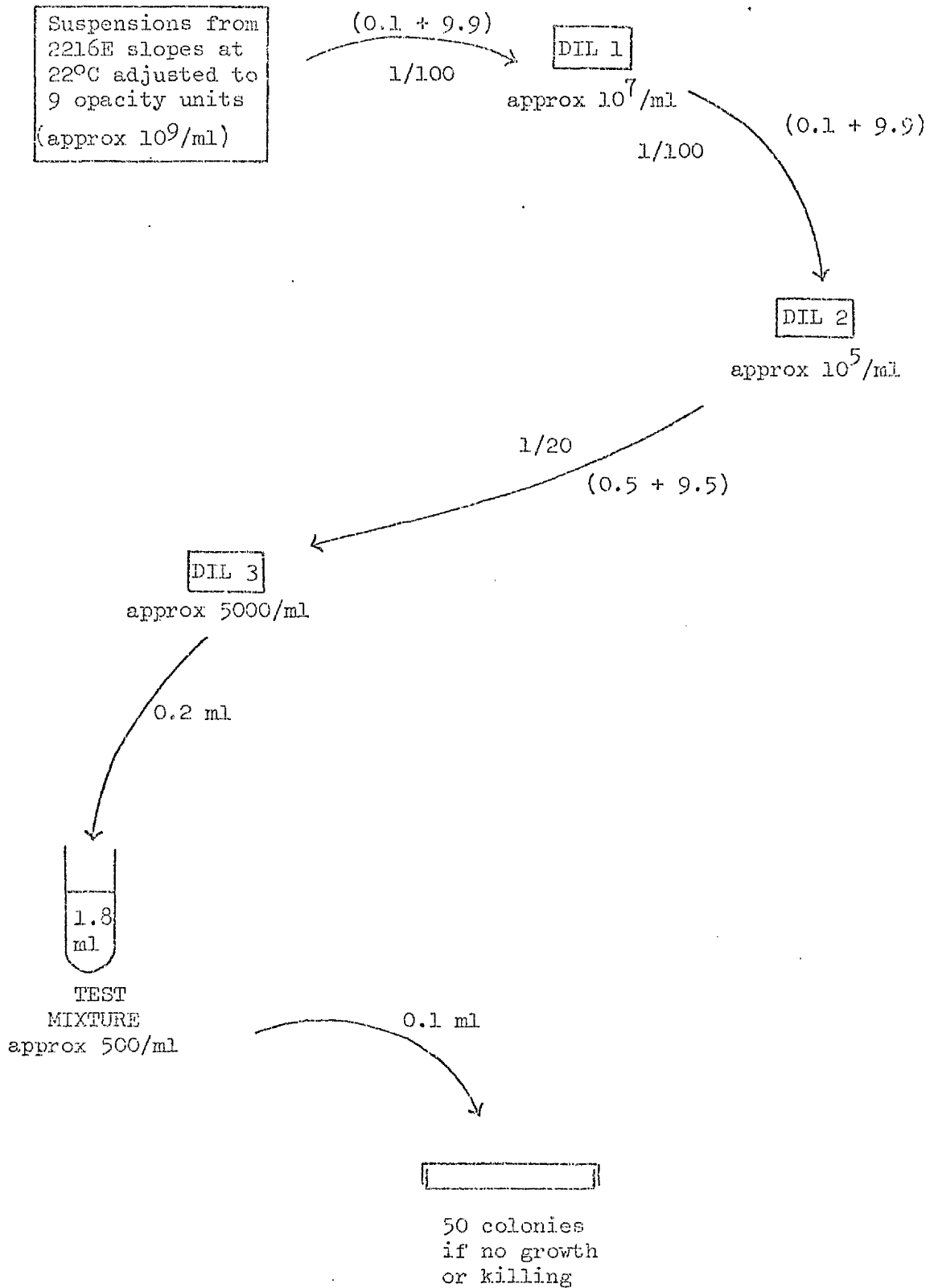
bench centrifuge. The supernatant was decanted into another sterile universal container which was placed in a boiling water bath for 15 min. S_0/B was allowed to cool before addition of bacteria. The test mixtures were incubated either at the ambient tank water temperature, $8^{\circ}C - 14^{\circ}C$, or at $10^{\circ}C$ in a thermostatically-controlled bath. The effect of temperature was observed by incubating the test mixtures at $4^{\circ}C$, $10^{\circ}C$ and $22^{\circ}C$.

To follow changes in bacterial count samples (0.2 ml) were taken at zero time (from S_0/B only), 4 h, 24 h and 48 h, after vigorous mixing by shaking the tube and repeatedly sucking and expelling the fluid with a 1 ml pipette, and 0.1 ml spread on dried 2216E agar plates using the tip of the pipette to spread the liquid.

After 2-days incubation at room temperature, the plates were examined for growth of strain 111 and the number of colonies counted and recorded. Accurate counts could be obtained with up to 350 colonies per plate because of the uniformity of size of the colonies. Beyond this number, counts were either estimated by eye or designated "almost confluent" (a.c.) when growth was not uniformly thick across the agar, or "confluent" (c) when there was thick uniform growth.

ANTIBACTERIAL TESTS WITH CELL-FREE EXTRACTS

Preparation of extracts and test procedure

Coelomic fluid was drained from urchins as previously

described into sterile 250 ml plastic centrifuge bottles.

Usually, the fluid from 2 urchins was pooled in one bottle but occasionally only one urchin was used per bottle. The fluid was transported on ice from Millport to the Microbiology Department, Garscube Estate, and within 1 - 2 h of collection was centrifuged at 5000 rpm (4000 g) for 40 min at $4^{\circ}C$ to sediment the coelomocytes (Figure 6). (This preliminary centrifugation was omitted when preparing unconcentrated cell extract). The supernatant was decanted into sterile bottles. The cells were then resuspended in 1/20 of the volume of supernatant S_0 and transferred to 50 ml centrifuge tubes which had been sterilised with boiling water. The cells were disrupted by sonicating on ice 3 times for 2 min with 30 sec intervals at full power on the MSE 100 watt Ultrasonic Disintegrator. To minimise contamination, the probe (end diameter 1/8") was immersed in sterile 3.2% saline which was sonicated for 30 sec before each run. After sonication, the fluid was inspected by naked eye for reduction in opacity and microscopically for absence of intact cells. The lysate was centrifuged at 10,000 rpm (12,000 g) for 50 min at $4^{\circ}C$ and the clear, orange-yellow supernatant decanted into sterile universal containers and designated S_1 or when concentrated, S_2. The process of sonication and centrifugation was repeated with the residue from S_2 resulting in an orange-yellow S_3 fraction.

The 3 fractions were stored either in the cold room at $4^{\circ}C$ or in the deep freeze at $-20^{\circ}C$.

Figure 6.   Flow diagram for processing of coelomic fluid
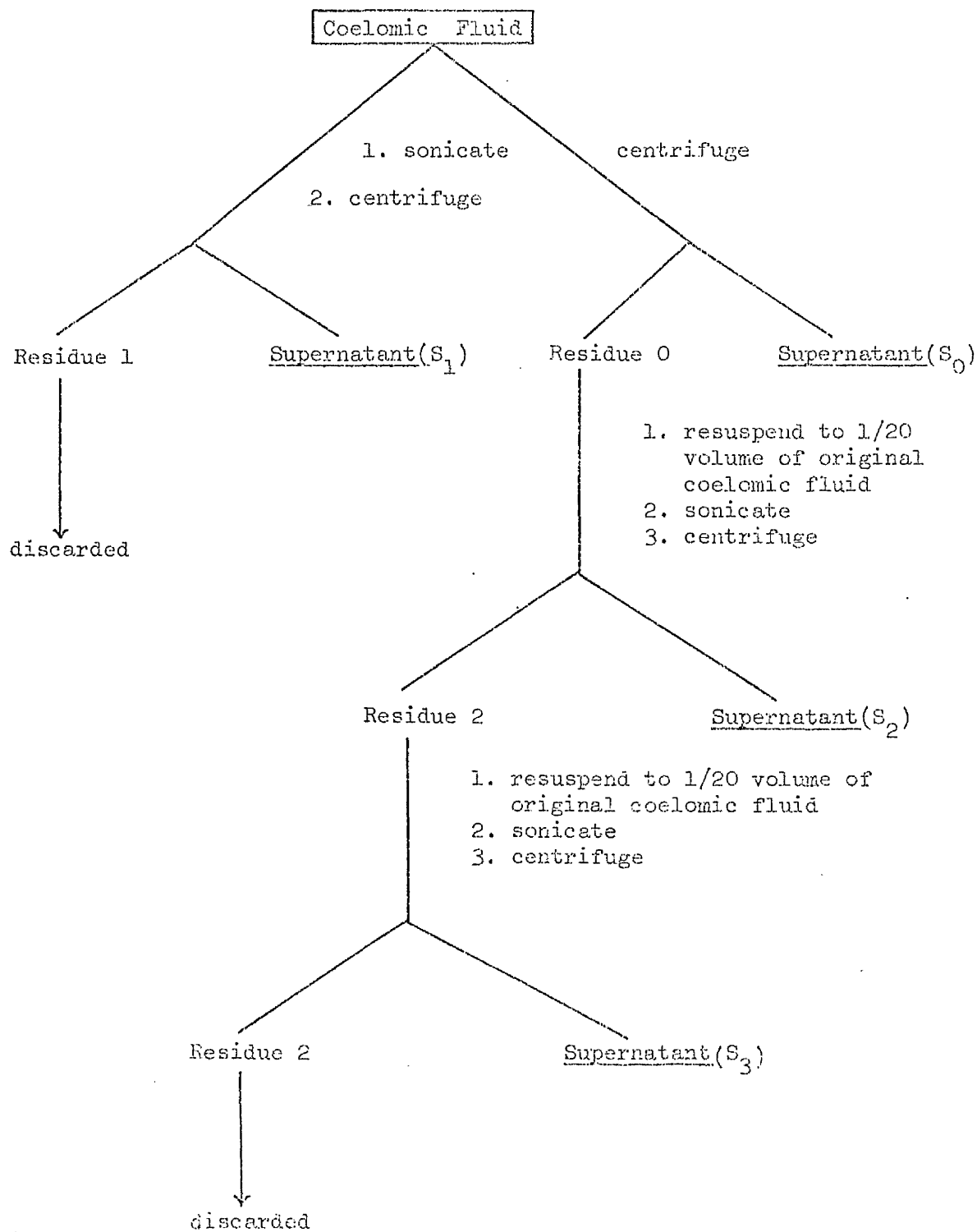
Dialysis of 5 ml of S_2 was carried out at $4^{\circ}C$ against 500 ml of supernatant S_0 for 36 h. Initially, no precautions were taken against contamination and so the dialysed fraction was centrifuged and filtered through a 20 mm Millipore filter before use. Latterly the visking tubing was first boiled in distilled water, S_0 was Millipore filtered before dialysis and a sterile flask was used. Sterilisation of S_2 after dialysis was not then necessary.

Heat-stability of S_2 was tested by placing 1.8 ml samples in 16 x 150 mm test tubes at room temperature $(20^{\circ}C)$, $37^{\circ}C$, $56^{\circ}C$ and $100^{\circ}C$ for 30 min. The samples were allowed to cool before testing.

Sonic extracts were tested for antibacterial activity in the same way as for fresh coelomic fluid. The inoculum (0.2 ml), containing 5×10^3 bacteria per ml was added to 1.8 ml of the test fluid which was kept on ice and after mixing transferred to a thermostatically-controlled water bath at $10^{\circ}C$. The control fluid used was S_0/B. Samples were taken at zero time (from S_0/B only), 4 h, 24 h and 48 h after vigorous mixing of the test mixture and 0.1 ml was spread on dried Marine 2216E agar plates and incubated at room temperature for 2 days before counting the colonies.

Estimation of protein in extracts

Protein estimations were made on S_2 by the method of Lowry et al. (1951) using crystalline bovine serum albumin (Sigma) in

distilled water as the reference standard.   To 1 ml of
the test sample, 5 ml of freshly prepared reagent C was added
and mixed well.   This was allowed to stand for 10 min before
addition of reagent D and immediate mixing.   After 30 min at
room temperature the absorbancy of the samples at 750 nm was read.
The blank was a distilled water sample treated in the same way as
the extract samples.

RESULTS

NORMAL BACTERIAL FLORA OF E. ESCULENTUS

Identification of isolates

To survey the normal bacterial flora of the sea urchin, samples were taken from the peristomial membrane, the gut and the coelomic fluid. The actual viable count from the peristomial membrane and the gut was usually of the order of 2.5×10^5 bacteria per peristomial membrane and 2×10^7 bacteria per 3 cm gut section. A total of 85 bacterial isolates from the 3 sites and, for comparison, 26 strains from sand and seawater were identified to generic level by the scheme of Shewan et al. (1960). Figure 7 shows the numbers of each genus identified as a percentage of the total from each site. Overall, the main genera found were Pseudomonas and Vibrio. From seawater/sand and the peristomial membrane there was a high percentage of Gram-positive bacteria but none was found in the gut or the coelomic fluid. From the gut and coelomic fluid the predominant genus was Vibrio followed by Pseudomonas, Aeromonas and Flavobacterium in that order. Pseudomonas predominated from seawater/sand and the peristomial membrane, but Vibrio, Aeromonas, Flavobacterium, Acinetobacter and Moraxella were also found to varying extents from both sites.

Bacterial count in coelomic fluid

In the early stages of this investigation, difficulty was experienced in obtaining sterile coelomic fluid for in vitro studies of antibacterial activity. Consequently, the practise was established of routinely performing a sterility test on the

Figure 7. Distribution of bacterial genera from different sites

of E. esculentus and from seawater and sand.

coelomic fluid of each urchin by spreading 0.1 ml of fluid on a 2216E agar plate. On most occasions, the tank temperature and the bacterial count per 0.1 ml of tank water were also monitored. Table 4 shows the bacterial count per 0.1 ml coelomic fluid expressed a) as the fraction of the total number of urchins having sterile fluid, b) as the fraction of the total number of urchins having confluent growth and c) as the median count for each sampling date over a period of 11 months. These counts are compared with the bacterial count of the tank water in which the urchins were kept. After 9th October 1975 urchins were no longer kept in a small tank but collected from various tanks in the Marine Station and so temperature measurements and bacterial counts were not taken.

A total of 188 urchins was studied. Of this total, 108 of the urchins gave sterile coelomic fluid samples while only 7 of the 188 urchins showed confluent growth. On only 3 occasions did the median count exceed 4 colonies per 0.1 ml.

The counts obtained from the tank water were invariably much higher than those from the coelomic fluid and showed considerable fluctuation within the range of 176 colonies per 0.1 ml to confluent growth. There did not appear to be any correlation between the counts of the tank water and either the median count of coelomic fluid or the fraction of samples showing confluent growth.

Table 4.   Bacterial counts per 0.1 ml of coelomic fluid and
aquarium tank water over a period of 11 months.

| Date | Number of urchins | | Median count | Count of tank water per 0.1 ml |
| | with sterile coelomic fluid | with confluent growth | per 0.1 ml | |
	total	total		
2nd April 1975	1/3	0/3	3	320
15th April 1975	2/3	0/3	0	500
8th May 1975	0/3	0/3	4	a.c.
15th May 1975	3/3	0/3	0	c.
22nd May 1975	3/5	0/5	0	c.
8th June 1975	1/6	0/6	2.5	a.c.
12th June 1975	4/6	0/6	0	a.c.
18th June 1975	2/4	0/4	0.5	a.c.
26th June 1975	2/4	0/4	0.5	262
3rd July 1975	5/7	1/7	0	297
10th July 1975	6/7	0/7	0	400
16th July 1975	3/7	0/7	3	600
22nd July 1975	4/7	1/7	0	a.c.
23rd July 1975	3/7	0/7	74	500
24th July 1975	1/7	5/7	c.	235
29th July 1975	5/7	0/7	0	300
4th Sept. 1975	4/7	0/7	0	500
18th Sept. 1975	4/5	0/5	0	176
9th Oct. 1975	7/7	0/7	0	296
27th Oct. 1975	5/7	0/7	0	
3rd Nov. 1975	3/7	0/7	0.5	
12th Nov. 1975	0/4	0/4	27.5	
19th Nov. 1975	4/6	0/6	0	
17th Dec. 1975	6/11	0/11	2	
13th Jan. 1976	5/12	0/12	4	
27th Jan. 1976	8/12	0/12	0	
12th Feb. 1976	11/12	0/12	0	
26th Feb. 1976	6/12	0/12	0.5	
Total	108/188	7/188		

Figure 8 compares the median count of coelomic fluid with the tank water temperature for each sampling date. The temperature which ranged from 8.2 - 14°C rose steeply from April to July and dropped gradually from August to November. The median counts of coelomic fluid showed 2 definite peaks in July and November with maxima of confluent growth and 27.5 colonies per 0.1 ml respectively. With these exceptions the counts fluctuated between 0 and 4 colonies per 0.1 ml.

Effect of maintenance of urchins

The effect of maintenance of urchins was examined by counting the number of colonies from 0.1 ml of coelomic fluid of a total of 24 urchins, of which 9 were kept for a minimum time of 1 month in a cage on the seabed, 6 in an aquarium tank and 9 taken from the seabed. Table 5 shows the counts per 0.1 ml of coelomic fluid. From caged urchins, the counts ranged from 0 to 39 colonies with a median count of 1. Urchins kept in the tank ranged from 0 to 4 colonies (median 0) and from the seabed the range was from 0 to 15 colonies (median 1).

CLEARANCE OF BACTERIA INJECTED INTO E. ESCULENTUS

Early in this investigation an experiment was done in which a mixture of 8 marine bacteria (prepared for a marine phage isolation exercise) was injected into 2 specimens of E. esculentus. The estimated dose was about 10^9 cells and clearance of these organisms was monitored by withdrawing a sample of coelomic fluid

Figure 8.   Median bacterial count per 0.1 ml of coelomic fluid

in relation to the aquarium tank water temperature

over a period of one year.

O————————O   median bacterial count per 0.1 ml

O — — — — — O   aquarium tank water temperature

BACTERIAL COUNT PER 0.1 ml

TEMPERATURE °C

DATE 1975 - 1976
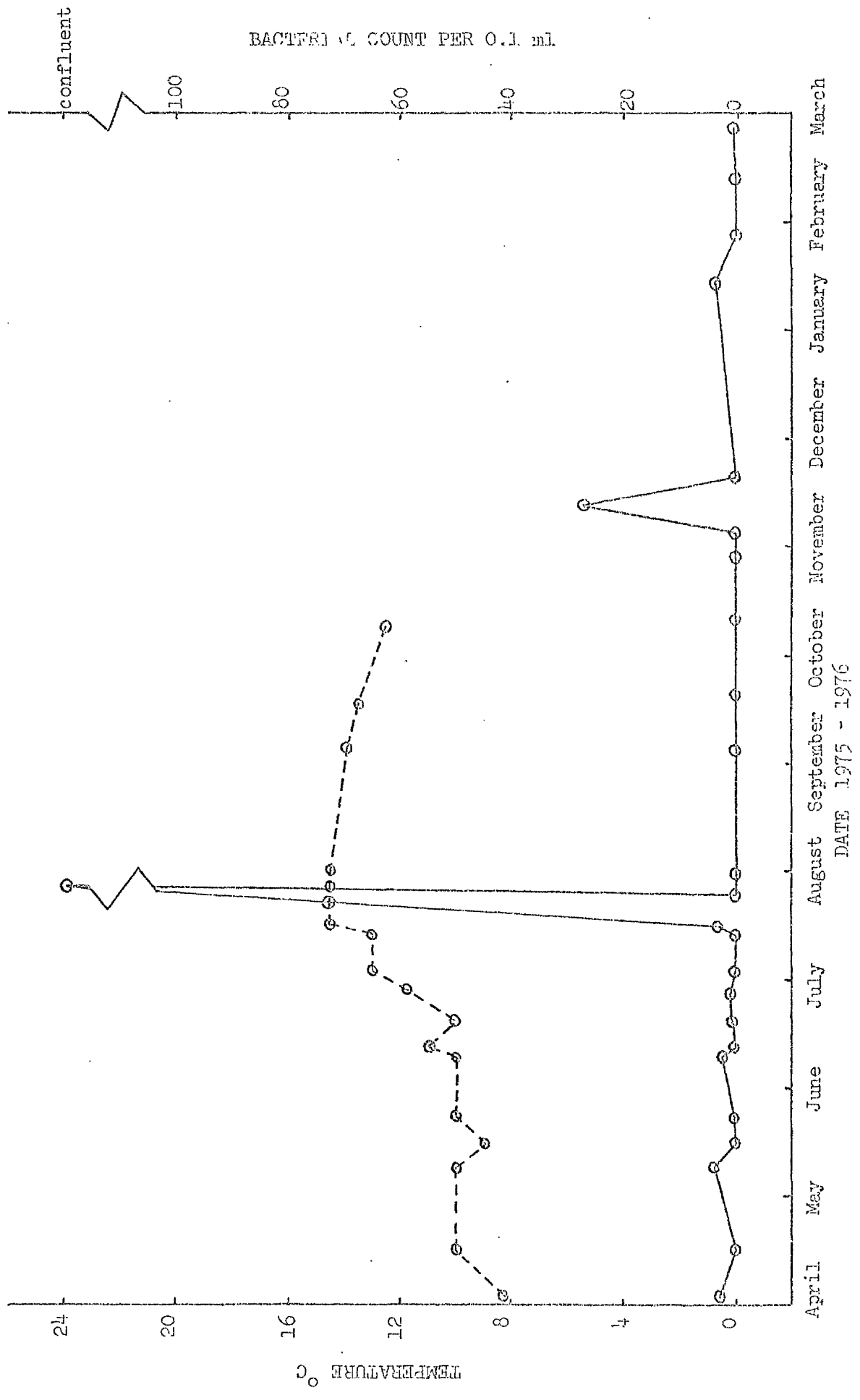
Table 5.   Bacterial count per 0.1 ml of coelomic fluid from individual urchins

maintained in different conditions.

Maintenance	Count per 0.1 ml coelomic fluid (individual urchins)										Median	Range
Cage on seabed	39	4	1	2	1	0	1	0	1	1	1	0 – 39
Aquarium tank	4	0	0	0	0	1	0				0	0 – 4
Freshly picked from seabed	1	15	13	0	3	1	2	0			1	0 – 15

at 2 days. The animals were also kept to observe possible ill effects of the inoculation. The samples of coelomic fluid withdrawn at 2 days showed no bacteria on the culture plates and the urchins remained healthy.

In a second experiment a mixture of 3 marine strains (from among the 8 used previously) was injected in a 0.5 ml dose of 0.5 x 10^7 bacteria per ml (equal numbers of each) into 5 specimens of E. esculentus. At the same time 0.5 ml of the mixed suspension was inoculated into a control vessel containing 100 ml of 1/20 dilution of 2216E medium in seawater. This vessel was intended to mimic the volume in the live urchins whose coelomic cavities were estimated to contain about 100 ml. The urchins and the control bottle were placed in the aquarium tank at 8^oC and samples taken for viable counting at 24 h and 48 h from each urchin and at zero time, 24 h and 48 h from the control bottle.

Table 6 shows that in the control bottle all 3 strains grew and increased in count from 1.5 - 2.2 x 10^4 initially to 10^6 - 2.8 x 10^7 at 48 h. Mean generation times of 4 h, 5 h and 7 h were calculated for the 3 strains growing in a 1/20 dilution of 2216E agar in seawater at 8^oC.

In contrast to the above, samples taken from the urchins showed that strain 111 was reduced in count to undetectable levels at 24 h except for one urchin from which a single colony was obtained. The other 2 strains gave similar results and the 48 h

Table 6.　　Growth of 3 marine bacterial strains at 22°C

in a 1/20 dilution of 2216E agar in seawater

Bacterial strain	Viable count per ml at		
	0	24 h	48 h
111	1.7×10^4	1.2×10^6	2.8×10^7
230874/1	1.5×10^4	5.6×10^5	5.0×10^6
091274/2	2.2×10^4	2.5×10^5	1.0×10^6
Total count per ml	5.4×10^4	2.0×10^6	3.4×10^7

samples were likewise free from any of the injected bacteria.
This experiment showed that E. esculentus has a powerful bacterial
clearance mechanism in the coelomic cavity.

ANTIBACTERIAL ACTIVITY OF COELOMIC FLUID IN VITRO

Results of a typical experiment

Simple tests for studying antibacterial activity of
coelomic fluid in vitro were carried out by adding 0.2 ml of
bacterial strain 111 to 1.8 ml of freshly withdrawn coelomic fluid
and to S_0/B as a control.   Viable counts were made at time 0, 4 h,
24 h and 48 h, and the number of colonies per 0.1 ml recorded
after 2 days incubation of the plates, as in Table 7.

The viable count in the control fell slightly at 4 h but
increased rapidly at 24 h to almost confluent growth and at 48 h
to confluent growth.   This result, with very few exceptions,
was typical of that obtained for the growth of strain 111 in S_0/B.
In the coelomic fluid samples, the count of strain 111 generally
dropped, sometimes dramatically, and was usually below the count
from S_0/B at 4 h.   The count was usually still lower at 24 h and
frequently at 48 h, there were fewer than 2 colonies per 0.1 ml.
Sometimes however, as with Echinus No. 6 in Table 7, the count
decreased at 4 h and 24 h but at 48 h there was an increase.

The survival index

As each experiment had a different bacterial count at

Table 7. A typical experiment showing the antibacterial

activity of coelomic fluid from individual

urchins

Echinus	Number of colonies per 0.1 ml at			
No.	0	4 h	24 h	48 h
1		46	1	0
2		26	2	1
3		35	4	0
4		2	1	2
5		17	4	1
6		32	3	22
S_C/B	46	39	a.c.	c.

0 time, it was necessary in order to compare separate experiments to standardise the results. This was done using the Survival Index which is defined as:

$$\text{Survival index} = \frac{\text{viable count at time}_t}{\text{viable count at time}_0} \times 100$$

Growth of strain 111 in control fluid

As mentioned above, strain 111 had a very characteristic growth pattern in the control fluid S_0/B. Table 8 summarises the results of 45 experiments with the count expressed as the survival index within the ranges shown. It can be seen that variation in index occurred only at 4 h. Only 8 indexes were less than 60. In the range 0 - 79 there were 22 indexes and between 80 and 159, 14 indexes. Only 1 index was greater than 160. At 24 h and 48 h all the indexes were greater than 160.

Variation in activity between urchins

Between the months of May and November 1975 antibacterial tests were carried out to discover whether there was a variation in activity between urchins. Antibacterial tests were performed in the usual manner in vitro with a total of 35 urchins.

Figure 9 is a series of three scatter diagrams of the date of the experiment plotted against the survival index at 4 h, 24 h and 48 h. Each point represents an individual urchin. At 4 h, the activity was fairly constant with index values between 0 and 42 over the 6 month period. There was no correlation between

Table 8. Frequency-distribution of the survival indexes

of strain no. 111 in S_0/B control fluid at $10^\circ C$

Sampling Time (h)	No. of tests with index in range			
	0 – 59	60 – 79	80 – 159	> 160
4	8	22	14	1
24	0	0	0	45
48	0	0	0	45

Figure 9.   Relationship of the sampling date to the survival

indexes at 4 h, 24 h and 48 h.

Samples of coelomic fluid were taken from a total

of 35 urchins over a period of 6 months and the

sampling date plotted against the survival indexes

of strain no. 111 in coelomic fluid from

individual urchins.

SURVIVAL INDEX

DATE 1975
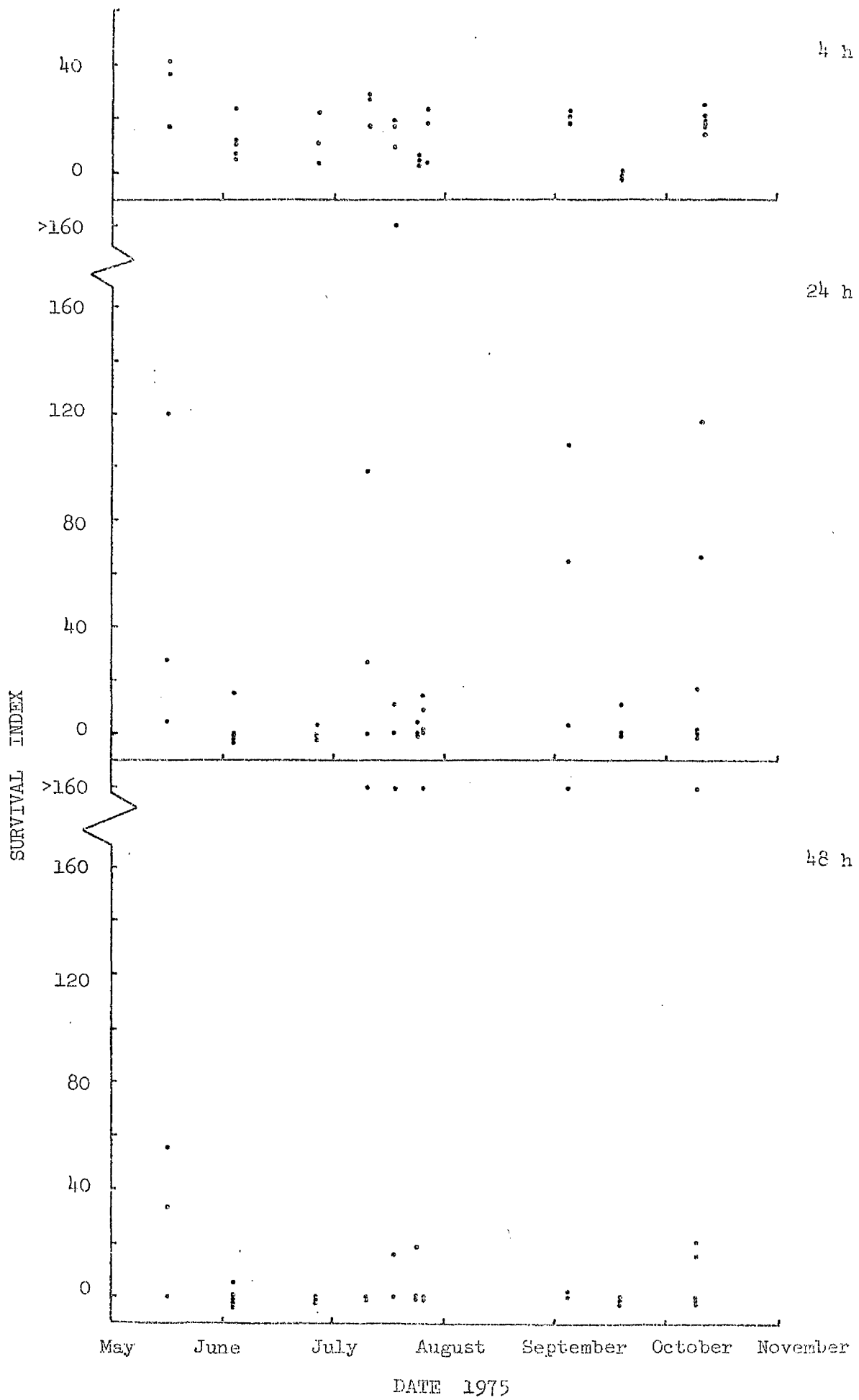
the date and the survival index at any sampling time. At 48 h
however, all the indexes were either less than 60 or greater than 160.

A comparison in the form of scatter diagrams of the
initial count at time 0 and the survival index at 4 h, 24 h and
48 h is given in Figure 10. The initial count ranged from 46 - 244
bacteria per 0.1 ml. For all values of initial count, the
survival indexes at 4 h for the urchins were dispersed irregularly
between the values 0 - 42 and there was no apparent correlation.
At 24 h and 48 h there was an irregular distribution with again no
apparent correlation.

It was considered of interest to find if there was any
correlation between survival indexes at the different sampling
times. Figures 11, 12 and 13 show scatter diagrams of the 4 h
indexes against the 24 h indexes, the 4 h indexes against the 48 h
indexes and the 24 h indexes against the 48 h indexes. These
showed that within the range 0 - 42 of the 4 h indexes, the 24 h
and 48 h indexes were extremely variable and that there was no
correlation. Comparison of 24 h indexes and 48 h indexes showed
no obvious pattern.

Variation in activity in serial samples of coelomic fluid

Since it was shown that the antibacterial activity of
coelomic fluid samples varied between individual urchins, it was
of interest to find if activity also varied within an individual
urchin over a period of time or if it remained at a constant level
in each urchin. A total of 15 urchins were tested for this

Figure 10.   Relationship between the initial bacterial count
             and the survival indexes at 4 h, 24 h and 48 h.

             The initial count (per 0.1 ml) is plotted against
             the survival indexes of strain no. 111 in
             coelomic fluid samples from individual urchins.

Figure 11.   Relationship between the survival indexes of
             strain no. 111 in coelomic fluid samples at
             4 h and 24 h.

Figure 12.   <u>Relationship between the survival indexes</u>

<u>of strain no. 111 in coelomic fluid samples</u>
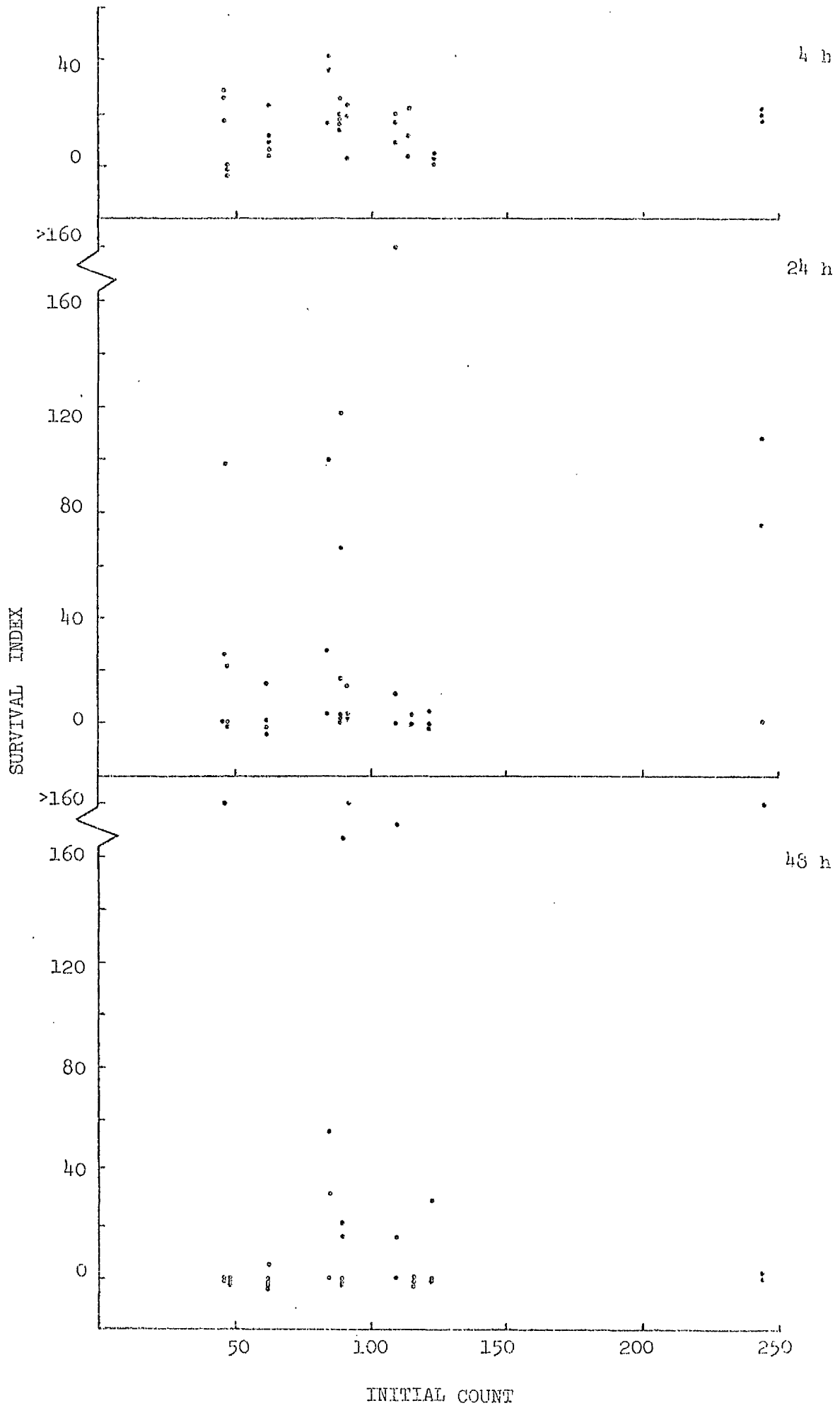
<u>at 4 h and 48 h.</u>

Figure 13.   Relationship between the survival indexes

of strain no. 111 in coelomic fluid samples

at 24 h and 48 h.
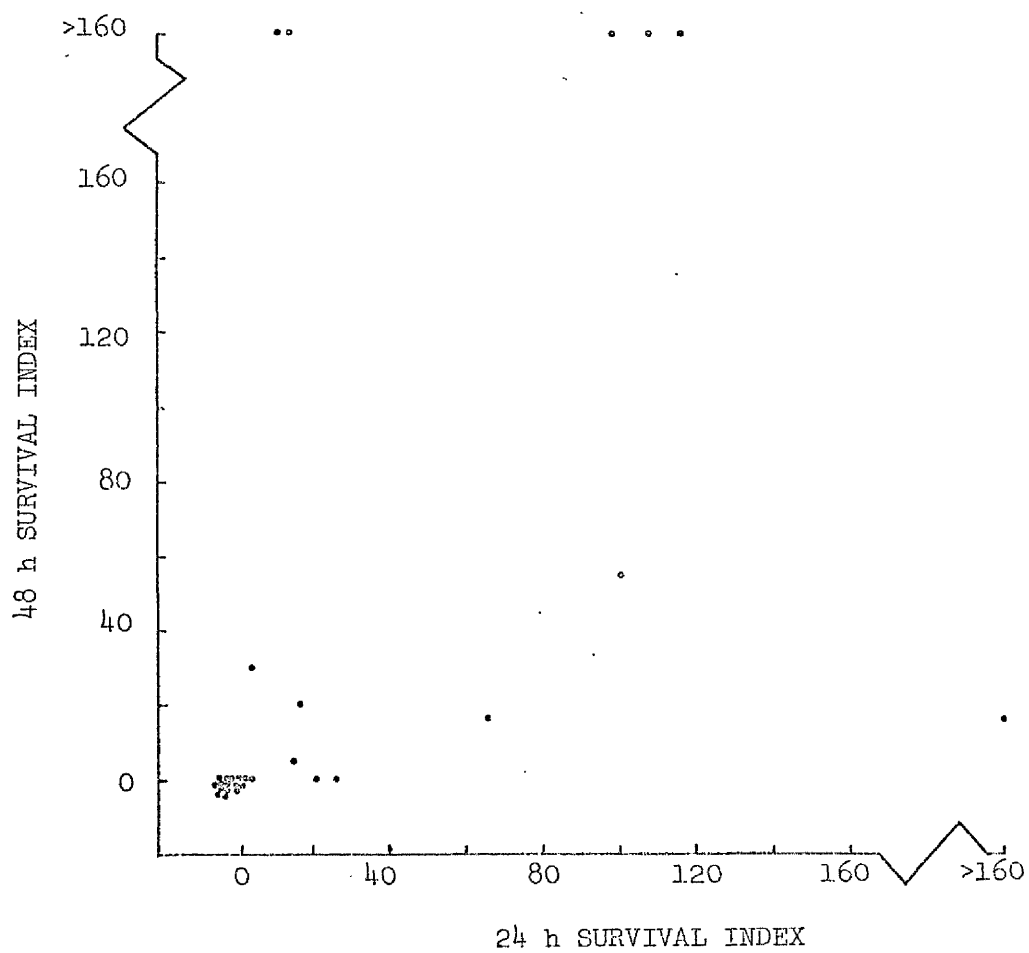
variation. Results of antibacterial activity were obtained from new urchins and at 1 - 14 days later, a second sample of coelomic fluid was examined for antibacterial activity. The results of this study are shown in Figures 14, 15 and 16 in the form of scatter diagrams representing the survival index of the first sampling plotted against that of the second sampling for each urchin at 4 h, 24 h and 48 h.

At 4 h, the coelomic fluid from the first sampling seemed definitely superior and showed little variation with index values between 3 and 37 but the index values of the second sampling were much more variable and showed no correlation with the first sampling. At 24 h this situation was reversed, with the exception of one urchin, and between the index values of 0 and 21 for the second sampling the first samples were quite variable. At 48 h however there was an irregular distribution of points although most were at the origin and no correlation could be seen.

Effect of incubation temperature

To test the effect of the incubation temperature on the antibacterial activity of coelomic fluid, test mixtures were incubated at $4^{\circ}C$, $10^{\circ}C$ and $22^{\circ}C$. Unfortunately, due to the bimodal distribution of indexes especially at 48 h, a simple graph showing the average indexes would give a false representation of the results. Figure 17, therefore, presents the survival indexes of 2 tests with a total of 6 urchins in the form of a histogram.

Figure 14.   <u>Relationship of the survival indexes of</u>

<u>strain no. 111 at 4 h in serial samples of</u>

<u>coelomic fluid.</u>

Two samples of coelomic fluid were taken from

individual urchins and the survival indexes of

the test organism from the first sampling were

plotted against the survival indexes of the second
sampling.
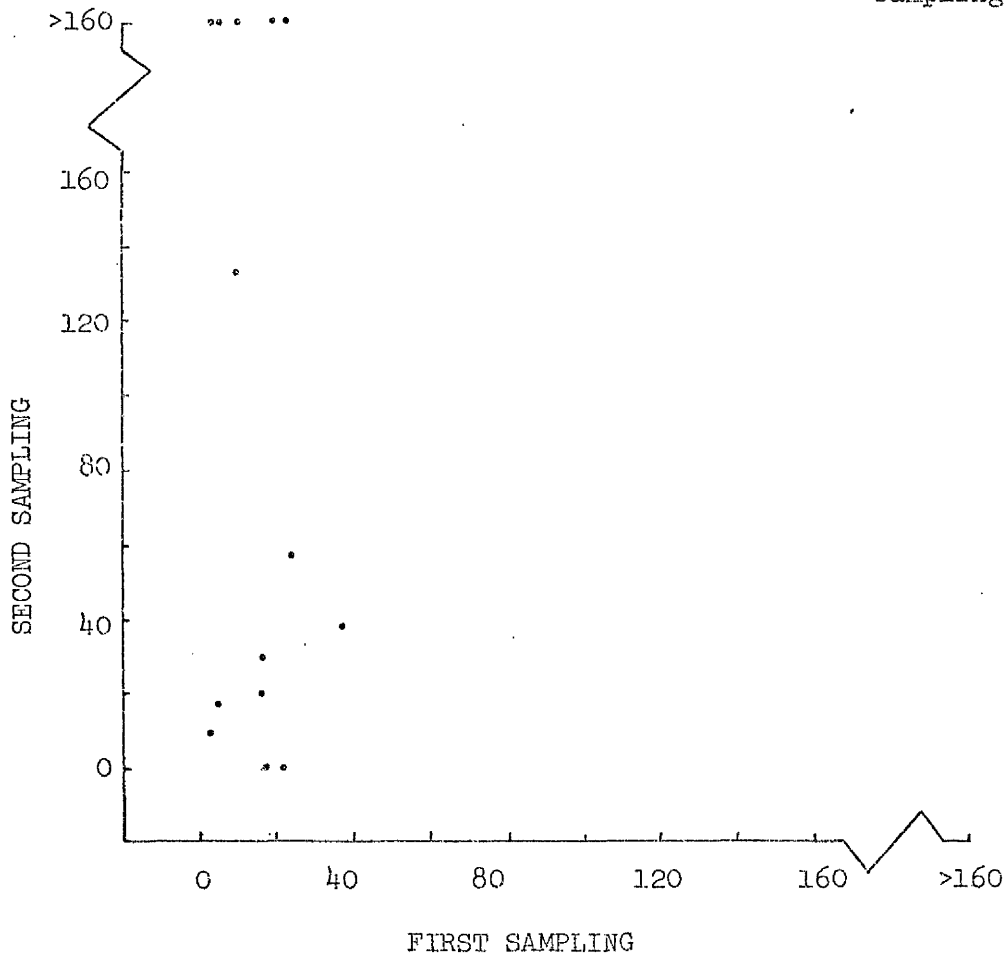
Figure 15.   Relationship of the survival indexes of strain

no. 111 at 24 h in serial samples of coelomic fluid.

Two samples of coelomic fluid were taken from

individual urchins and the survival indexes of the

test organism from the first sampling were plotted

against the survival indexes of the second sampling.
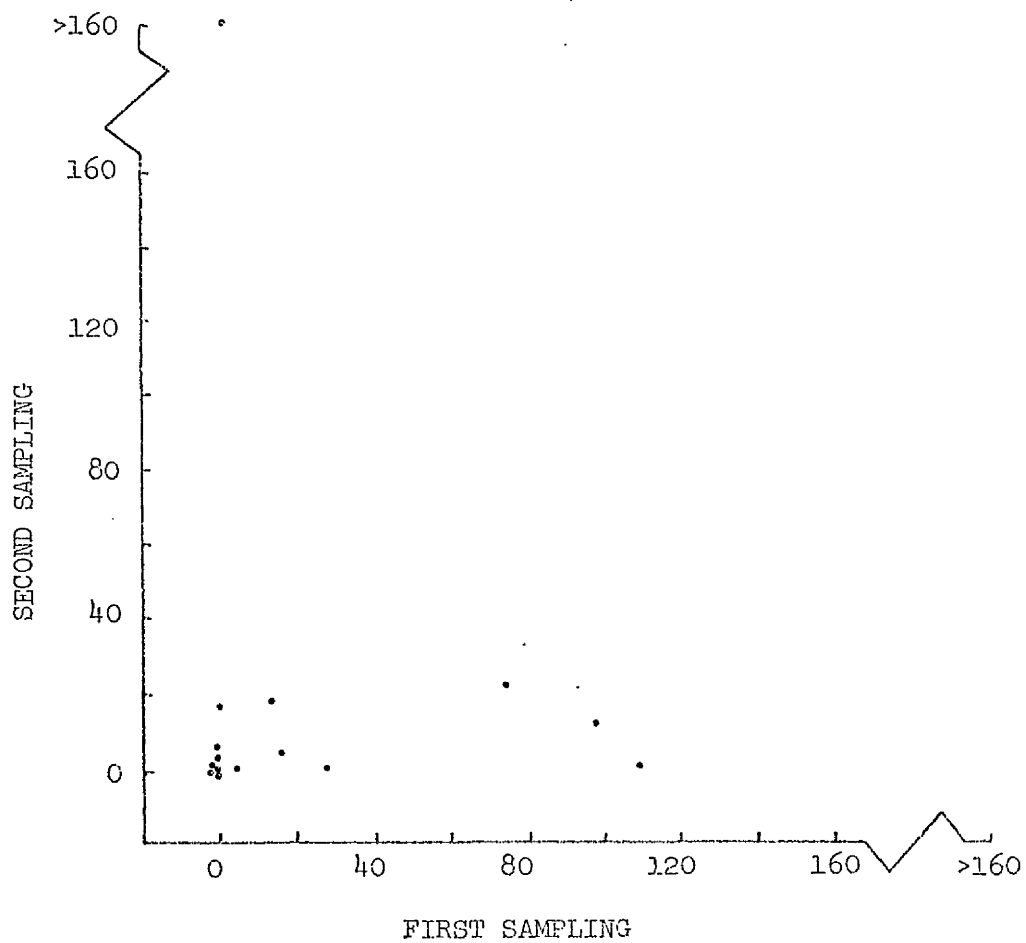
Figure 16.   Relationship of the survival indexes of strain

no. 111 at 48 h in serial samples of coelomic fluid.

Two samples of coelomic fluid were taken from

individual urchins and the survival indexes of

the test organism from the first sampling were

plotted against the survival indexes of the

second sampling.
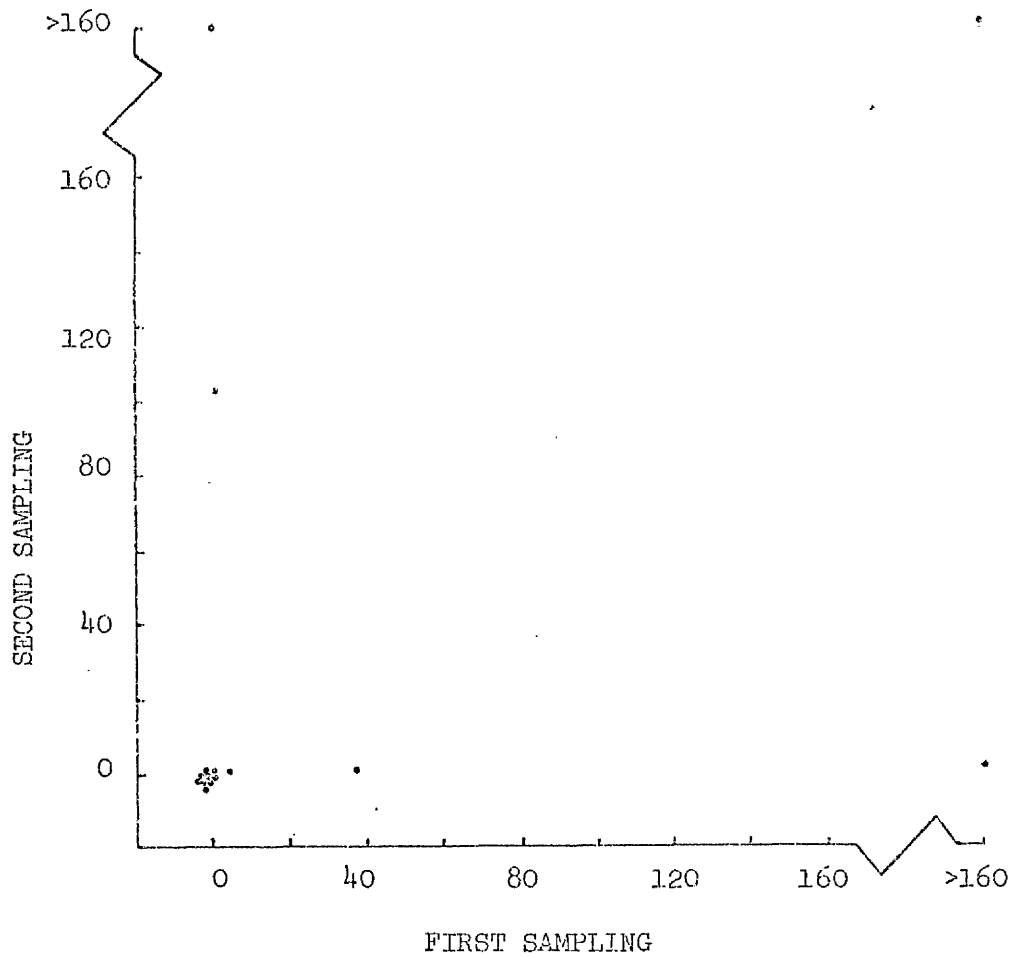
Figure 17. Frequency-distributions of survival indexes of
strain no. 111 in coelomic fluid at 4 h, 24 h
and 48 h at different incubation temperatures.
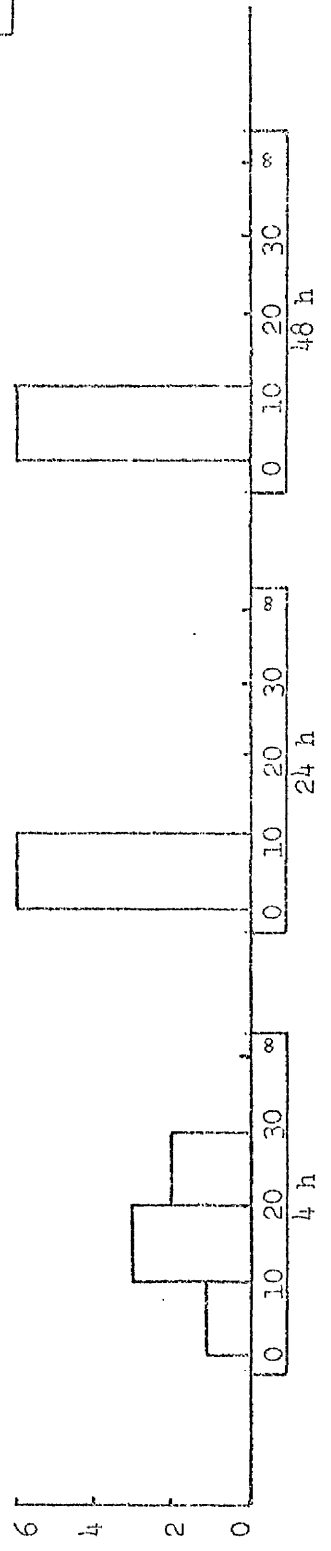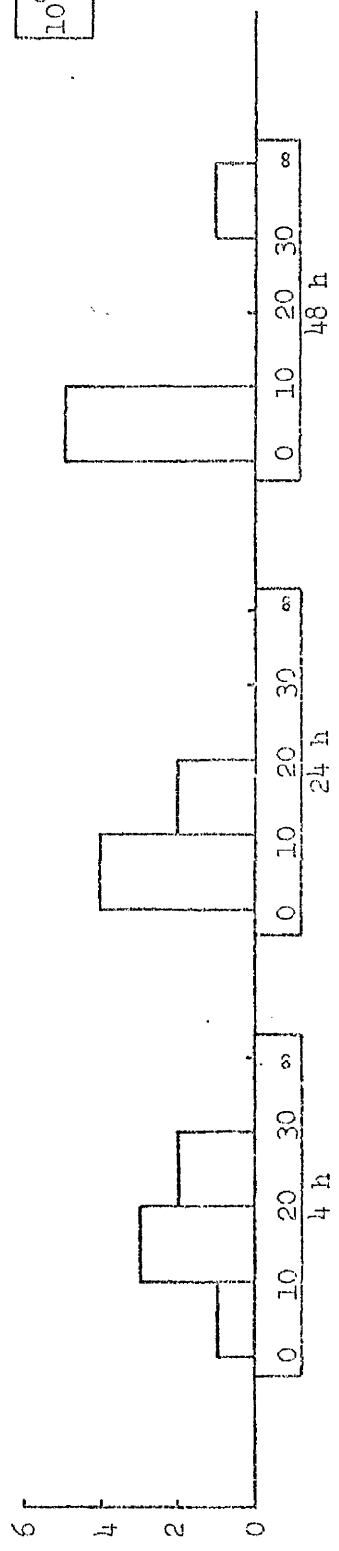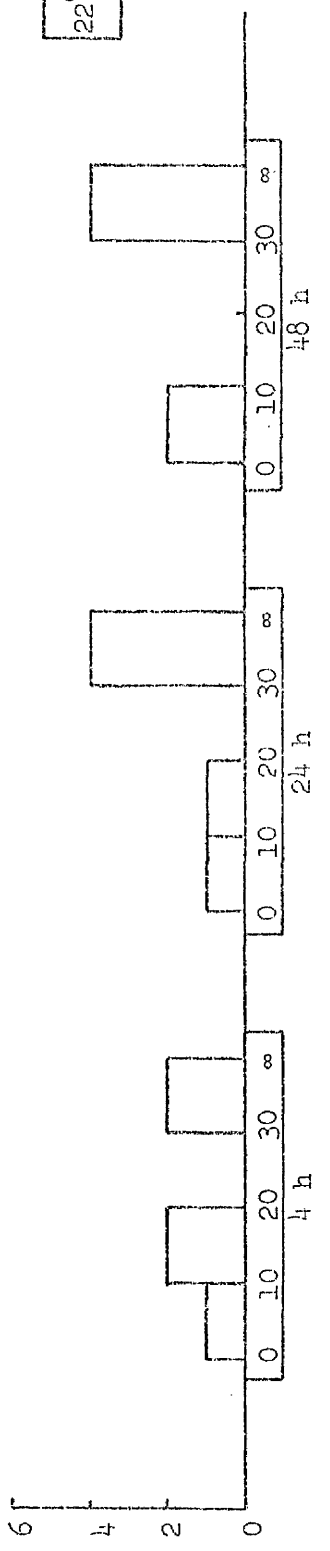
At 4 h the results for incubation at 4°C and 10°C were similar but at 22°C the antibacterial effect was reduced. At 24 h all the samples incubated at 4°C had survival indexes of less than 10 while at 10°C the survival indexes ranged from 0 - 19. Incubation at 22°C again showed a detrimental effect with two-thirds of the survival indexes greater than 30. At 48 h, all the indexes at 4°C were within the range 0 - 9; 5 of the indexes at 10°C were within the range 0 - 9 with only one index greater than 30 while at 22°C only 2 indexes were in the range 0 - 9 and 4 were greater than 30. Incubation at 4°C therefore appeared to produce the highest activity and incubation at 22°C, the lowest.

The effect of temperature on the growth of strain 111 in S_0/B is shown in Table 9. Each survival index represents the average of the 2 tests. At 4°C there was a marked lag phase of 24 h before there was appreciable growth. At 10°C and 22°C growth was apparent after 4 h, with growth at 22°C faster than at 10°C.

Effect of pre-clotting of coelomocytes

Coelomocytes form clots within a few seconds of removal of fluid from an urchin. The antibacterial activity of coelomic fluid in which the coelomocytes were allowed to clot for 2 h is compared with the activity of the corresponding freshly drawn fluid at 10°C in Figure 18. The survival indexes are given in the form of a histogram representing a total of 21 urchins. A

Table 9.   Survival indexes of strain no. 111 in S_O/B control fluid at 4^OC, 10^OC and 22^OC.

Incubation	Average survival index at		
Temperature ^{O}C	4 h	24 h	48 h
4	76	59	a.c.
10	86	a.c.	c.
22	78	c.	c.

Figure 18.   Frequency-distributions of survival indexes of

strain no. 111 in fresh coelomic fluid and pre-

clotted coelomic fluid at different times.
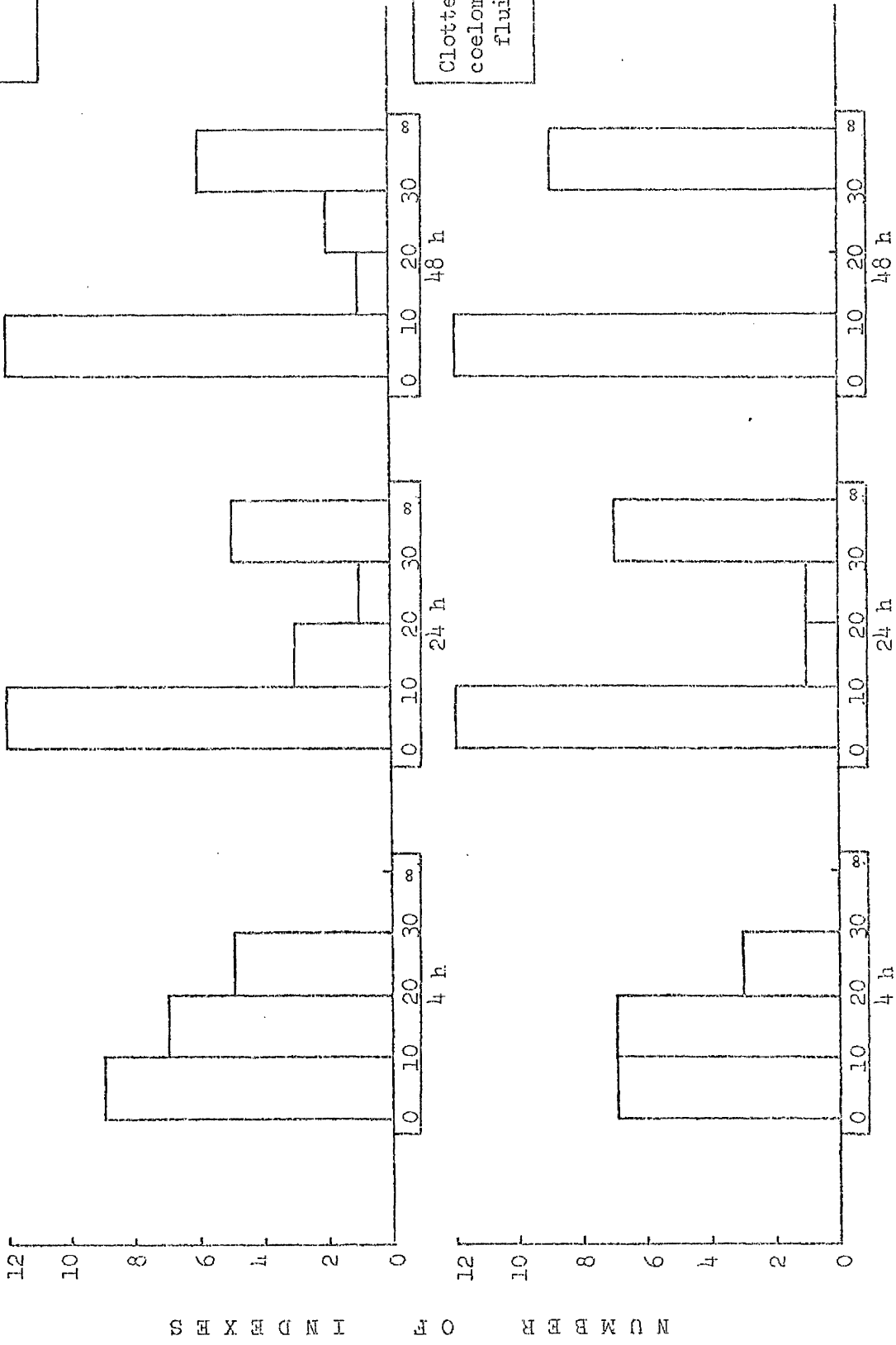
variable survival index was seen throughout in both the fresh
and pre-clotted coelomic fluid but the pre-clotted coelomic fluid
showed slightly less activity with more indexes falling in the
range 30 to infinity than with fresh coelomic fluid. This effect
was particularly pronounced at 48 h.

Comparison of activity of coelomic fluid and cell-free supernatant

From a total of 24 urchins samples of coelomic fluid
and its cell-free supernatant S_0 were inoculated with strain 111.
The survival indexes of these experiments are shown as a histogram
in Figure 19. It is clear that S_0 was much less active than
coelomic fluid; at 4 h coelomic fluid indexes were all in the
range 0 - 39 and the supernatant indexes, with one exception, in
the range 40 to infinity. At 24 h the majority of the supernatant
indexes ranged from 80 to infinity whereas most of the coelomic
fluid indexes were in the range 0 - 19 with only 3 in the range
80 to infinity. At 48 h, all the supernatant indexes were in the
range 80 to infinity compared with only 5 coelomic fluid indexes
and it appeared that the supernatant of coelomic fluid acted as a
growth medium for strain 111.

ANTIBACTERIAL ACTIVITY OF COELOMOCYTE EXTRACT

Comparison of fresh coelomic fluid and unconcentrated
coelomocyte extract

Having found that the antibacterial activity in
coelomic fluid was associated with the coelomocytes, it was decided

Figure 19. Frequency-distributions of survival indexes of strain no. 111 in coelomic fluid and supernatant S_0 of coelomic fluid at 4 h, 24 h and 48 h.

to see if activity would still be present in a sonic extract of coelomocytes or if it was necessary to have whole cells for activity.

A coelomocyte lysate was prepared by sonication and a clear extract designated S_1 obtained by high speed centrifugation. The antibacterial activity of this fluid was compared with that of fresh coelomic fluid taken from the same urchin. A total of 12 urchins were used for this test. Figure 20 shows a histogram of the survival indexes in coelomic fluid and S_1. At 4 h both the coelomic fluid and S_1 samples showed considerable variation in activity but S_1 appeared to be slightly more active than coelomic fluid with a higher proportion of indexes in the range 0 - 9. The 24 h indexes showed a more marked difference with S_1 appearing considerably more active than coelomic fluid. At 48 h however the antibacterial activities were similar.

Figure 21 presents the survival indexes in coelomic fluid plotted against those in S_1 at 4 h, 24 h and 48 h. Each point represents results from one urchin. At 4 h there seemed to be a positive correlation between activity in coelomic fluid and activity in S_1. Results at 24 h showed no correlation, with the indexes of S_1 constant within the range 0 - 19 while the indexes of coelomic fluid varied considerably from 0 to greater than 160. The indexes at 48 h showed no correlation and no definite distribution pattern.

Comparison of coelomic fluid and coelomocyte extract
concentrated 20 times

The antibacterial activity of a concentrated

Figure 20.   Frequency-distributions of survival indexes of

strain no. 111 in coelomic fluid and

unconcentrated extract S_1 at 4 h, 24 h and 48 h.

Coelomic
fluid
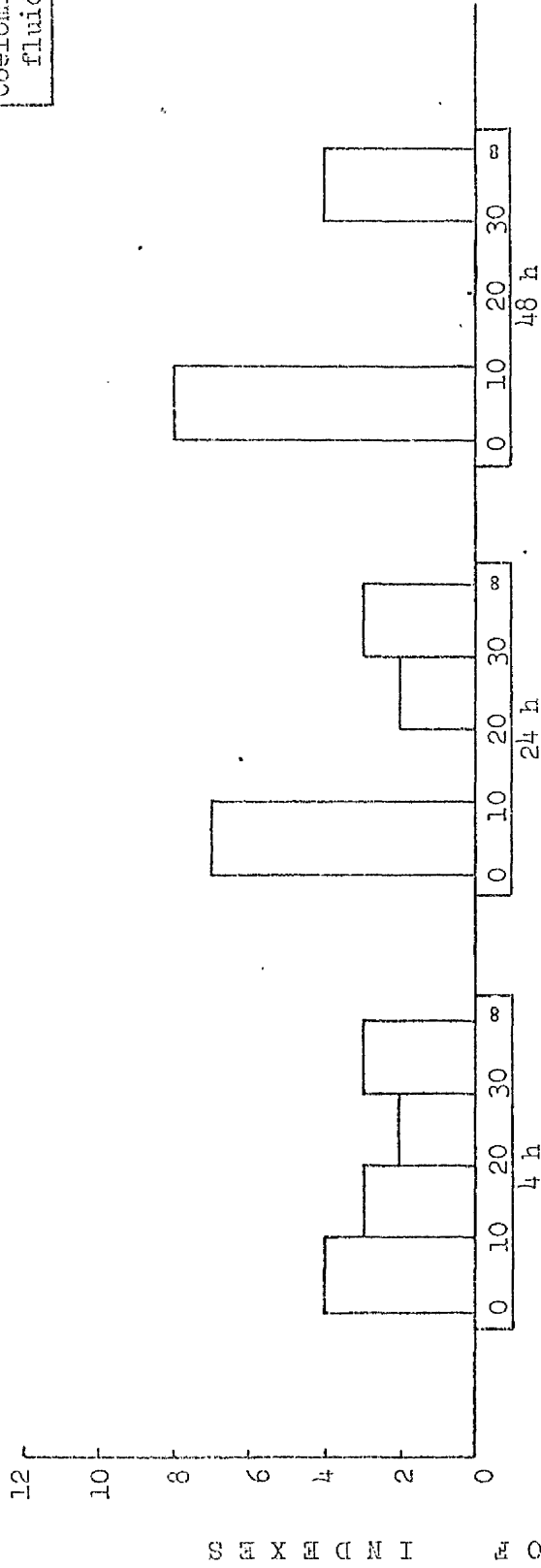
Unconcentrated
extract S₁

S U R V I V A L   I N D E X

N U M B E R   O F   I N D E X E S

0   10   20   30   8
4 h

0   10   20   30   8
24 h

0   10   20   30   8
48 h

12
10
8
6
4
2
0

12
10
8
6
4
2
0
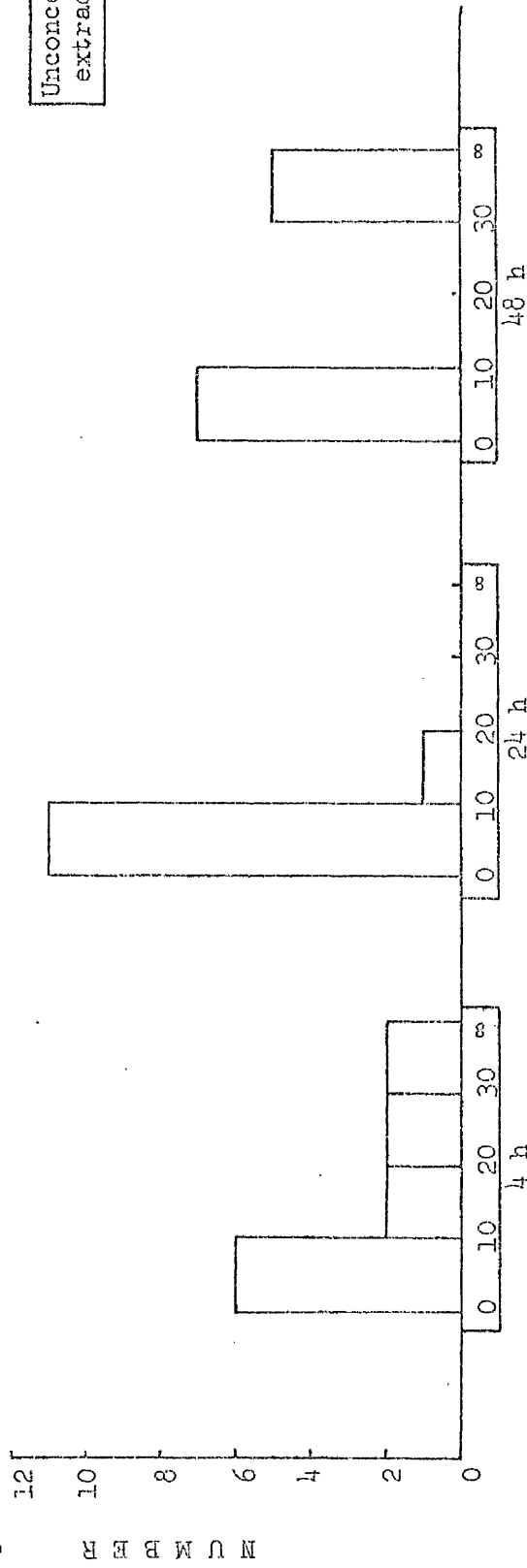
Figure 21.   Relationship between the survival indexes of

strain no. 111 in coelomic fluid and

unconcentrated extract S_1 at 4 h, 24 h and 48 h.

40

4 h

>160

160

24 h

120

80

40

S$_1$ SURVIVAL INDEX

0

>160

1.60

48 h

120

80

40

0

0   40   80   120   160 >160

COELOMIC FLUID SURVIVAL INDEX
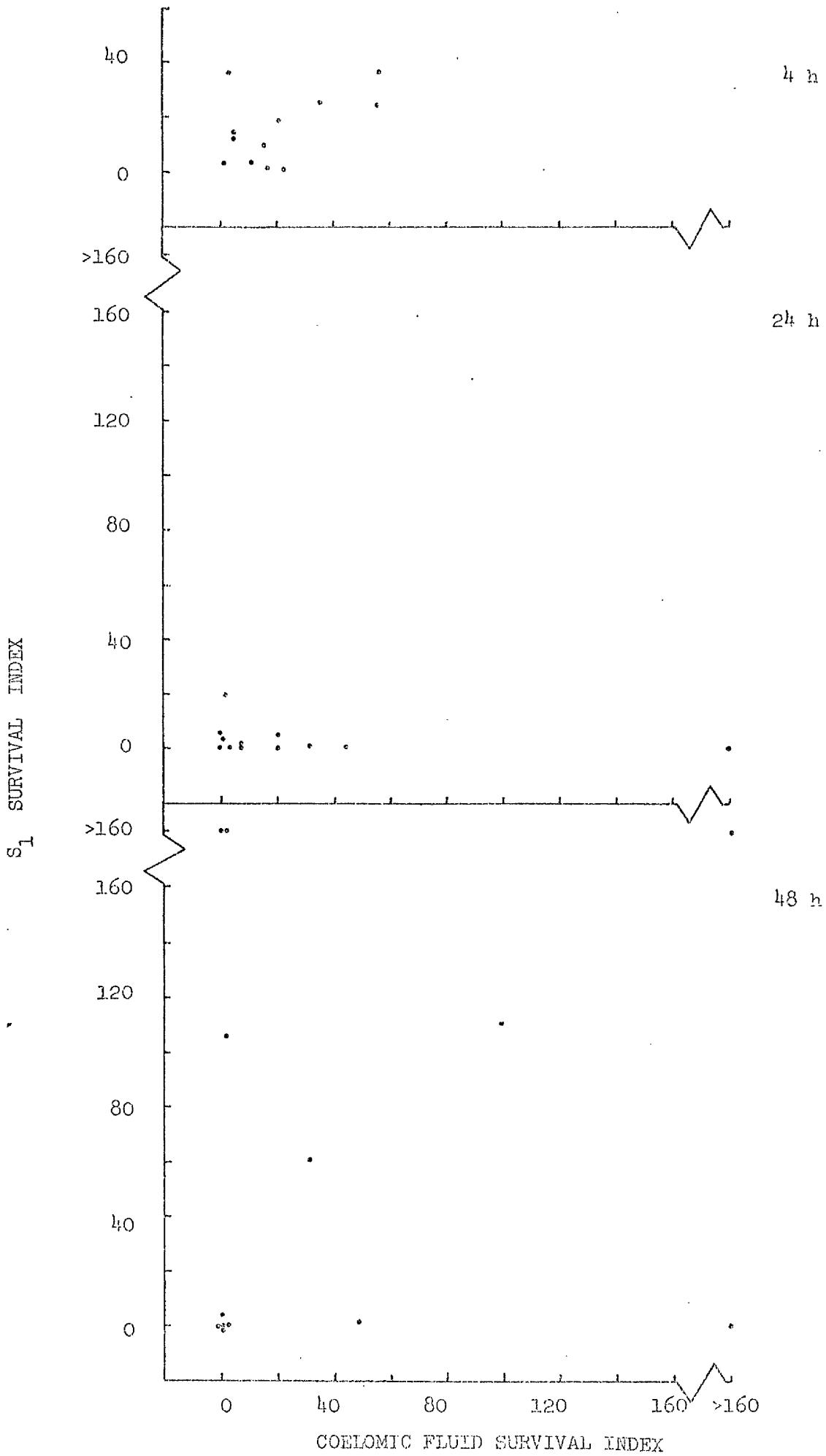
coelomocyte extract, S_2, in which the cells had been suspended in 1/20 the volume of supernatant S_0 prior to sonication was compared with that of the corresponding coelomic fluid from 12 urchins. The survival indexes of these tests are presented as a histogram in Figure 22. The indexes in both coelomic fluid and S_2 at 4 h and 24 h showed variation in activity but it can be seen that the coelomic fluid was slightly more active than S_2. At 48 h coelomic fluid definitely appeared to be more active than S_2.

Figures 23, 24 and 25 show scatter diagrams of the survival indexes in coelomic fluid plotted against those in S_2, each point representing one urchin. At 4 h there appeared to be no correlation in activity. The indexes in coelomic fluid ranged from 3 - 50 while those in S_2 ranged from 6 - 113. Indexes at 24 h showed no correlation but S_2 was more variable than coelomic fluid. Results at 48 h showed that S_2 was very variable; activity ranged from 0 to greater than 160 while indexes of coelomic fluid with one exception ranged from 0 - 1.

Table 10 compares the survival indexes in 6 samples of S_2 and the protein concentration per ml of the concentrated extract (before addition of bacteria) as determined by the Lowry method. The protein concentration varied from 1.94 - 3.86 mg/ml but did not appear to be related to the antibacterial activity of the extract.

Serial extracts of coelomocytes

From the residue obtained in the production of extract

83

Figure 22.   Frequency-distributions of survival indexes of

strain no. 111 in coelomic fluid and

concentrated extract S_2 at 4 h, 24 h and 48 h.
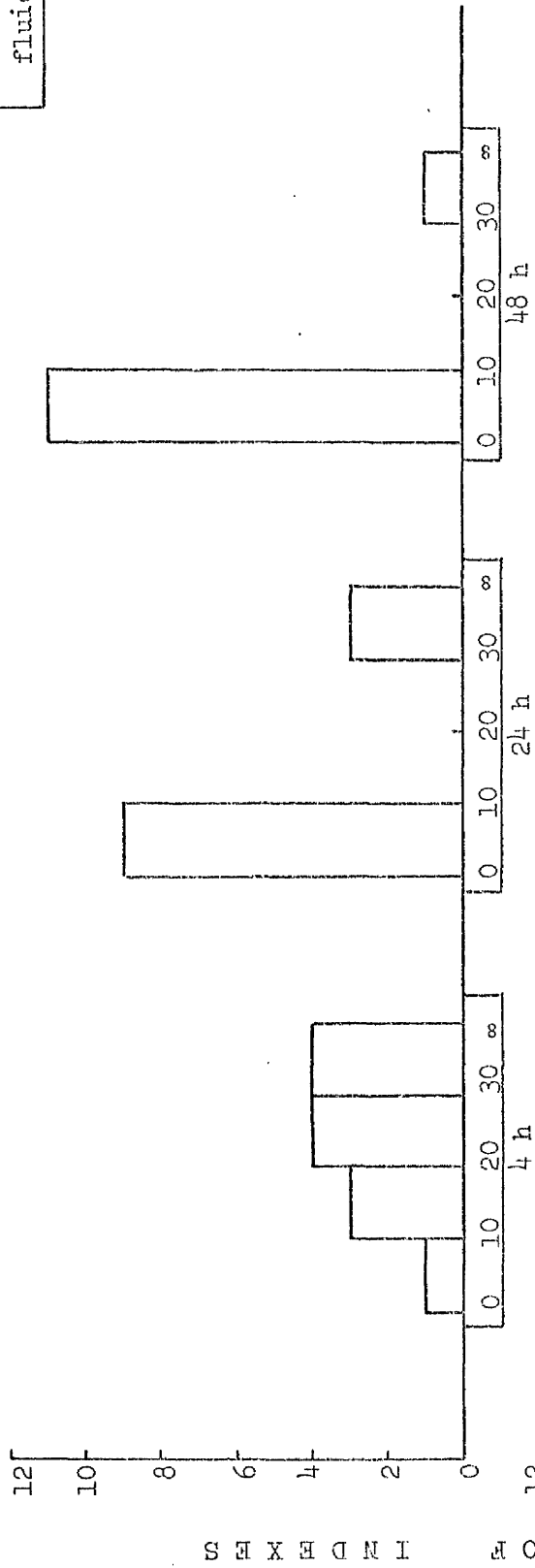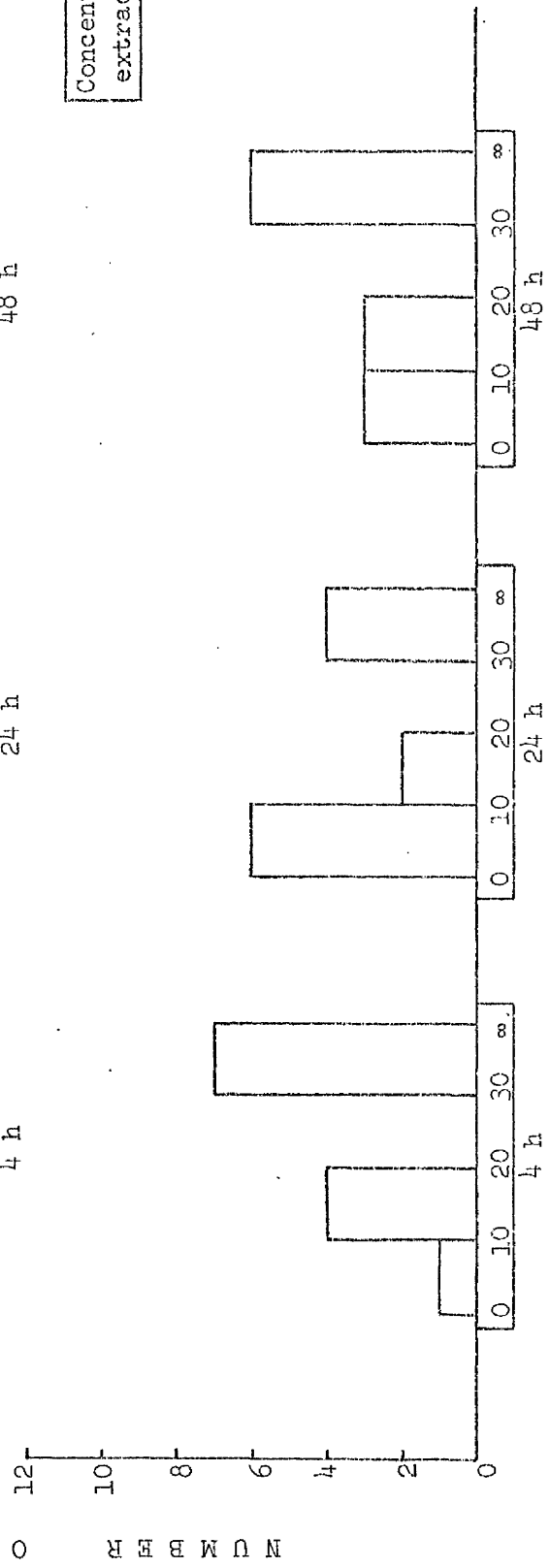
Figure 23. Relationship between the survival
indexes of strain no. 111 in
coelomic fluid and concentrated
extract S_2 at 4 h.
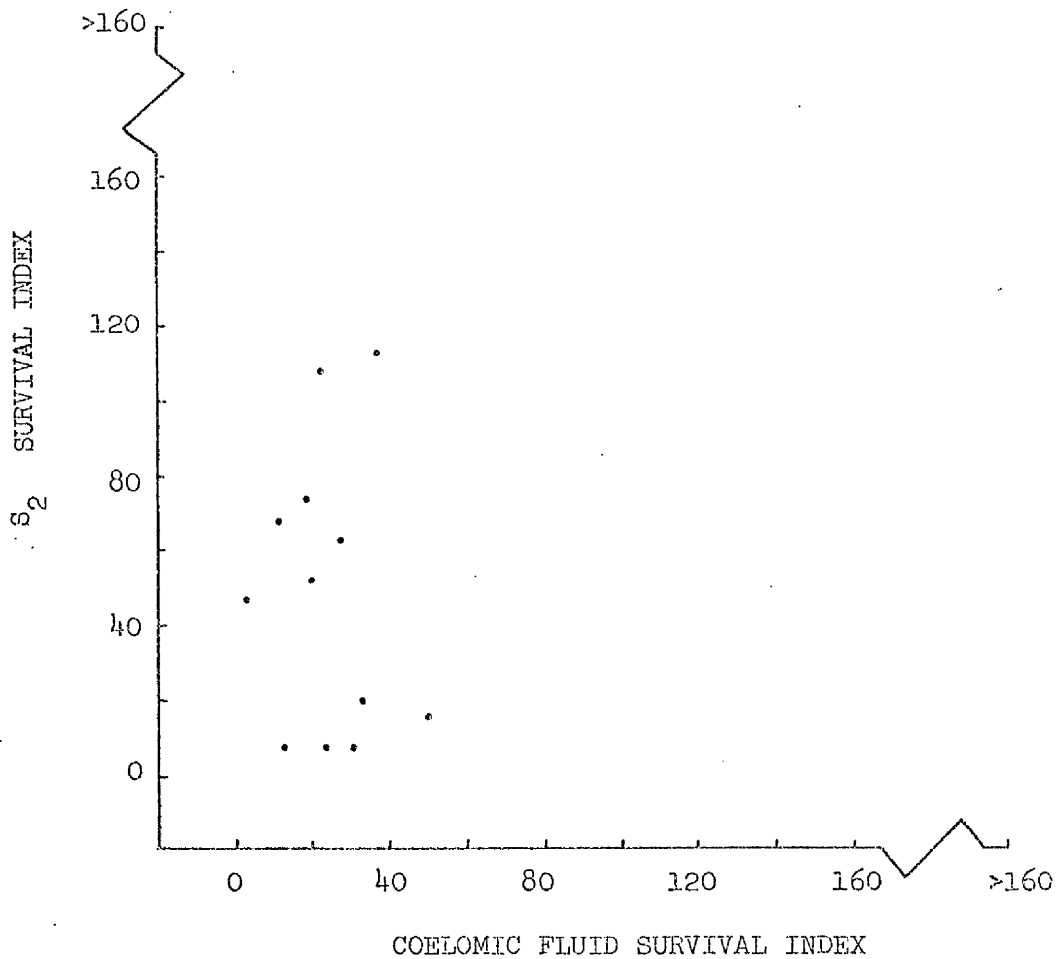
Figure 24.   Relationship between the survival indexes

of strain no. 111 in coelomic fluid and

concentrated extract S_2 at 24 h.

COELOMIC FLUID SURVIVAL INDEX
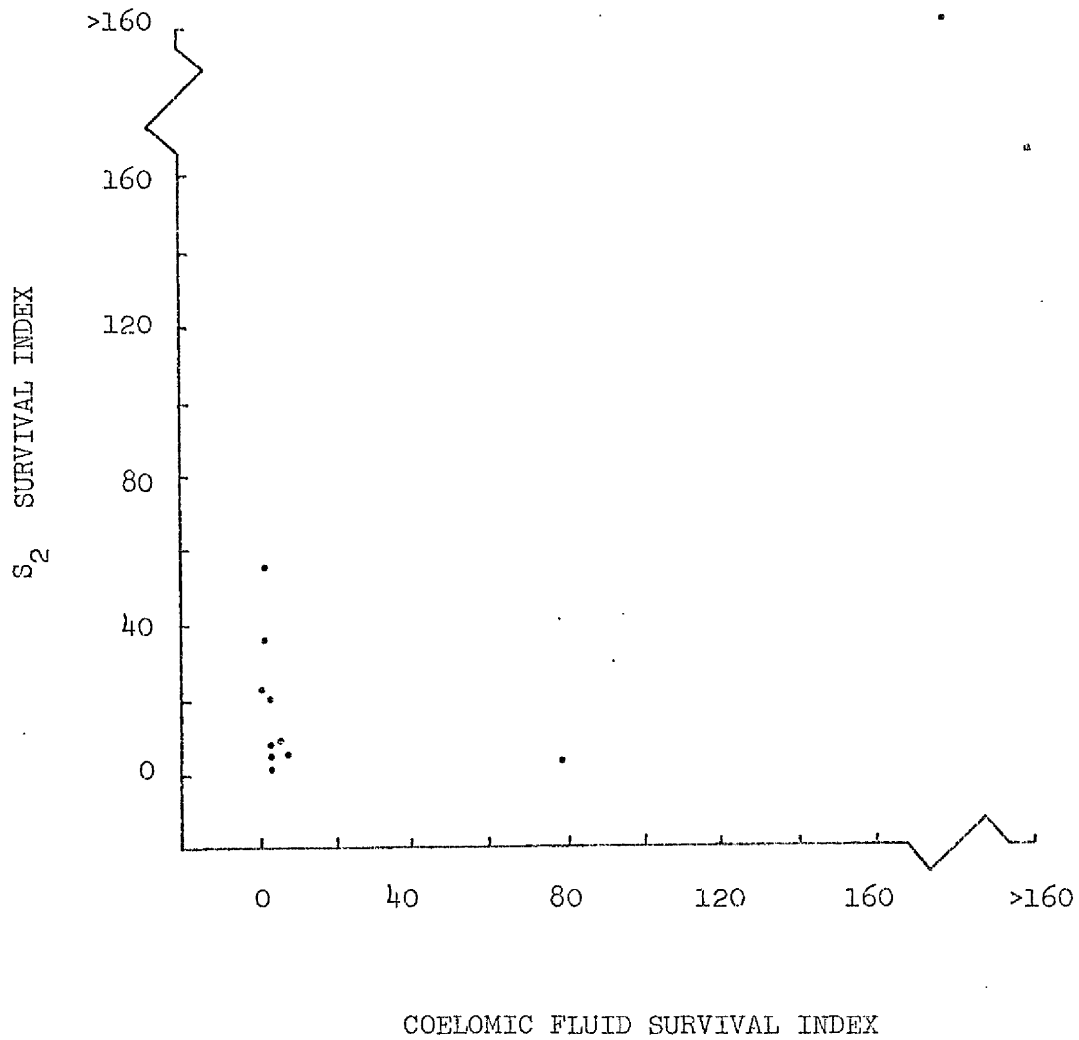
Figure 25.   Relationship between the survival indexes

of strain no. 111 in coelomic fluid and

concentrated extract S_2 at 48 h.
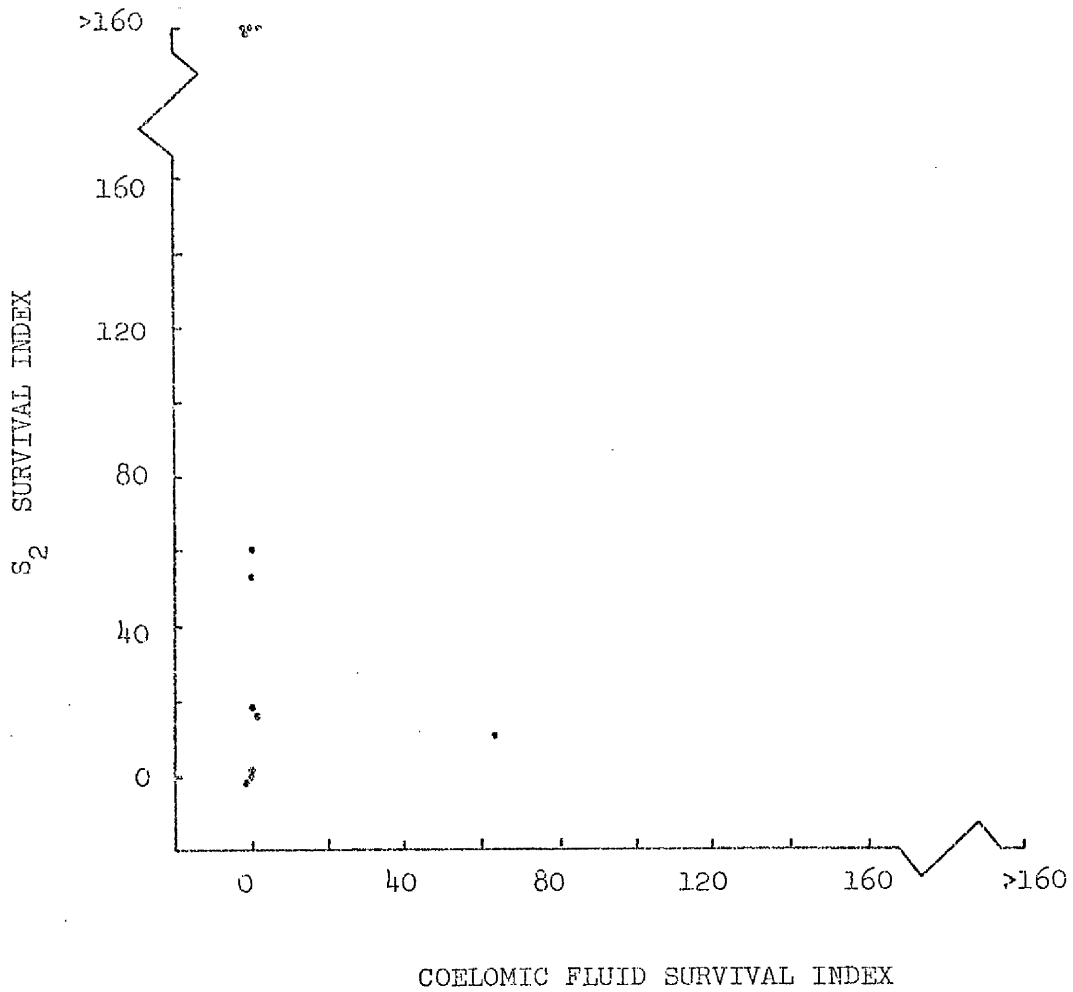
Table 10.   Survival indexes of strain no. 111 in extract

S_2 in relation to the initial protein

concentration.

Survival index			Initial protein concentration
4 h	24 h	48 h	mg/ml
< 1	< 1	< 1	1.94
< 1	< 1	< 1	3.86
13	< 1	< 1	2.02
36	104	a.c.	3.26
11	17	141	2.09
4	17	6	2.12

S_2, a further extract S_3 was prepared by sonication and centrifugation and a comparison of the antibacterial activity of these 2 fractions was made. Figure 26 presents the survival indexes in the form of a histogram representing a total of 14 tests. At 4 h there was little difference between the 2 fractions but at 24 h S_2 appeared slightly more active than S_3 while at 48 h S_2 was definitely more active. There was however, some residual antibacterial activity in the S_3 fraction.

Effect of storage of extract at $4^{\circ}C$ and $-20^{\circ}C$

Samples of S_2 were prepared from a total of 12 urchins and pooled before storage. Half the lysate was stored at $4^{\circ}C$ and the remainder frozen at $-20^{\circ}C$ and after 36 h tested for antibacterial activity. Frozen S_2 always formed a precipitate on thawing. This was resuspended before use. Table 11 shows the survival indexes at 4 h, 24 h and 48 h of pooled S_2 stored at $4^{\circ}C$ and at $-20^{\circ}C$ on three separate occasions. The indexes at 4 h all showed antibacterial activity. At 24 h S_2 stored at $4^{\circ}C$ appeared slightly less active in two cases than S_2 stored at $-20^{\circ}C$ and at 48 h one index of S_2 stored at $4^{\circ}C$ was very much higher than the corresponding index at $-20^{\circ}C$. The ranges of the indexes were as follows:

	$4^{\circ}C$	$-20^{\circ}C$
4 h	4 - 44	6 - 17
24 h	< 1 - 24	< 1 - 6
48 h	< 1 - 391	< 1 - 38

Figure 26.   Frequency-distributions of the survival indexes of

strain no. 111 in concentrated extract fractions

S_2 and S_3 at 4 h, 24 h and 48 h.
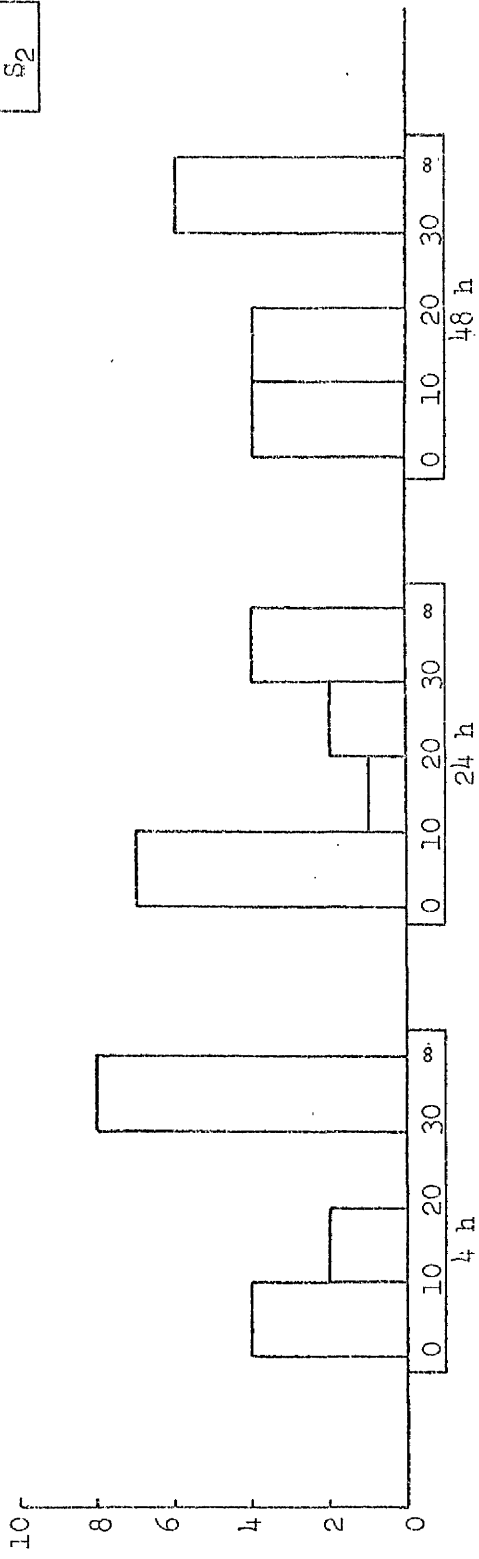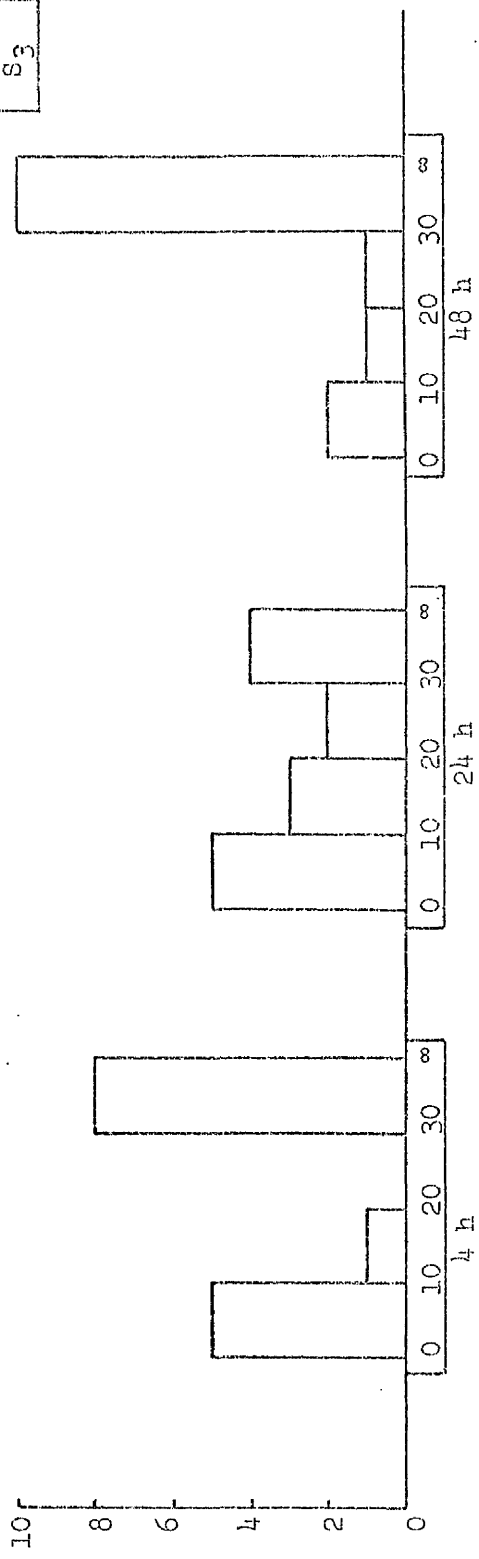
Table 11. Survival indexes of strain no. 111 in S_0/B control fluid

and extract S_2 stored at $4^{\circ}C$ and $-20^{\circ}C$

Test No.	S_0/B			S_2 stored at $4^{\circ}C$			S_2 stored at $-20^{\circ}C$		
	4 h	24 h	48 h	4 h	24 h	48 h	4 h	24 h	48 h
1	81	a.c.	c.	23	24	<1	17	<1	<1
2	89	a.c.	c.	44	19	19	6	6	38
3	89	a.c.	c.	4	<1	391	9	4	4

The range of indexes was much smaller for S_2 stored at $-20^{\circ}C$

and it would appear that storage at $4^{\circ}C$ caused a reduction of

antibacterial activity.

Effect of freezing

In contrast to the preceding section which shows the

survival indexes of the same bacterial suspension in extract

samples, this section compares the survival indexes of the test

organism in freshly-prepared extract and in the corresponding

extract after storage at $-20^{\circ}C$ for 36 h. It must be noted

that these tests were carried out using different bacterial

suspensions.

In 6 cases the extract prepared from separate pairs

of urchins was used and in 3 cases pooled extract, each prepared

from 12 urchins, was tested. Table 12 shows the survival indexes

at 4 h, 24 h and 48 h. All the samples showed antibacterial

activity at 4 h and with 2 exceptions the indexes of fresh S_2

were lower. At 24 h 3 samples of fresh S_2 showed an increased

index with corresponding increases in S_2 stored at $-20^{\circ}C$. Again

the indexes of fresh S_2 were generally lower. At 48 h, 2 of the

indexes of fresh S_2 and 3 of the indexes of frozen S_2 were greater

than 160. Of the remaining indexes there was little difference.

It appears that freezing had little effect on the antibacterial

activity of S_2.

Effect of dialysis

To obtain information about the molecular weight of the

Table 12.   Survival indexes of strain no. 111 in S_0/B

control fluid, fresh extract S_2 and extract

S_2 stored at $-20^{\circ}C$ for 36 h.

Test No.	S_O/B (mean)			Fresh S_2			S_2 stored at -20°C		
	4 h	24 h	48 h	4 h	24 h	48 h	4 h	24 h	48 h
1	43	a.c.	c.	<1	<1	<1	3	<1	<1
				<1	<1	<1	14	18	<1
				15	<1	<1	35	43	<1
				36	104	a.c.	71	205	a.c.
				11	17	192	41	73	a.c.
				4	17	6	8	119	a.c.
2	73	a.c.	c.	39	13	6	17	<1	<1
3	70	a.c.	c.	27	22	560	19	4	21
4	75	a.c.	c.	2	3	9	9	4	4

active substance, concentrated extract S_2 was dialysed at $4^{\circ}C$ for 36 h. Dialysis was carried out against supernatant S_0 to avoid changing the ionic composition of the material. The antibacterial activity was compared with that of the starting material which had been kept at $4^{\circ}C$ for 36 h. Altogether 4 such experiments were done; on each occasion the S_2 extract had been obtained from the pooled coelomic fluid of 12 urchins, and the survival indexes at 4 h, 24 h and 48 h are shown in Table 13.

At all sampling times the indexes of S_2 stored at $4^{\circ}C$ were lower than those of dialysed S_2. Dialysed S_2 retained some activity, however, compared with the S_0/B control fluid but growth of strain 111 occurred in all the dialysed S_2 samples. These results indicate that a low molecular weight substance is required for full expression of antibacterial activity.

Effect of heat treatment

To determine the heat lability of the antibacterial principle, samples of pooled extract S_2 which had been stored frozen for 24 - 36 h were heated at $20^{\circ}C$, $37^{\circ}C$, $56^{\circ}C$ and $100^{\circ}C$ for 30 min. After cooling the activity of each was compared with that of unheated extract.

A noticeable change in colour occurred on heating of the extract. The normal orange colour remained at $20^{\circ}C$ and $37^{\circ}C$ but at $56^{\circ}C$ the extract turned green and at $100^{\circ}C$, dark muddy brown but again became dark green between 4 h and 24 h of incubation.

Table 13.   Survival indexes of strain no. 111 in S_0/B control fluid, extract S_2 stored at 4°C for 36 h and extract S_2 dialysed at 4°C for 36 h.

Test	S_0/B			S_0 stored at 4°C			S_2 dialysed at 4°C		
No.	4 h	24 h	48 h	4 h	24 h	48 h	4 h	24 h	48 h
1	81	a.c.	c.	23	24	<1	36	124	a.c.
2	89	a.c.	c.	44	19	19	100	125	a.c.
3	89	a.c.	c.	4	<1	391	70	207	a.c.
4	76	a.c.	c.	3	14	40	92	91	a.c.

After treatment at 100°C for 30 min and subsequent cooling the pH of the extract dropped from pH 6.2 - pH 6.0.

When compared with the indexes of the S_0/B controls in Table 14 those of the heat treated extract were antibacterial but not as clearly so when compared with the indexes of the untreated frozen S_2. On two occasions, the frozen S_2 indexes increased at 48 h and this was seen also with S_2 at 20°C and 37°C but not at 56°C. On the third occasion when the index of frozen S_2 fell to a low value at 4 h and decreased at 24 h the index of extract treated at 56°C did not show as significant a drop at 4 h, decreased at 24 h but increased slightly at 48 h. The index of extract treated at 100°C however, although not falling as much at 4 h decreased to a low value at 48 h.

Table 14. Survival indexes of strain no. 111 in S_O/B control fluid, frozen and thawed extract S_2 and heat treated frozen and thawed extract S_2.

Test No.	S_O/B			Frozen S_2			S_2 20°C			S_2 37°C			S_2 56°C			S_2 100°C		
	4 h	24 h	48 h	4 h	24 h	48 h	4 h	24 h	48 h	4 h	24 h	48 h	4 h	24 h	48 h	4 h	24 h	48 h
1	81	a.c.	a.c.	6	6	38	6	31	131	25	19	875	31	38	6			
2	89	a.c.	a.c.	<1	4	98	2	4	13	28	15	98	23	9	6			
3	89	a.c.	a.c.	9	4	4							57	20	41	63	26	11

Extract which had been stored frozen was heated at 20°C, 37°C, 56°C and 100°C

for 30 min before testing.

DISCUSSION

NORMAL BACTERIAL FLORA OF E. ESCULENTUS

The first part of this investigation was to define
the normal bacterial flora of E. esculentus, and bacteria from
different sites on urchins were isolated and identified. The
genera found were Vibrio, Pseudomonas, Aeromonas, Flavobacterium,
Acinetobacter and Moraxella. Gram-positive bacteria were also
isolated but further identification was not undertaken.

The distribution of genera varied according to the
different sites of isolation. The percentages of genera found
from the peristomial membrane closely resembled those obtained
from seawater and sand while the distribution of genera from
coelomic fluid and the gut were similar. This indicated that
the bacterial flora of the peristomial membrane was dependent on
that of the surrounding environment, which is not surprising as
urchins are bottom feeders, i.e. they graze off the seabed. The
bacterial flora of the gut and the coelomic fluid however
differed from that of seawater and sand and therefore were, at
least partially, independent of external environment.

Although the bacterial flora of E. esculentus has not
been described by previous workers, the same genera of bacteria
have been reported from other invertebrates. It has also been
noted by Colwell and Liston (1962) and Beeson and Johnson (1967)
that the bacterial species found varied according to the organ
from which they were isolated. Leifson et al. (1964) showed
that the bacterial flora of seawater consisted mainly of

Pseudomonas while in the gut of several marine animals Vibrio
predominated.

Bang and Lemma (1962) suggested that the coelomic
fluid of the sea star is normally sterile and that bacterial
infection only occurs when the sea stars are unhealthy. Tubiash,
Sizemore and Colwell (1975) however, thought that it was more
probable that the haemolymph of normal healthy blue crabs
contained an indigenous bacterial population. However,
considering that the counts per 0.1 ml coelomic fluid (Table 4)
showed that two-thirds of urchins had sterile fluid, it is more
likely that the coelomic fluid of E. esculentus is normally
sterile and that the bacteria isolated represented infecting
organisms introduced by damage to the animals by, for example,
ripping off tube feet during handling. The organisms found
could have been those which were capable of overcoming the natural
defence mechanisms of the urchins hence the apparent dissimilarity
with the bacterial flora of the external environment. It has
been shown that sea urchins rapidly phagocytose Gram-positive
bacteria (Johnson, 1969c) and this could account for the lack of
these organisms in the coelomic fluid. Alternatively since the
bacterial flora of the coelomic fluid resembles that of the gut
rather than seawater and sand, it would seem possible that the
coelomic fluid may become contaminated by gut bacteria.

There was an increase of the numbers of bacteria in the
coelomic fluid on the 22nd, 23rd and 24th July 1975 corresponding
to the highest recorded tank water temperature thus suggesting a

temporary heat-stress effect on count. It should however be noted that these counts are taken from the same urchins on 3 consecutive days and it is possible that the high count may have resulted from damage during serial sampling.

There was little difference in the count per 0.1 ml from urchins maintained in different ways which justified the convenience of using urchins kept in an aquarium tank rather than fresh urchins collected immediately prior to use.

ANTIBACTERIAL ACTIVITY OF E. ESCULENTUS

Preliminary experiments to establish whether E. esculentus could clear bacteria injected into the coelomic fluid were encouraging. After 24 h none of the injected bacteria could be found. Assuming that the injected bacteria did not localise at the point of inoculation but spread evenly throughout the coelomic cavity, these results are indicative of a strong antibacterial activity.

There is no previous published work on the in vivo clearance of bacteria by E. esculentus but injection of a Vibrio sp. into the coelomic fluid of the sea star, Asterias, showed that bacterial numbers gradually decreased and disappeared within 3 days (Bang and Lemma, 1962). The mechanism of clearance was not however elucidated from these experiments.

To study further the antibacterial activity of E. esculentus an in vitro method was employed. Coelomic fluid

had an easily demonstrable antibacterial activity in vitro against the marine pseudomonad strain 111 and although there were quantitative differences in antibacterial activity of coelomic fluid from different urchins none of the 188 urchins examined had fluid completely lacking in this activity. In some tests, survival indexes increased between 24 h and 48 h. This could be due to a limited amount of antibacterial substance present in the coelomic fluid or to the activity being slowly degraded. Either of these suppositions would lead to the unhindered multiplication of surviving bacteria.

The variation between urchins was unrelated to the date when the animals were sampled and to the size of the initial bacterial count. Variation in activity was also seen between consecutive samples of coelomic fluid taken a few days apart from the same urchin. These observations suggested that the antibacterial activity was present at a basal level in all urchins but in some cases was altered perhaps by recent bacterial infection or change in the physical environment.

The antibacterial activity was dependent on the temperature of incubation of the test mixtures. Coelomic fluid incubated at $4^{o}C$ was slightly more antibacterial than fluid incubated at $10^{o}C$ and much more active than fluid incubated at $22^{o}C$. However, before a final conclusion is reached, the growth of the test organism in S_{0}/B at these temperatures must be taken into account. At $4^{o}C$ there was a decrease in the survival index of the organism until growth became apparent after 24 h.

Presumably a corresponding decrease would occur in the coelomic fluid samples which, combined with antibacterial activity would greatly depress the survival indexes and so make the fluid incubated at 4°C appear more antibacterial. At 10°C and 22°C however growth of the test bacterium was evident after 4 h and so the factor which caused a decrease of survival index in the fluid after this time could only be attributed to the antibacterial activity. It may be that at 22°C a critical stage was reached at which the antibacterial activity of the coelomic fluid could no longer deal with the fast growth of the bacterium at this temperature and so the activity appeared to be reduced. At 4 h however when the survival index of the test bacterium dropped equally in the control for each temperature, the coelomic fluid samples at 22°C showed less activity than those at 4°C and 10°C and so the antibacterial activity itself may also have been affected at this temperature. It is probable then that 10°C, which is near the normal ambient temperature of the animals, is best for studying antibacterial activity.

Clotting of coelomocytes of echinoderms is a well-recognized phenomenon and although it is a mechanism for repair of damage, it possibly also has a role in host defence such as encapsulation of invading objects (Bang and Lemma, 1962; Johnson, 1969c). Clotting of the coelomocytes of E. esculentus fluid was seen soon after removal of the fluid from the urchin, and after 2 h a dense clot was formed. The antibacterial activity of fluid to which the test bacterium was added immediately was

only slightly higher than the corresponding fluid which had been allowed to clot for 2 h before addition of the bacteria. This suggested that it was not necessary for the coelomocytes to be distributed evenly throughout the test mixture in order for the antibacterial activity to take effect, and that although it was better to use fresh fluid, a slight delay in addition of bacteria made negligible difference.

The control fluid used throughout the investigation was S_0/B. It was used because it was found to promote growth of the test bacterium. Since it was simply the cell-free supernatant of coelomic fluid which had been boiled, it was therefore a good comparison for antibacterial activity in the whole coelomic fluid as presumably the salts and nutrients in S_0/B are the same as those in whole coelomic fluid. A characteristic of the growth of the test bacterium in S_0/B was the decrease in survival index at 4 h. The reason for this is not clear. It may be that the effect was due to the transfer of the bacterium from a 2216E agar slope, rich in nutrients, to S_0/B which is relatively poor in nutrients. The change in temperature from $20^{\circ}C$, the temperature used to grow the test culture, to $10^{\circ}C$, the incubation temperature of the test mixture, may also have affected the survival index.

Comparison of the antibacterial activity of coelomic fluid and cell-free supernatant S_0 of coelomic fluid showed that the activity resided in the coelomocytes. In fact, the supernatant acted as a growth medium for strain 111 although it

is possible that there was some residual activity at 4 h and
24 h in some cases.   Generally however the growth of the test
bacterium in supernatant paralleled growth in S_0/B control fluid.

Published work with other echinoderms is very limited.
Johnson and Chapman (1971) tested the coelomic fluid of a sea-
cucumber and a sand dollar for antibacterial activity against a
variety of bacteria using a viable counting method.   Their
conclusions were that there was a bacteriostatic system in both
animals but that none of the bacteria was killed.   They also
concluded that the activity was probably associated with the
coelomocytes but their results do not show clearly that the
cell-free supernatant is acting as a growth medium.   The control
fluids which they used were autoclaved seawater and bovine
albumin seawater and as such neither properly represented the
conditions of the coelomic fluid.   Neither of these controls
promoted good growth of the organisms, survival being especially
poor in seawater.

Although Johnson (1969c) observed lysis of some bacteria
in hanging drops of coelomic fluid, it seems unlikely in
E. esculentus that the antibacterial activity in the coelomic
fluid was due to the release of a bacteriolytic enzyme because in
most cases the survival index did not reach zero until 24 h or 48 h.
An enzyme would be expected to produce an effect much more quickly
than this.   Also, on no occasion when test mixtures were
observed by phase-contrast microscopy were there signs of bacterial
cell lysis such as unusual morphological shapes or ghosts.   Neither

was agglutination of bacterial cells seen which could also account for a drop in survival index. If agglutination were taking place the time course of the tests would also possibly have been different showing a dramatic clearance initially and a levelling off, instead of which most cases showed a continuous decrease in index.

Phagocytosis has been found to be of major importance in Pacific urchins as a defence against Gram-positive bacteria (Johnson, 1969c). Johnson et al. (1970) observed by electron microscopy that a Gram-negative bacterium was also phagocytosed although this was not visible by light microscopy. It is therefore possible that phagocytosis was involved in the anti-bacterial activity in E. esculentus coelomic fluid.

Another possibility exists which involves the release of a substance from the coelomocytes affecting the metabolism of the bacteria thus rendering them non-viable. By phase-contrast microscopy it was seen that many bacteria lost motility after exposure to coelomic fluid. Johnson (1969c) observed that red spherule cells released echinochrome in the vicinity of bacteria inoculated into a hanging drop of coelomic fluid. She found that bacteria lost motility in drops prepared from the fraction of coelomic fluid which contained mainly red and colourless spherule cells while in the fraction containing mainly leucocytes and vibratile cells, motility was not affected. Test mixtures in this investigation often turned yellowish presumably also due to release of echinochrome, and it may be that this substance can affect bacterial metabolism.

Johnson has shown however that purified echinochrome did not have an inhibitory effect on 3 of her test bacteria but she pointed out that since echinochrome occurs naturally associated with a protein, her results did not rule out the possibility of the pigment/protein complex being antibacterial.

Antibacterial activity was also found in the cell-free coelomocyte extract and did not seem to be dependent on the initial protein concentration. The activity of the extract showed the same time course as coelomic fluid in that there was a continuous decrease in survival index. Unconcentrated extract was only slightly less active than whole coelomic fluid and there appeared to be correlation, at least at 4 h, between activity in coelomic fluid and activity in the extract. When the extract was concentrated 20 times however, the antibacterial activity did not increase but seemed slightly less active than unconcentrated extract. This apparent inhibition of activity could have been a concentration effect. The extraction procedure appeared quite efficient, there being only little residual activity in S_3 compared with S_2.

Antibacterial activity in S_2 was not stable at 4° but since the ambient temperature of $\underline{E.}$ $\underline{esculentus}$ was $8 - 14^{\circ}C$, enzymes from the cell extract are likely to be active at $4^{\circ}C$ and so it is possible that the antibacterial substance was degraded at this temperature. Freezing caused precipitation in the extract although there appeared to be little detrimental effect on activity when frozen only once. It is not known whether removal

of this precipitate has an effect on the activity of the extract or if repeated freezing results in a loss of activity.

Dialysis of S_2 greatly reduced the activity but the dialysed extract still retained some activity. This suggested that perhaps two antibacterial substances were present, one of which was non-dialysable and therefore probably a high molecular weight molecule and the other substance, either a low molecular weight molecule or a large molecule which required a small cofactor for activity. Since dialysis was performed for 36 h against 100 times the volume of S_2 it is unlikely that residual activity was due to incomplete dialysis or to leakage from the visking tubing since this was checked before use.

The antibacterial substance was heat stable with the activity only slightly impaired by boiling for 30 min. It is therefore unlikely to be a protein as most proteins are destroyed at this temperature. The colour change noted on heating was probably due to oxidation and reduction of echinochrome rather than a change in pH.

If the antibacterial activity of the whole coelomic fluid was due to phagocytosis, it is unlikely that the activity of the extract represented enzymes released from lysosomes after sonication because of a) the heat stability and b) the slow time course of the tests. If lysosomal enzymes were responsible for activity one would expect the activity of the extract to be faster than that of the coelomic fluid but this was not the case.

It has been inferred that haemagglutinins may have a role in defence against bacteria other than as opsonins in phagocytosis (Acton and Weinheimer, 1974) but in E. esculentus the activity of the haemagglutinin was abolished by heating at $60^{\circ}C$ for 30 min (Parker, 1974) and so it cannot be involved in the activity of the extract after boiling.

The results can be explained by assuming that there were two substances present one of which was dialyzable, heat stable and more active than the other which was non-dialyzable and heat labile. Their combined activities could produce a bactericidal effect but when only one was present activity was impaired. The results could also be explained in terms of an active substance/cofactor complex such as could occur with the protein/echinochrome complex where either part of the complex is inactive alone. On dialysis much of the complex could dissociate and the cofactor would be dialysed leaving only a small amount of active complex accounting for the residual activity after dialysis. On heating at $56^{\circ}C$ or $100^{\circ}C$ the complex could also dissociate but slowly re-associate and regain activity hence the higher survival index at 4 h in heat-treated S_2.

As with coelomic fluid, the survival indexes of tests on S_2 sometimes showed an increase at 48 h but more frequently than with coelomic fluid. It may be that the antibacterial substance or substances are limited; in coelomic fluid there could be some synthesis of new material but there could be none in the extract and so there would be apparently less activity at 48 h.

Antibacterial activity has been shown in a number of marine invertebrates. In molluscs, where the principal defence against bacteria appears to be phagocytosis, clearance of injected bacteria has been shown to depend on the ambient temperature. Pauley, Krassner and Chapman (1971) using live suspensions of 4 marine bacteria injected into the sea hare, Aplysia, and Foley and Cheng (1975) studying in vitro phagocytosis by the oyster, Crassostrea, and the clam, Mercenaria, of heat-killed bacteria noted that the elimination of bacteria was slower at low temperature than at high temperature. Feng (1966) studying the fate of bacteria injected into the oyster found however that at $9^{\circ}C$ animals were able to control infection with a viable Pseudomonas-like bacterium but died at $23^{\circ}C$. Molluscs do not appear to possess a natural or induceable humoral response to bacteria (Weinheimer et al., 1969b; Johnson and Chapman, 1970b; Pauley et al., 1971) although it has been shown that lysosomal enzymes can be released into the body fluid which possibly kill susceptible bacteria (Cheng and Rodrick, 1975; Cheng, 1976).

The sipunculid worms from which echinoderms may be descended, have been shown to possess a strongly antibacterial coelomic fluid for a wide range of bacteria in vitro (Johnson and Chapman, 1970a) and activity was shown to exist in the cell-free fluid as well as the whole coelomic fluid. Krassner and Flory (1970) showed that the antibacterial factor was stable after heat treatment of $50^{\circ}C$ for 10 min and was dialyzable. The fluid of a tunicate was also shown to have antibacterial activity for a range

of bacteria but the fluid was not as active as that of
sipunculids and in some cases the cell-free fluid appeared to
have no activity compared with the control (Johnson and Chapman,
1970b). Like that of the spiny lobster (Evans et al., 1968)
the fluid of the tunicate was not active against Gram-positive
organisms.

Unlike the antibacterial activity of fluids of other
invertebrates, the substance present in E. esculentus fluid was
cell-associated. Although sipunculids, echinoderms and tunicates
are all possibly related, it would be unwise to assume that their
immune systems are also therefore phylogenetically linked. The
antibacterial factors found in the fluid of sipunculids and in
the extract of E. esculentus could be related as it has been shown
that they are both dialyzable. It has been shown that the anti-
bacterial activity of sipunculids and tunicates varies between
bacterial strains used. This may also be the case with echinoderms
and so it is possible that strain 111 does not elicit the strongest
response from E. esculentus fluid hence the apparent inactivity
of cell-free fluid.

Much work has still to be done on the nature of the
antibacterial activity of E. esculentus. It would be interesting
to discover the range of bacteria susceptible to the activity since
there is evidence that antibacterial activity in marine invertebrates
differentiates between types of bacteria, for example Gram-positive
and Gram-negative bacteria (Johnson, 1969c; Johnson and Chapman,

1970b). It has also been shown that the bactericidal activity of certain marine invertebrates can be enhanced (Stewart and Zwicker, 1972) and it may be that injection of live or heat-killed bacteria may increase the activity of E. esculentus coelomic fluid. Further characterisation of the active substance or substances in the coelomocyte extract is also necessary.

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APPENDIX

MARINE BROTH 2216 (Difco)

Peptone	5 g
Yeast extract	1 g
Ferric citrate	0.1 g
Sodium chloride	19.45 g
Magnesium chloride dried	5.9 g
Sodium sulphate	3.24 g
Calcium chloride	1.8 g
Potassium chloride	0.55 g
Sodium bicarbonate	0.16 g
Potassium bromide	0.08 g
Strontium chloride	0.034 g
Boric acid	0.022 g
Sodium silicate	0.004 g
Sodium fluoride	0.0024 g
Ammonium nitrate	0.0016 g
Disodium phosphate	0.008 g
Distilled water	1 l

The dehydrated medium was rehydrated by suspending 37.4 g in 1 l of distilled water. It was heated to boiling for 1 - 2 min and autoclaved for 15 min at 15 lb/in^2.

MARINE AGAR 2216E   (Difco)

Peptone	5 g
Yeast extract	1 g
Ferric citrate	0.1 g
Sodium chloride	19.45 g
Magnesium chloride	8.8 g
Sodium sulphate	3.24 g
Calcium chloride	1.8 g
Potassium chloride	0.55 g
Sodium bicarbonate	0.16 g
Potassium bromide	0.08 g
Strontium chloride	0.034 g
Boric acid	0.022 g
Sodium silicate	0.004 g
Sodium fluoride	0.0024 g
Ammonium nitrate	0.0016 g
Disodium phosphate	0.008 g
Agar	15 g
Distilled water	1 l

The dehydrated medium (55.1 g) was suspended in 1 l of cold distilled water, heated to boiling to dissolve the medium completely, and sterilised in the autoclave for 15 min at 15 lb/in^2.

MARINE OXIDATION/FERMENTATION MEDIUM (Difco)

Casitone	1 g
Yeast extract	0.1 g
Trishydroxymethylaminomethane	0.5 g
Boric acid	0.011 g
Ammonium sulphate	0.5 g
Disodium phosphate	0.004 g
Ammonium nitrate	0.0008 g
Sodium chloride	9.7 g
Magnesium chloride	4.4 g
Sodium sulphate	1.6 g
Calcium chloride	0.9 g
Potassium chloride	0.275 g
Sodium bicarbonate	0.08 g
Potassium bromide	0.04 g
Strontium chloride	0.017 g
Sodium silicate	0.002 g
Sodium fluoride	0.0012 g
Phenol red	0.01 g
Agar	3.0 g
Distilled water	1 l
Glucose	10% (w/v)

The dehydrated medium (22 g) was suspended in 1 l of distilled
water, heated to boiling and dispensed in 100 ml amounts before
autoclaving for 15 min at 15 lb/in^2. Glucose (10 ml), which
was sterilised separately by Seitz filtration, was added to each
100 ml aliquot. The medium was mixed thoroughly and dispensed
aseptically in 5 ml amounts into sterile 16 x 150 mm test tubes.

REAGENTS FOR PROTEIN ESTIMATION (Lowry et al., 1951)

Reagent A :   2% sodium carbonate (Na_2CO_3) in 0.1 N

sodium hydroxide.

Reagent B :   0.5% copper sulphate ($CuSO_4.5H_2O$) in

distilled water. 1% aqueous solution

of potassium sodium tartrate. Mix

equal amounts of each solution.

Reagent C :   1 ml of reagent B added to 50 ml of

reagent A. Discarded after one day.

Reagent D :   1 N Folin-Ciocaltau reagent (B.D.H.).