Attempts to Develop A Marine Molluscan Cell Line
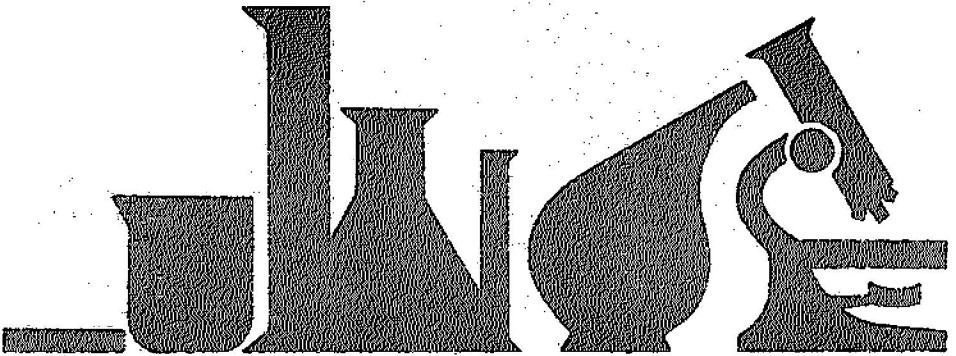
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ATTEMPTS TO DEVELOP A MARINE
MOLLUSCAN CELL LINE

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The development of a marine molluscan cell line is proving to be among the more recalcitrant research problems for biologists and microbiologists. While there has been success in obtaining amoebocytes in culture, and even maintaining them for long periods, researchers so far have been unable to determine the conditions that would sustain a cell line indefinitely.

Any such effort involves balancing a number of variables, among them, handling the culture, determining the best sources of tissues, choosing physical parameters of the nutrient media, and deciding on culturing methods and nutrient formulations.

While methods for establishing primary cultures of mammalian and avian cells have been successful, analogous methods for developing molluscan cell cultures have been unsuccessful. Thus, microbiologists like Dr. Hetrick and his colleagues have had to chart their attempts through intuition that comes from the experience of what does not work, what almost works and what looks promising. In the process, useless paths are identified and new methodologies are established.

This, in part, is the value of the work reported on here, the record of the numerous though unsuccessful attempts to develop cell lines using the adult oyster *Crassostrea virginica*, larval oysters of *C. virginica* and the soft-shell clam, *Mya arenaria*. While Dr. Hetrick and his co-researchers focused their studies on the development of suitable nutrient formulations-- they identify the most promising ones--they also developed culturing methods, techniques of tissue preparation and decontamination, and procedures for preparing larval oysters that should prove valuable to other researchers. Their discussion of the best sources of tissues of *C. virginica*, the relative success of enzymatic dissociation of oyster tissue, and the suggestions for further investigations should serve continuing research efforts.

Eventually, molluscan cell lines will be developed and when they are, scientists will have more sophisticated means for studying the intracacies of shellfish disease, nutrition and biochemistry. This information will provide the practical biological information needed by aquaculturists and resource managers.

Merrill Leffler

INTRODUCTION

While the establishment of cell cultures in vitro from the tissues of a wide variety of mammalian, avian, piscine and invertebrate species has been accomplished, the establishment of a molluscan cell line has proved to be a considerably more difficult task. The only successful cell line that has been established is from the embryos of a freshwater snail *Bromphalaria glabrata* by Hansen (1976).

The value of growing molluscan cells in culture has long been recognized. Such a tissue system would provide a valuable research tool in the study of molluscan diseases, nutrition and biochemistry. To take one example: human enteroviruses and hepatitus viruses are known to be concentrated by molluscs which reside in polluted waters; if these molluscs are con-sumed raw, human disease can result. While it is generally believed that human viruses do not multiply in the molluscs but rather the molluscs serve as a vehicle for their transmission, direct experimental evidence for such an assumption cannot be undertaken since there is no marine molluscan cell line available for the critical studies.

For the past fifteen years, researchers have work-ed on the problem of initiating a cell line from a marine mollusc, such as the oyster or clam. While establishment of oyster primaries has been reported (Brewster and Nicholson, 1979), no continuing marine molluscan line has been established. Our work during the past three years has centered on developing a cell line from oyster tissues of *Crassostrea virginica* and clam tissues of *Mya arenaria.*

Primary cultures of oyster cells were obtained from heart, mantle and gonadal tissues of *Crassostrea virginica.* Following decontamination of the oysters, tissues were removed, minced into small fragments and planted into culute dishes. Cells continued to migrate from the fragments for several days and would eventually form a confluent monolayer of densely packed cells in the vessel.

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Depending on the tissues and media employed, confluency
was achieved 7 to 21 days following culture initiation.
Several cell types could be detected in the cultures;
however, the granular amoebocyte predominated. Subcul-
tivation of these primary cultures was not successful;
nor did trypsinization of oyster tissue fragments con-
sistently yield satisfactory cultures.

Efforts were also made to establish a cell line
from oyster embryos. Methods were developed whereby
contaminant-free oyster embryos could be obtained for
use in cell culture. Eggs and sperm from sexually
mature oysters were dissected out, decontaminated
and then mixed in proportionate amounts to achieve egg
fertilization. When the embryos reached the 16 to 32
cell stage, the embryos were dissociated by trypsini-
zation to obtain a cell suspension for culture. The
embryo cells that attached to the culture vessel
differed from the adult oyster cells in that they were
agranular, and fibroblast-like, having long cellular
processes. Since mammalian tumor cells grow more
luxuriantly in culture than normal cells, we attempted
to induce malignant growth in adult oysters by inject-
ing chemical carcinogens, both with and without acti-
vation by rat liver homogenates. No tumors developed,
and cultures prepared from tissues of these animals
were similar to those from untreated oysters.

Hemocytes from both normal and neoplastic clams
(*Mya arenaria*) were established in culture. Cells
were obtained from the hemolymph of both types of
clams by bleeding from the adductor muscle region.
Normal cells spread out and attached to the culture
vessel while the neoplastic cells did not attach but
remained suspended. Neoplastic cells were maintained
for 3 months while the normal cells were maintained
in healthy condition for 5 months. However, sub-
cultivation of the primary cultures was not successful
with either cell type.

In summary, although it was possible to maintain viable molluscan cells in culture for extended periods, significant cell mitosis, leading to the development of a cell line, was not achieved. A total of 138 media formulations and a wide variety of culture initiation and maintenance regimes were evaluated in an attempt to stimulate division of mulluscan cells in vitro. None of the techniques, however, were successful in promoting significant mitosis.

EFFORTS ON OYSTER CELL CULTURE--ADULT OYSTERS

In attempting to initiate a marine molluscan cell line, there are a number of variables to work with, among them, source of tissue, physical parameters of the media, handling of the cultures, culturing methods and nutrient formulations. While we adjusted several of these variables over the course of our work, for example, experimenting with different sources of tissues of *Crassostrea virginica* in hopes of inducing significant cell mitosis, our primary emphasis has been on developing optimum nutrient formulations.

Oysters used for culture were collected with dredges from various locations in the Chesapeake Bay by personnel from the University of Maryland's Horn Point Environmental Labs (HPEL). Once specimens were collected, they were maintained at HPEL in natural bay water. During the months in which the HPEL oyster hatchery was in operation (April-October), oysters were maintained in flowthrough bay water tanks in the hatchery. Water temperatures in the flow through systems were controlled to stay at 20°C or less. During the remainder of the year, oysters were kept in trays and hung from the HPEL pier in the Choptank River.

When oysters were to be used for culture, they were taken from the trays and scrubbed thoroughly in tap water to remove all fouling organisms. Shell surfaces were disinfected, oysters were opened aseptically and the desired tissues were dissected out. After the tissues were subjected to a series of de-contamination procedures, they were minced and dispensed into culture vessels containing test media.

The types of cultures established were primarily explant cultures, with oyster amoebocytes migrating from the tissue fragments and attaching to the culture

surface. In addition, a smaller number of cultures
were initiated from cell suspensions obtained on
enzymatic dissociation of a tissue fragment.

Initiation of Cultures--Tissues Used

In an effort to find the best source of poten-
tially dividing cells, a variety of oyster tissues
were cultivated in vitro. These included the mantle,
gonad, adductor muscle, heart, labial palps and
pericardial membrane. Oysters have very little con-
nective tissue, with the presence of molluscan colla-
gen still in question. All the tissues are engorged
with amoebocytes, the actively phagocytic blood cells
of the oyster.

In addition to culturing "healthy" tissues,
several attempts were made to cultivate cells from
diseased oysters as a potential source of proliferating
cells. Specimens exhibiting gross pathological
abnormalities were obtained from the HPEL oyster
histology lab. On several occasions, mantle tissues
containing watery cysts were cultured.
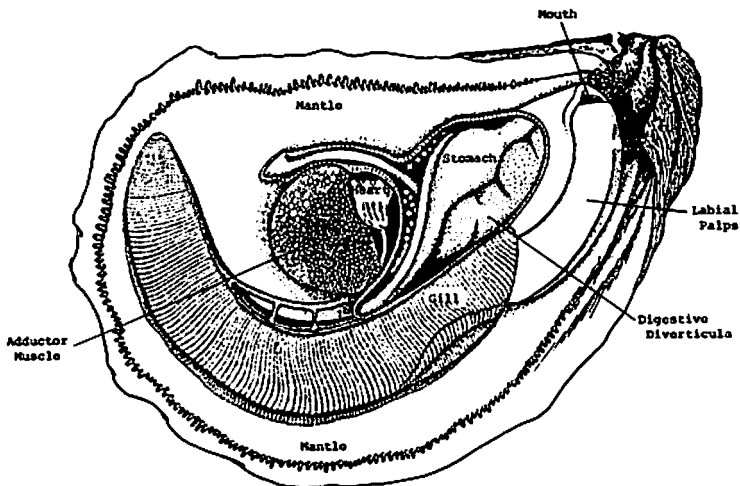
Figure 1. Diagrammatic sketch of oyster and internal
organs

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Another source of tissue included heart and mantle tissue from 0.5 inch oyster spat. However, because of the extremely small size of these tissues, accurate dissection was difficult, and all such cultures were lost due to microbial contamination.

With the exception of the oyster adductor muscle, all tissues from which cultures were initiated provided numerous amoebocyte-type cells which readily attached to the culture vessel surface. The pericardial membrane, being a relatively small mass of tissue, did not yield a large number of cells. The labial palps, as a highly ciliated tissue, yielded many ciliated, motile cell forms, as well as typical amoebocytes. No advantage could be seen in regularly using the palps in culture. None of the tissues exhibiting pathology were a source of proliferating cells.

The mantle, gonad and heart tissues provided the most suitable cultures, in terms of numbers of cells, apparent health of cells and ease of initiation of the cultures. The majority of cells cultured from these tissues were granular amoebocytes.

The mantle tissue, although a good source of numerous cells, was somewhat less desirable in culture than the heart tissue because it secretes a slimy material which interferes with attachment of the amoebocytes to the vessel surface.

The developing gonad of the oyster does not exist as much of a distinct mass of tissue; instead it is more a film of follicle cells which develops between the mantle tissue and digestive system. The majority of the mass of cells in the gonad are either egg or sperm, with minimal connective tissue or defined structure. The cells cultured from the gonad were, primarily, granular amoebocytes.

Heart tissue was also a source of agranular amoebocytes which, in culture, appeared fibroblast-like. In heart explant culture, other fibroblast-like contractile cells would also attach to the culture vessel; these cells would continue to pulsate regularly in culture, while attached to the vessel surface.

Initiation of Cultures—Processing of Tissues

Decontamination. One problem encountered in attempting to grow oyster cells in culture was microbial contamination of certain tissues that, in the water environment, is generally protected against such contamination. For example, when the oyster is open and pumping, the heart and those tissues enclosed within the pericardial sac, as well as parts of the oyster gonad, are not in constant contact with the water and are less likely to be as heavily contaminated by microorganisms as the mantle and gill, which are in direct contact.

Microorganic contaminants include a variety of zooplankton, fungi and bacteria. While fungi and bacteria can be eliminated by using antimycotic and antibiotic mixtures, the protozoan contaminants pose somewhat more of a problem. Extensive rinsing of the excised tissues and mild chemical treatments were found to satisfactorily remove most opportunistic, surface contaminants; systemic parasites, or protozoan stages of such parasites, often embedded in the tissues, were not as readily eliminated.

Decontamination procedures were the first steps in processing the oyster tissue for culture. Oysters to be used were removed from the holding tanks and scrubbed thoroughly in tap water. Outer shell surfaces were disinfected by soaking the oysters in 5% bleach for one minute. Surfaces were permitted to air dry. Once external surfaces were cleaned, oysters could either be used directly for culture or could be de-

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purated in aerated lab aquaria.

Depuration in lab aquaria involved surface cleaning each oyster, as mentioned above, and placing them, left valve down, in aerated artificial bay water. Six to ten oysters were placed in five gallons of water in a 15 gallon aquarium. Each day the oysters were scrubbed and the water changed. Most oysters were depurated in this manner for 3 to 4 days prior - to use.

For heart tissue that was to be used in culture, depuration was unnecessary, as uncontaminated healthy cultures could be obtained from oysters taken directly out of their natural habitat. In control experiments using mantle tissue, no differences could be detected between the extent of protozoan contamination in oysters depurated for 7 days and those oysters taken directly from the natural environment; it was felt, however, that several days of depuration could reduce potential protozoan populations and clean some of the sediments from the oyster tissue. Thus, in most instances, mantle tissue underwent brief depuration.

Depuration in lab aquaria was found to be essential in obtaining protozoa-free cultures from oysters which had been maintained in the refrigerator, out of their natural habitat even for one day. Regular pumping by the oysters, possibly maintaining their aerobic metabolism, seems to be important in keeping the protozoa population down. It must be remembered that expensive depuration periods in artificial bay water containing no nutrients, or accidental introduction of traces of unwanted chemicals, may cause the oysters to close down and stop pumping, effecting the same problem as in refrigerated oysters.

Following depuration, the oysters were opened and the desired tissues dissected out and further decontaminated by rinsing the tissues six times in bay water containing penicillin (100 units/ml), streptomycin (100 µg/ml) and neomycin (100 µg/ml). In

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rinsing, the tissues were swirled vigorously in the
tube to mix, allowed to settle out and the superna-
tant then discarded.

Following rinsing, the tissues were treated to
remove any remaining protozoa. Heart tissue, since it
is relatively "clean," was not treated. Other tissues
were treated for 60 seconds with 10^{-5} M hyamine hydrox-
ide and rinsed immediately in bay water. This level
of hyamine hydroxide was found to have no harmful
effects to the tissues being cultured, or the resulting
cells obtained. Treatment of the tissues with 10%
ethanol made up in bay water was found to have no effect
on the protozoa observed in oyster tissue. In repeated
tests, using split samples, resulting cultures were
protozoa-contaminated or not contaminated, regardless
of the original tissues, exposure to 10% ethanol. It
should be mentioned, however, that since the microbial
population of the oyster reflects that of its surround-
ing environment, it is apparent that decontamination
procedures may not be universally effective.

Fungus and bacterial contaminants were effectively
eliminated from the oyster tissues by soaking the
tissues in antibiotic-antimycotic wash media. The
antimicrobial wash treatments consisted of three 45-
minute washes: tissues were soaked for 45 minutes in
the antibiotic wash media, allowed to settle to the
bottom of the tube and the supernatant decanted off.
Following the third wash, the tissues were rinsed
three times in filter-sterilized bay water. Following
the decontamination procedures, the tissues were
minced into smaller fragments, and again rinsed three
times in filtered bay water. The fragments were then
either placed directly in culture vessels containing
test media, or subjected to further chemical or
physical dissociation procedures, prior to culturing.

Dissociation. While the majority of cultures
were initiated by using minced fragments of oyster
tissue planted directly in the culture vessel, some

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cultures were initiated from cell suspensions that were the result of dissociation of the whole tissue. Both physical and enzymatic methods of dissociation were used.

As a means of physical dissociation, wire sieves with various screen sizes were tested on all types of oyster tissue cultured in the lab. Enzymatic dissociation, however, was more successful. Reports on invertebrate tissue culture indicate that the routine methods for enzymatic dissociation of vertebrate tissue yield variable results when used on invertebrate tissue. Trypsin has been used successfully to dissociate insect and molluscan tissues for cell culture (Farris, 1968; Hansen, 1976; Kurtti, 1976). Hansen (1976) emphasized the importance of developing a suitable trypsin solution for use in the establishment of the snail cell line. The use of carbohydrates in the trypsin diluent has been reported to aid in dissociation, and is consistently incorporated in many invertebrate tissue trypsin diluents. Throughout the two years of work on oyster cell culture, a variety of trypsin solutions were tested.

The enzyme used was a commercially prepared trypsin solution (2.5%) in normal saline (GIBCO). The trypsin concentrations tested, as well as the time of exposure, were varied with consideration to the tissues being dissociated and the diluent components. The range of concentrations tested was 0.05% to 0.25% trypsin. The length of trypsinization was regulated by macroscopic observation of the tissue fragments during trypsinization, and microscopic observation of the resulting cell suspension. Although trypsin treatment was tested on mantle, muscle and palps fragments, the tissues best suited to trypsinization were the heart and gonad tissues.

In general, the best cultures obtained were those established in the following diluents with diluent G providing the best results.

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Table 1. Formulations of trypsin diluents used in
          oyster cell culture (in g/ℓ, unless other-
          wise indicated)

Trypsin diluent D   Trypsin diluent G

Na gluconate 1.50   Na gluconate 1.86
NaCl   6.00   NaCl   3.50
KCl   0.38   KCl   0.19
Na_2HPO_4 0.10 Na_2HPO_4 88   mg/ℓ
$NaHCO_3$ 0.05 $NaHCO_3$ 63   mg/ℓ
Glucose   0.50   Glucose   0.50
Galactose   0.50   Galactose   0.50
Trehalose   0.50   Trehalose   0.50
EDTA   38   mg/ℓ   EDTA   38   mg/ℓ
Phenol red   5   mg/ℓ

        pH 7.6   pH 7.6

                 Trypsin diluent E

               (physiological saline)

            NaCl   8.50

Diluent G was constructed, as was diluent D, as a
modification of Hansen's trypsin formulation. Ad-
justments were made in salt concentrations in attempts
to effect the appropriate osmolality for the oysters
being used. Following trypsinization and rinsing,
cells would adhere firmly to the culture vessel and
remain attached for several months.

     Cultures initiated from trypsinized tissues
were found to remain healthy for longer periods if a
small trypsinized fragment was also added to the cul-
ture. The fragment did not appear to serve as a sig-

nificant source of cells, but rather functioned, perhaps, in more rapidly conditioning the medium. (See "Maintenance of Cultures")

Possibly the most significant observation of the effect of trypsinization was its potential for altering the population of attached cells. In explant cultures, the primary cell type which migrates from the tissue and attaches to the vessel surface is the granular amoebocyte. While trypsinization of the tissue fragment also yields some granular amoebocytes, other fibroblast-like cells also attach in culture.

Trypsinization of heart tissue yielded individual, fibroblast-like contractile cells, which would attach to the culture vessel. These single cells would remain attached at the ends, and continue to "beat" in culture for up to six weeks. While these individual contracting cells were also observed occasionally in some heart explant cultures, they represented a very small percentage of the attached cell population. Under certain conditions, these individual contracting cells would seem to replicate, such that a small ball of beating cells, attached to the culture vessel, would develop.

Trypsinization of gonad tissue also provided a source of fibroblast-like cells differing from the typical granular amoebocytes observed in explant cultures. Some of the cells were small with very long, thin, protoplasmic processes, resembling the only attached cell type obtained from culturing oyster larvae. For convenience, cells with this characteristic microscopic appearance are termed "larval-type" cells. Such larval-type cells were obtained in repeated tests using gonad tissue trypsinized with 0.25% trypsin diluted in saline.

While trypsinization of the tissues did not always provide a source of non-amoebocyte cells, dissociation of the tissue fragment by trypsin treatment may provide a means of evaluating cell types other than the amoebocytes, in cell culture.

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Incubation. Following decontamination and disso-
ciation procedures of the oyster tissue, the tissue
fragments or cells were placed into culture vessels
containing test media. A variety of plastic and glass
culture vessels were tested; no differences in cell attach-
ment are found among them. The Costar 24 well cluster
dishes (approximately 2 cm^2 per well) were found to be
the most desirable for the oyster cell culture work.
Cells attached readily to the well surface, the multiple
wells providing an efficient means of comparing a va-
riety of test media on a single batch of oyster tissue;
the small well was suited for the high tissue-to-media
ratio that was desired for initiation of invertebrate
tissue cultures. All cultures were incubated at 15°C.

Maintenance of Cultures

Immediately after initiation of an explant culture,
amoebocytes began to migrate from the tissue fragment,
and, within minutes, attached to the culture surface.
After 24 hours, each tissue fragment in the culture
vessel exhibited a dense "halo" of attached amoebocytes.
Within several days, the outer edge of amoebocytes be-
came less dense, as amoebocytes migrated from the frag-
ment, and began to form a sparse layer of cells through-
out the culture vessel. Within 7 to 14 days, as more
amoebocytes migrated from the fragments, the cells be-
came more and more densely packed on the vessel surface,
until finally a confluent monolayer of amoebocytes was
achieved. Confluency was reached, generally within 7 to
21 days, depending upon the tissues and media employed.
Explant cultures were refed every 3 to 5 days by removing
50% of the spent media and adding 50% fresh media.

Cultures which were initiated from trypsinized
tissue were maintained by refeeding with 25 to 50% fresh
media every 7 days. Basically, the primary cultures
resulting from these explants or trypsinized tissues
were used in one of the following ways in attempts to
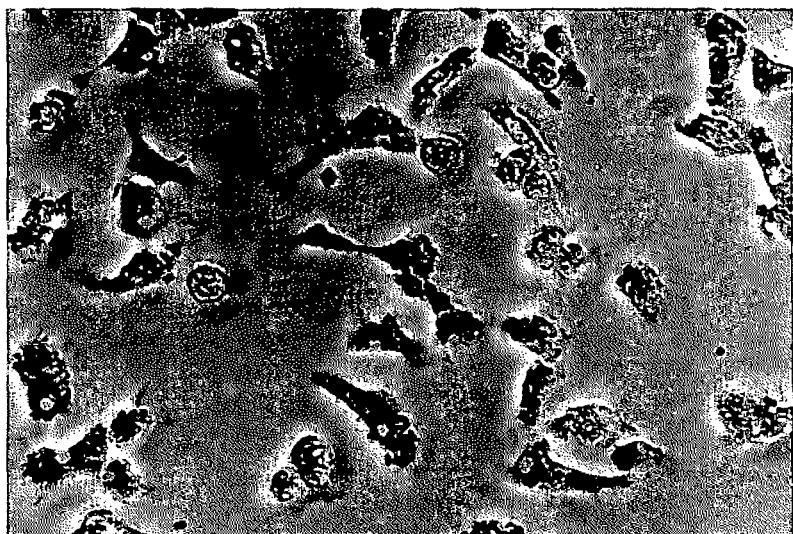establish an oyster cell line:

Figure 2.   Photomicrograph of granular amoebocytes
from an oyster mantle explant culture
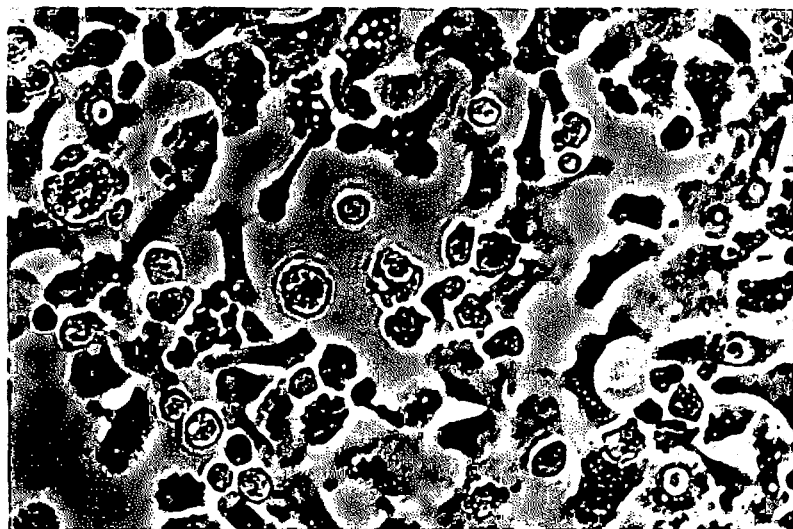after 24 hours. (1150x)

Figure 3.   Photomicrograph of granular amoebocytes
from an oyster mantle explant culture
after 24 hours. (500x)

. Cultures were maintained for extended
  periods (6 months) and observed for
  indications of foci formation or sig-
  nificant cell alterations or mitosis.

. Cultures were manipulated (by physical
  or nutritional alterations) in attempts
  to induce cell division.

. Cultures were used to test a variety of
  media formulations and specific nutri-
  ents to determine the effects of each
  one on oyster cell viability, attach-
  ment and, potentially, cell division.

Tissue Fragments. Early in our investigations,
it became apparent that the presence of small tissue
fragments in the culture vessel was important to the
health of the cells in culture. The length of time
cells would remain viable in culture was extended if
an oyster tissue fragment was included in the cul-
ture vessel. It seemed as though the fragments were
metabolizing in culture, as ciliated mantle tissue would
continue to show ciliary activity and heart fragments
would continue to contract in culture. The importance
of the fragment to the cells in culture is not known.
It is possible, of course, that the cells were direct-
ly using some metabolite of the fragment. Secondly,
it is possible that the fragments were serving to con-
dition the media, or to help maintain a suitable pH.
It is also possible that the fragments provided a con-
tinuing source of "fresh" cells, making the culture
appear to stay healthy longer, but not significantly
increasing the life span of any one cell.

Pulsing. One technique employed by Hansen in
development of the snail cell line was "pulsing" the
cultures with periods of serum-free media. Her re-
ports indicated that refeeding the primaries with
serum-free media, followed in 2 to 3 days by refeed-
ing with complete media, seemed to stimulate cell
division. This procedure was attempted several times

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in our lab using oyster heart explant cultures. While one heart culture seemed to give evidence of an increase in the number of small agranular cells following refeeding of the culture with the serum-free treatment, this was never reproduced with other cultures.

Several cultures of heart, mantle and gonad were subjected to changes of the media, alternating from poorer "starvation" media, such as 100% bay water, into a nutrient rich media. No such alterations in media stimulated cell division.

Trypsinization. Traditional methods of passaging vertebrate cell cultures, such as trypsinization, were attempted on primary cultures of oyster amoebocytes. Observation of the culture three days after trypsinization revealed very few cells attached; most remained floating, but viable, in the media.

Hansen, in her work on the initiation of the snail cell line, noted the appearance of small foci or colonies of cells which developed in a low percentage of the primary cultures. These colonies, which appeared to contain dividing cells, would stop growing after a short period and the entire primary culture would eventually deteriorate. Hansen postulated that these colonies stopped growing due to contact inhibition caused by the other non-proliferating cells of the culture. To eliminate the potential problem of contact inhibition, Hansen suggested an "early trypsinization" procedure to remove the non-dividing cells which might have surrounded a colony of dividing cells, blocking continued growth of the colony. Using this procedure on oyster tissues, we did not observe dividing cells, probably because the oyster cells subjected to this trypsin treatment were merely clusters of migrating amoebocytes rather than true foci of dividing cells.

In addition to the efforts to stimulate cell division by early trypsinization methods, trypsinization was also used to attempt to disperse cells from clumps

of beating cells sometimes observed in heart cultures. As mentioned previously, when heart tissue was trypsinized and the resulting cells cultured, single, contractile cells would attach to the vessel surface and continue to beat. It was found that when these cultures were maintained in certain types of media, especially those containing oyster extracts, these single beating cells would seemingly divide and would appear, microscopically, as a small cluster of 2 to 4 cells, all beating and attached to the original cell. Within several weeks after initiation of the culture, a larger clump of beating cells could be observed, attached to the original cell. The number of these spheres of cells varied from culture to culture, and media to media. As the clumps of cells became larger, they often detached from the surface of the vessel and continued to beat, suspended in the media. These beating cells could be distinguished from the minute pieces of heart tissue by the configuration of the cells. Although these contractile cells were initially detected only in cultures initiated from trypsinized heart tissue, they were also later observed in explant cultures. The observation that these cell spheres arose from cell division of single contractile cells is speculation, based only on microscopic examination of the cultures. The observations were made repeatedly over a two-year period; as media formulations seemingly improved, the frequency of occurrence of these clumps of cells also increased.

We felt that if these clumps of beating cells really represented dividing cells in culture, it might be possible to dissociate the cells, perhaps with repeated treatments, to eventually obtain a monolayer of attached, dividing cardiac cells. Numerous efforts were made to achieve dissociation of these clumps of cells. Cultures were easiest to work with when the clump was small enough such that it was still attached to the vessel. Thus, it was thought possible to flush the culture with mild trypsin, in order to remove the surrounding amoebocytes, and then attempt to dissociate the spheres with more harsh trypsinization.

However, definitive results were never obtained on the use of these clumps of cells as a source of possible dividing cells for culture. Relatively harsh trypsin treatment was needed to cause dissociation; perhaps use of other proteolytic enzymes could produce more effective results.

Although discussion of maintenance regimes used on the oyster cell cultures has seemed to emphasize use of trypsin on the cultures, relatively few of the cultures initiated were treated with trypsin. Many cultures were held, with minimal handling, to observe effects of the various media on the cultures. As indicated, cultures of cells and tissue fragments could be held for extended periods. Tissue fragments would remain active in culture for up to a year. Heart fragments, if left in culture for several weeks without refeeding, would develop cilia, sometimes around the entire periphery of the fragment, and the fragment would become mobile in the culture vessel. Oyster cells in culture would maintain optimum health and attachment for about six weeks, after which time increasing percentages of the cells would detach from the vessel surface. Cells would remain viable in suspension, and low levels of cells would remain attached for six to eight months.

Media Tested

One of the difficulties encountered in working on marine molluscs is that very little is known about the nutritional requirements and metabolism of the animals. Biochemical studies have been done on various molluscan species to determine lipid and fatty acid distribution, and amino acid and carbohydrate content. Enzymes of many of the metabolic pathways found in vertebrates, such as those for glycolysis and pentose-phosphate pathways, have been detected in those molluscs studied. However, the metabolic capabilities of these animals and their resultant nutritional requirements has not been established.

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As filter feeders, the oysters pose a problem to in-
vestigators wanting to do traditional nutrition ex-
periments. Although there is some controversy sur-
rounding the oyster's feeding capabilities, it is
thought that the oyster is able only to ingest par-
ticles of a particular size range, and not able to
utilize dissolved nutrients. Thus, classical nutri-
tion experiments have been hindered as researchers
must prepare chemically defined diets in a capsule
form. Recently, Epifanio (1979) attempted to identi-
fy "good" and "poor" algal diets for oysters and then
compare the biochemical analyses of these "good" and
"poor" foods. Analyses of the algae included fatty
acid and amino acid content. Unfortunately, no con-
clusive information pinpointing specific nutritional
requirements for the oyster could be obtained.

As a result of this dearth of information on
oyster biochemistry, developing media for in vitro
cultivation of oyster cells took on a "kitchen sink"
approach. Quantities of undefined nutrients were
added to media in the hope of including all ingredi-
ents necessary for oyster cell division. The danger
in this approach, of course, is that along with the
growth requirements one may also include toxic or
inhibitory substances. In addition, it is possible
that excessive concentrations of carbohydrates or
amino acids may become inhibitory, serving as meta-
bolic regulators to "shut down" necessary metabolic
pathways.

Much of the media was prepared from commercial-
ly available components used routinely in vertebrate
and invertebrate tissue culture; however, some algae
and oyster extracts, prepared in our lab, were also
employed in the media. A wide variety of formulations
were tested in an effort to determine the suitability
of each for supporting oyster cell maintenance and,
hopefully, stimulating cell division and growth.

It was necessary to adjust all media tested
to the osmolarity appropriate for oyster cells.

Since oysters exist in nature as osmoconformers, the osmolarity of the oyster hemolymph is nearly equal to the osmolarity of the water in which the oyster is maintained. Oysters are capable of rapid internal osmolarity adjustments, and can conform to a wide range of salinities (2 ppt to 30 ppt). Oysters used in our lab, generally, were maintained at the salinity of the Choptank River which ranges from 6 ppt in the spring up to 12 to 13 ppt in the fall (ranging in osmolarity from approximately 175 to 275 mOsmoles The osmolarity of the media used on the oyster cells was adjusted by the use of filter sterilized bay water. Water was collected from the area from which the oysters were taken and, thus, had the osmolarity equivalent to that found in the oysters. Since a large percentage of the oyster media was composed of bay water, and the majority of commercially prepared components used were 280 to 340 mOsmoles the appropriate osmolarity for oyster cells was not too difficult to achieve. Exact duplication of osmolarity was not thought to be too critical due to the apparent tolerance of oyster cells in nature to rapid changes in environmental salinities. The use of natural bay water in the oyster culture media also aided in supplying the proper ion balance required for the oyster cells, and possible trace elements necessary for growth.

A wide variety of commercially prepared media were tested for each one's suitability for oyster cell culture. Many of the media components were individually screened on developing oyster larvae to detect possible toxicity or inhibition of cell division. While it was recognized that the results obtained on oyster larvae may not necessarily duplicate media effects on oyster amoebocytes, the technique provided a rapid screen for media components, on a source of known dividing cells.

The various media formulations were primarily tested on explant cultures of oyster tissue. A batch of homolgous tissue, generally from five to six oys-

ters, was processed together as a pool of tissue.
After the final fragments were obtained, several
fragments were placed in each of 15 to 20 to cm^2
wells. One ml of test media was added to each well,
with triplicate wells being used for each media.
Dishes were incubated, and the response of the ex-
plant cultures to the various test media, over at
least a two month period, was noted. Initial experi-
ments were done comparing 4 to 5 media on a given
batch of oyster tissue, each media varying from the
other by only one component. It soon became evident
that the response of explant cultures to slight vari-
ations in media compositions was very difficult to
assess. With few exceptions, alterations in media
formulations elicited negligible alterations in cul-
ture response. Amoebocytes from mantle tissue were
maintained equally well (for four to six weeks) in
bay water, as in highly complex media. As a result
of such observations, more gross alterations were
made in media compositions for comparison purposes.
Again, it should be emphasized that evaluation of
the media was subjective, based only on microscopic
observation of the cultures; minimal differences
were detected between most media formulations tested.
Confluent monolayers of oyster amoebocyte primaries
could be obtained in nearly all the media evaluated.

In general, the oyster cell media formulations
which included percentages of insect tissue culture
media yielded poorer culture responses than other
formulations. Duplication of Hansen's snail cell
culture medium, with adjustments made for osmolarity,
also produced poor results on the oyster cell cul-
tures.

Review of the compositions of the "best" media
reveals that most formulations include oyster or
algae (*chlorella*) extracts. Although the use of oys-
ter and algae estracts as supplements to the media
did not repeatedly produce marked differences in
culture results, it was observed that all "interest-
ing" cultures obtained during the past two years

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(i.e., all those observed to have potentially divid-
ing cells) were maintained in media which contained
low percentages of these extracts. The clumps of
beating cardiac cells described previously were ob-
served with greater frequency in cultures maintained
in media containing algae extract, oyster hemolymph
and/or oyster larvae extract. Despite the somewhat
encouraging results obtained with media containing
oyster and algae extracts, no significant mitosis,
leading toward development of a cell line, was ob-
tained.

In addition to the use of oyster hemolymph in
the culture medium, efforts were made to construct
media more nearly consistent with the amino acid com-
position of the hemolymph. Evidence from insect tis-
sue culture indicates that such duplication of hemo-
lymph composition was unnecessary for successful
cultivation of insect cells. However, since other
formulations were unsuccessful in stimulating oyster
cell division, attempts at hemolymph duplication be-
came desirable. While no reports were found on the
free amino acid content of hemolymph from *Crassos-
trea virginica*, several studies presented amino acid
data on oyster tissue. Lynch (1965) has studied how
the free amino acid concentrations in the adductor
muscle of *Crassostrea virginica* change with respect
to salinity. As is true in other marine inverte-
brates, amino acids function in osmoregulation. As
the salinity of the water environment increases, the
free amino acid content of the oyster tissue also in-
creases significantly. Thus, the free amino acid
composition measured from molluscan tissue is depen-
dent on the salinity from which the mollusc was taken.
While the concentrations of most amino acids are sig-
nificantly higher (10 to 100 times) in marine molluscs
than in mammals, certain of the amino acids deviate
more dramatically than others. Using the values re-
ported by Lynch, for oysters taken from 12.5 ppt, the
free amino acid concentrations of alanine, arginine,
glutamic acid, glycine, histidine, proline and taur-
ine in the tissues are significantly higher than the

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concentrations found in mammalian tissue culture media.

One of the amino acids found in high concentrations in oyster tissues, but not found in most mammalian or insect culture media, is taurine. Taurine is thought to play a role in osmoregulation, although it possibly functions in metabolic processes. Taurine was tested as an additive in several culture media; however, no significant positive effect was noted. Mitogens have been used by other investigators (Hansen, 1976; Tripp, 1966) in attempts to induce cell mitosis in molluscan cells; however, no cell division was induced by the mitogens in their efforts. Pokeweed mitogen was tested as a media supplement in several of our oyster cell cultures. Again, no response was observed.

Other "stimulators" of cell mitosis were also employed in the culture media. Multiplication Stimulating Activity (MSA -- Collaberative Research, Inc.), isolated from serum, is thought to be the protein factor responsible for supporting mammalian cell division in vitro. Media were supplemented with MSA and tested on different batches of oyster heart tissue. No clear cut distinction could be made between the MSA-treated and non-treated cultures; however, the cultures maintained in MSA had a higher frequency of the clumps of beating cells.

A report by McKeehan and Ham (1976) indicated that clonal growth of human and chick fibroblasts was enhanced by coating of the culture vessel with polylysine. Thus, polylysine coated surfaces were tested for use in oyster cell culture. Techniques employed in coating the vessels were those described by McKeehan and Ham (1976). Tests were run using 0.2, 0.4 and 0.8 µg polylysine coating per 2 cm^2 well. The treatments used were not toxic to the cells (as was the case with the neoplastic clam cells), nor did they enhance cell attachment or cell growth.

The dry mass of a typical mammalian cell contains
a sizeable portion of lipids. This is also true of
invertebrate cells; the range in *C. virginica* is from
2.4% to 3% (Sampugna et al., 1972). However, most
synthetic, commercially prepared tissue culture me-
dia contain little or no lipid. A number of estab-
lished cell lines, both vertebrate and invertebrate,
have been shown to be capable of synthesizing all the
lipids that they need for growth, but they preferen-
tially utilize exogenous supplies of lipids when
available (Stegemen and Teal, 1973). In the absence
of the essential fatty acids, these cells continue to
multiply, but with grossly altered intracellular lipid
compositions (Bailey and Dunbar, 1973). The preced-
ing evidence indicates the probable importance of
supplying a source of lipids to tissue culture media
for primary cultures of mollusc tissue.

Additionally, feeding studies with *C. virginica*
have indicated that it does have an essential fatty
acid requirement for the linolenic and linoleic series
fatty acids (Trider and Castell, 1980). However, most
reports on molluscan lipid and sterol metabolism show
conflicting results. Idler and Teshima (1972) found
that cholesterol was a major sterol in oysters and
that it could be synthesized from squalene. In con-
trast, Trider and Castell (1980) report that the re-
sults of a ^{14}C-acetate metabolism experiment suggest
that *C. virginica* cannot synthesize its own sterols.

We investigated the possibility that lipids,
sterols and fatty acids must be added to the basal
media for oyster primary cell cultures in order to
encourage proliferation of the cells. Accordingly,
a variety of combinations and concentrations of these
components were tested.

Lipids were obtained from oysters by extraction with
organic solvents. Whole oyster lipids have an average
chain length of 18 to 22 carbons, which makes them quite
insoluble in aqueous media. The lipids were also found
to be insoluble in ethanol, acetone, dimethyl sulfoxide

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(DMSO), TWEEN and sodium deoxycholate (NaDOC). The
solubility problem was solved by sonicating the whole
oyster lipids into fetal bovine serum, which formed
an emulsion that was dispersible in the basal tissue
culture media formulations. The stock concentration
of the oyster lipid-FBS emulsion was 10% oyster lipid.

A variety of concentrations ranging from 3% oys-
ter lipid content to 0.001% oyster lipids were tried
in several of the basal media formulations. The low-
er concentration spectrum, 0.01% to 0.001%, seemed to
result in primary oyster heart cultures with increased
numbers of fibroblast-like cells, but no source of
proliferating cells was ever found. It should be not-
ed that lipid concentrations higher than 0.01% seemed
to have toxic effects on the cells, evidenced by ex-
cessive alkalinity of the media and rapid deteriora-
tion of the cultures.

The next type of supplement tested was whole
oyster sterols. The extraction procedure for obtain-
ing oyster sterols is very similar to the procedure
for obtaining whole oyster lipids. An additional
column chromatographic step was used to separate com-
plex sterol mixtures as their acetates. Solubility
was also a problem with the whole oyster sterol pre-
paration. The oyster sterols were sonicated to FBS,
as was done with the whole oyster lipids. No dis-
cernible difference was noted in the condition of the
oyster heart primary cultures supplemented with whole
oyster sterols and those supplemented with whole oys-
ter lipids.

Similar results were obtained with commercially
prepared sterols and fatty acids. Linoleic acid and
linolenic acid were also tried. Again, although
there seemed to be a slight improvement in culture
quality, all of the cultures supplemented with choles-
terol, essential fatty acids and lipids eventually
deteriorated.

Insulin has been a useful addition to culture media for vertebrate and invertebrate cell lines and seems to be necessary for some vertebrate cell lines which are propagated in serum-free media (Higuchi and Robinson, 1973). Cells supplemented with insulin in the "physiological range," however, showed no discernible difference from untreated control cultures.

Several other unconventional supplements were tested. Putresine has been shown to stimulate the clonal growth of Chinese hamster ovary cells and it has been identified as a component of conditioned media that promotes growth of human fibroblast cells. L-dopa (L-β-3,4 dihydroxyphenylalanine) seems to be necessary for the attachment of oyster spat to surfaces. It was also tested for possible growth promoting activity. Neither the addition of putrescine or L-dopa seemed to have any beneficial effects on oyster heart primary cell cultures.

Since reducing agents seem to be essential for successful maintenance of insect cell lines, glutathione (GTT) and ascorbic acid concentrations of the basal media were increased. Again, there seemed to be no discernible difference between control cultures and those fed with the test media. It should be noted that the GTT and ascorbic acid levels were adjusted differently depending on the composition of the basal media employed. This was because NCTC-135, a component of all the basal media formulations, contains low levels of GTT and ascorbic acid.

Another group of media supplements traditionally used as "serum extenders" includes bovine amniotic fluid, bovine embryo extract, chicken embryo extract, whole egg ultrafiltrate and lactalbumin hydrolysate. There has been little rationale for using these ingredients in addition to serum, other than the fact that they seem to work in instances where serum alone does not seem to be effective in promoting growth of cultured cells. With the exception of chicken embryo extract (GIBCO) which seemed to have

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toxic effects on oyster heart primary cell cultures, all of the other supplements increased culture quality significantly. Addition of bovine embryo extract seemed to be as beneficial for the primary cultures as adding oyster hemolymph or oyster heart extract. This indicates a need for nutritional or growth factors not present in fetal bovine serum. Although addition of these serum extenders did not stimulate proliferation of cells in the primary cultures, one or more of these supplements should be routinely added to media intended for primary culture of mollusc tissues since they do seem to extend the average survival time of the cultures.

Attempts at Transformation

Although neoplasia has been most extensively studied in humans and laboratory mammals, the existence of tumors in fish and shellfish has been recognized for almost a century. The first recorded incidence of a solid tumor in an oyster was reported by Ryder in 1887. Even though research in this area has been scant, there is limited information available on several types of fairly common cellular abnormalities occurring in shellfish (Rosenfield,1976). In addition, Yevich and Barszcy (1977) have examined neoplastic growths occurring in the soft shell clam, *Mya arenaria*, in relation to petroleum contamination. Epizootic neoplasma of a hematopoeitic origin and with possible environmental etiology have also been studied by Mix (1976) and Mix et al. (1979) in several species of bivalve molluscs in Yaquina Bay, Oregon.

However, since neoplasms have not been induced, transplanted or transmitted in molluscs under laboratory conditions, the biologic nature of these diseases remains unknown. Some evidence indicates that the reported neoplastic diseases of molluscs appear to represent proliferative disorders. Additionally, since the occurrence of reported tumors in molluscs has been associated with petroleum wastes and chlorinated hydrocarbons, it was felt that exposure to con-

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centrated sources of carcinogens might induce a
culturable source of proliferating mollusc cells.

Three different carcinogens were used in ex-
periments ranging over eight months. Benzo(a)pyrene
(BAP) and 3-methylcolanthrene (3-MCA) were chosen be-
cause they are chemicals that are present in pollut-
ed marine estuarine environments. Additionally, BAP
has been directly correlated with the prevalence of
the "large cell" neoplastic condition in molluscs
collected from Yaquina Bay in Oregon (Mix, 1978).
N-methyl N' nitrosoguanidine (NTG) was also used,
because unlike BAP and 3-MCA, NTG is a direct act-
ing mutagen, needing no metabolic activation to a
highly carcinogenic intermediate form.

After injection with 1, 10 or 100 μg of a car-
cinogen, oysters were placed in holding tanks with
five gallons of aerated, artificial sea water. The
uninoculated controls and the controls injected only
with the solvent DMSO or Tris-maleic buffer were
placed in a separate tank. The water in each tank
was changed every other day for a minimum of eight
weeks. (It should be noted that oysters generally
could not be held for longer than 10 weeks because
of an increase in mortality due to starvation.)

At the end of this time, the oysters were care-
fully opened and visually examined for any gross
morphological changes. Interestingly, none of the
oysters had any localized necrotic areas even at
the injection site and most appeared to be in good
condition. Tissue samples were taken from the in-
jection site and from the mantle and labial palps.
The heart and pericardial membrane were also pro-
cessed. The tissues were prepared for explant cul-
ture and for trypsinization by the previously de-
scribed procedures.

Since we were primarily interested in finding
a source of proliferating cells in molluscs, no
histopathological studies were done. The only dif-

ference between cultures established from treated oysters and those established from the control oysters was a predominance of atypical amoebocytes. Some of these amoebocytes were three to four times the size of "normal" oyster amoebocytes. These cells may be analogous to Mix's observations of neoplastic "large cells" in oysters collected from a diseased population in Yaquina Bay. However, none of the cultures contained cells that showed any significant mitotic activity.

The effect of adding carcinogens directly to oyster primary cell cultures was also explored. Primary oyster cell cultures were derived from trypsinized heart tissue. Initially, BAP and 3-MAC were added to the cell cultures without metabolic activation. When no observable differences between the treated and control cultures were noted, the chemicals were treated with a rat liver microsomal (S-9) mix in an attempt to metabolically activate the compounds. A small number of the "large" granular amoebocytes were seen in treated cultures after 10 to 15 days of exposure, but much less than were noted from the cultures established from oysters that were treated with carcinogens *in vivo*.

Use of NTG in the culture media produced similar results. A few "large cells" appeared in the treated cultures, with none in the control cultures. Concentrations of NTG higher than 0.1 µg/ml of culture media had toxic effects on the primary cultures. Several of the NTG treated primary cultures were passaged using the modification of Hansen's "early trypsinization" techniques described earlier. Unfortunately, although many of the passaged cells reattached, a population of dividing cells was never attained.

Summary

Primary cultures of oyster cells were obtained by cultivating tissue from the adult American oyster, *Crassostrea virginica*. Cultures could be initiated

from a variety of tissues, with the oyster heart, mantle and gonad providing the best results. Cultures were established by first decontaminating the oyster tissue, mincing the tissue into small fragments, and either using the tissue directly in explant culture or dissociating the fragments further by trypsinization. Explant cultures were established as cells would migrate from the tissue fragment and attach to the culture vessel. Cells would continue to migrate from the fragment for several weeks, eventually forming a confluent monolayer of densely packed cells in the culture vessel. Confluency was achieved 7 to 21 days following culture initiation, depending on the tissues and media employed. Several cell types could be detected in the oyster cell cultures; however, granular amoebocytes predominated.

While it was possible to maintain viable cells in culture for extended periods, significant cell mitosis, leading toward development of a cell line, was not achieved. While in a few cultures, observational evidence indicated possible low levels of cell division, such cultures never led to establishment of a cell line. Over a two year period, a variety of culture maintenance regimes and media were tested in an effort to stimulate cell division of the oyster cells. None of the techniques tested, however, were successful in promoting significant mitoses.
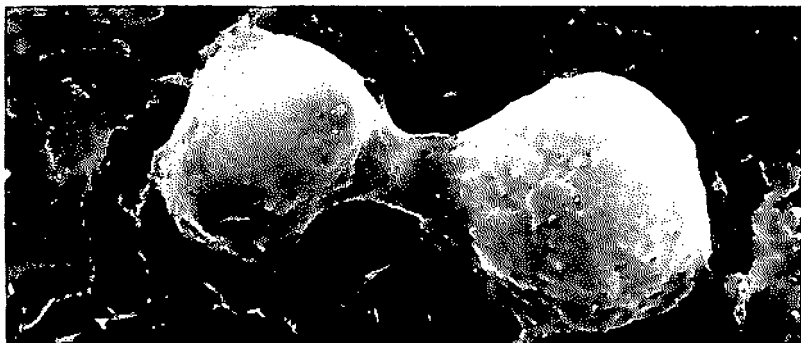
Figure 4.   Scanning electron micrograph of a rare
            mantle cell apparently undergoing divi-
            sion. (9700x)

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Efforts to initiate a marine molluscan cell line included work not only with adult and neo-plastic molluscan tissue but also with embryonic tissue from the American oyster, *Crassostrea virginica*. The relative ease of initiating cell cultures from embryonic material has been demonstrated repeatedly in vertebrate and other invertebrate tissue culture efforts.

Before describing the actual use of oyster embryos in cell culture, it is important to describe the biology of *Crassostrea* reproduction, and the methods available for achieving healthy oyster embryos. Oysters differ from some molluscs in that they have no secondary sex characteristics, and their sex can be determined only during the reproductive season by examining the gonad for the presence of ova or spermatozoa. During the reproductive season, gonads form a large, indistinct, non-encapsulated organ, bordered by the digestive diverticula on the inner side and by the mantle epithelium on the other, encompassing (bilaterally) the entire visceral mass. The sexually mature gonad forms a network of highly branched follicles lying within a thin sheath of connective tissue. The lumen of the follicles are lined with germinal epithelium from which the sex cells arise. During development and maturation of the oyster gonad, the mature sex cells remain stored in the follicles until they are released, at spawning, into the epibranchial chamber and on into the environment. The fully ripe gonad forms a milky colored mass of tissue, up to several mm thick, consisting primarily of densely packed sex

cells, with a small percentage of connective tissue and follicular cells.

At the end of the reproductive season, whether or not spawning has taken place, large numbers of phago-cytic cells enter the gonad and digest any remaining sex cells. The connective tissue layer between the follicles becomes disorganized. After reabsorption of the gonad is complete, the organ consists only of a few follicles lined with a thin layer of germinal epithelium. During this period, the gonad is indis-tinct, not visible to the naked eye.

While members of the genus *Crassostrea* are rarely hermaphroditic, i.e., having both mature ova and spermatozoa within the gonads at the same time, these oysters can change sex from one reproductive season to the next. Occasionally, they will even change within a given season. The primordial gonad of *Crassostrea* is bisexual, in that it contains germinal cells for both sexes. The factors regulating the development of an oyster as a male or a female are not understood. It is known(Galstoff, 1964) that in *C. virginica*, spermatogonia develop more rapidly and require less metabolic activity than ovogonia; thus, a young oyster gonad will often take on a predominately male appear-ance. In general, oysters in their first breeding season will develop as a male.

C. virginica are oviparous, releasing mature eggs and sperm into the environment, where fertilization and embryogenesis takes place. A few oyster species (*Ostrea edulis, O. lurida*) are larviparous organisms. The release of mature sex cells (spawning) by adult oysters is stimulated by a gradual rise in water temperature, until a threshold level is reached. The coordinated release of eggs and sperm by an entire oyster population within a specific area is thought to occur by the oysters' response to chemical stimuli in the water. Addition of a sperm or egg cell suspension into water containing sexually ripe oysters often

triggers a spawning response. Other chemical stimuli,
such as pollutants or low salinity, can inhibit the
spawning response in sexually ripe oysters.

In the Chesapeake Bay region, the reproduction
season for *Crassostrea* is generally from April-October;
however, there is much variation throughout the length
of the Bay. Gonad development usually begins in April
when water temperatures rise above 10°C. The rate of
gonad development depends primarily on environmental
conditions, such as water temperature, salinity and
food availability; consequently, much variation exists
in the spawning period. In the lower Bay area, where
salinity is high, spawning may begin in mid-May, while
in the Choptank River area, spawning may not occur
until mid-June. After the initial spawning the oyster
will continue to develop gonad, and may release eggs or
sperm at low levels throughout the summer, or as a
second major event in late August or September. By late
September and October, the oysters have begun reabsorbing
gonadal material and dissection of the gonad at this
time will reveal large numbers of amoebocytes and
partially disrupted ova or immobilized sperm. In general,
male oysters remain sexually active later into the fall
than do female oysters.

Embryogenesis and oyster larvae development is
described thoroughly by Galstoff (1964) and, thus,
it will suffice to mention briefly only those events
which are of significance to cell culture efforts.
Following egg fertilization, there is much variation
in the timing of the developmental sequences, as
environmental conditions and variability of oyster
populations play a large role in determining develop-
mental rates. In general, at 28°C the first cleavage
is noted at about one hour following egg fertilization.
After the initial cleavage, cell division continues
rapidly for several hours and the embryo appears as
merely a microscopic ball of cells. During this
period, the diameter of the developing embryo remains
relatively constant--35 to 40 u. Within 3 to 4 hours
post fertilization, gastrulation takes place, rapidly
followed by the formation of cilia and "hatching" of
the embryo from the vitelline membrane into a troco-

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phore stage larva. Once larvae have reached the
trocophore stage, they are covered with a layer of
ciliated cells and have developed a rudimentary gut,
capabel of nutrient absorption. Continued development
of the larvae is possible in the absence of external
nutrient sources, however, and in sterile Bay water at
28°C, the trocophore stage larvae will develop to
become straight hinge larvae within 24 hours. The term
"straight hinge" refers to the readily apparent bi-
valve shell which has formed on the larvae. The
straight hinge larvae are rapidly motile, swimming with
the valves open somewhat and ciliated membrane (velum)
protruding from the open shell. If nutrients are not
available for the larvae at this stage, death will
result. Straight hinge larvae measure approximately
60 to 70 μ across the widest dimension of the shell. No
oyster larvae older than straight hinge larvae were
used in our molluscan cell culture work.

unfertilized
egg
NUCLEUS

fertilization
SPERM

initiation of
division

two-cell
stage
YOLK

four-cell
stage

early segmen-
tation

late segmen-
tation

beginning
of shell
formation
SHELL
CILIA
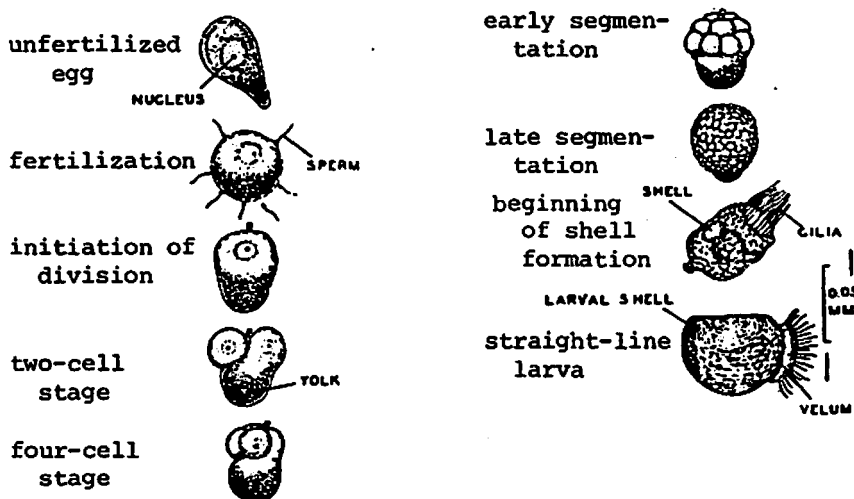LARVAL SHELL
0.03
MM

straight-line
larva
VELUM

Figure 5.   Early developmental stages of the oyster.
(Adapted from Galtsoff, 1964.)

Methods for Obtaining Oyster Embryos for Cell Culture

Adult oysters to be used for production of oyster larvae were collected from a variety of locations throughout the Chesapeake Bay early in the reproductive season, prior to natural spawning (early May). These oysters were held in flowthrough salt water tanks in the Horn Point Environmental Laboratory (HPEL) oyster hatchery, at water temperatures not exceeding 20°C. This temperature is adequate for continued slow development of the gonads, but is too low for spontaneous natural spawning. By holding oysters in this manner, it was possible to keep them sexually ripe for an extended period throughout the summer months.

While this method provided an efficient means of extending the oyster reproductive season, maintenance problems did exist. The adult oysters kept in the tank were dependent upon naturally occurring phytoplankton in the water supply for their food source. Phytoplankton levels of the Choptank River were often low--sufficient for maintenance of oyster populations but suboptimal for good gonad development. In addition, the hatchery conditions were suitable for rapid transmission of systemic parasites, and many oysters contracted infections with *Tricephalum* sp., a parasite whose larval stages are within the gonad of adult oysters. While these problems reduced the numbers of oysters available for larvae production, the problems were not prohibitive.

Primarily two methods were used for obtaining oyster embryos:

1) Induction of natural spawning by sexually mature adult oysters, in a controlled laboratory environment.

2) Dissection ("stripping") of gonadal material from sexually mature oysters, then proportionately mixing the resulting ova and spermatozoa to achieve fertilization.

Induced Spawning. Oysters were spawned by methods used in the HPEL oyster hatchery, with some modification to reduce contamination of the resulting embryo suspension. Sexually ripe oysters (10 to 12 oysters) were removed from the 20°C holding tanks and, in the lab, were scrubbed in tap water and dipped in a 5% bleach solution to disinfect shell surfaces. The oysters were then placed in Nalgene tubs, each containing about 6 L autoclaved Bay water. Over a 3 hr period, the water temperature of the tubs was gradually increased from 22°C to 28°C by the addition of prewarmed, autoclaved Bay water. If specific oysters were observed not to be actively pumping during this period, they were removed from the tubs and replaced with other oysters, when available. Once the water temperature had stabilized at 28°C, a suspension of oyster eggs or sperm, prepared by dissecting gonadal tissue and suspending it in sterile Bay water, was carefully pipetted into the tubs around the open oyster valves. Small quantities of the suspension could be pipetted in every 10 min or so, until spawning was initiated. During this period, it was essential not to jar the oyster tubs, causing the oysters to close, or to pipet the sperm suspension too vigorously, also causing the oysters to close. Depending upon the sexual ripeness of the oysters being used, minimal quantities of the suspension (3 to 5 ml) were necessary to induce spawning.

Oysters were observed carefully during this period for indications of spawning activity. A stream of white suspension flowing from the oyster indicated release of sex cells into the water. Although spawning behavior is generally different for the male and female oyster, for the inexperienced observer it is necessary to microscopically examine the released material to definitively determine the sex of the spawning oyster. Once spawning was initiated in the original Bay water tubs, all female spawning oysters were placed in a clean tub containing sterile 28°C Bay water, and permitted to complete

spawning in this container. Likewise, male oysters were transferred to a separate, fresh tube for completion of spawning. In this manner it was possible to collect separate egg and sperm suspensions which could later be mixed in proper proportions to obtain healthy embryos. If oysters are permitted to spawn completely in the original container, the proportion of sperm will, most probably, be too great for the number of eggs, and polyspermy will result, yielding abnormally developing embryos.

Once spawning was complete, the oysters were removed from the "male" and "female" spawning tubs. The spawned eggs and sperm were mixed to achieve fertilization within 1 to 2 hrs after initiation of spawning. The egg suspension was adequately concentrated by permitting the eggs to settle to the bottom of the container before pouring off the majority of liquid. The eggs were then poured into sterile one liter beakers to achieve several million eggs per beaker. The suspension was diluted to 500 ml with sterile Bay water. The amount of sperm suspension added to the eggs was critical to obtaining a good yield of healthy embryos, but was difficult to standardize or quantify, because yield not only depended upon the density of the suspension, but also upon the apparent viability of the sperm. With experience, it was possible to add sperm to the egg suspension, mix and observe the mixture microscopically to determine if the sperm/egg ratio was sufficiently high. More sperm could be added if necessary.

In addition to obtaining oyster embryos from oysters spawned in our lab, oyster embryos were occasionally obtained from the HPEL oyster hatchery. Hatchery oysters were induced to spawn in a manner similar to that described above, except the procedure was carried out on a larger scale and river water, filtered through a 1 μ cartridge filter, was employed instead of autoclaved water.

Figure 6. Unfertilized egg of the American oyster embryo 2 hours post-fertilization. (360x)

Figure 7. Unfertilized egg of the American oyster embryo 3 hours post-fertilization. (450x)

    Inducing natural spawning of oysters to obtain oyster embryos for cell culture had both its advantages and disadvantages. While natural spawning of the oyster yielded high percentages of healthy embryos, the suspension of embryos was generally contaminated with the internal microflora of the adult oysters used. In addition, while it was possible to induce spawning in sexually ripe oysters, the methods were far from foolproof, and natural spawning was not always achieved when desired. Induction of spawning in very ripe oysters was relatively easy; however, the chances of success were reduced as the sexual ripeness of the oyster diminished.

Dissection of Gonads. The second method for ob-
taining oyster embryos for cell culture involved
dissection of gonadal material. This method differs
from spawning only in the means of getting the oyster
eggs and sperm. Oysters to be used for dissection
were scrubbed thoroughly in tap water, dipped in a 5%
bleach solution and depurated for two to three days in
lab aquaria containing artificial Bay water. During
this period, water was changed daily and oysters and
tanks were scrubbed with each water change.

Prior to dissection of the oysters, shell sur-
faces were again scrubbed and disinfected by dipping
into a solution of 5% bleach. Surfaces were permitted
to air dry. Oysters were opened aseptically and the
mantle tissue, covering the gut and gonad, was cut and
laid back on itself, thus exposing a thin layer of
connective tissue which covers the gonad. Using the
tip of the scalpel, the connective tissue was nicked
and a small sample of gonadal material was examined
microscopically to determine the sex and health of the
oyster. Any oysters found to be harboring gonadal
parasites were not used. It was generally desirable to
use at least two males and four to six females to
obtain suitable quantities of oyster embryos.

Once a sufficient number of male and female
oysters had been identified, dissection of the
specimens was continued. Using sterile scalpel and
forceps, the connective tissue sheath surrounding the
gonad was severed, exposing the follicles and sex
cells. The eggs or sperm were carefully scooped or
scraped out with the edge of the scalpel and suspended
in sterile Bay water.

One advantage of obtaining embryos by this
method, instead of by natural spawning, was that it
provided a more reliable means of achieving embryos
on a specific day. By dissecting out the eggs and
sperm, it was possible to obtain oyster embryos, even
when using adult oysters with minimal gonadal develop-

ment. In this manner, it was feasible to extend the natural reproductive season--using oysters which may have contained mature ova and spermatozoa, but which may not have been sufficiently ripe to spawn naturally. In addition, this method provided a suspension of oyster embryos with much less microbial contamination than found with embryo suspensions from spawned oysters.

Decontamination. Once a suspension of developing oyster embryos had been obtained, it was necessary to eliminate the protozoan, fungal and bacterial contamination from the suspension. Decontamination procedures involved, primarily, rinsing of the embryos several times to reuce debris and contaminant populations, then treating the embryos with a series of antimicrobial agents. The primary constraint on processing of the embryos was time. Three to four hours post egg fertilization, the embryos developed into trocophore larvae, having invaginated and developed a rudimentary gut cavity. In addition, the trocophore stage larvae were covered with a layer of ciliated cells.   We felt that attempts to culture embryonic oyster tissue should be made on early, pretrocophore stage embryos to avoid the difficulties associated with deciliation and decontamination of invaginated, actively feeding, organisms. Thus, it was necessary to restrict decontamination procedures to a minimal length of time.

Protozoa posed the greatest decontamination problem for two reasons: there was no known specific antiprotozoan chemical treatment which could be applied to kill protozoa without harming developing embryos; and there was less of a size differential between the embryos and some protozoan forms, than between embryos and bacterial or fungus contaminants, thus making size differentiation as a means of eliminating these contaminants impractical. The following treatments were tried with varying degrees of success. Hyamine hydroxide (10^{-5} M in bay water) did not have any readily observable deleterious effects on the oyster

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embryos, nor did the treatments eliminate all protozoa
contaminants. Brief treatments (30 seconds) of the
embryos with higher concentrations of hyamine hydro-
xide (10^{-4} M) were found to be detrimental to the
embryos. Acriflavin, an antiprotozoan agent, was
tested in embryo suspensions but was found to be un-
successful. Mycostatin (100 to 200 unites/ml) was
effective in eliminating fungal contaminants but, due
to its insolubility in the medium, was not a very
desirable treatment. Once the embryos had been washed
in a mycostatin suspension, it was difficult to sep-
arate the mycostatin from the embryos, and often the
resulting culture contained much residual mycostatin;
however, there was no evidence of direct toxicity.

Bacteria were effectively eliminated from the
embryo suspension by a series of washes in antibiotic-
containing media, as used on the adult oyster tissue.
Oyster embryos were subjected to three 30 minute wash
periods in the antibiotic wash media. For treatment,
embryos were lightly pelleted in a 50 ml centrifuge
tube and supernatant fluid discarded. Antibiotic
media was added and the pellet resuspended by gently
swirling the tube. Embryos were permitted to soak in
the wash media for 30 minutes, with the tube being
swirled every 10 minutes to resuspend the embryos.
After 30 minutes, the embryos were pelleted by low
speed centrifugation. Supernatant was discarded and
fresh antibiotic wash media was added to the tube. The
procedure was repeated for a total of three washes.

While the combination of the above mentioned
treatments--embryo rinsing, 10^{-5} M hyamine hydroxide
rinse, antibiotic and antimycostatic washes--did yield
decontaminated embryos suitable for tissue culture,
the percentage of contaminant-free cultures obtained
was only 50% to 75% of those initiated.

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One technical problem involved in the decon-
tamination procedures of oyster embryos was centri-
fugation. Since processing time of the embryos was
limited, low speed centrifugation of the embryos,
rather than natural settling by gravity, was used at
all the wash steps to more rapidly and completely
separate out the larvae. We discovered early in
our work that unfertilized eggs, or embryos prior to
the four cell stage, could not be centrifuged even at
low speed, without causing abnormalities in the
development of those embryos. Thus, processing and
decontamination of the embryos generally did not begin
until the embryos had reached the four cell stage. This
further limited the time period during which embryos
could be prepared for culture, and, often embryos had
reached the ciliated, trocophore stage before they were
dissociated for use in culture.

The overall protocol for obtaining and decontam-
inating embryos for culture, as outlined below, was
very successful in yielding contaminant-free embryo
suspensions. In over 100 cultures initiated using
these methods, none were discarded because of contam-
ination problems. In addition, relatively high
percentages of healthy embryos could be obtained, free
from tissue and cell debris.

Procedure for obtaining and processing oyster
embryos for cell culture were as follows (all procedures
were carried out at room temperature):

1. Sexually ripe oysters were collected from
   holding tanks and scrubbed thoroughly in
   tap water. Oysters were dipped in 5 %
   bleach solution for one minute to disinfect
   shell surfaces.

2. Oysters were depurated 2 to 3 days in aerated,
   15 gallon aquaria using distilled water with
   artificial sea salts added to yield the
   proper salinity. Approximately 8 oysters
   were placed in one aquarium containing 5

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gallons of water. Water was changed daily, and oysters and aquaria were scrubbed and disinfected with bleach on a daily basis. Increasing the depuration period beyond 3 days increased the risk of natural spawning taking place in the aquaria, and potentially decreased the vigor of the oyster gonadal material.

3. Immediately prior to use, oysters were removed from the aquaria and were again scrubbed and disinfected with 5% bleach. Using aseptic techniques, oysters were opened, care being taken not to cut into any soft tissue. Excess liquid was drained from the oyster, and the mantle tissue covering the gonad was cut back.

4. Using a sterile scalpel, the connective tissue surrounding the goand was cut, and a small portion of the gonadal material was examined microscopically to determine the sex of the oyster, and the apparent health of the eggs or sperm present. Once the sex of the oyster was determined, the oyster was set aside and the procedure was repeated with the remaining oysters to be used.

5. Once all the oysters had been identified as to sex, the eggs were carefully dissected from the female and put into a 50 ml cen- trifuge tube containing filtered bay water with penicillin added (100 units/ml). Depending upon the ripeness of the female and the corresponding number of eggs obtained, the eggs from one or possibly two females were processed together as one "batch" in a 50 ml tube. The total volume of eggs settling to the bottom of the tube did not exceed 0.5 ml.

6. The eggs were permitted to settle to the
    bottom of the tube (15 to 20 minutes). Eggs
    should not be centrifuged as centrifugation
    will damage them and result in poor fertili-
    zation. The majority of healthy eggs settled
    to the bottom of the tube fairly rapidly,
    while those eggs with disrupted membranes
    remained in the upper portion of the tube.
    Following settling, the upper 1/2 superna-
    tant was discarded and the eggs were re-
    suspended in the remaining supernatant.
    The mixture was poured into a sterile
    gauze-covered 250 ml beaker (to remove
    large tissue fragments), and rinsed through
    with filtered bay water with penicillin to
    yield approximately 50 ml/beaker. The
    gauze was removed from the beaker and the
    eggs allowed to settle (10 to 15 minutes).
    Again, the uppermost portion of supernatant
    was poured off and the eggs rinsed with
    additional filtered bay water containing
    peniciliin.

7. The rinsing procedure was repeated, as
    indicated above, using the following rinse
    solutions:

        Filtered bay water + gentamycin (100 µg/ml),
        two times.
        Filtered bay water without antibiotics,
        two times.

    The entire rinsing procedure took about
    90 minutes.

8. Once the eggs had been thoroughly rinsed
    free of debris, gross contamination and
    unhealthy eggs, and had been suspended in
    filtered bay water containing no anti-
    biotics, they were ready for fertilization.
    Sperm was dissected out from the male

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animals in a manner similar to that used
in stripping the eggs from the female.
A sperm suspension was prepared in filtered
bay water without antibiotics. A pool of
sperm from at least two males was most
desirable.

9. A few drops of the sperm suspension were
then added to the beaker of washed eggs,
and the beaker swirled gently to mix.
Microscopic examination of the mixture was
continued throughout the fertilization pro-
cess, to determine ratio of motile sperm
to eggs, and extent of fertilization. If
poor fertilization was obvious after 30
minutes, additional sperm was added. Under
normal lab conditions, using the procedures
described, cell division was observed 60
minutes after mixing the eggs and sperm.
Percentage of developing embryos obtained
was generally 75% to 98% of the washed eggs.

10. Once cell division had been observed, the
developing embryos were permitted to settle
to the bottom of the beaker. The super-
natant was poured off and the embryos
were poured into a 50 ml centrifuge tube
and again allowed to settle. Once the
supernatant was removed, antibiotic wash
media were added to the tube. The embryos
were bathed in the antibiotic wash media
for a total of 30 minutes, with one change
of media after 15 minutes.

11. Following the antibiotic wash, embryos were
rinsed one time in filtered bay water. By
this time, the developing embryos could be
centrifuged at low speeds without adversely
affecting the health of the embryos. After
rinsing, the embryos could then be disso-
ciated for use in cell cultures.

Processing Oyster Embryos for Cell Culture

After decontamination of the embryos, it was essential to dissociate the embryos to obtain a suspension of cells suitable for cell culture. In cases when pre-trocophore stage larvae were used, preparation of a cell suspension involved only dissociation of the intercellular bonds. However, preparation of later stage larvae for culture involved both shell removal and deciliation prior to dissociation.

Farris (1968) and Hansen (1976) both reported on the difficulty associated with dissociation of molluscan tissue. Although dissociation is possible, reaggregation of molluscan cells often occurs, and a cell suspension is difficult to maintain. This re-aggregation is apparent in both embryonic tissue and in adult tissue. These investigators found that dissociation was more complete when a combination of physical and enzymatic treatments were employed.

In working to obtain a cell suspension from oyster embryos, a variety of methods were tested, including procedures reported by Farris (1968), Hansen (1976) and Brewster and Nicholson (1979).

Shell removal. The shells of the straight hinge stage oyster larvae consist of a thin matrix of $CaCO_3$. Although the shell layer is relatively thin, its removal was found to be very difficult. The problems associated with shell removal were one reason why earlier stage embryos were more frequently employed in tissue culture efforts.

Bonar (1979, personal communication) recommended chemical means for removing shell, such as low pH citrate buffer. One minute treatment of the larvae with sodium citrate buffer (pH 4.0, pH 5.0, pH 6.0) stopped larval motility and had no apparent effect on the shells. Extended exposure of the larvae to the low pH citrate buffer resulted in larval death.

We felt that perhaps the oyster larvae could be enzymatically separated from the shell, since some of the larvae/shell attachment sites are thought to be protein or glycoprotein. Treatment of the straight hinge larvae with 0.25% trypsin in diluents D or E for 30 minutes at room temperature (p.11) had no effect on the larvae. Treatment of the larvae with 0.01% hyaluronidase in 0.85% NaCl for 60 minutes caused the shells to close, but the ciliary activity of the larvae inside the shells was still evident. Since no positive effects of the chemical treatments were noted, other methods were tested in efforts to remove the larval shells.

Hansen (1976) and Brewster and Nicholson (1979) were successful in using physical methods for removing shell from molluscan larvae. Hansen, for example, removed the shells from snail larvae by vigorously aspirating through a fine tip Pasteur pipet. Signifi-cant removal of shell from the oyster larvae could not be accomplished by these methods; although small shell pieces would sometimes break off, complete sep-aration of the oyster from the shell did not occur.

The most successful method for removing shell was physical abrasion. Six day old, straight hinge larvae were obtained from the HPEL oyster hatchery. A small portion of the larvae were poured onto a stain-less steel wire sieve, having a mesh opening of 44 μ. The sieve was placed over a sterile petri dish. Using a glass pestle, the larvae were gently rubbed over the screen, and pieces washed through with sterile bay water. When the petri dish was filled with suspension, the shell pieces were permitted to settle to the bottom (2 to 3 minutes) and the supernatant fluid examined microscopically. The supernatant was found to contain individual and clumps of cells, shell-free ciliated larval fragments and cell debris. Few shell fragments of larvae with shells were observed in the sieved preparation. In using this method, some cells were disrupted; possibly, by using larger larvae and/ or larger mesh sieves, the cell loss could be minimized.

Deciliation. Although it was desirable to dis-
sociate the embryos prior to cilia development, many
times the embryos processed for culture had, or were
beginning to develop, cilia before the cultures could
be initiated. On dissociation of the ciliated embryos,
the resulting suspension contained a high percentage of
ciliated cells. These cells could not easily be sep-
arated from the non-ciliated cells. When put in
culture, the ciliated cells were highly motile, keeping
the culture agitated and making settling and attach-
ment of other cells more difficult. As a result, it
was most desirable to remove the cilia or layer of
ciliated cells prior to dissociation of the larvae.

It was known (Bonar, personal communication) that
deciliation of some marine invertebrates was accomp-
lished by an abrupt change in the salinity of the
suspending medium. Trocophore stage oyster larvae
which were maintained in bay water at 10 ppt were
pelleted and resuspended in artificial sea water at
30 ppt. Microscopic observation of the larvae revealed
that, while a brief (30 sec) adjustment period was
nedessary, normal motility of the larvae resumed almost
immediately in the 30 ppt sea water. The larvae did
not appear to be physiologically shocked by the abrupt
osmolarity change, and continued to be motile after
1 hr in the higher salinity. No deciliation was
observed. A more abrupt change in salinity (up to 45
ppt) was also tried, with no effect on removal of
cilia.

Chemical deciliation was attempted, using a
method described by Thompson et al. (1974) for non-
lethal decilaition of the protozoa Tetrahymena; but
the treatments seemed to have a negative effect on
the viability of larvae.

No efficient means of deciliation of the oyster
larvae was found. The primary emphasis was to use
early stage embryos for culture so that cilia would
not pose a problem. Using the methods described
previously, in which oyster embryos were obtained by

fertilizing previously decontaminated eggs, it was
possible to routinely culture early stage embryos.

Dissociation. Although physical methods of
dissociating the embryos for culture were tested,
enzymatic treatments were the most effective. A
variety of trypsin concentrations, trypsin diluents,
exposure times and temperatures were evaluated to
select optimum procedures. Trypsin solutions and
diluents are the same formulations as those used on
adult oyster tissue dissociation.

The effect of trypsin on the embryos was greatly
dependent upon the age and extent of development of
the embryos. As would be expected, very early stage
embryos could be dissociated into single cells by
using low trypsin concentrations (0.025%) while higher
concentrations (0.05% to 0.25%) were necessary for
older embryos and larvae. The length of exposure to
the trypsin was varied, and was determined by micro-
scopic monitoring of the embryo suspension during the
trypsinization procedure.

Problems did arise in culturing the suspension
of disaggregated cells. First, dissociation was
generally not complete and, thus, small fragments of
developing embryos were cultured along with the cell
suspension. As a result, one day following initiation,
the cultures would contain ciliated pieces of larvae,
or nearly normal larvae, swimming through the culture.
In addition, the embryonic oyster cells were capable
of reaggregation or "regeneration"; thus, cultures
which were initiated from a suspension of single, non-
ciliated embryonic cells, would, within 24 hours,
develop ciliated larval tissue and single ciliated
oyster cells.

Culture Methods

Once oyster embryos had been decontaminated,
dissociated and rinsed free of trypsin, the resulting
suspension was inoculated into culutre vessels and

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test media were added. The oyster embryo cell cultures
which were obtained differed from the adult cell
cultures in extent of cell attachment and types of
cells. Within 24 hours after culture initiation, only
a very small percentage (1% to 10% under optimal con-
ditions) of the cells in culture would be attached to
the culture vessel surface. The other cells would
remain viable in suspension. Often, 24 hours after
initiation of the culture, many of the non-attached
cells in the culture would be ciliated and motile in
the vessel. Many clusters of ciliated cells could be
observed, as well as ciliated fragments of larval
tissue.

     The attached embryo cells differed in appearance
from the majority of cell types observed in the adult
oyster cell cultures and, thus, for convenience, were
termed "larval-type cells." These cells were agranular
and fibroblast-like or spindle shaped, with long thin
processes. The oyster larval-type cells resembled
pictures of the snail cell line presented by Hansen
(1976). In addition to the attached larval-type cells
there were also clusters of attached rounded cells
and what appeared to be attached fragments of larval
tissue. These attached fragments were sometimes cili-
ated and would often detach from the vessel surface,
due to the strong ciliary activity of the fragment.

     The general fate of the embryo cell cultures,
which was altered by changes in culture conditions and
media, was that the larval-type cells would remain
attached for about 7 days, after which most cells
would remain viable (and some ciliated) in suspension
for about one month. After that period, cell lysis
would occur.

     Basically, the same culture maintenance proce-
dures and evaluations used on the adult oyster cultures
were also applied to the oyster embryo cultures.
Embryo cells were inoculated into a series of test
media and a control medium whose culture effects were
known from previous cultures. Effects of the test

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media and culture conditions were evaluated with
respect to extent of embryo cell attachment, apparent
health of attached cells and length of time cells
remained viable. All cultures were observed for in-
dications of foci formation or cell division. Gener-
ally, the media formulations found to produce the best
results on oyster primaries were those employed as test
media for the embryo cultures.

Cultures were most often incubated at 15^OC;
however, some cultures were maintained (in the dark)
at room temperature (22^OC to 25^OC). No temperature
effects could be detected in controlled experiments,
in which split cultures were incubated at both 15oC
and room temperature.

While some embryo suspensions cultured contained
embryo fragments and thus were not homogeneous sus-
pensions of individual cells, an effort was made to
adjust cell densities prior to initiation of cultures.
Inoculum sizes tested varied from 0.25 x 10^5 cells/ml
to 5 x 10^5 cells/ml.

Day to day maintenance of the culutes was deter-
mined primarily by daily microscopic inspection of the
individual cultures. The cultures were usually rinsed
within 3 days after initiation of the culture, then
rinsed with 50% fresh media every 7 days. In most
cases, early rinsing of the culture was important to
remove some of the ciliated larval pieces which devel-
oped in the culture vessel within the first 24 hours
after culture initiation. As was noted with the
adult oyster cell cultures, frequent handling of the
cultures, after the initial rinsing, seemed to reduce
culture vigor.

Occasionally, transfer of cell suspensions from
the original culture vessel to fresh wells was made.
Cells appeared to deteriorate more rapidly in the
transferred cultures than in the original cultures.
Since only small percentages of embryo cells attached,

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no efforts were made to transfer attached cells by
trypsinization.

Since one of the main problems associated with
culture of the embryonic cells was the negligible
cell attachment, efforts were made to enhance the
attachment properties of the cells. Human fibronectin
(HF, Collaborative Reasearch), a serum protein thought
to be one of the factors responsible for mammalian
cell attachment, was tested for its suitability in
enhancing cell attachment of molluscan cells and was
found to improve attachment significantly. In several
controlled experiments in which treated and non-
treated vessels were used for culturing embryo cell
suspensions, attachment was always improved in the HF
treated vessels, regardless of medium used.

In addition to use of purified HF to enhance cell
attachment, efforts were made to improve attachment by
increasing serum content of the medium. A preliminary
observation indicated that high concentrations of FBS
in the media did improve initial attachment of the
larval-type cells, but did not improve overall health
of the culture. In fact, cultures maintained in media
with high FBS (>15%) seemed to deteriorate more
rapidly than cultures maintained in lower concentra-
tions of FBS.

Collagen coated vessels were also tested for
their suitability for molluscan cell cultures. Methods
for preparing the collagen and coating the flask and
well surfaces were those described by Michalopoulos
and Pitot (1975), although collagen in our lab was not
from rat tail fibers, but was collagen Type III. The
collagen coated vessels were only used on two batches
of embryonic oyster cells; none of these cultures
initiated showed a significant improvement in numbers
of attached cells over the non-treated cultures.

There was some indication from work with adult
oyster cultures that culture vigor was improved when
actively contracting tissue fragments were included
in the culture. In addition, embryonic oyster cell

cultures seemed to remain viable somewhat longer when
low percentages of oyster extract was added to the
growth media. Thus, some efforts were made to estab-
lish a "feeder layer" type system, whereby oyster
embryo cells could take advantage of possible growth
support factors from adult tissue fragments. In order
to use adult tissue fragments in cultures of embryonic
cells, however, it was important to partially compart-
mentalize the fragment and to immobilize the amoebo-
cytes which would otherwise migrate from the fragment
and overtake the embryo cell culture. To accomplish
this compartmentalization, an agar overlay was used.
The agar immobilized the fragment, but provided a
substrate through which substances could diffuse from
the fragment and on into the embryo cell culture medium.

        To culture embryo cells on the agar overlay,
sterile pieces of glass coverslips were carefully
pressed onto the agar surface. The embryo cell sus-
pension in test medium was then gently pipetted onto
the surface, taking care not to dislodge the coverslip
and cause it to float. Overlay cultures were then
incubated and maintained as for other embryo cultures.

        Initial results on the agar overlay method for
culturing oyster embryo cells were encouraging, and
the method warranted further investigation. As was
observed in the adult oyster cell cultures, addition
of a tissue fragment to the embryo cultures increased
the length of time the embryo cells would remain
viable in culture. In the traditionally prepared
cultures, embryo cells would remain viable for about
one month, while in the agar overlay cultures, which
contained a tissue fragment, embryo cells would
remain viable up to two months. Embryo cell cultures
initiated in agar overlay wells which did not contain
fragments (control wells) remained viable for only
one month.

Use of Oyster Larvae for Bioassays

        In addition to employing oyster embryos as a cell

source for tissue culture, the embryos were also used as a tool for bioassay of various media components. Individual components could be tested on a suspension of developing oyster larvae to determine if the component at the concentration tested was toxic or inhibitory to cell division or continued larval development. The biological response monitored was morphogenesis of the trocophore stage larvae to the straight hinge larvae. In sterile bay water, trocophore larvae develop to straight hinge larvae in 18 to 24 hours. The morphological changes which accompany this development are easily recognizable by microscopic observations at 125X. While an external nutrient supply is not essential for this morphogenesis to occur, inhibitory substances in the suspending medium can incur abnormal development, or larval death and lysis or dissociation. The developing trocophore stage larvae are necessarily exposed to the suspending medium, since they cannot close down within an impermeable shell, as adult oysters can. Although this bioassay method did not aid in determining whether particular substances were beneficial to the larvae, it did provide a rapid, first step evaluation system for determining maximal tolerance levels for media supplements, antiobiotics, decontamination treatments and growth factors.

Summary

Efforts were made to establish a molluscan cell line by using oyster embryos as a source of rapidly dividing cells. It was found most desirable to use oyster embryos within 4 hours post egg fertilization; however, later stage larvae (trocophore, straight hinge) were occasionally employed. Methods were established whereby contaminant-free oyster embryos could be obtained for use in cell culture. In most cases, eggs and sperm from sexually mature oysters were dissected out, decontaminated, then mixed in proportionate amounts to achieve egg fertilization. Further decontamination was continued during the early stages of embryogenesis; once the embryos had reached the 16 to 32 cell stage, the embryos were

dissociated by trypsinization to achieve a cell
suspension for culture. Cultures were incubated
in a variety of test media at 15°C.

Embryo cells which did attach to the culture
vessel differed from the majority of cell types ob-
served from the adult oyster tissue: these "larval-
type cells" were agranular and fibroblast-like, having
long, thin cellular processes.

Problems associated with the culture of oyster
embryo cell included cilia development and larval
tissue reaggregation within 24 hours after initiation
of the cultures. A homogeneous culture of single,
non-motile cells was difficult to maintain. In
addition, only relatively few of the single, non-
motile embryo cells would firmly attach to the culture
vessel.

Procedures which were found to improve the embryo
cell cultures included coating the vessel surface with
human fibronection which significantly improved larval
cell attachment. Also, length of culture maintenance
was improved by culturing embryo cells in culture
vessels which contained a metabolizing fragment of
adult tissue embedded in agar. While viable embryo
cells could be maintained in culture for up to two
months, no significant cell division, leading toward
development of a cell line was observed.

While a variety of culture media were tested in
an effort to establish and oyster cell line, the best
culture results were observed from cultures maintain-
ed in Media #204, 205, 211, 214 and 219:

Media #204
```
   Bay water                 74
   FBS                       10
   Algae extract              5
   Oyster larvae extract      5
   Oyster hemolymph (HI)      5
   Glucose                    2.5 mg/ml
```

Media #205
```
   NCTC 135                  10
   Bay water                 63
   FBS                       10
   Algae extract              5
   Oyster larvae extract      5
   Oyster hemolymph           5
   Glucose                    5 mg/ml
```

Media #211
```
   L-15                      30
   Bay water                 43
   FBS                       10
   Oyster larvae extract      5
   Oyster hemolymph           5
   Algae extract              5
   Glucose                    5 mg/ml
```

Medium #214
```
   L-15                      30
   Bay water                 49
   FBS                       10
   Whole egg ultrafiltrate    5
   Yeast extract              0.4 mg/ml
   Glucose                    5   mg/ml
```

Medium #219
```
   L-15                      30
   Bay water                 43
   FBS                       10
   Whole egg ultrafiltrate    5
 · Algae extract              2.5
   Oyster larvae extract      2.5
   Glucose                    5    mg/ml
   Galactose                  0.65 mg/ml
   Yeast extract              0.4  mg/ml
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Attempts to Culture Clam Cells

Our efforts to initiate a marine molluscan cell line included using neoplastic tissue of the soft-shelled clam, *Mya arenaria*. This attempt was based on the relative ease of initiating cell lines from malignant tissue of vertebrates. While neoplastic disorders have been reported to occur in molluscs, the malignancies are found infrequently, and solid tumors are very rare. Brown (1977) has described a neoplastic disorder of the hemopoetic tissue which occurs with high frequency (30% to 40%) in certain populations of *Mya arenaria* in New England. Neoplastic cells are obtained from these diseased clams most readily from the hemolymph; however, large concentrations of these cells can also be observed in the gill.

Clams were collected by the Marine Pathology Lab of University of Rhode Island (URI) from Allen's Harbor in Narragansett Bay, near Davisville, Rhode Island. They were diagnosed as neoplastic by the Marine Pathology Lab, and classified as to the severity of the disease. To accomplish this, a sample of hemolymph was withdrawn from each clam, and the cytopathology of the hemocytes examined by phase miscroscopy. The severity of the disease was noted for each clam, based on the percentage of neoplastic cells in the cell population examined.

Clams were maintained in URI labs in recirculating artificial sea water systems, at 4°C, until packed for shipment. Once the clams were received by our lab, they were maintained at 13°C, in 15 gallon aerated aquaria, using a maximum of 12 clams in 5 gallons of water per aquarium. The water was adjusted to a salinity of 25 ppt using artificial sea salts (Marine Mix) added to either distilled water or natural bay water.

In general, for extended maintenance of the clams, bay water at 25 ppt was used. Water was changed and aquaria cleaned every 2 to 3 days. Due to the potential infectious nature of this neoplastic disease, all materials used in handling the clams were autoclaved prior to disposal, and all water was disinfected with bleach.

Clams were maintained on a diet of Purina Trout Chow, approximately 1 to 2 grams of food being added to aquaria each time the water was changed. Food was not added to aquaria in which clams were being depurated for use the following day. Rubber bands were fitted snugly around the clam's shell, to ease the energy requirements needed for the clam to keep its valves shut, and to potentially increase the length of survival of each clam.

Efforts at culturing neoplastic cells from the soft-shelled clam focused on, first, decontamination procedures to obtain cells free from microbial contaminants, suitable for use in cell culture, and, second, determination of nutritional requirements for long-term maintenance and eventual growth of cells. In general, cultures of clam cells were prepared by disinfecting the external surface of the clam by wiping with 70% ethanol, and bleeding the clam from the posterior adductor muscle. The cells obtained could then be rinsed, decontaminated and dispensed into culture vessels containing test media.

One advantage of obtaining the neoplastic cells from the hemolymph in this manner is that once the clams are bled, they can be returned to the aquaria, and used again as a source of cells 2 to 3 days later. Depending upon the size of the clam and the skill of the technician bleeding the clam, approximately 0.5 ml of hemolymph can be withdrawn with no harm to the clam.

One characteristic of the neoplastic cells, which in some ways makes them more difficult to decontaminate, is that they do not attach to culture vessel surfaces, but remain in suspension. Normal clam hemocytes will

attach to the culture vessels, and can be separated
from the neoplastic cells, to some extent, merely
by attachment properties.

Decontamination

   The primary problem encountered in attempting to
use neoplastic clam cells in cluture was protozoal
contamination. Although identification of the various
protozoal contaminants was not pursued, three types
were encountered most frequently: (1) an amoeboid
type, about the same size as the clam hemocytes,
(2) a very small flagellated type and (3) a larger
ciliated·type, about 8 to 10 times the size of the clam
cells.   These protozoa were often detected in freshly
drawn hemolymph samples and, occasionally, in the
aquarium water in which the clams had been maintained.

   A variety of treatments, as described below, were
tested directly on the contaminants in an effort to
determine which treatment could serve most effectively
in removing the protozoa from the clam cells.   Any
treatment found to destroy the protozoa was then tested
on clam cells to determine its toxicity.   Some of the
treatments mentioned below may have been effective in
eliminating one type of contaminant; however, since it
was necessary to eliminate all types of protozoal
contaminants, only the treatments on the most resistant
protozoan forms are presented.   During the course of our
cell culture efforts, one protozoan type became the most
prevalent, and was treated individually.

   Treatments and their results included the following:

   1.   With various concentrations of hyamine hydro-
        xide in sea water, all protozoan types were:
        irreversibly immobilized together with 100%
        mortality of cells (0.1 mM, 30 sec); not
        affected although cell viability was (0.05 mM,
        10 min); not affected (0.01 mM, 10 min).

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2. Ten percent ethanol in sea water seemed to slow protozoan motility but was reversible when ethanol was removed.

3. Using mycostatin (400 units/ml) in antibiotic wash medium, protozoa were still motile after 2 hrs.

4. Heat treatment of 37°C for 30 min effectively lysed the most common protozoa, the amoeboid type; clam cell viability appeared to be un-affected. It was not determined, however, whether or not this treatment may have slightly shortened the length of time these cells remained viable.

5. With acriflavin at 50 ug/ml in sea water for as much as 60 min, protozoan population was reduced but not eliminated. With acriflavin at 500 ug/ml in sea water for 20 min, all motile forms of protozoa were eliminated; when this treatment was combined with incuba-tion at 37°C, no motile protozoa were observed--many protozoa appeared partially lysed. Vi-ability checks performed on cells one day after treatment for 30 min with acriflavin indicated 95% viability, compared with 100% viability for untreated controls. However, after one week in culture, cells treated with acriflavin appeared unhealthy, while the untreated controls still appeared healthy.

In addition to testing anti-protozoan chemical treatments which could be used directly on cell suspensions, several treatments which could be used in the clam aquaria were also tested. Perhaps, with less concentrated but prolonged treatments of the aquarium water, contaminant populations might be more effectively reduced while having a less direct effect on the viability of the cells to be cultured.

Attempts to separate the protozoan contaminants from the clam cells by differential centrifugation were also made. Researchers at the Marine Pathology Lab of the University of Rhode Island had indicated success in obtaining contaminant-free cells by using sucrose gradients. While it was expected that subjecting the cells to sucrose gradients would greatly reduce cell viability and render them unsuitable for culture, we also felt that these gradients could be used as a preliminary test for the effectiveness of differential centrifugation to separate cells from protozoan contaminants. If the sucrose gradients were successful in that respect, perhaps Ficoll-Hypaque gradients could be set up to parallel the same separation.

During the four month period in which efforts were made to establish a cell line from the soft clam, no single treatment was found that would eliminate protozoa contaminants from a cell suspension. This is not to say that contaminant-free cultures were not established; rather, protozoan-free cultures were not obtained with 100% efficiency. As work on the clams continued, it became apparent that certain individual clams consistently yielded contaminated cultures, while other individuals were generally "clean." Moreover, the longer that clams were maintained in our lab aquaria, the less diverse the protozoa population of the clams became. The only protozoa type that remained fairly prevalent throughout was the heat sensitive type, and it was effectively eliminated from the cell cultures by the 30 minute, 37°C, treatment. As a result, for the final month of work on clam cell culture, using the methods developed to process the cells, less than 5% of the cultures were lost due to protozoan contamination.

Fungus contamination did not seem to be a problem with the clam hemocyte suspension, although the cells could be washed with a mycostatin suspension (200 units/ ml) without ill effect to the cells. Antibiotic washes were used to eliminate bacterial contamination from the cell suspension. A series of three, 1 hr antibiotic

washes, using a combination of seven antibiotics in a
salt solution, was generally sufficient to eliminate
bacteria.

Culturing Methods

While our major effort was to initiate a cell line
from the neoplastic cells of diseased clams, some work
was also done on culturing "normal" cells from the less
severely diseased clams and culturing tissue explants
from diseased and normal clams. As mentioned previously,
the most readily observable difference between the normal
and neoplastic cells was the difference in attachment
capabilities--the normal hemocytes will attach and
spread out on a glass or plastic (polyethylene) surface,
while the neoplastic cells will not attach to glass, and
will remain rounded in suspenion. Thus, the processing
of these two types of cells for use in culture was
necessarily different.

Cells from the soft clam were obtained by bleeding
the clam from the posterior adductor muscle. In contrast
to oysters, soft clams are unable to close off completely
from the external environment and it was not possible
to disinfect the external surfaces with a thorough
treatment, such as dilute bleach. Instead, the
outer shell surfaces and the external mantle tissue
area which covers the adductor muscle were gently wiped
with either 70% ethanol or O-syl disinfectant. Some-
times even mild disinfectant treatment inhibited healing
of the wounds to the mantle tissue incurred on repeated
bleedings of the clam.

Once the cells were obtained, if neoplastic, they
were handled as suspension cultures. We discovered
very early that although these cells do not attach to
glass slides or normal tissue culture vessels, a
significant percentage of the neoplastic cells do ad-
here rather firmly to polyethylene surfaces, such as
centrifuge tubes or bacteriological petri dishes.
Thus, if the cells were rinsed in 15 ml polyethylene
centrifuge tubes, many of the cells were lost, as
they would adhere to the tube surface and could not be

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aspirated or scraped off. This adhesion problem was
overcome by using only centrifuge tubes that had first
been rinsed with fetal bovine serum (FBS).

Initially, the neoplastic cells were processed by
rinsing the cells in 15 ml FBS-pretreated centrifuge
tubes. The freshly drawn hemolymph sample was put into
the tube and 10 ml sea water (25 ppt) containing peni-
cillin (100 units/ml), streptomycin (100 µg/ml) and
neomycin (100 µg/ml) were added. The suspension was
mixed, and cells spun out at 1000 rpm for 10 min. The
supernatant was discarded and the cells were resuspended
in 5 to 10 ml of sea water with antibiotics (as above),
and again spun at 1000 rpm for 10 min. The cells were
then subjected to a series of antibiotic washes. Cells
were resuspended in antibiotic wash media and allowed
to soak for 60 min. After this period, the cells were
pelleted and the supernatant fluid discarded. The
antibiotic wash treatment was repeated 2 more times, for
60 min each time. Initial experiments were done including
mycostatin (100 units/ml and 200 units/ml in the anti-
biotic wash media. However, since mycostatin is non-
soluble in the media, the mycostatin particles were
interspersed with the cells in suspension. While it was
desirable to remove the high levels of antibiotics from
the cells after the antibiotic washes, removing the mycos-
tatin from the cell suspension proved to be very diffi-
cult. After a series of tests in which cells were
processed with and without mycostatin, it was deter-
mined that fungal contamination of the clam cells was
a negligible problem; thus, treatment of the cell
suspension with mycostatin was stopped.

Following the antibiotic wash treatment, cells
were again rinsed two times in filter sterilized
sea water containing antibiotics. Throughout the
rinsing procedure, samples of the cell suspension were
examined microscopically. If any of the amoeboid type
of protozoa were detected, the suspension was incubated
at 37°C for 30 minutes, in an effort to eliminate the
contaminants.

Cells were dispensed into culture vessels contain-
ing test media. Both 16 x 110 mm Ambitubes (Lux Scien-
tific) and 2 cm^2 wells in the 24 well cluster dishes

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(Costar) were employed as culture vessels. Since no readily observable difference was noted between the cultures tested at 10°C, 15°C and 20°C, 15°C was selected as the temperature for use in future culture attempts.

Early results with the cultures initiated from neoplastic cells indicated that the cells were not dividing in culture, and were deteriorating and lysing after several weeks. It was felt that perhaps the methods used in preparing the cells for culture, such as repeated centrifugations, might be too harsh. Two other methods were used to remove the debris and microbial contaminants from the cell suspension, without subjecting the cells to extensive handling. In the first method, it was reasoned that if cell attachment could be enhanced, the cells could be rinsed free of suspended contaminants directly in the culture vessel. To effect such cell attachment, polylysine coated culture surfaces would be used. In the second method, it was thought that rinsing the cells directly in the culture vessel, without inducing cell attachment, might allow the cells to settle to the bottom of the vessel, leaving the motile protozoa suspended in the wash fluid.

This second technique was the most efficient in providing the greatest numbers of healthy and uncontaminated cell cultures. The resulting cultures appeared, microscopically, as a confluent, or nearly confluent, layer of loosely attached, rounded cells, and remained as such for at least two weeks after culturing.

The same result was also obtained by altering the methods slightly to use the complete antibiotic wash media (containing all seven antibiotics), rather than the series of antibiotic wash media. It shoud be noted that while this method was found successful in providing contaminant-free cultures, it was used only on clams that had been maintained in our aquaria for a few weeks up to several months. It is not known whether this mild rinsing treatment would be successful when

using highly contaminated specimens. It should also be mentioned that the end result of the cultures initiated in the manner described above, in terms of length of culture maintenance, did not seem significantly different from cultures initiated with centrifuged cells.

Cultures were monitored by microscopic examinations and viable cell counts, using trypan blue dye exclusion techniques. Counts were determined only on the cultures that appeared most healthy. In some cases, cultured neo-plastic cells would attach to some degree, and since counts on just the suspended cells in these cultures would have been inaccurate, cell counts were not made.

Culture inocula were varied in cell densities, with a range from 0.25 x 10^5 cells/ml to 5 x 10^6 cells/ ml. Cell counts performed within the first 7 days after culture initiation indicated no significant change in cell count from the inoculum. Cell viability was generally close to 100%. Cell counts taken on 10 to 14 day cultures indicated a significant drop (30% to 50%) in cell densities, while cell viabilities, as deter-mined by trypan blue, remained fairly high (80% to 95%). While the cell density of the cultures always dropped significantly from that of the original culture, low levels of viable cells remained in ·the cultures for up to three months.

Cells were maintained by refeeding the cultures 5 to 7 days after initiating the culture, followed by re-feeding approximately every 10 to 14 days. Cultures were refed by aseptically withdrawing 50% of the spent media from the culture vessel, and adding 50% fresh media. A variety of culture maintenance regimes were tested. Frequent handling of the culture appeared to induce more rapid deterioration of the cells. Centrif-ugation (700 rpm for 8 min) of the cultured cells to remove them from suspension for refeeding also caused deterioration, whether the cells were refed with 100% fresh media or the 50% conditioned/50% fresh mixture.

Samples of cells taken directly from the clam, at various times during the processing, and after several days in culture, were fixed on microscope slides and stained with Giemsa. All slides were examined with a light microscope under high power (1250X) to determine if deterioration of the neoplastic cells could be detected at any one point in the sequence. No obvious difference between the health of the cells in any of the stained samples could be noted.

In addition to the efforts towards culturing neoplastic cells, work was also directed toward culturing "normal" cells from the less severely diseased clams. Some of these cells were truly normal hemocytes while others exhibited attachment properties of normal cells but were beginning to express some of the pathology of the neoplastic cells.

Cells were obtained by bleeding the clam from the posterior adductor muscle. Freshly drawn hemolymph was placed directly in the culture vessel and cells were permitted to attach (approximately 30 min). Although the normal cells do attach to the culture surface, they do sometimes detach on repeated rinsings. It was found that attachment of the normal cells could be enhanced by adding low levels of oyster hemolymph or clam homogenate to the rinse media. Once the cells were attached, they were allowed to soak for two 30 min rinses in sea water containing penicillin, streptomycin and neomycin, plus 1% oyster hemolymph or clam homogenate.

After removing the final rinse solution with a Pasteur pipet, antibiotic wash media (complete) was added. After 90 min the first antibiotic wash was removed from the cells with a Pasteur pipet. The antibiotic wash treatment was repeated for a second 90 min period. Following this period, the antibiotics were rinsed from the cells with sea water, which was subsequently drained from the culture. Test media were then added to the cells and the cultures incubated at 15°C.

Depending on the inoculum size, immediately after
initiation of the culture, and for 7 to 10 days, the
cultures sometimes appeared as confluent or nearly
confluent monolayers of clam hemocytes on the culture
surface. Obviously, if fewer cells were used, the
monolayer would not be complete. The cells spread out
as typical "amoebocyte" cells but were mostly agranular.
After a week in culture, some of the cells began to
round up and eventually slough off. However, viable
"normal" cells were maintained in culture up to 3 months;
in general, greater percentages of "normal" cells
remained viable in culture for longer periods than did
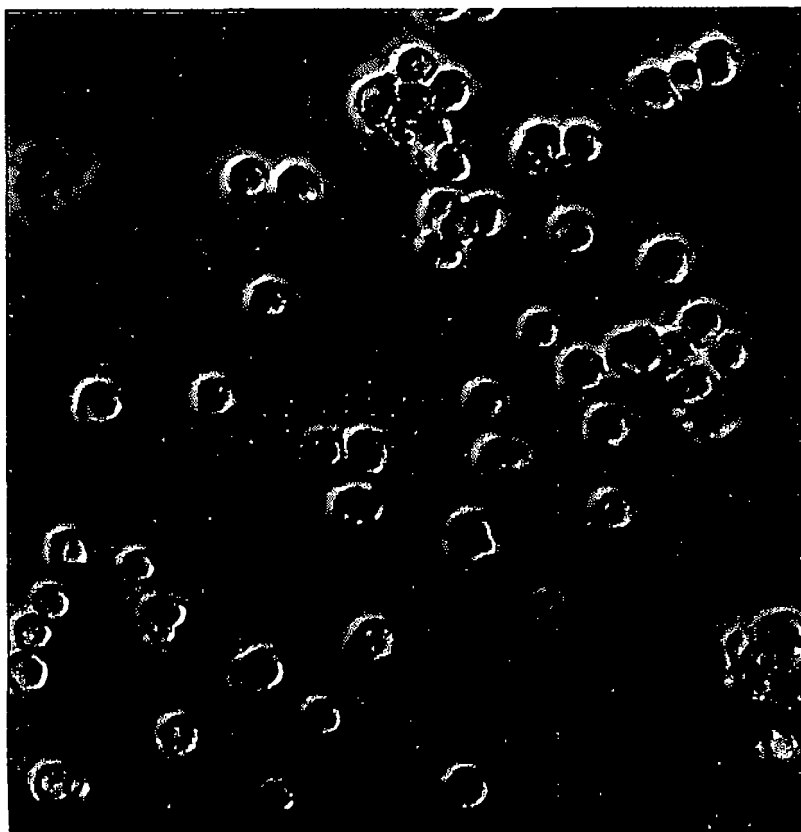the neoplastic cells in culture.

Figure 8. Seven day old cell cultures of neoplastic
hemocytes from the soft-shelled clam, *Mya
Arenaria*. (720x)

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Maintenance of the "normal" culutres was primarily
the same as for the neoplastic cultures. Again, it was
observed that more frequent handling of the cultures was
detrimental to the cells. While these "normal" cells
did appear to stay healthier in culture longer than did
the malignant cells, no significant cell mitosis was
evident.

In addition to the hemocyte cultures initiated
from the soft clam hemolymph, a few cultures were
also established from tissue explants. Heart and gill
tissues were used from both non-diseased and neoplastic
clams. Mantle tissue proved to be a poor source of
migrating cells. Unlike the mantle from the oyster,
which is soft and fleshy tissue, the mantle from the
clam is exposed to the external environment at all times
and is very tough.

Both the gill and heart tissue used were found to
be highly contaminated, more so than the parallel
tissues of the oyster. Unlike the heart tissue of the
oyster, which is not exposed to external contaminants
since it is completely encased in the pericardial sac,
the heart and gill tissues of the soft clam are all
directly exposed to the sea water environment. Another
factor leading to greater contamination, at least of
the heart tissue, is that the clam intestine passes
directly through the heart and must be severed to
remove the heart. In addition, because the clams being
handled were, at most, 1 in. in shell length, the hearts
(of the larger clams) were approximately 1 mm across,
a factor which made them difficult to remove.

Once the tissues were excised from the clam, they
were rinsed 3 times in sea water containing antibiotics.
In attempts to remove protozoa contaminants, the tissue
was rinsed for 15 sec in a dilute iodine solution pre-
pared in sea water. The tissue was immediately rinsed
free of the residual iodine with sea water. Tissue
fragments were then treated with antibiotic wash media
(complete), using a series of three 60 min washes.
Following the antibiotic wash treatments, the fragments

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were washed 2 times with sea water containing anti-
biotics, and minced into 1 mm fragments. The smaller
fragments were again rinsed 2 times with sea water and
dispensed into culture vessels, to which test media
were added. Cultures were incubated at 15°C.

Gill tissue was highly ciliated, and fragments
were quite "motile" in the culture vessel. Many
individual cells were suspended in the culture media,
but very few attached, possibly due to the constant
ciliary activity and resultant agitation of the culture
media. Polylysine coated culture surfaces were tested
in an effort to increase cell attachment, using the
high levels of polylysine found to enhance neoplastic
cell attachment. No significant difference was de-
tected between cell attachment in cultures with
polylysine coated or non-coated surfaces.

In addition to the ciliated fragments, there were
also many individual motile cell forms, which were
thought to be ciliated cells dissociated from the
larger tissue fragment. The cilia remained active
in culture in a variety of media tested; however, most
cultures were discarded within several weeks after
initiation due to their undesirable nature and ab-
sence of attached cells. Some cultures were also
lost to bacterial contamination. It was possible,
however, to use the gill tissue in a type of physio-
logical assay, to test the ciliary response to a
variety of test media.

The clam heart fragments cultured did provide a
source of fibroblast-like cells, which attached to the
culture vessel. The response of the cells and fragments
to culture seemed to be somewhat delayed. Cells did
not migrate from the fragment for several days after
initiation of the cultures; fragments, sometimes
beating when first introduced into the culture, would
stop beating for several days, only to re-establish a
regular contraction pattern after the initial acclima-
tion period. The cells which migrated from the tissue
explant and attached were, presumably, ameobocytes,

although they appeared spindle-shaped and agranular. The numbers of cells which migrated from the explant were not large, although low levels of migration continued throughout the period during which the fragments were maintained. Transfer of the fragments to fresh culture vessels often induced a new "spurt" of cell migration. Some cultures were lost as a result of bacterial contamination. One culture was maintained for 5 months, with fibroblast-like cells attached, and fragments beating, before it was lost to microbial contamination.

Media Formulations--Nutrients and Supplements Tested

The components incorporated into the media used on the clam cell cultures were those common to established cell lines. A variety of commercially available nutritional supplements, as well as clam homogenates and algal extracts which were prepared in our lab, were tested. Cultures were prepared, and test media added; each medium was evaluated against a standard "control medium" and rated as to its effect on the health of the cells in culture.

One important physical parameter to be adjusted in the media prepared was osmolality. *Mya arenaria* exist in the estuarine environment as osmoconformers, adjusting their internal osmolality to that of their external environment. Thus, cells taken from clams which had been maintained at 25 ppt must be maintained in media with an equivalent osmolality. Since the majority of clams used for cell culture in our lab were obtained from water at 25 ppt, and 690 mOsmoles, the media were prepared at approximately that osmolality.

The osmolality of the media was adjusted, primarily, with the use of filter sterilized natural sea water, obtained from Indian River Inlet, Delaware. It was felt that the use of natural sea water in the media would help maintain the appropriate osmolality and would also provide the proper ion balance and trace elements necessary for cell maintenance. Artificial

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sea water, prepared from Marine Mix, as well as natural sea water from Naragansett Bay, were also tested in media, and compared with the sea water normally used in the lab. No difference could be detected in the cultures maintained in any of the various salt water bases used; thus, the natural sea water from Delaware was routinely employed.

Since the majority of media components used were for mammalian tissue culture, at approximately 290 mOsmoles, it was necessary to use a relatively high percentage of sea water in the media to effect the proper osmolality. The osmolality of the natural sea water was, at times, increased by addition of artificial sea salts, so that a lower effective concentration of sea water could be used in the media to obtain the necessary osmolality.

National Cancer Institute Tissue Culture Medium 135 (NCTC 135), a commercially available, rich tissue culture media was employed as a base for some of the clam media. Glucose was added as the carbohydrate source. While glucose is the primary carbohydrate in molluscan hemolymph, galactose and trehalose are also frequently detected. Thus, both galactose and trehalose were tested in a few preparations.

Initially, the media that were tested on clam cells were those formulations found to yield the best results on oyster cells, with adjustments made for osmolality. These "oyster" media did not appear to produce optimal results in the clam cells. A media formulation reported by Cecil (1976) for use on surf clam cell culture also yielded poor results.

Emphasis was placed on the use of molluscan homogenates in the culture media. While molluscan cells could be satisfactorily maintained in a wide variety of culture media, no cell mitosis was evident. Previous work using oyster homogenates on oyster cell culture indicated no toxic effects of the homogenates on the cells. It was thought that perhaps use of clam homogenates or hemolymph might supply undefined but

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necessary "growth factors" for clam cell division. A variety of clam homogenates were tested in the media for the ability of each to induce cell growth.

Homogenates were prepared by dissecting the desired tissues into a tarred petri dish, and determining the wet weight of the excised tissues. These tissues were then minced, and forced through a wire mesh screen, with frequent rinsings with filtered sea water. The homogenate was prepared in sea water to yield a 25% (w/v) suspension. Particulates were centrifuged out at 1500 x g for 15 min, and the resulting supernatant was filtered through a 0.45 µ Millipore filter. The resultant crude homogenate was aliquoted into small volume sizes and stored at -20°C until used in media.

Two types of clam homogenates were prepared. One homogenate ("B" -- whole) included all the clam tissues with the exception of syphon and adductor muscle. The second homogenate ("C") was prepared with tissues excluding the syphon, adductor muscle and digestive system of the clam. Both homogenate types were tested in the media as heat-inactivated (56°C for 30 min) and untreated extracts.

Clam hemolymph was collected by bleeding the clams from the posterior adductor muscle. Cells and large particulates were removed by centrifuging at 1500 x g. The resulting supernatant was filtered through a 0.45 µ Millipore   filter and stored at -20°C until used in media.

While full evaluation of the value of hemogenates was not complete due to contamination problems, we learned that the homogenates did not seem to cause any microscopically observable harmful effects on the cultures. Homogenates, generally at the level of 10% (w/v), were added as a supplement to other media; however, they did not seem to induce an appreciable difference in the health of the cells or length of maintenance of the cells in culture. In no instance was mitosis evident.

Some attempts were made to construct media more nearly parallelling the free amino acid concentrations found in clam hemolymph. As has been found with many marine molluscs, *Mya arenaria* is thought to regulate its intracellular osmolality by fluctuating the free amino acid pools in the tissue. In studies involving *Mya arenaria* at salinities of 2 ppt through 30 ppt (Virkar and Webb, 1970), total free amino acid concentrations increased from 45.9 mmoles/kg tissue water at 2 ppt to 350.7 mmoles/kg tissue water at 30 ppt. The amino acids found to contribute most to the total free amino acid pool were alanine, glycine, taurine, arginine, glutamic acid and ornithine. Using the information presented by Vikar and Webb for free amino acid concentration in the adductor muscle of *Mya*, several media were constructed. Since the mammalian tissue culture media we used (NCTC 135) has much lower amino acid concentration than those reported for invertebrate tissue, it was necessary to add significant quantities of certain amino acids. Stock solutions of the amino acids were prepared in distilled water and filter sterilized. Most stocks were prepared at concentrations near saturation. Due to its relative insolubility, glutamic acid could not be prepared at a high enough concentration in a stock solution for subsequent dilution in media preparation. Thus, to achieve a sufficiently high final concentration of glutamic acid in the formulation, it was necessary to dissolve this amino acid directly in the prepared media. No obvious positive effects were noted.

Another supplement which was used in the media was Ca^{++}, added in the form of $CaCl_2$. Since calcium has been known to play a role in the regulation of some metabolic processes, it was felt that increasing or decreasing the levels of this ion might induce some cell growth. Clam hemolymph is known to have a Ca^{++} concentration of 12mM, while sea water has a calcium content of about 10mM (Pierce, personal communication). Calculations indicated that the majority of media used on the clam cells had a much lower Ca^{++} content than that found in sea water; therefore, a

stock solution of CaCl$_2$ was prepared such that Ca^{++} could be added to the media to yeild concentrations equivalent to those found in the clam hemolymph (476 mg Ca^{++}/L). Otherwise, identical media with or without Ca^{++} were tested on clam cell cultures. The additional calcium did have a noticeable effect on the neoplastic cells, in that it enhanced cell attachment; moreover, "monolayers" of neoplastic cells, which could not readily be aspirated off, were obtained. Calcium did not have any positive effect in terms of culture maintenance. In general, cultures supplemented with calcium seemed to deteriorate more rapidly than cultures in identical media without Ca^{++}.

Other supplements which were tested on the clam cells included dimethylsulfoxide (DMSO) and 2-mercaptoethanol (2-ME). DMSO was added to test media in attempts to increase the permeability of the clam cells and possibly increase nutrient transport. DMSO was used at concentrations of 0.5% (v/v) in the media. As a reducing agent, 2-ME was added to the media (0.4 mM). Concentrations used were those suggested in written or verbal reports. Neither supplement produced visible effects on the cells in culture.

At the recommendation of the Molluscan Cell Culture Workshop, polyvinyl pyrrolidone (PVP) was added to the culture media to aid in protecting fragile cells. PVP was used in the media at concentrations of 0.01% to 0.05% and found to exert no effect at those concentrations.

Mitogens have reportedly been tested on molluscan cells in the past, but to no avail; however, another type of "growth stimulator" was tested on clam cell cultures in our lab. The seeds of *Ulex europeaus* are known to contain a lectin. Extracts of these seeds induce mitogenic responses in some cells (Roberson, personal communication). While no purified extract was available, some whole *Ulex* seeds were surface sterilized by soaking overnight in a solution of pennicillin, streptomycin and fungisone. Seeds were then rinsed and crushed with forceps. The crushed

seeds were then added to healthy 14 day cultures of
neoplastic clam cells (one crushed seed per well).
The cultures were monitored for three additional weeks,
but mitosis was not evident.

   In general, it was difficult to distinguish clear
cut responses of the cells to the various media tested.
Clam cells could be maintained for several weeks in
sea water, as well as in some of the nutrient media
constructed. In none of the media was significant cell
division detected. However, the media yielding the
healthiest looking cells, maintained in culture for the
longest periods, include the following:

Media #901
    NCTC 135   10
    Sea water   71
    FBS   8
    Whole egg ultrafiltrate   5
    NEAA   2
    Glucose   2.5 mg/ml
    Galactose   1.3 mg/ml
    Trehalose   0.5 mg/ml

Media #905
    NCTC 135   10
    Sea water   87
    FBS   5
    Glucose   2.50 mg/ml
    Glutamine   0.15 mg/ml

Media #908
    NCTC 135   10
    Sea water   77
    FBS   5
    Whole egg ultrafiltrate   5
    NEAA   2
    Glutamine   0.15 mg/ml
    Glucose   2.50 mg/ml

Media #921
    Sea water   83
    FBS   5
    Clam homogenate B (HI)   8
    Yeast extract   0.4 mg/ml
    Glucose   1.3 mg/ml

Media #922
    Sea water   91
    FBS   5
    Yeast extract   0.4 mg/ml
    Glucose   1.3 mg/ml

Media #924
    Sea water   84
    FBS   5
    Clam homogenate B (HI)   10
    UDP-glucose   0.2 µg/ml
    Glucose   1.3 mg/ml

Media #925
    Sea water   94
    FBS   5
    UDP-glucose   0.2 µg/ml
    Glucose   1.3 mg/ml

Summary

Hemocytes from both normal and neoplastic soft-
shell clams, *Mya arenaria*, were maintained in culture
for extended periods. Cultures were obtained by first
depurating the clams in laboratory aquaria containing
artificial sea water. Cells were collected from the
clam hemolymph by bleeding the clam from the adductor
muscle. Cells were rinsed and treated with anti-
biotics to remove debris and contaminating organisms.
By adjusting the densities of the culture inocula
(2.5×10^6 cells/ml per 2 cm^2 surface for normal cells),
it was possible to achieve a confluent layer of cells
in the culture vessel. Normal cells spread out and
attached to the vessel surface, while neoplastic cells

did not attach but settled to the bottom of the vessel.
Such cells remained healthy, on microscopic examination,
for about 3 to 6 weeks after which time the cultues
began to deteriorate. Low levels of viable cells
remained in the cultures for extended periods. Neo-
plastic cells were maintained for nearly 3 months,
while normal clam cells were maintained in relatively
healthy condition for 5 months. Little difference
could be detected between the effects of the various
culture media tested. No cell division was apparent
in any of the clam cell cultures initiated.

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