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Methods examined for the preparation of cells from tissues of salmonid fishes for primary cultivation were the fragment (explant) and enzyme dispersion techniques. Both methods were employed for the cultivation of a variety of tissues from Pacific salmon, Oncorhynchus sp., and the steelhead trout, Salmo gairdneri gairdneri. A technique was devised whereby embryonic cells of these fishes were prepared in primary culture and maintained with periodic fluid changes and transfers over long periods of time. These preparations, referred to as stock cultures, were used as a cell source for much of the experimental work reported here.

The optimum concentration of cell inoculum for primary culture of embryonic cells was found to be one to one and one-half million per ml of culture medium. The optimum concentration of cell inoculum for subcultures of the primary cultures

was found to be 600,000 per ml of medium.

Several enzymes were compared with trypsin for the preparation of primary cell cultures. All were able to cause separation of cells from the tissue mass; however, these test enzymes were found to be more toxic to the cells than trypsin.

The first of a series of experiments to determine factors influencing cell growth involved the comparison of five tissue culture media. Eagle's minimum essential medium supplemented with 20 percent agamma calf serum was found to be more growth stimulatory for embryonic cells than any of the other preparations tested.

The addition of 25 percent by volume of Eagle's basal medium with 20 percent agamma calf serum removed from an actively growing culture to 75 percent by volume of this same medium failed to stimulate more cell growth than did Eagle's basal medium prepared with all fresh components. There appeared to be no growth stimulatory factors for these cells present in the fluid of actively growing cultures.

Antibiotics have been employed at concentrations as high as 1000 units of penicillin, 1000 micrograms of streptomycin and 100 units of Mycostatin per ml of medium for the control of microorganisms without any indication of toxicity. Cultures were routinely carried for long periods of time at concentrations of 100 units penicillin, 100 micrograms streptomycin and 25 units Mycostatin without obvious harmful effects.

Vitamin B_{12} was found to inhibit growth of steelhead trout embryonic cells at concentrations of 0.5, 1.0, and 2.0 mg per liter in Eagle's basal medium supplemented with 20 percent agamma calf serum.

Oxaloacetic acid was also incorporated into this same medium at 2.5, 5.0, and 10.0 millimolar concentrations. This compound was found to inhibit the growth of coho salmon embryonic cells.

The use of blood serum to supplement media was investigated. Homologous fish serum was toxic for the steelhead embryonic cell cultures tested. Horse serum failed to support growth of these same cells. Human and calf serum, with and without gamma globulin, were compared and the agamma calf serum was superior to the other three as a growth stimulant for fish cells.

The growth of coho salmon embryonic cells was inhibited when incubated in an atmosphere containing concentrations of one and three percent CO_2.

Steelhead trout embryonic cells were found to grow best at 18° and 23° C. Twenty-eight and 35° C. were both lethal for these cells. Cultures incubated at 4° and 13° C. failed to grow. Twenty-five degrees centigrade was believed to be inhibitory for these cultures but not lethal.

Observations indicated that the pH of the medium used to culture chinook salmon embryonic cells increased in alkalinity

shortly after planting, followed by a slow decrease requiring approximately 28 days to reach values near neutrality.

Cells of hepatoma tissue excised from adult rainbow trout were cultured and studied during the course of this work.

A number of cultures have been grown under conditions of continuous cultivation. Five of these preparations are still viable and two have exceeded one year in culture.

METHODS FOR THE IN VITRO CULTIVATION OF CELLS FROM THE TISSUES OF SALMONID FISHES

by

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METHODS FOR THE *IN VITRO* CULTIVATION OF CELLS FROM THE TISSUES OF SALMONID FISHES

INTRODUCTION

The experiments performed in this research were designed to develop techniques for the *in vitro* cultivation of cells from tissues of salmonid fishes. Tissue culture, the common name applied to this relatively new field of biology, has been defined as "a group of loosely related techniques wherein cells or tissues from higher plants and animals are kept alive, and in some cases growing after separation from their source" (48, p. 3). The cultivation of normal and abnormal tissue cells from warm-blood vertebrates has become indispensable to both human and veterinary medicine as a tool for the study of animal viruses. In addition, research biologists in the fields of cytology, histology, embryology, physiology, and cancer research have employed cell cultures in a variety of studies. It was the objective of this investigation to modify existing methods or develop new techniques which would be optimal for the preparation of monolayer cell cultures from fish tissues.

One purpose of such a program was to make available to fish pathology, laboratory methods for culture, maintenance and study of viral agents infectious for salmonid fishes. The need for such techniques became obvious while experimenting with a virus infectious for the sockeye salmon, *Oncorhynchus nerka*. This virus was isolated from stocks of juvenile sockeye salmon

experiencing a heavy mortality at the Oregon Fish Commission's Willamette River Fisheries Station. Tissue containing active virus was lyophilized in the laboratory and resuspended for use in in vivo experiments. Because of the lack of cell cultures all work was restricted to the intact living animal. Research did reveal that the disease afflicting these fish was caused by a filterable agent, later shown to be a virus specific for the sockeye salmon. This virus appeared to be the same as that described by Rucker et al (36, p. 35-46). Work with the agent was limited because of the lack of suitable laboratory techniques for its study. The research was then redirected toward the development of reliable cell culture methods which could subsequently be utilized for the detection and study of viruses infectious for fishes.

Because of the economic importance of Pacific salmon, Oncorhynchus sp., and steelhead trout, Salmo gairdneri gairdneri, cultivation of cells from their tissues was emphasized. However, other species of fish were used in certain experiments where it seemed advantageous. The occurrence of epidemic hepatoma in the rainbow trout, Salmo gairdneri, offered an excellent and interesting source of cells for study. Information concerning the cultivation of cells from cold-blooded animals is not abundant and of the existing material very little is quantitative in nature. In this work attempts were made to determine the best methods for the preparation of cells for cultivation and to further ascertain the proper environment

for growth by means of quantitative experimentation. The two techniques examined most extensively for the preparation of tissues and cells for cultivation were the fragment (explant) and enzyme dispersion methods. Media, growth factors, gaseous atmosphere, temperature, and size of inoculum were of major concern with regard to the proper environment for cell growth. Preliminary experiments were started late in 1959 and experimentation was terminated in July 1963.

REVIEW OF THE LITERATURE

The *in vitro* cultivation of tissue is a comparatively new biological technique. Paul states that tissue culture developed from some of the methods of embryology which were in use during the last century. The experiments performed by Wilhelm Roux in 1885, whereby the medullary plate of a chick embryo was kept alive for several days in warm saline, has been credited by Paul as the first reported successful explantation (33, p. 1).

Other experiments followed this work. However, it was Harrison who first devised a simple and effective technique that would permit explanted tissue to continue growth and development outside the animal. Employing fragments of frog embryonic tissue from the walls of the neural tube, suspended in hanging drops of clotted lymph from adult animals, the development of nerve fibers was observed and studied (18, p. 140-143). Harrison's experiments indicated the value of these techniques for certain types of experimentation.

The development of more precise methods for the cultivation of animal tissues and cells was one of many important contributions of Carrel. Having a full knowledge of aseptic techniques he undertook tissue culture in the manner of a surgical operation. The successful results obtained by Carrel included keeping cells alive and in a state of active multiplication for years. It is interesting to note that this was accomplished without the assistance of antibiotics (32, p. 4). Methods for

growing chicken leucocytes on plasma clots containing chick embryo juice or extract were also studied. He determined that chick embryo juice was stimulatory for these cells but that homologous serum was inhibitory (2, p. 365-377). The discovery of the effect of embryo juices contributed greatly to the success of his explants and long term cultivation experiments.

Fragments of breast muscle of 12-day-old chick embryos were cultivated by Parker in single flasks on plasma clots and coagulum consisting of plasma and Tyrode's solution. The cultures were periodically removed and cut into smaller fragments, then these fragments were subcultured by the same method. He was able to maintain some of these cultures in this manner for a period of one year (31, p. 121-130).

Rous and Jones discovered that individual living tissue cells could be obtained in suspension by dispersion of cells growing on a plasma clot by means of the enzyme trypsin. When these cells were removed with the enzyme, washed and replanted on fresh plasma they put out processes and cell proliferation resumed. After growth had progressed this process could again be repeated (34, p. 549-555).

Fragments of monkey kidney cells were planted directly on glass without the aid of plasma clots by Morann and Melnick. The technique used was basically the same as that described by Gey in 1933 and was employed here for the cultivation of poliomyelitis virus. The fragments of monkey kidney tissue were fixed directly to the glass and the cells grew out in a thin

sheet around these explants. This modification had as an advantage the elimination of the troublesome clot (28, p. 558-563).

Recently tissue culture has assumed the status of a major technique in the virus laboratory. There are three primary reasons behind this increase in utilization of these methods.

The first is the general recognition by virologists about 1950 that many viruses during multiplication in cultured cells produce degenerative changes which can be easily distinguished. This then meant that for certain types of experimentation and study with cytopathogenic viruses, intact animals were no longer required.

The second circumstance responsible for the present increased use of tissue cultures was the development of antibiotics. The addition of these substances to the tissue culture medium made it possible to prepare cultures on an almost unlimited scale. Before the advent of antibiotics the number of cultures which could be prepared and maintained was very limited because of the stringent precautions required to avoid bacterial contamination.

Finally the usefulness of the method was greatly enhanced during 1952-53 by the utilization of trypsin for the dispersion of cells. This involved the revival and modification of the technique described by Rous and Jones (1916). Treatment of tissue with this enzyme produced suspensions of cells and

afforded methods for the preparation of large numbers of uniform cultures (9, p. 209-211).

Younger has described in detail the procedure required to prepare primary cultures of monkey kidney cells by enzyme dispersion. The techniques employed gave quantitatively standardized cell suspensions which could be used in the preparation of large numbers of replicate cultures. Trypsin was used at a concentration of 0.25 percent in phosphate buffered saline. The process was carried out at 37° C. and utilized a 10-minute pre-enzyme treatment followed by successive 10-minute exposures with the enzyme to obtain cells for planting. The cells were harvested, washed, and standardized in suspension prior to planting (58, p. 202-205).

Fogh and Lund were able to establish a cell line (FL strain) from human amniotic membrane tissue. This cell line was developed by trypsinization of the primary source and cultivated without embryo extract. Initial subcultures experienced a sharp reduction in cells. By concentrating the remaining cells in glass culture tubes they were able to maintain the epithelial-like cells through a critical period after which the cultures could be transferred and grown without difficulty. These cells had been under cultivation for eight months and had received 30 transfers at the time this material was reported. Cultures were routinely cultivated in LY medium with 20 percent pooled human serum (11, p. 532-537).

A technique for the cultivation and continuous propagation of skin tissue from small biopsy specimens has been described by Hsu and Kellogg. Biopsy specimens as small as two to three mm in diameter were used to initiate cell lines. They found McCoy's medium 5_a satisfactory as a general medium. Calf and fetal calf serum were both effective in this medium. The serum was added to give concentrations of 20 percent for primary cultures and 10 percent for the subsequent subcultures. Fragments of tissue were placed on one surface of a culture bottle. Perforated cellophane was cut the same dimension as the surface bearing the fragments and placed over the tissue. The growth medium was then added and the cultures incubated at 37° C. When thick growth was present the cellophane was removed and discarded. Cells were freed from the glass with 0.2 percent trypsin and two bottles were reduced to one in order to maintain a high cell concentration in the inoculum. Very successful results were obtained using this method for the propagation of these cultures (21, p. 221-235).

The nutrition of cells under cultivation has received increased attention with the improvement in methodology for the preparation of cell cultures and the advent of chemically defined media. The present information on this complex subject has been extensively reviewed by Morgan in which he has noted the nutritional requirements for cells investigated in this manner (29, p. 20-45).

Increased observations of viral infections in fishes has resulted in a stimulated interest in fish tissue culture. Watson has furnished an excellent review of virus infections in fish (43, p. 331-341). In studies regarding the propagation of lymphocystis disease of fish a filterable agent has been demonstrated (46, p. 249-256). Infectious pancreatic necrosis (IPN) of trout has been found to be a very important virus infection of trout (50, p. 61-65). Guenther et al. has studied the etiology of the sockeye salmon virus disease already mentioned in this paper (17, p. 1-6) and Watson, Guenther and Royce have reported on the hematology of fish infected with this agent (42, p. 27-39). Histopathologic changes in infected sockeye salmon have also been described from specimens obtained during the advanced stages of an epizootic occurring in a salmon hatchery (56, p. 85-90). Wolf has furnished an informative review of all pertinent literature concerning this viral agent (47, p. 1-3).

Certain of the early experiments involving the culture of fish tissues employed the embryos of Fundulus heteroclitus as a cell source. Explants of these tissues were cultured in diluted sea water containing Fundulus bouillon. Growth was obtained; however, the cultures were short-lived (6, p. 221-234). Goodrich employed the same system as described above for the study of cell behavior in tissue cultures prepared from embryos of Fundulus (12, p. 252-261). Chlopin prepared

explant cultures from tissues of pike and crucian carp in rabbit plasma diluted with homogeneous extract (3, p. 97-102).

The pituitary gland of dogfish shark, skate, flounder, sculpin, and the angler fish, Lophius piscatorius, were prepared and cultured, employing the explant method, by Lewis and MacNeal. Growth was obtained in autoplasma or homoplasma to which chick plasma had been added. Tissue from the dogfish and skate gave best results under these conditions of cultivation (23, p. 14-16).

Grand and Cameron have successfully cultured abnormal tissues obtained from melanotic growths of hybrid Mexican killifish. The medium selected was composed of one part fowl plasma, three parts fish serum (carp or goldfish) and one part chick embryo extract made up in Holtfreter's solution. The explants were incubated at 24° to 25° C. (13, p. 171-175).

Schlumberger has cultivated cells of cutaneous tumors present on goldfish. Explants were cultured by the hanging-drop and roller tube method. The medium was composed of chicken plasma, beef or chick embryo extract for clot, with blood serum from human umbilical cord and Tyrode's solution as the liquid phase (38, p. 287-294).

Soret and Sanders have successfully propagated eastern equine encephalomyelitis virus in the embryos of Gambusia sp. Whole embryos were removed aseptically from gravid females and placed in roller tubes. The embryo continued to develop

and served as cell cultures for the growth of this virus (40, p. 526-529).

Grützner has developed techniques for the cultivation of embryonic and adolescent tissue of the warm water aquarium fishes--Macropodus opercularis (Linne'), fish of paradise and Lebistes reticulatus (Peters), the guppy. These cells grew rapidly when cultured at 26-28° C. It was possible to maintain the cultures several weeks for observation and study (16, p. 8-29). This same author has subsequently utilized these techniques with the tissues mentioned above to cultivate the virus responsible for lymphocystis disease in fish and the virus causing carp-pox (15, p. 81-101). In addition to these accomplishments, Grützner has also perfected a method for the preparation of long-term cell cultures from liver and kidney tissue of Tinca vulgaris, the tench. This was done by means of enzyme dispersion of the cells from these organs for primary planting. Subculture of these initial plants have been successful (14, p. 195-202).

Wolf and his associates have recently contributed a great deal of information concerning the cultivation of fish cells. The principles and methods of tissue culture and how they might be applied to investigations in fish pathology have been clearly described (48, p. 3-7). Experiments to determine methods for the preparation of explant or fragment cultures of trout and goldfish tissues have been successful. Trout tissues

were cultured for as long as 65 days at 19° C. in medium composed of 20 percent serum, 30 percent SM 199, 45 percent Earle's solution and 5 percent embryo extract. Goldfish tissue grew more vigorously than did comparable trout tissue (49, p. 455-458).

Tissues of six species of freshwater boney fishes, a frog and a turtle were prepared for cultivation by means of low temperature (4-6° C.) trypsinization. The period of exposure to the enzyme lasted overnight. These cells were harvested and resuspended in the nutrient fluid. All cells grew well in a preparation consisting of 10 percent human cord serum, 5 percent whole egg ultrafiltrate, 30 percent medium 199, 0.5 percent lactalbumin hydrolysate, 10 percent Hanks' balanced salt solution, 45 percent Earle's balanced salt solution (which contained one-half the prescribed $NaHCO_3$) and antibiotics (400 units each penicillin and streptomycin and 50 units of Nystatin per milliliter) (53, p. 1890-1891). Development of the RTG-2 cell line from gonad tissue of the rainbow trout has been a most important contribution. These cells were prepared in primary culture by means of the cold trypsinization method and cultured in the same medium just described (52, p. 1065-1066). The techniques devised by this group have been extensively employed for the study of infectious pancreatic necrosis virus in these cell culture systems (51, p. 64-68) (54, p. 105-108).

Malsberger and Cerini have employed the RTG-2 cell line to study the characteristics of IPN virus in this culture system (25, p. 1283-1287).

Fish cell culture systems were employed by Parisot, Yasutake and Bressler for study and diagnosis of IPN virus (30, p. 63-66).

Members of the staff of the Wyoming Game and Fish Laboratory employed explant cultures of rainbow trout tissues for attempted diagnosis of a suspected viral infection in stocks of trout residing in Shoshone Lake (57, p. 1-4).

Clem, Moewus and Sigel have cultured cells from several marine fishes by both the explant and enzyme dispersion technique. Tissues of the yellow-stripped grunt, Haemulon flavolineatum, were used for most of the studies. Cells were not difficult to culture by the explant method; however, it was necessary to increase the amount of NaCl to ensure growth of the enzyme dispersed marine fish cells. Various modifications of Eagle's basal medium with animal sera were employed in this study (4, p. 762-766).

The culture of marine fish tissues has also been investigated by Townsley, Wight and Scott. Cell proliferation was obtained with explant cultures grown in medium 199 plus 10 percent human serum. Tissues cultured and examined were fin, spleen, heart, kidney, gonad, brain, uterus, and thymus. These tissues were obtained from mature Atlantic cod, Gadus

morhua; white perch, Roccus americanus; winter flounder, Pseudopleuronectes americanus; thorny skate, Raja radiata; American goosefish, Lophius americanus; pollock, Pollachius virens; and the shorthorn sculpin, Myoxocephalus scorpius. Flounder kidney tissue prepared by mechanical disruption was maintained through several transfers over a period of several months (41, p. 679-684).

EXPERIMENTAL MATERIALS AND SUPPLIES

Chemicals

All reagents and solutions were prepared using reagent grade chemicals made up in deionized or double distilled water. Chemicals used throughout this work were obtained from Merck and Company or J. T. Baker Chemical Company.

Glassware

Enzyme treatment of tissue for primary culture was carried out in 250 ml Pyrex trypsinization flasks (Bellco, Vineland, New Jersey). Erlenmeyer flasks were substituted for this purpose when required.

All containers used for the cultivation of cells were of Pyrex glass, including roller and Leighton tubes, milk dilution bottles (150 ml), and Roux flasks. The roller tubes (16 x 150 mm) and Leighton tubes (16 x 150 mm) were used in conducting quantitative growth experiments. The bottles and flasks mentioned above were employed for the preparation of stock cultures and for cells undergoing long-term growth experiments. All containers were capped with pure gum rubber stoppers with aluminium foil hoods.

Coverslips used for the preparation of stained slides were obtained from Bellco, Vineland, New Jersey. These slips were inserted in Leighton tubes and sterilized for use at planting. Coverslips were subsequently removed, stained, and the cells

viewed through the light microscope.

Rose chambers were used where it was desired to view, by means of phase microscopy, the development of living cells from planting to the formation of a confluent monolayer sheet.

Coverslips used in Leighton tubes and for the assembly of Rose chambers were cleaned by immersion in acid-alcohol (2 percent acetic acid in 95 percent ethanol) for twenty minutes. After removal from this solution the slips were rinsed thoroughly in double-distilled water.

Processing Glassware

All new glassware was placed in a solution of 25 percent sulfuric acid for one hour prior to use in the laboratory. A routine procedure for cleaning glassware was adhered to throughout this research. All items were boiled for 20 minutes in Labtone (Scientific Supplies Company) or Micro-Solv (Microbiological Associates, Inc.) followed by ten tap-water rinses and five deionized-water rinses. Culture bottles and tubes were given an additional three rinses in double-distilled water. To be sure all cell residue was removed from the culture tubes before re-use it was required that they be placed in 25 percent sulfuric acid for one hour prior to receiving the routine washing procedure.

Processing Rubber Materials

New gum rubber stoppers, tubing, and gaskets used in Rose

chambers were boiled 15 minutes in 0.5 N NaOH, rinsed in tap water, boiled in four percent v-v hydrochloric acid (10 ml concentrated HCl per 100 ml solution), rinsed in tap water, then thoroughly rinsed in deionized or double-distilled water. After this initial treatment, rubber products were washed by the same method already described for the cleaning of glassware.

Instruments

Stainless steel knives (Bard-Parker), scissors and forceps were used in the removal and preparation of tissues for cultivation. This equipment was cleaned by boiling in Micro-Solv for 15 minutes followed by rinses in tap and deionized water.

Filters

Seitz and Millipore filters were used when filter sterilization of solutions was required. Filter holders were washed in the same manner as were the instruments. The membranes employed with the Millipore filters did not require washing; however, the pads (60 mm in diameter) used in Seitz filters were washed as follows:

1. One hundred ml of one percent acetic acid was drawn through the filter pad.
2. One hundred ml of deionized water was then added.
3. After the water had passed the filter, 100 ml of one percent sodium bicarbonate was drawn through the filter pad.

4. This was followed by several washes in deionized water until the pH of the filtrate was no longer alkaline as determined by brom thymol blue.

This washing was very important because the asbestos disc which formed the filter pad contained large amounts of residual calcium and magnesium ions.

Sterilization of Equipment and Supplies

Glassware and instruments were sterilized in a dry heat oven for 12 hours at 110° C. Rubber supplies, filter holders and other items which could not be sterilized in the manner described above were autoclaved at 121° C. for 20 minutes.

Sterility Tests

All solutions used for the preparation of cell cultures were tested for sterility. The nutrient fluid used on cell cultures was checked before, during, and at the end of experimentation to ensure the preparations were free of contaminating microbes. These tests were made by adding a small amount of material to tubes containing thioglycollate broth prepared from Difco dehydrated thioglycollate medium and autoclaved at 121° C. for 20 minutes. These inoculated tubes of broth were then incubated at room temperature for ten days. If at the end of this time no growth appeared, the material tested was considered sterile.

Sterile Transfer Room with Cooler

An important facility for the preparation and manipulation of tissue and cell cultures was the availability of a small isolation room complete with a filtered air system to reduce contamination. In addition, this room was equipped with a cooling unit which was used as required to maintain temperatures in a range favorable for cold-blooded animals. During the preparation and handling of cultures the air temperature in the room ranged from 15-18° C. Temperature was never allowed to exceed 20° C.

Media

The determination of adequate growth-promoting media for fish cell cultures was an important aspect of this work. Five such nutrient fluids were utilized for the preparation of fish cell cultures and were experimentally compared as to growth-promoting properties for these cells. The five media employed are listed as follows:

1. Lactalbumin hydrolysate-yeast extract (LY)
2. Hanks' balanced salt solution-yeast extract medium (Hanks' YE)
3. Yeast extract-Eagle's medium-lactalbumin hydrolysate-peptone (YELP)
4. Eagle's Basal Medium (Eagle's BM)
5. Eagle's Minimum Essential Medium (Eagle's MEM)

The formula, preparation and compounding of these media were according to standard methods for the preparation of tissue culture materials and are indicated as follows:

1. Lactalbumin hydrolysate-yeast extract (LY) (27, p. 169)

NaCl	6.8000	grams	per	liter
KCl	0.4000	"	"	"
$CaCl_2 \cdot 2H_2O$	0.2650	"	"	"
$MgSO_4 \cdot 7H_2O$	0.2000	"	"	"
$NaH_2PO_4 \cdot H_2O$	0.1250	"	"	"
Dextrose	4.5000	"	"	"
$NaHCO_3$	1.1000	"	"	"
Lactalbumin[1] hydrolysate	5.000	"	"	"
Phenol red	0.0125	"	"	"

Double-distilled water to make one liter

The medium was then sterilized by filtration through a Seitz filter, dispensed into serum bottles and held at 35° C. for five days to observe for bacterial contamination. Bottles of media showing no contamination at the end of this period were stored at 10° C. until used. The complete medium was prepared by aseptically combining the following components in a sterile flask:

Lactalbumin hydrolystate medium	75.0	percent	by	volume
2.0 percent Difco yeast extract	5.0	"	"	"
Blood serum	20.0	"	"	"

[1] Nutritional Biochemical Corp.

Antibiotics were added to give the desired concentration in the medium. The final pH was adjusted to 7.3 ± 0.05 by the addition of sterile 1 N NaOH or 10 percent by volume of concentrated hydrochloric acid.

2. Hanks' balanced salt solution-yeast extract medium

Hanks' balanced salt solution

NaCl	8.0000	grams	per	liter
KCl	0.4000	"	"	"
$MgSO_4 \cdot 7H_2O$	0.2000	"	"	"
$CaCl_2 \cdot 2H_2O$	0.1855	"	"	"
Na_2HPO_4	0.6000	"	"	"
KH_2PO_4	0.6000	"	"	"
Glucose	1.000	"	"	"
Phenol red	0.0200	"	"	"

Double-distilled water to make one liter

This solution was dispensed in 50 to 100 ml volumes in Pyrex containers and autoclaved for 15 minutes at 121° C. Before use the pH was adjusted to 7.3 by the addition of a solution of sterile 1.4 percent $NaHCO_3$. The complete medium was then prepared by combining aseptically the following components:

Hanks' balanced salt solution	75.0	percent by	volume
2.0 percent yeast extract	5.0	"	"
Blood serum	20.0	"	"

Antibiotics were added to give the desired concentration

in the medium. The final pH of the medium was adjusted to 7.3 ± 0.05.

3. Yeast extract-Eagle's medium-lactalbumin hydrolysate-peptone (YELP) (27, p. 173)

Yeast extract	0.5 gram
Eagle's BM amino acid stock[2] (100X)	5.0 ml
Eagle's BM vitamin stock[3] (100X)	2.0 ml
Lactalbumin hydrolysate	2.5 grams
Bacto-peptone	5.0 grams
$NaHCO_3$	1.0 gram
Phenol red	0.0125 gram

Dilute to one liter in Hanks' BSS

The medium was then filter sterilized through a Seitz filter and tested for contamination in thioglycollate broth as described and stored at 10° C.

Complete medium was prepared by combining the following:

Yeast extract-Eagle's medium-lactalbumin hydrolysate peptone	80 percent by volume
Blood serum	20 percent by volume

Antibiotics were added to give the desired concentration in the medium. The final pH of the medium was adjusted

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[2,3]Microbiological Associates, Inc.

to 7.3 ± 0.05.

4. Eagle's Basal Medium (8, p. 501-504)

This medium was obtained from Microbiological Associates and has the following formulation:

Earle's balanced salt solution

NaCl	6.80 grams per liter
KCl	0.40 " " "
$CaCl_2$	0.20 " " "
$MgSO_4 \cdot 7H_2O$	0.20 " " "
$NaH_2PO_4 \cdot H_2O$	0.125 " " "
$NaHCO_3$	2.20 " " "
Glucose	1.00 " " "
Phenol red	0.02 " " "

Eagle's basal medium

L-Amino Acids	mM	Vitamins	mM
Arginine	0.10	Biotin	10^{-3}
Cystine	0.05	Choline	10^{-3}
Glutamine	2.00	Folic acid	10^{-3}
Histidine	0.05	Nicotinamide	10^{-3}
Isoleucine	0.20	Pantothenic acid	10^{-3}
Leucine	0.20	Pyridoxal	10^{-3}
Lysine	0.20	Thiamine	10^{-3}
Methionine	0.05	Riboflavin	10^{-4}
Phenylalanine	0.10		
Threonine	0.20		
Tryptophan	0.02		
Tyrosine	0.10		
Valine	0.20		

The Earle's balanced salt solution was held at 10° C. until use. The Eagle's basal medium vitamin solution was

concentrated 100X had to be kept frozen (-20° C.) until use. The L-amino acids as received were 100X concentrated and could be stored at 10° C.; however, they did not include glutamine. This amino acid was retained in a 100X concentrated stock solution and kept frozen at -20° C. It was added to the medium only at the time of final compounding just prior to use. The components other than glutamine were aseptically combined in the following proportions:

Eagle's basal medium (less glutamine and serum)	
Earle's balanced salt solution	980 ml
Eagle's basal medium amino acid mixture (100X)	10 ml
Eagle's basal medium vitamin mixture (100X)	10 ml

This preparation could be retained for two weeks at 10° C.

The final or complete medium as used in this work was prepared as follows, again with aseptic precautions:

Eagle's basal medium, as above	790 ml
Blood serum	200 ml
Glutamine (100X)	10 ml

Antibiotics were added to give the desired concentration in the medium. The final pH of the medium was adjusted to 7.3 $\pm$ 0.05.

5. Eagle's Minimum Essential Medium (7, p. 432-437)

This medium was also obtained commercially from

Microbiological Associates. Earle's BSS was again used here and has the same formulation as already indicated.

Earle's BSS -- same as indicated for Eagle's BM

MEM Essential L-Amino Acid	mg/liter	MEM Vitamin	mg/liter
Arginine	105	Choline	1.0
Cystine	24	Folic acid	1.0
Glutamine	292	Inositol	2.0
Histidine	31	Nicotinamide	1.0
Isoleucine	52	Pantothenate	1.0
Leucine	52	Pyridoxal	1.0
Lysine	58	Riboflavin	0.1
Methionine	15	Thiamine	1.0
Phenylalanine	32		
Threonine	48		
Tryptophan	10		
Tyrosine	36		
Valine	46		

MEM Non-Essential L-Amino Acid	mM		mM
Alanine	0.1	Sodium pyruvate	1.0
Asparagine	0.1		
Aspartic acid	0.1		
Glycine	0.1		
Glutamic acid	0.1		
Proline	0.1		
Serine	0.1		

The Earle's BSS was again retained at 10° C. and the MEM vitamin stock (100X) was kept frozen at -20° C. The L-glutamine stock solution was stored as indicated before and was not added to the medium until final compounding.

Sodium pyruvate stock solution (100X) was stored at 10° C. The Eagle's MEM Essential Amino Acids (50X) and MEM Non-Essential Amino Acid stock solutions were stored at 10° C until use. These components were aseptically combined in the

following proportions:

Eagle's Minimum Essential Medium (less glutamine and serum)

Earle's BSS	960 ml
MEM Essential Amino Acids (50X)	20 ml
MEM Non-Essential Amino Acids (100X)	10 ml
MEM vitamins (100X)	10 ml
Sodium pyruvate (100X)	10 ml

This preparation could be stored for two weeks at 10° C.

The final or complete medium added to the cell cultures was prepared as follows:

Eagle's Minimum Essential Medium, as above	790 ml
Blood serum	200 ml
Glutamine (100X)	10 ml

Antibiotics were added to give the desired concentration in the medium. Final pH of this medium was adjusted to 7.3 ± 0.05.

Yeast Extract

The yeast extract contained in LY, YELP, and Hanks'-YE was prepared by adding 2.0 grams of Difco yeast extract to 100 ml of Hanks' BSS. The pH of the balanced salt solution was adjusted to 7.4 with sterile 1.4 percent $NaHCO_3$. The solution was then filter sterilized by Seitz filtration, dispensed in screw cap tubes at 5 ml per tube and stored at -20° C.

Phenol Red Stock Solution

Phenol red (Difco) was prepared in a 0.2 percent stock solution for addition to the media. One gram of phenol red was placed in a flask and N/20 NaOH added slowly and mixed until the powder was dissolved and a deep red color prevailed. The material was then diluted to 500 ml in double-distilled water, stoppered and stored at 10° C. The phenol red solution was added to media by volume to give the required concentration. The presence of phenol red in the nutrient fluid made it possible to observe pH changes in the cultures.

pH Adjustment of Fluids

Solutions which were not strongly buffered were adjusted with 1.4 percent $NaHCO_3$ or 0.03 N hydrochloric acid in double-distilled water. These solutions were dispensed in screw cap tubes (5 ml per tube) and autoclaved for 15 minutes at 121° C. Adjustment of complete media or other strongly buffered solutions was accomplished with 1 N NaOH or 10 percent v-v hydrochloric acid (10 ml concentrated HCl per 100 ml solution). These solutions were prepared for use in the same manner as those mentioned above.

Blood Serum

All the media above required the addition of animal sera to ensure the growth of fish cells. Human and calf serum with and without gamma globulin were obtained from Hyland

Laboratories. These sera had been processed to remove the gamma globulin fraction. This was done to furnish a product free of toxicity for cells which might be injured by this material. Horse and additional human serum were obtained from Microbiological Associates. Fish serum was obtained from adult chinook salmon and steelhead trout. The adult fish were selected in order to obtain the quantity of blood serum required. The fish were bled by severing the tail in the region of the caudal peduncle and allowing the blood to flow into collecting tubes, which were refrigerated overnight at 10° C. to obtain maximum clot rectraction. The material was then centrifuged and the serum fraction removed. Fish serum was sterilized by Seitz filtration and dispensed aseptically in 50 ml serum bottles prior to storage at 10° C. Each bottle of serum was tested for sterility in thioglycollate broth.

Antibiotics

Antibiotics were employed in culture media for the control of contaminating microorganisms. A stock solution of sodium penicillin G and streptomycin sulfate was prepared in Hanks' BSS and sterilized by filtration in a Millipore filter equipped with a plain white HA 450 mμ pore size membrane. The solution, containing both antibiotics, was aseptically dispensed into serological tubes, in 1.0 ml volumes, tested for sterility, and stored at -20° C. The concentration of penicillin and streptomycin in this solution was 25,000 units and 25,000 micrograms

per ml respectively. The solution could then be added to the media in the proportion of 0.1 ml for each 25 ml of complete medium, giving a final concentration of 100 units penicillin and 100 micrograms streptomycin per ml of medium. Mycostatin (Nystatin, Squibb) was used for the control of fungi. It was obtained in sterile lyophilized bottles containing 500,000 units. The material in one vial was aseptically resuspended in 20 ml of Hanks' BSS and dispensed in serological tubes at 0.5 ml per tube. The concentration of this solution was then 25,000 units per ml, the same as that indicated for the other antibiotics.

The concentration of antibiotics used in the medium was dependent upon the source and kind of culture receiving the fluid. When preparing primary cultures from external organs which could not be obtained aseptically, penicillin and streptomycin were employed at concentrations of 1,000 units and 1,000 micrograms respectively per ml of medium. Mycostatin was introduced at 100 units per ml of medium for the control of fungi and yeast. These concentrations of antibiotics were maintained in the nutrient fluid of primary cultures for approximately one week. If no contamination had appeared by this time the antibiotic concentrations were reduced to 100 units and 100 micrograms of penicillin and streptomycin at the next change of nutrient fluids. The concentration of Mycostatin was also lowered at this time to 25 units per ml of medium. These levels of antibiotics were then maintained until the culture was terminated. When internal organs were the source of tissue

used to derive primary cell cultures, penicillin and streptomycin were initially added to give a concentration of 400 units and 400 micrograms respectively per ml of nutrient fluid. Mycostatin was added to the medium at a concentration of 50 units per ml of the growth medium. Lower levels of antibiotics could be utilized in this case as the internal organs were removed using absolute aseptic technique and presumably did not harbor large numbers of microbes. After one week of cultivation the antibiotics in these preparations were also reduced to the same levels indicated for the other primary cultures. Stock cultures were maintained and all growth experiments were conducted employing penicillin, streptomycin and Mycotatin at concentrations of 100 units, 100 micrograms and 25 units per ml of culture medium.

Enzyme Solutions

Trypsin (Nutritional Biochemicals Corporation 1:300) was used throughout this work for the preparation of primary cell cultures and for subsequent transfer of these cells during subculture. The trypsin solution was prepared in Hanks' BSS at a concentration of 0.25 percent in the following manner. The pH of Hanks' BSS was adjusted to 7.5 by the addition of a sterile 1.4 percent $NaHCO_3$ solution. The required amount of enzyme was added and the volume brought up to 1 liter. The flask containing the enzyme was then placed on a mechanical shaker at low speed for 30 minutes in a 35° C. incubator. After the trypsin had dissolved, the pH was again brought to 7.5 with $NaHCO_3$ and

the solution sterilized by filtration through a Seitz filter. It was then aseptically dispensed into serum bottles, tested for sterility and stored at -20° C. Other enzymes used during the course of this work were hyaluronidase, collagenase, papaine, pancrease, and receptor-destroying enzyme (RDE). These enzymes were prepared in the same manner as trypsin; however, in most cases the mechanical shaker was not required to get the compounds in solution.

Source and Kind of Tissue Used

It became apparent from the start of this research that problems of availability of tissue from anadromous salmon and steelhead trout would be encountered. Experiments had to be carried out utilizing one source and kind of tissue for cultivation and then changing when this material was no longer available. Salmon and steelhead trout were obtained from Oregon Fish Commission hatcheries. Rainbow trout and hepatoma tissue from this fish were made available by the Oregon State Game Commission's Roaring River Hatchery. Squawfish were taken by hook and line from the Willamette River near Corvallis.

May-Grünwald-Giemsa Stain

Mounted slides were prepared from stained coverslips on which monolayer cell cultures were grown. Cell morphology and the development of cultures from planting to the formation of a confluent monolayer sheet were observed, photographed and

studied. Stock May-Grünwald stain was made by dissolving 2.5 grams of dye in 1000 ml of absolute methanol and allowed to age for one month. Stock Giemsa was prepared by adding 1.0 gram of this dye to 66 ml of glycerol at 55-60° C. for 1.5 to 2.0 hours, followed by the addition of 66 ml of absolute methanol. The staining procedure (27, p. 134) was as follows:

1. Wash the coverslip with cells three times in BSS.
2. Fix for five minutes in absolute methanol.
3. Stain for ten minutes in stock May-Grünwald stain.
4. Stain for 20 minutes in dilute Giemsa solution (1:10 dilution of stock in deionized water).
5. Quickly rinse in acetone two times to remove moisture from tissue. Do not let coverslip dry.
6. Clear by rinsing three times in acetone-xylol (2:1), three times in acetone-xylol (1:2) followed by a rinse in fresh xylol.
7. Mount on slide in mounting medium.

Versene Solution

To obtain complete removal of cells from tube cultures used in the various quantitative growth experiments conducted with fish cells, a solution containing Versene was employed. This compound was much more efficient in this process than was trypsin (0.25 percent). Cells removed from the glass under these conditions were used to obtain a total count per culture tube and were never transferred or subcultured. The solution

used had the following formula:

NaCl	8.0 grams
KH_2PO_4	0.2 grams
KCl	0.2 grams
Na_2HPO_4	1.15 grams
Versene	0.2 grams
Double-distilled water	1000 ml

The solution was then sterilized by autoclaving at 121° C. for 15 minutes. The pH of this solution was adjusted to 7.4 (27, p. 176).

Crystal Violet Stain

Suspensions of cells removed from culture tubes with Versene for enumeration were diluted 1:2 with a crystal violet dye solution composed of 0.1 percent crystal violet and 0.1 molar citric acid in deionized water. The procedure stained nuclei deeply, facilitating counting of cells in a hemocytometer (26, p. 116).

Enumeration of Cells

The hemocytometer count was the only feasible means of obtaining routine cell counts on the small tube cultures employed in these studies. The method was both time consuming and subject to substantial error. A single count required approximately 10 to 15 minutes to complete and was probably subject to never less than 10 percent error. Cell enumeration

was required to determine the degree of cell liberation from fish tissues by various enzymes tested and for establishing the proper cell inoculum for primary, stock and experimental cultures. The experiments to determine the effects of various treatments of cultures on cell growth were evaluated by this counting technique. In general the method used here was the same as that employed in other investigations which depend on counts made in a hemocytometer as the measure of cell concentration or proliferation (37, p. 773-795). Counts were made by removing 1 ml of the cell suspension to be counted and diluting this with 2 ml of the 0.1 percent crystal violet dye solution already described. This solution was shaken 25 times to break up clumps of cells and to obtain a uniform suspension of material. The hemocytometer and coverglass were cleaned in 95 percent ethanol and polished dry with a soft lint-free cloth. The coverglass was then placed firmly on the hemocytometer so that it covered both counting chambers. The two chambers of the hemocytometer were carefully filled with the solution of cells to be counted by means of a capillary pipette. The cells were counted in all nine of the one square mm areas of each chamber. The process was always repeated so that for any cell suspension examined there were values obtained from four chambers. The mean of these four counts was calculated and the cell concentration per ml of the original suspension was determined as indicated below (26, p. 116).

$$\frac{\text{Mean no. of cells counted}}{\text{Mean no. of squares counted}} \times 10{,}000 \text{ (hemocytometer volume)}$$

$$\times 3 \text{ (dilution)} = \text{number of cells per ml of the original suspension}$$

Some variation was introduced in this method because of the particular requirements for certain measurements. Enumeration of cell suspensions obtained from tissue treated with enzymes for the preparation of primary cell cultures and for experiments designed to reveal the ability of certain enzymes to disperse cells from the tissue mass was performed in the following manner. Only cells showing both nuclei and cytoplasm were counted. This was done in an attempt to provide a suspension, used for seeding cultures, which was based as nearly as possible on a viable cell count. The convention adopted for counting clumps of cells was as follows: Clumps in which the individual nuclei were surrounded by a large volume of cytoplasm were treated as aggregations of single cells and every visible cell was counted. Clumps in which the nuclei were close together with only a little cytoplasm visible were merely counted as one cell.

When transferring stock cultures or preparing the inoculum for quantitative growth experiments from these stocks, again only whole cells (nuclei plus cytoplasm) were counted. Clumps of cells were treated in the same manner as above; however, they were not numerous in these preparations. Cells from these stock cultures were removed from the containers with the

addition of trypsin (0.25 percent) as already described.

Determination of the total number of cells in the culture tubes used for the quantitative growth experiments were handled somewhat differently. These were terminal cultures established only for the purpose of observing, at intervals over a set period, the effect of given treatments on groups of cultures prepared in the same manner. These cultures were grown in roller or Leighton tubes and contained 1 ml of nutrient fluid. The medium was decanted from the tubes and the sheet of cells gently washed with 3 ml of Earle's BSS. The wash solution was decanted and all tubes were inverted to drain off the fluid. One ml of the Versene solution (0.02 percent) was then placed in each tube and allowed to remain in contact with the sheet of cells for approximately 30 minutes. The cells were removed from the glass by the action of this agent and a cell suspension suitable for counting was obtained by shaking the tube for three to four minutes to break up the clumps. On several occasions glass beads were employed to assist in destruction of these aggregates of cells. Versene was used to free the cells from the glass for counting as it is much more effective than trypsin. Two ml of a 0.1 percent crystal violet staining solution plus citric acid was added to the tubes containing Versene and cells, thus diluting the 1 ml of Versene threefold. After shaking the suspension 25 times the hemocytometer was filled in the same manner already described. Because the dead cells and debris were removed in washing it was assumed only

live cells remained in these cultures; therefore, when counts were made all nuclei were enumerated whether the cytoplasm was present or not. Four counts were made for each tube and there were four culture tubes for each treatment at any given interval. The value obtained in these counts was expressed in cells per ml of nutrient fluid and because there was only 1 ml of fluid in each tube the value indicated the total number of cells present in that culture.

These growth experiments were conducted over a period of two to four weeks with cell counts made at predetermined intervals. The base line or zero determination was of special importance and is described in detail here. A given cell inoculum cannot be assumed to contain all viable cells capable of growing in a cell culture system. It was desired, however, to know the viable cell population in a given culture at the start of each experiment so that the growth pattern could be measured and followed more precisely. To accomplish this the base line or zero cell count was employed. Experiments were carried out to allow for a set of four replicate tubes for each treatment to be removed 18 to 24 hours after start of the test. The cells were attached to the glass by the end of this time, but growth had not been initiated. This cell count presumably represents the viable cells inoculated in each culture at planting and the value is referred to as the base line cell count or zero time count. This procedure has been a very important factor with regard to the growth experiments related in this report.

Statistical Methods

Variation in cell counting and that encountered in the biological systems of the cultures themselves made it necessary to determine statistically significant differences between the mean cell counts obtained in growth experiments. The technique employed to accomplish this is referred to as the least significant difference between means. Three ranges of cell counts were selected for testing and have been listed below.

10,000 - 99,999 cells per culture tube
100,000 - 499,999 " " " "
500,000 - 1,000,000 " " " "

Twenty-five sets of counts representing the total number of cells in each of four culture tubes were selected randomly from the growth experiment data for each of the three ranges used in this determination. The analysis for any given range was therefore based on 25 observations which were composed of the total number of cells present in each of four culture tubes (Table 1). The calculations required to determine the least significant difference have been indicated (Table 1). Differences in the mean cell counts of four replicate cultures must be greater than 25,950 cells per culture tube for the first range, 64,540 cells per culture tube for the second range, and 109,966 cells per culture tube for the third range in order to be statistically significant. Data was then compared and significance determined between mean cell counts on the basis

of these values. When comparing two means which did not fall in the same range the least significant difference value for the lower mean cell count was used. It should also be noted that the word significant has been reserved and used only when referring to data which has been statistically evaluated. This word as used here means statistically significant.

Statistical procedures used in estimating the variation in growth among four replicate cultures and in estimating the least significant difference between the means of four such replicates was based on methods described in Mainland (24, p. 159 and p. 204).

Table 1. Statistical treatment of three ranges of cell counts to determine the least significant difference.

Range 10,000-99,999 cells per culture tube

Obs.#	Total number of cells in each of four replicate culture tubes				Mean no. cells per culture tube	Deviation from mean			
	1	2	3	4		1	2	3	4
1	45,000	60,000	11,700	98,300	53,800	8,800	6,200	42,100	44,500
2	11,700	13,300	10,000	56,000	22,800	11,100	11,500	12,800	33,200
3	67,700	91,700	76,000	68,300	75,900	8,200	15,800	100	7,600
4	83,300	75,000	93,300	64,300	79,000	4,300	4,000	14,300	14,700
5	90,000	98,300	83,300	95,000	91,600	1,600	6,700	8,300	3,400
6	70,000	85,000	89,300	91,000	83,800	13,800	1,200	5,500	7,200
7	67,300	74,000	50,700	66,000	64,500	2,800	9,500	13,800	1,500
8	68,300	86,000	87,300	71,700	78,300	10,000	7,700	9,000	6,600
9	43,000	23,000	37,300	43,000	36,700	6,300	13,700	600	6,300
10	59,300	51,700	43,300	55,300	52,000	7,300	300	8,700	3,300
11	42,700	61,700	40,000	48,300	48,200	5,500	13,500	8,200	100
12	64,000	61,700	87,300	53,300	66,600	2,600	4,900	20,700	13,300
13	89,300	73,300	88,300	67,300	79,600	9,700	6,300	8,700	12,300
14	65,000	64,300	55,000	54,300	59,700	5,300	4,600	4,700	5,400
15	76,700	46,700	45,700	36,700	51,500	25,200	4,800	5,800	14,800
16	12,700	23,300	15,000	14,000	17,300	4,600	6,000	2,300	3,300
17	24,000	41,000	43,300	29,300	34,400	10,400	6,600	8,900	5,100
18	81,700	58,300	58,300	47,300	61,400	20,300	3,100	3,100	14,100
19	43,300	80,700	69,300	71,700	66,200	22,900	14,500	3,100	5,500
20	94,300	98,300	39,300	90,000	80,500	13,800	17,800	41,200	9,500
21	20,000	20,000	22,000	26,000	22,000	2,000	2,000	0	4,000
22	84,300	99,300	72,700	73,700	82,400	1,900	16,900	9,700	8,700
23	55,000	36,700	91,700	83,300	66,700	11,700	30,000	25,000	16,600
24	46,000	56,700	68,300	44,000	53,800	7,800	2,900	14,500	9,800
25	94,300	86,700	72,700	79,300	83,300	11,000	3,400	10,600	4,000

(continued next page)

Table 1 (continued)

Calculations: Range 10,000-99,999

Combined sum of squares of the deviations = 18,738,110,000

Divisor (sum of degrees of freedom) = 4 - 1 x 25 = 75

$$\text{Variance} = \frac{18,738,110,000}{75} = 249,841,466.67$$

$$\text{Estimated standard deviation} = \sqrt{249,841,466.67} = 15,806.37$$

Standard error of difference between means =

$$\pm \sqrt{\left(\frac{15,806.37}{\sqrt{4}}\right)^2} \times 2 = \frac{15,806.37}{2} \times 1.414 = 12,975$$

In Fisher's table of t with 75 degrees of freedom, and P = 0.05 t = 2.0

Least significant difference = 12,975 x 2.0 (t value) = >25,950

(continued next page)

Table 1 (continued)

Range 100,000-499,999 cells per culture tube

Obs.#	Total number of cells in each of four replicate culture tubes				Mean no. cells per culture tube	Deviation from mean			
	1	2	3	4		1	2	3	4
1	172,300	110,000	227,300	137,300	161,700	10,600	51,700	65,600	24,400
2	222,700	253,300	201,700	222,700	225,100	2,400	28,200	23,400	2,400
3	152,700	146,000	184,300	146,000	157,300	4,600	11,300	27,000	11,300
4	397,300	333,300	380,000	375,000	371,500	25,800	38,200	8,500	3,500
5	342,700	380,700	345,000	326,700	348,800	6,100	31,900	3,800	22,100
6	465,000	437,300	248,300	366,000	379,200	85,800	58,100	130,900	13,200
7	331,700	448,300	315,000	360,300	373,800	42,100	74,500	58,800	13,500
8	211,700	174,300	313,300	211,000	227,600	15,900	53,300	85,700	16,600
9	265,000	346,700	367,300	313,300	323,100	58,100	23,600	44,200	9,800
10	254,000	220,000	204,000	191,700	217,400	36,600	2,600	13,400	25,700
11	125,000	140,000	109,300	100,700	118,800	6,200	21,200	9,500	18,100
12	356,700	332,000	296,700	426,700	354,000	2,700	22,000	57,300	72,700
13	224,300	152,700	270,000	231,000	219,500	4,800	66,800	50,500	11,500
14	224,000	376,000	233,300	275,000	277,000	53,000	99,000	43,700	2,000
15	141,000	124,300	160,700	137,700	140,700	300	16,400	20,000	3,000
16	203,300	210,700	162,700	212,700	197,400	5,900	13,300	34,700	15,300
17	278,300	324,000	348,300	297,300	312,000	33,700	12,000	36,300	14,700
18	256,300	189,300	232,700	246,700	231,200	25,100	41,900	1,500	15,500
19	148,300	151,700	129,300	131,700	140,200	8,100	11,500	10,900	8,500
20	392,700	421,000	360,000	421,700	398,800	6,100	22,200	38,800	22,900
21	365,000	417,300	428,300	435,000	411,400	46,400	5,900	16,900	23,600
22	411,700	418,300	356,000	339,300	388,800	22,900	29,500	32,800	49,500
23	456,700	421,700	344,000	420,700	410,800	45,900	10,900	66,800	9,900
24	150,700	333,300	323,300	296,700	276,000	125,300	57,300	47,300	20,700
25	370,000	350,700	411,000	384,300	379,000	9,000	28,300	32,000	5,300

(continued next page)

Table 1 (continued)

Calculations: Range 100,000-499,999

Combined sum of squares of the deviations = 156,253,830,000

Divisor (sum of degrees of freedom) = 4 - 1 x 25 = 75

$$\text{Variance} = \frac{156,253,830,000}{75} = 2,083,384,400.00$$

$$\text{Estimated standard deviation} = \sqrt{2,083,384,400.00} = 45,644.10$$

Standard error of difference between means =

$$\pm \sqrt{\left(\frac{45,644.10}{\sqrt{4}}\right)^2} \times 2 = \frac{45,644.10}{2} \times 1.414 = 32,270$$

In Fisher's table of t with 75 degrees of freedom, and P = 0.05 t = 2.0

Least significant difference = 32,270 x 2.0 (t value) =)64,540

(continued next page)

Table 1 (continued)

Range 500,000-1,000,000 cells per culture tube

Obs.#	Total number of cells in each of four replicate culture tubes				Mean no. cells per culture tube	Deviation from mean			
	1	2	3	4		1	2	3	4
1	574,300	714,300	698,300	695,300	670,500	96,200	43,800	27,800	24,800
2	687,700	709,300	695,000	528,300	654,800	32,900	54,500	40,200	126,500
3	751,700	921,000	758,300	661,000	773,000	21,300	148,000	14,700	112,000
4	634,000	646,000	516,000	591,700	596,900	37,100	49,100	80,900	5,200
5	632,700	540,700	550,700	564,000	572,000	60,700	31,300	21,300	8,000
6	865,000	742,300	662,000	577,300	711,800	153,200	30,500	49,800	134,500
7	723,300	679,300	690,000	830,000	730,700	7,400	51,400	40,700	99,300
8	710,700	661,700	709,300	629,300	677,800	32,900	16,100	31,500	48,500
9	863,300	951,700	821,700	772,700	852,100	11,200	99,600	30,400	79,400
10	860,700	712,700	860,000	852,700	824,000	36,700	111,300	36,000	28,700
11	786,700	583,300	550,000	541,700	615,400	171,300	32,100	65,400	73,700
12	564,000	563,300	592,700	506,000	556,500	7,500	6,800	36,200	50,500
13	618,300	745,000	679,300	765,000	701,900	83,600	43,100	22,600	63,100
14	736,300	688,300	676,000	717,300	711,200	25,100	22,900	35,200	6,100
15	756,000	930,000	787,300	926,700	850,000	94,000	80,000	62,700	76,700
16	670,700	974,300	890,000	769,300	826,100	155,400	148,200	63,900	56,800
17	711,700	715,000	621,700	595,000	660,900	50,800	54,100	39,200	65,900
18	643,300	594,000	673,300	606,700	629,300	14,000	35,300	44,000	22,600
19	670,700	752,700	751,700	641,700	704,200	33,500	48,500	47,500	62,500
20	885,000	816,700	890,000	783,300	843,300	41,700	26,600	46,700	50,000
21	810,700	961,700	690,000	845,000	826,900	16,200	134,800	136,900	18,100
22	782,700	781,700	787,300	793,300	786,200	3,500	4,500	1,100	7,100
23	511,700	800,700	711,700	751,700	693,500	181,800	107,200	18,200	58,200
24	718,300	649,300	633,600	702,700	674,700	43,600	25,400	41,100	28,000
25	511,700	635,000	596,700	522,700	566,500	54,800	68,500	30,200	43,800

(continued next page)

Table 1 (continued)

Calculations: Range 500,000-1,000,000

Combined sum of squares of the deviations = 453,610,760,000

Divisor (sum of degrees of freedom) = 4 - 1 x 25 = 75

$$\text{Variance} = \frac{453,610,760,000}{75} = 6,048,143,466.67$$

$$\text{Estimated standard deviation} = \sqrt{6,048,143,466.67} = 77,769.81$$

Standard error of difference between means =

$$\pm \sqrt{\left(\frac{77,769.81}{\sqrt{4}}\right)^2} \times 2 = \frac{77,769.81}{2} \times 1.414 = 54,983$$

In Fisher's table of t with 75 degrees of freedom, and P = 0.05 t = 2.0

Least significant difference = 54,983 x 2.0 (t value) = >109,966

EXPERIMENTAL RESULTS

Methods for Preparation of Cultures

Techniques for the *in vitro* cultivation of tissues from Pacific salmon and steelhead trout were lacking and only a small amount of published material concerning the cultivation of fish cells was available compared to that for other animals. Several methods for the preparation of cell cultures from tissues of salmon and steelhead for use in experimentation were examined. Two techniques, the fragment (or explant), and enzyme dispersion methods showed the most promise and were used throughout this work.

Fragment Method

This was the first technique examined for the preparation of fish cell cultures. The two major advantages to be found in this method are its reliability and the ease of preparation of the tissues for cultivation. The fragment or explant method also has certain disadvantages which should be pointed out. The presence of the fragment is troublesome in cultures which are to be maintained over several weeks. The fact that these cultures cannot be transferred or subcultured as easily as the enzyme dispersed monolayer cultures is also a hindrance. A variety of fish tissues have been cultured by this method (Table 2). Embryonic, yolk-sac fry, fin and hepatoma tissue

Table 2. Cells cultured by the fragment method.[1]

Species	Tissue
Coho salmon, *Oncorhynchus kisutch*	Yolk-sac fry
" " " "	Embryonic
" " " "	Embryo heart[2]
Sockeye salmon, *Oncorhynchus nerka*	Fin
" " " "	Yolk-sac fry
" " " "	Embryonic
Chinook salmon, *Oncorhynchus tshawytscha*	Tumor-like growth
" " " "	Yolk-sac fry
" " " "	Embryonic
" " " "	Fin
" " " "	Gill
Steelhead trout, *Salmo gairdneri gairdneri*	Yolk-sac fry
" " " " "	Embryonic
Rainbow trout, *Salmo gairdneri*	Hepatoma
" " " "	Embryonic
" " " "	Fin
Squawfish, *Ptychocheilus oregonensis*	Fin
" " "	Cornea

[1] All of the above were primary cultures in roller tubes and were considered successful if a definite outgrowth of new cells could be seen around the fragments microscopically.

[2] Kept beating five days after excised from animal.

all produced excellent cultures when prepared in this way. The following steps were employed in order to establish primary cell cultures using the fragment method.

1. The fish were killed just before use by severing the vertebral column immediately back of the head and placing them in a cold solution of 1:1 million Roccal for one minute to aid in controlling bacterial contamination. At the end of this period the animals were removed and bled by cutting the tail at the caudal peduncle. This was followed by a quick rinse in sterile cold distilled water to remove the Roccal and blood. The animals were then dried with sterile gauze to remove any excess moisture after the rinsing process.
2. The desired organ was excised using the absolute aseptic technique and rinsed quickly in a sterile cold, balanced salt solution at pH 7.4. It should be noted that when external organs, embryonic or yolk-sac fry were the source of the tissues to be used for cultivation, the Roccal treatment and distilled water rinse were omitted. When these tissues were employed the first step was the rinse in a cold balanced salt solution at pH 7.4.
3. The tissue was then cut into fragments, approximately 1-4 mm square with stainless steel Bard-Parker knives.

From this point on it was required that the fragments of tissue not be rinsed or allowed to come in contact with any fluid.

4. The fragments were then placed on the sides of the lower one-third of the roller tubes. This was done by using a pair of long curved-tipped stainless steel forceps. Most tissue fragments were found to stick to the glass if there was no excessive amount of fluid on the fragment.

5. The tubes containing the tissue were covered with loose foil hoods and placed at 18° C. for four and one-half to five hours. This allowed the tissue to become firmly stuck to the glass. The drying time was most important for if not observed, the fragments of tissue would not remain adhered to the glass when nutrient fluid was added.

6. At the end of the drying period, 1 ml of the complete nutrient fluid to be used was added to each culture tube. The foil hoods covering the tubes during drying were replaced with rubber stoppers and the hoods were fitted over the stopper and lip of the tube to assist in protecting the opening from contamination.

7. The tubes were then placed on a revolving roller drum turning at about 12 revolutions per hour in an 18° C. incubator.

8. The nutrient fluid was changed 24 hours after incubation had been initiated. Subsequent fluid changes were made every four to six days until the cultures were terminated.
9. Fragments could be observed for growth by examination with the compound microscope. Magnification was limited to that obtained with the low-power objective because of the thickness of the culture containers. The time required for new cells to appear was dependent on the tissue used. The newly grown cells could be seen extending out in a monolayer sheet around the fragment (Figure 1). Cells have been cultured by this method in stationary containers such as Leighton tubes and milk dilution bottles without difficulty.

Enzyme Dispersion Method

This technique was found preferable to the fragment method for the preparation of primary cultures of fish cells used in this work. Enzyme dispersion of the tissue produced a suspension of cells and small clumps of cells which after adhering to the glass surface of the culture container grew and ultimately formed a confluent monolayer sheet. A number of fish tissues were cultured by this method (Table 3). The procedure generally followed for the preparation of these cultures during this research is described as follows:

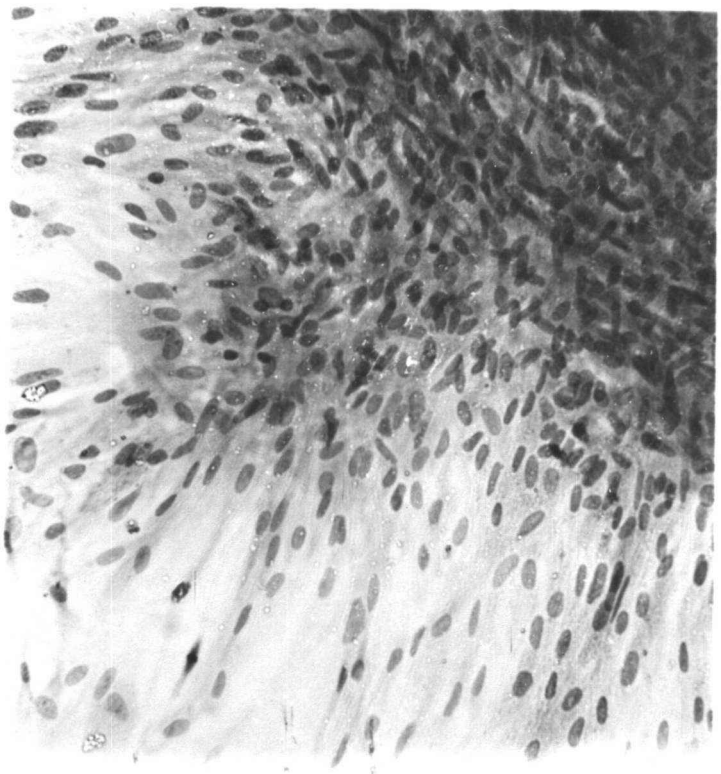

Figure 1. Coho salmon embryonic cells cultured by the fragment method on a coverslip and stained with May-Grünwald-Giemsa. The explant can be seen in the upper right side of the photo and is surrounded by a sheet of newly grown cells. Mitotic figures are evident in the cell sheet. The culture had been incubated five days at 18° C. in Eagle's basal medium with 20 percent agamma calf serum. (X 133)

Table 3. Cells cultured by the enzyme dispersion method using trypsin at 15° C.[1]

Species	Tissue
Sockeye salmon, *Oncorhynchus nerka*	Embryonic
" " " "	Yolk-sac fry
" " " "	Kidney
" " " "	Fin
" " " "	Air bladder
" " " "	Gonad
Chinook salmon, *Oncorhynchus tshawytscha*	Embryonic
" " " "	Yolk-sac fry
" " " "	Kidney
" " " "	Fin
" " " "	Air bladder
" " " "	Gill
Coho salmon, *Oncorhynchus kisutch*	Embryonic
" " " "	Yolk-sac fry
Steelhead trout, *Salmo gairdneri gairdneri*	Embryonic
" " " " "	Yolk-sac fry
Rainbow trout, *Salmo gairdneri*	Embryonic
" " " "	Yolk-sac fry
" " " "	Kidney
" " " "	Fin
" " " "	Hepatoma

[1]The above primary cultures were started in roller tubes or 150 ml culture bottles and were considered successful if a monolayer sheet of cells grew over the glass surface.

1. The fish was killed and placed in a 1:1 million Roccal solution for one minute unless external organs, embryonic or yolk-sac fry were to be used as the source of cells. When these tissues were employed the first step was a rinse in cold Hanks' balanced salt solution, and the Roccal treatment was omitted entirely.
2. The fish was bled in the same manner already described in the fragment method.
3. The organ desired was then removed and washed in cold Hanks' BSS to remove blood or other tissue fluids.
4. The tissue was then minced into fragments about 3 to 5 mm square, weighed on a torsion balance to the nearest tenth of a gram, and placed in a standard trypsinizing flask equipped with a Pyrex covered magnetic mixing bar.
5. Next the fragments were washed in the nutrient fluid to be used during cultivation. This was done by adding about 40 ml of medium for each gram of tissue in the flask and stirring, slowly enough to avoid foaming, for five minutes with a laboratory magnetic stirrer. At the end of five minutes the flask was removed from the magnetic stirrer and the tissue allowed to settle. The supernatant fluid was then decanted off and discarded.

6. The tissue preparation was then ready for treatment with the enzyme. Because trypsin at a concentration of 0.25 percent and pH 7.4 in BSS was the enzyme most commonly used for this purpose, it will be described here. The above trypsin solution was added to the flask containing the tissue and agitated slowly by means of a magnetic stirrer for 15 minutes. The temperature during manipulation and trypsinization of the tissue was maintained at 15° C. This operation is termed the pre-enzyme treatment. The ratio of tissue to trypsin should be known and was generally set at 1:20 to 1:50 (20 to 50 ml of trypsin solution for each gram of tissue). At the end of the pre-enzyme treatment the tissue was allowed to settle and the trypsin solution decanted and discarded. This discarded portion has been found to contain most of the toxic materials produced as a result of the contact of enzyme with tissue in the case of monkey kidney (1, p. 575).
7. An equal volume of fresh trypsin solution was then added and the mixing process continued for 30 minutes. At the end of this period the large tissue fragments remaining were allowed to settle and the trypsin solution containing cells and small clumps of cells was decanted off. At this point fresh trypsin could again be added to the fragments of

tissue remaining and the enzyme treatment repeated. This process could be continued until all the tissue had been reduced to cells suitable for planting. Subsequent viable cell yields were as good or perhaps better than the initial preparation (1, p. 575).

8. The solution of trypsin and cells was then centrifuged for 20 minutes at 600 r.p.m. The trypsin solution was removed by pipette and discarded. Care was taken not to disrupt the packed cells at the bottom of the centrifuge tube.

9. The cells were then washed by resuspending them in the nutrient solution complete with serum to be used during culture. Cells were resuspended by adding a few drops of nutrient fluid to the Rockefeller centrifuge tube. The tube was then shaken to disrupt the packed cells. The remaining volume of fluid was then added to the tube and the suspension of cells was completed by pipetting the fluid and cells back and forth several times. This suspension was then centrifuged for 20 minutes at 600 r.p.m. The fluid fraction was drawn off and discarded.

10. After completion of the wash the cells were resuspended again in the complete nutrient solution to be used during cultivation. The total number of whole cells was determined by means of a hemocytometer. Four counts were made and the mean value determined

for calculation of the cell concentration in the solution.

11. The total volume of cell suspension was then adjusted to give the desired cell concentration for planting. The concentrations used for seeding primary cultures in this work were from 1 to 1.5 million cells per ml of nutrient fluid. This concentration range was found to be optimal for trypsinized cells as described later in this section.

12. After the cells were suspended in the nutrient solution at the desired concentration they were ready for planting in any of the various containers used for cells culture. These included roller and Leighton tubes, which received 1 ml of cell suspension, and 150 ml Pyrex milk dilution bottles which received 8 ml of cell suspension.

13. After planting, the containers were placed on their sides in an 18° C. incubator for 24 hours. During this period they had to be left in a stationary position so that the suspended cells and clumps of cells could settle out and become attached to the glass.

14. The nutrient fluids were changed at the end of this 24-hour period. The cultures at this point could be handled and viewed under the microscope (100X).

Primary cell cultures of embryonic, yolk-sac fry, fin and hepatoma tissue were not difficult to establish by this method. Cell cultures of kidney, air bladder, gonad and gill tissue have not been dependable when prepared by this technique, and have often failed to grow. Many attempts to culture cells of normal liver tissue by means of enzyme dispersion have all failed.

An additional method examined combined both the fragment and enzyme dispersion methods and is here described because of the promise shown for the cultivation of hepatoma cells. Tissue was prepared by the fragment method as already indicated. After growth was obtained in the form of a sheet of cells around the fragments, trypsin was added and the cells and clumps of cells removed from the glass. The fragments of tissue were allowed to settle to the bottom of the container and the suspension of cells decanted off and washed in the usual manner. Planting of these cells followed the same procedure as described for the enzyme dispersion method. Excellent cultures of hepatoma cells have been prepared by this process.

Stock Cultures

The problems of working with anadromous fishes have already been related. Seasonal limitations on availability made it necessary to change from one species to another as a source of tissue for cultivation. The initial work was

accomplished by relying on primary cultures, which required maintaining or having access to intact animals for each tissue culture prepared. These problems became more acute when embryonic tissue was selected as the source of cells for the quantitative growth experiments. This material possessed certain properties which made it very desirable for these experiments. Embryonic tissue was easily cultured by the enzyme dispersion method, and produced excellent monolayer cultures. These attributes were, of course, most important when conducting such tests. The main disadvantages included the fact that the entire embryo was used so that a mixed cell population derived from several tissues was obtained and, secondly, embryonic cells from a single species were available for only a short period of time. The latter problem was overcome by preparing a large number of primary cultures in bottles. These cultures were incubated and after growth was complete were also stored at 18° C. for future use. After the initial 24-hour fluid change, the media changes were made at two- to three-week intervals. When growth became heavy enough to warrant, these preparations were subcultured and expanded when possible. This was accomplished by removing the cells from the glass with trypsin (0.25 percent), followed by pooling, washing, and resuspension in nutrient fluid. The cells were then replanted in bottles at the desired concentration. Preparations of this type were referred to as stock cultures and were maintained in 150 ml milk dilution bottles

(8 ml fluid per bottle). Cells required for experimentation were removed from bottles by trypsinization, as described above for subculturing, pooled, washed, resuspended, counted, the volume adjusted to give the proper cell inoculum and then planted in roller, Leighton tubes, or other culture vessels.

The development of this method of stock culture preparation by trypsin dispersion of embryonic tissues of salmon and steelhead trout made available for the first time a practical means of preparing large numbers of bottle cultures of such cells which could be easily maintained over long periods and serve as a reservoir from which any number of smaller cultures could be prepared at will for experimental purposes. It became apparent as a practical procedure only after experimental work had indicated the importance of a very heavy cell inoculum of 1.0 to 1.5 x 10^6 cells per ml of medium was required for planting primary cultures of trypsinized tissue as described later in this section. Most of the quantitative cultural work reported in ensuing pages was made possible by the use of this method. Stock cultures of this type have been maintained for periods of a few months to as long as one year with only the changes of nutrient fluid at two- to three-week intervals, and transfer to new culture vessels, with or without expansion at intervals of one to two months. One reason for the infrequent attention required appeared to be the fact that these cells did not produce a toxic pH in the culture fluid even after two or three weeks incubation.

These stock cultures (subcultures) were extremely valuable to this study for the reasons already indicated. In order to examine microscopically the development of these stock preparations from planting to the formation of a confluent monolayer sheet, coverslip and Rose chamber cultures of coho salmon embryonic cells were employed. These cells were grown in Eagle's basal medium with 20 percent agamma calf serum and incubated at 18° C. The inoculum employed was 300,000 cells per ml of medium. Leighton tubes containing coverslips were planted from stock cultures in the same manner used when preparing cultures for growth experiments. The coverslips were then removed at various intervals, stained with May-Grünwald-Giemsa, and observed and photographed in the light microscope using the 10X and 40X objectives with a Leitz Ortholux microscope and Leica camera with a 10X ocular and 1/3X adapter.

A group of slides was prepared from cultures incubated for 1, 5, 10, 14, and 21 days. The one-day preparation revealed individual or small groups of cells scattered over the surface of the glass. A few cells had begun to spread out over this surface and processes were evident; however, most cells were seen as small spherical dark stained bodies (Figure 2). Five days after planting the cytoplasm was no longer pulled in toward the nucleus but had spread out and cell processes were quite obvious (Figure 3). At this point cell division had begun. Mitotic figures could be observed and there appeared to be

Figure 2. Coho salmon embryonic cells cultured by the enzyme dispersion method on a coverslip and stained with May-Grünwald-Giemsa. The cells shown in the following photographs had been transferred (subcultured) four times since their original isolation in primary culture. This culture was incubated for 24 hours in Eagle's basal medium with 20 percent agamma calf serum. Cells had attached and were beginning to spread out over the surface of the glass. (X 133)

Figure 3. Coho salmon embryonic cells prepared in the same manner as in Figure 2. These cells were incubated for five days at 18° C. The cells had produced processes and small colonies were forming. (X 133)

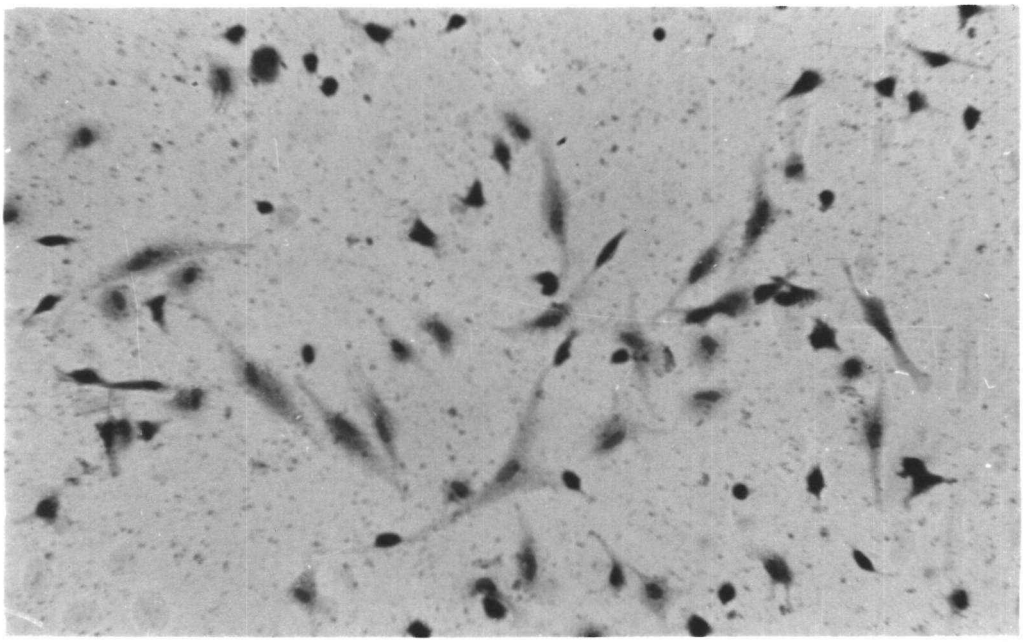

Figure 2.

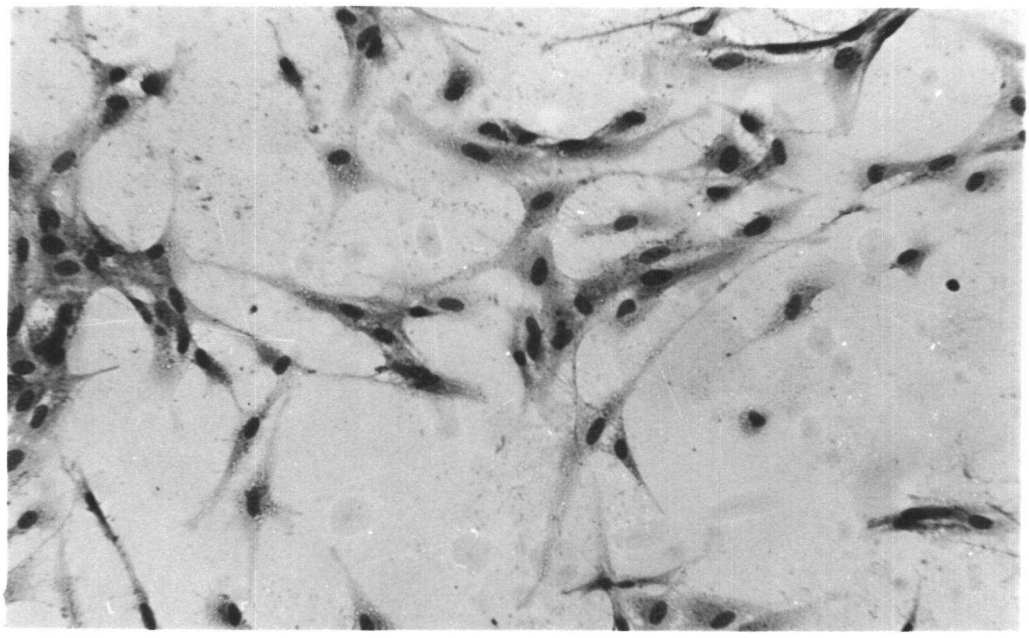

Figure 3.

some colony formation. By ten days, colony formation had advanced and there was a fusing of these colonies in certain areas to form larger groups of cells (Figure 4). Mitotic activity had also increased. A continuous monolayer sheet of cells was evident after two weeks incubation and mitotic activity was very obvious (Figure 5). Cultures receiving three weeks incubation showed heavy growth. Cells were observed forming thickened clumps in areas over the sheet of cells (Figure 6). Mitotic figures were observed indicating active cell division was still in progress.

In order to better demonstrate cellular detail in these cultures at the various incubation periods mentioned above, the entire sequence of photographs was repeated at a higher magnification. Examination of the one-day-old culture again indicated some cells had already started to spread out while most of them were spherical and showed little activity at this point (Figure 7). The same sequence of events indicated above for each time interval were observed and photographed at this higher magnification (Figures 8,9,10 and 11).

Cultures prepared in Rose chambers for observation with the phase microscope had an additional advantage over the coverslip technique. Here cells could be viewed in the unstained living state and a single preparation followed from planting to the formation of a continuous sheet of cells. Coho salmon embryonic cells were planted in Rose chambers and

Figure 4. Coho salmon embryonic cells prepared the same as in Figure 2. The cells shown here had been incubated for ten days at 18° C. Colony formation had advanced to the point where some fusing occurred; however, a continuous sheet of cells was not yet evident. (X 133)

Figure 5. Coho salmon embryonic cells prepared in the same manner as in Figure 2. This culture was incubated for 14 days at 18° C. Continued growth had produced a confluent monolayer sheet of cells. Mitosis was still active at this time and two mitotic figures can be seen near the middle of the photo. (X 133)

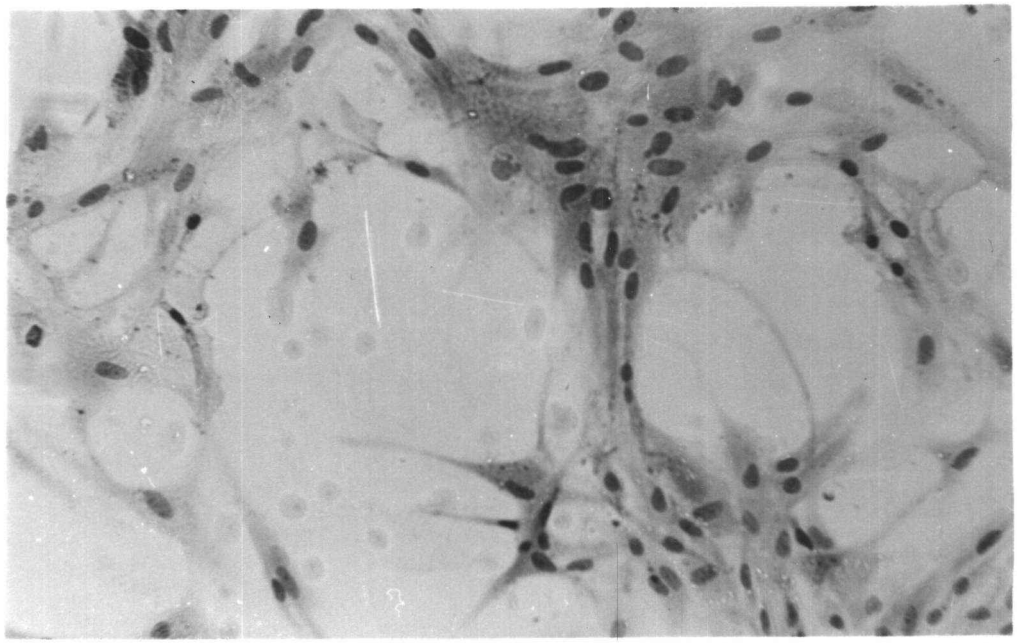

Figure 4.

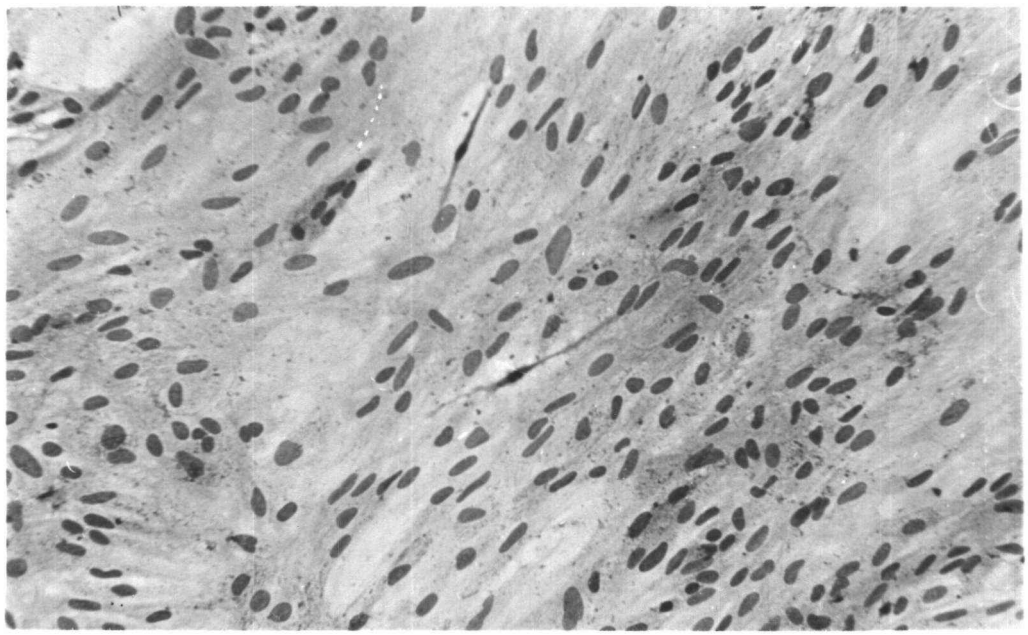

Figure 5.

Figure 6. Coho salmon embryonic cells prepared in the same manner as in Figure 2. This photograph, the last in the sequence shown at this magnification, indicates the pile-up of cells as cell division and growth continues. This culture was incubated for 21 days at 18° C. (X 133)

Figure 7. Coho salmon embryonic cells prepared in the same manner as in Figure 2. This sequence of photographs reveals the same series of events at a higher magnification. Cells shown here were incubated for 24 hours at 18° C. The cell in the center of the figure had spread out on the glass. Other cells present were round and did not show a great deal of activity at this point. (X 533)

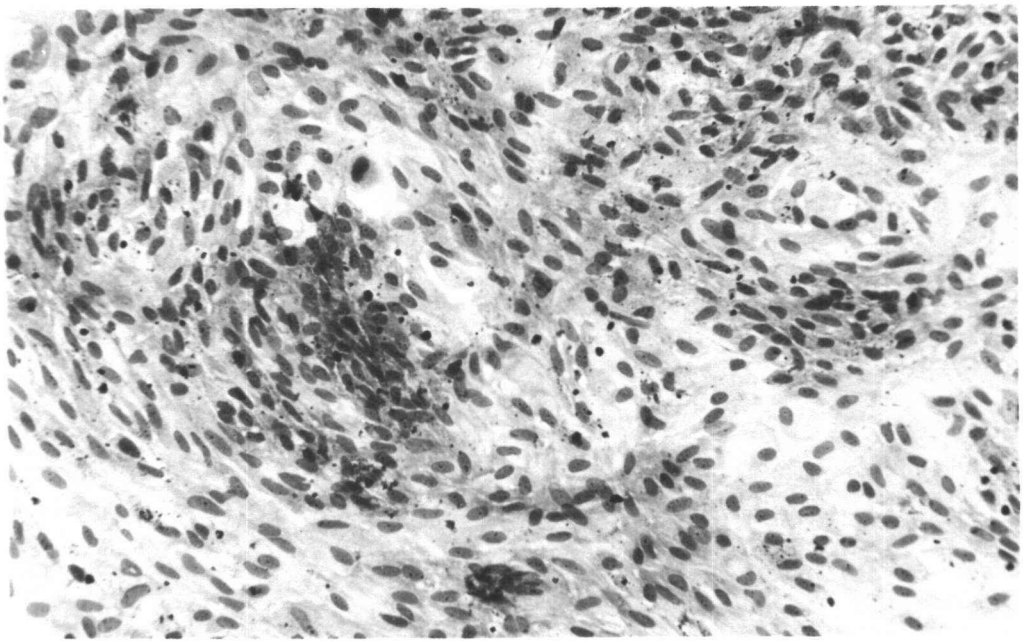

Figure 6.

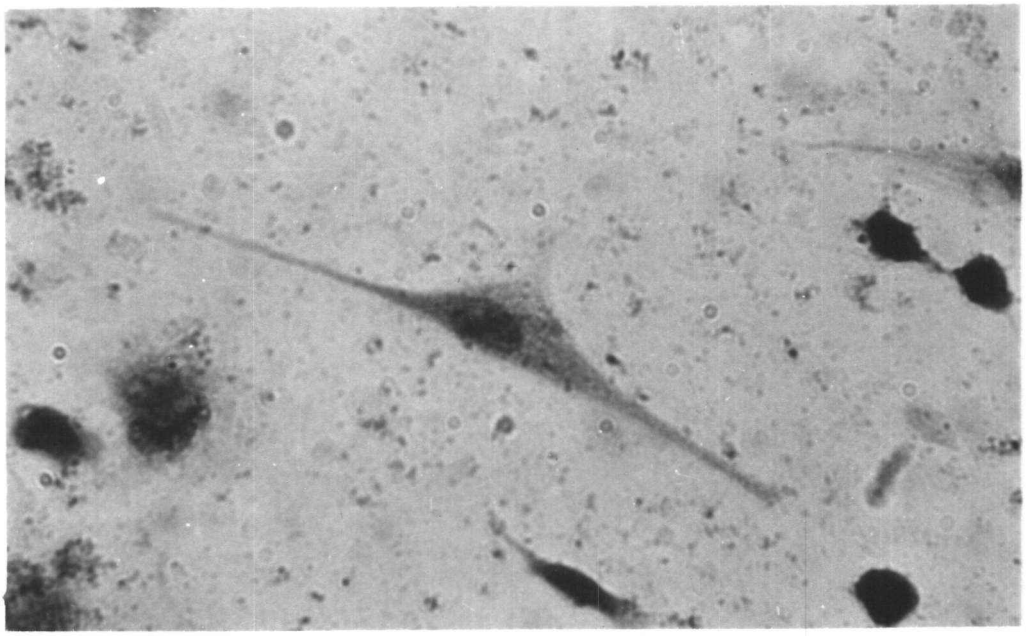

Figure 7.

Figure 8. Coho salmon embryonic cells prepared in the same manner as in Figure 2 and incubated at 18° C. for five days. The cells had spread out here and small colonies were beginning to form. (X 533)

Figure 9. Coho salmon embryonic cells prepared in the same manner as in Figure 2 and incubated at 18° C. for ten days. At this point cell division and growth had become quite active. Large colonies had formed and were fusing into areas which tended to become confluent. Note the recently divided cell in the upper portion of the photograph. (X 533)

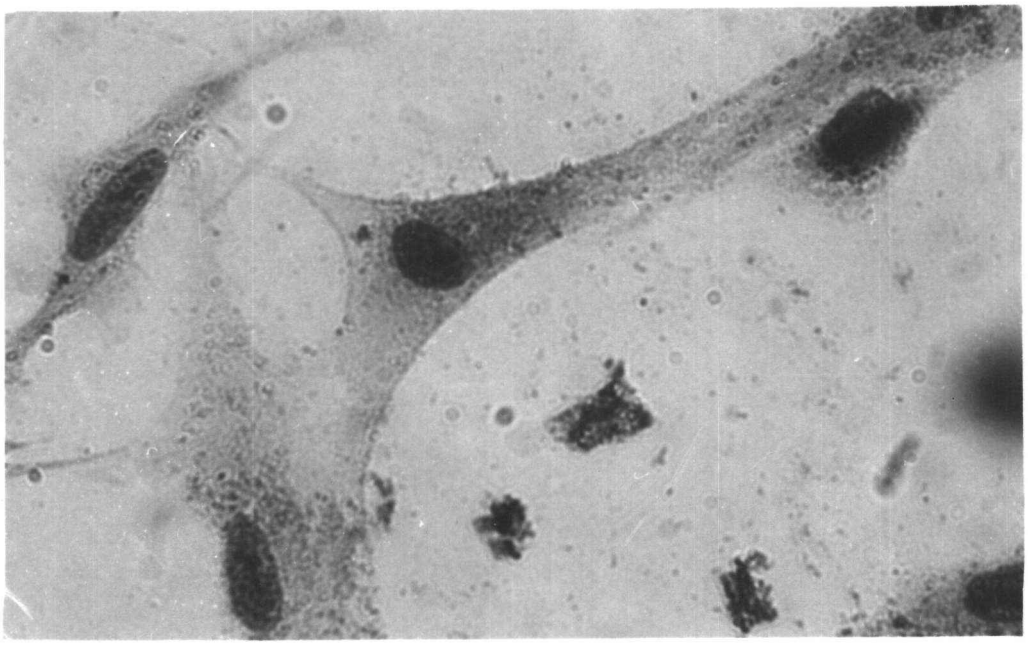

Figure 8.

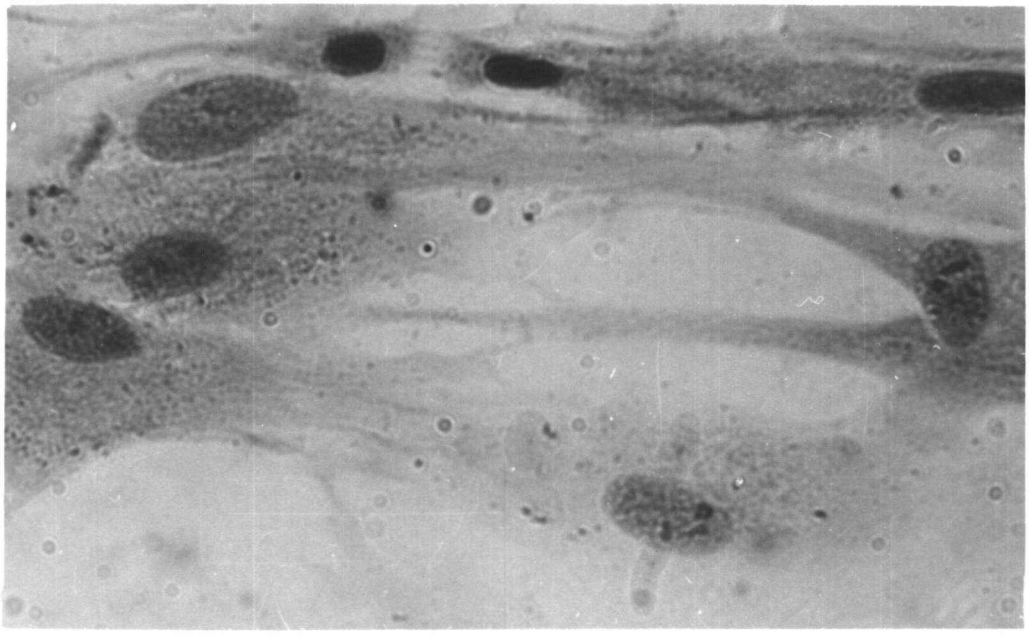

Figure 9.

Figure 10. Coho salmon embryonic cells prepared in the same manner as in Figure 2 and incubated at 18° C. for 14 days. A confluent monolayer sheet of cells had formed and has extended over the entire surface of the coverslip. (X 533)

Figure 11. Coho salmon embryonic cells prepared in the same manner as in Figure 2 and incubated at 18° C. for 21 days. Growth was no longer monolayer as the cells had started to overlap and mound up. At this point growth was quite heavy. Note the nuclei imposed over each other in certain areas. (X 533)

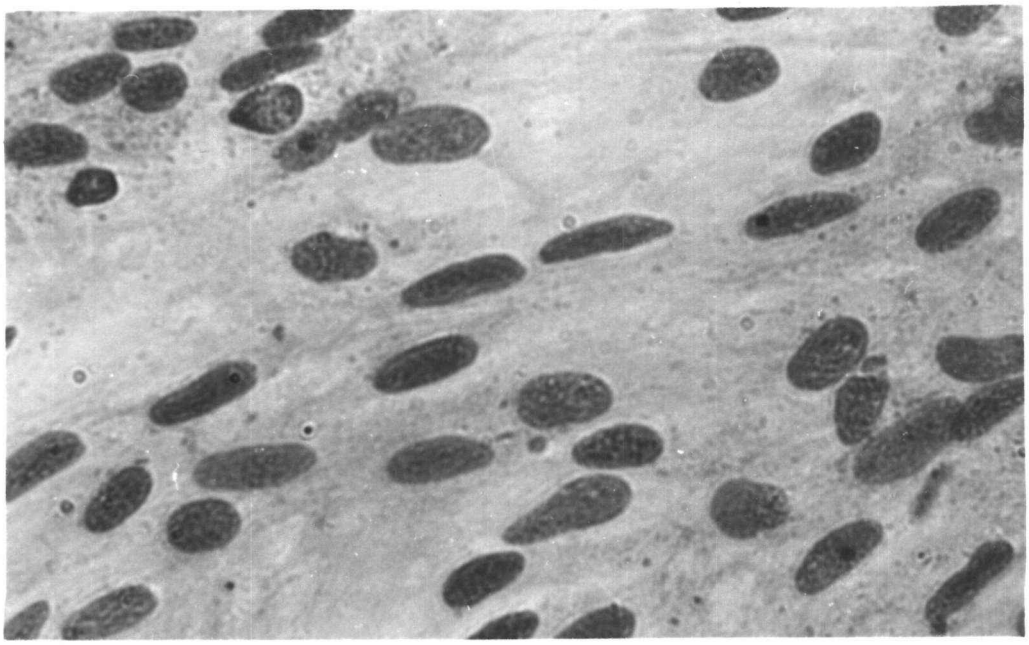

Figure 10.

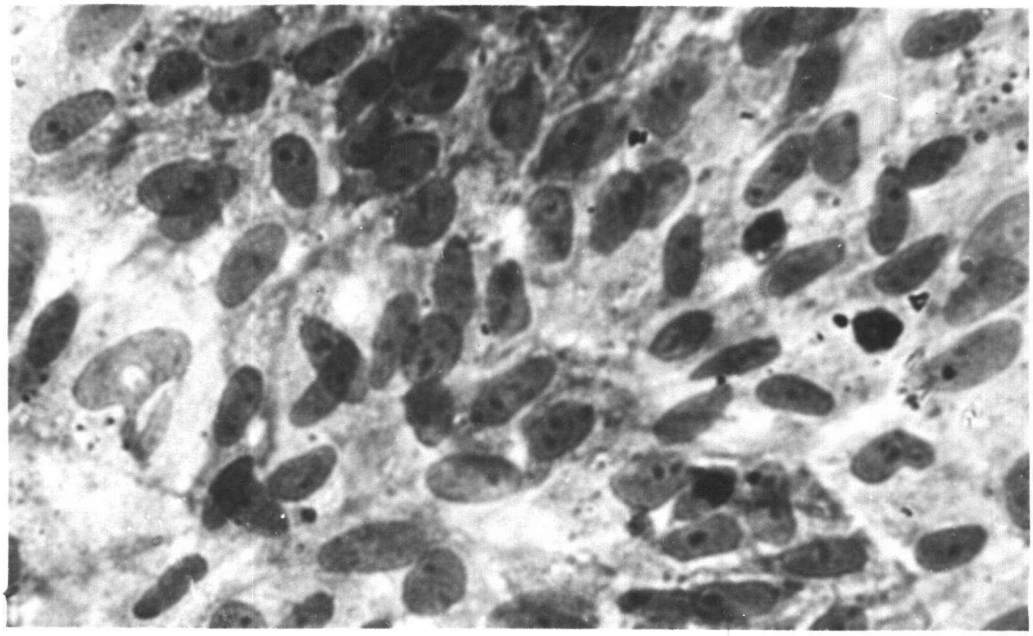

Figure 11.

observed over a two-week period. These cultures were grown in Eagle's basal medium and the cell inoculum was approximately 200,000 cells per ml of the nutrient fluid.

Twenty-four hours after seeding the chambers, individual cells could be seen attached to and scattered over the glass surface. Many cells had already spread out and processes were evident (Figure 12). Five days after initiation of the cultures, cell division was indicated by the presence of mitotic figures and the formation of small colonies (Figure 13). Growth continued to advance and after ten days of incubation large colonies of cells were observed (Figure 14). Certain areas had fused in what appeared to be initial formation of a sheet of cells. Confluent monolayer sheets of cells were formed by the end of 14 days (Figure 15). Cell division was very active at this point as mitotic figures were quite evident.

The predominant morphological cell type appearing in the cultures of coho salmon embryonic cells shown in Figures 2 through 15 appears to resemble fibroblasts. Epithelial-like cells have been observed often in small colonies surrounded by the longer spindle-shaped cells thought to be fibroblasts. The epithelial-like cells were also found to be more numerous in primary cultures than in subcultured groups of cells which had undergone several transfers.

Figure 12. Coho salmon embryonic cells cultured in Rose chambers for observation in the phase contrast microscope. The cell cultures were grown in Eagle's basal medium with 20 percent agamma calf serum and inoculated with approximately 200,000 cells per ml of nutrient fluid. The culture shown in this sequence of photographs was also prepared from cells which had been transferred (subcultured) four times since they were first isolated in primary culture. The cell shown here had been under incubation for 24 hours at 18° C. The nucleus and paired nucleoli are clearly visible in the center portion of the cell. Cytoplasmic granules can be seen extending around the nucleus. The cytoplasm has already spread out. (X 533)

Figure 13. The same Rose chamber culture of coho salmon embryonic cells as in Figure 12 after five days incubation at 18° C. Small colonies of cells have formed by this time. (X 533)

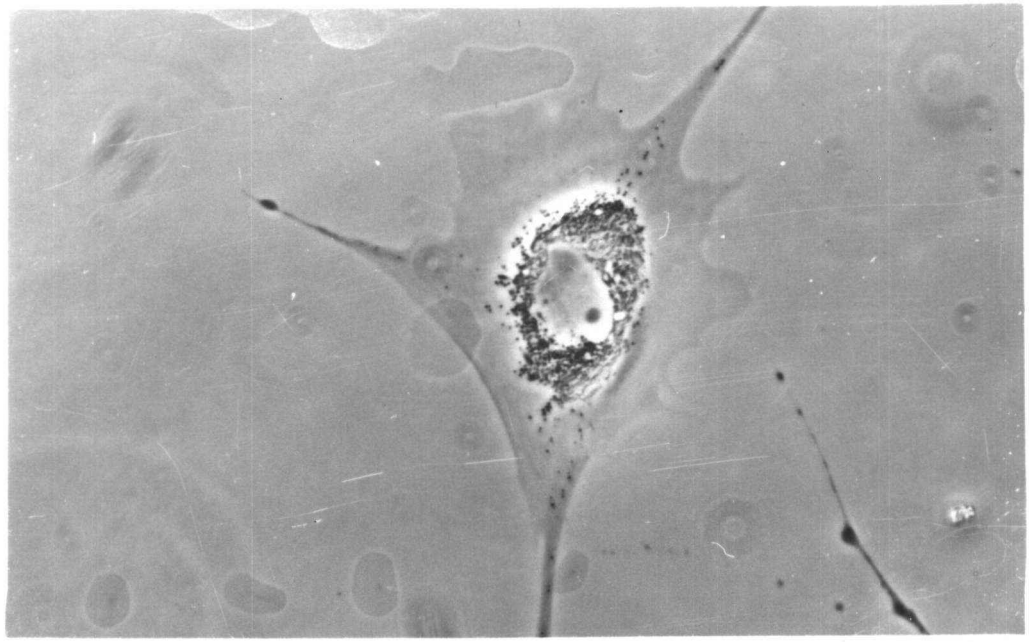

Figure 12.

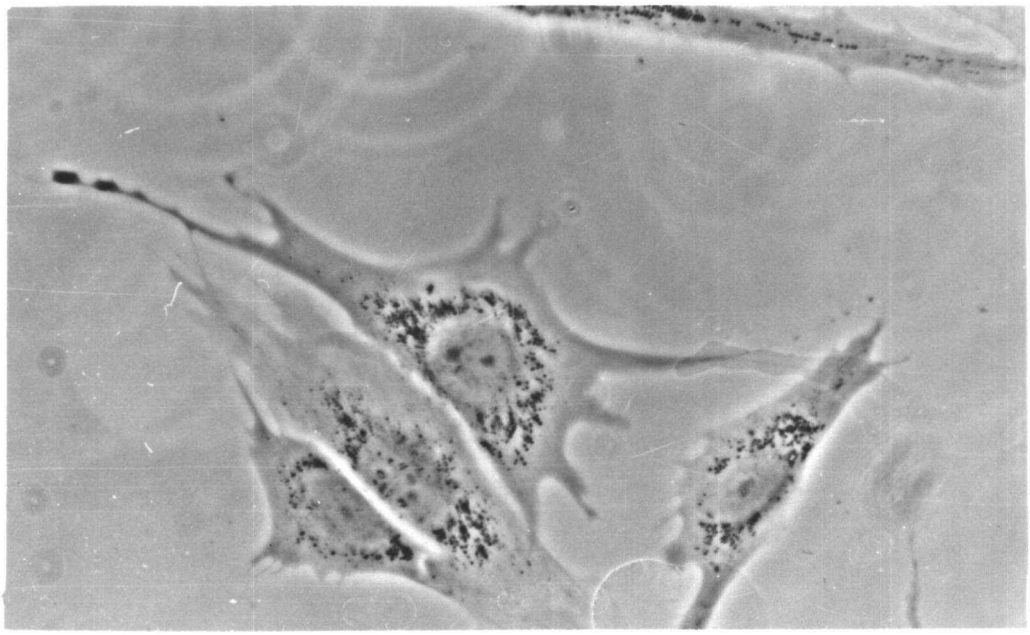

Figure 13.

Figure 14. The same culture of coho salmon embryonic cells indicated in Figures 12 and 13. This photograph was taken after 10 days incubation at 18° C. The cells have now formed larger colonies on the glass surface of the chamber as the growth process continues. (X 533)

Figure 15. The same Rose chamber culture of coho salmon embryonic cells shown in Figures 12, 13, and 14. This is the last in this sequence of photographs. The incubation period at this point was 14 days. The cells have now all but completed the formation of a confluent monolayer sheet of cells. (X 533)

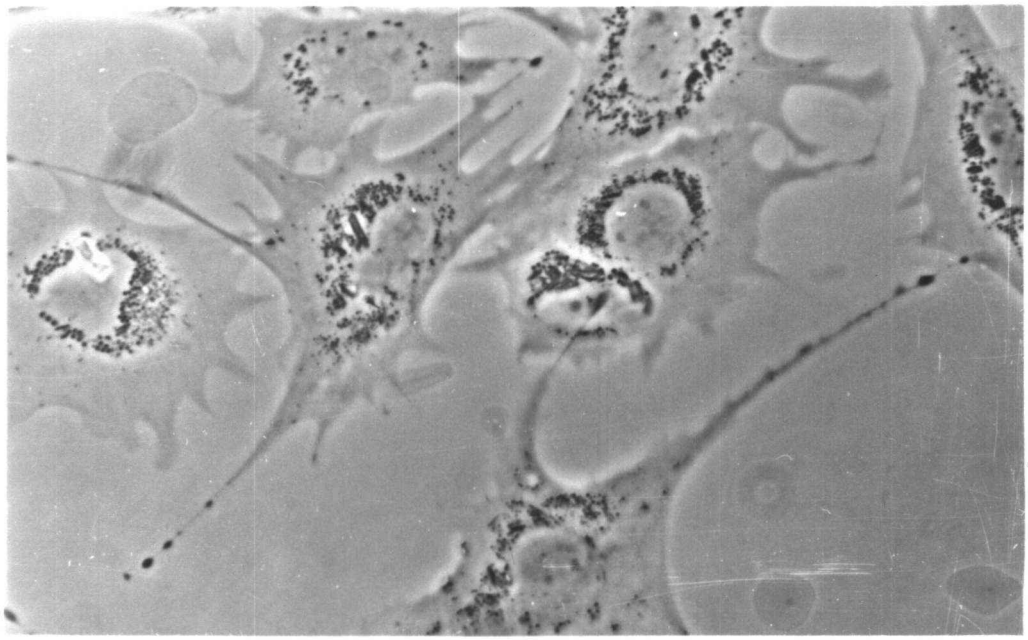

Figure 14.

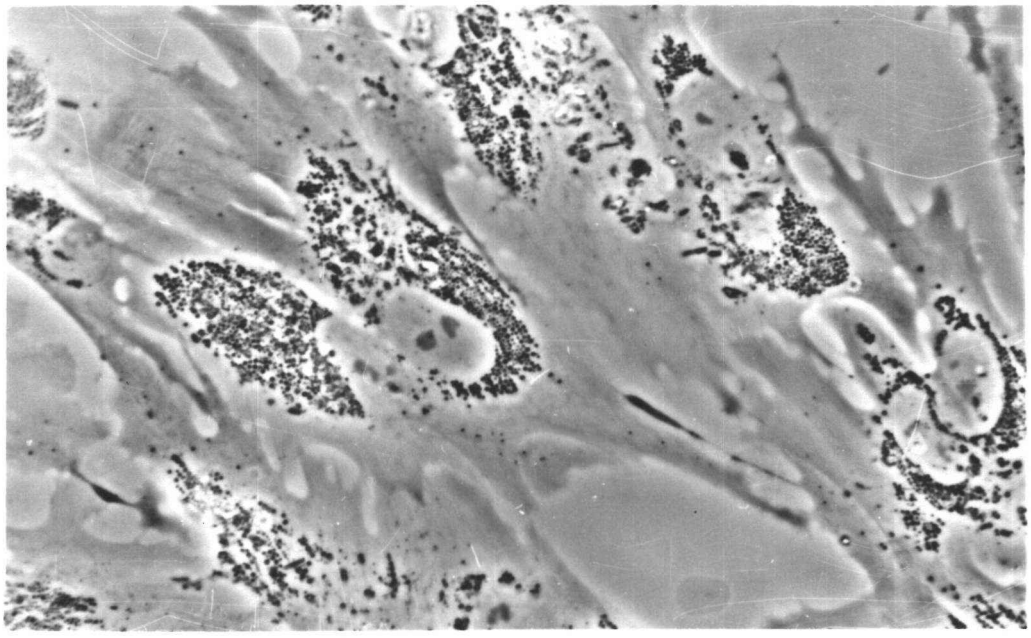

Figure 15.

Comparison of Several Enzymes for the Preparation of Primary Cultures

Treatment of Tissue with Enzymes for Cell Dispersion

A series of experiments were conducted to determine the degree of cell liberation from fish tissues exposed to various enzymes. Tissue of chinook and sockeye salmon were tested and in one case embryos of steelhead trout were used. The enzymes examined were trypsin, collagenase, hyaluronidase, pancrease, papain and the receptor-destroying enzyme (RDE). The latter is present in filtrates of Vibrio cholerae (22, p. 36). This enzyme has been shown to be active against mucoproteins. The RDE used in these experiments was obtained as a purified lyophilized preparation from the Beringwerke Corporation in Germany. Trypsin was utilized exclusively at a concentration of 0.25 percent. No further examination of the concentration of this enzyme was attempted as it has been used at this level for the preparation of cell cultures from a number of animal tissues. Preliminary experiments with fish cell cultures indicated trypsin (0.25 percent) was not acutely toxic. The remaining enzymes, however, were tested at more than one concentration.

All enzymes were prepared in Hanks' BSS and sterilized by filtration through a Millipore filter. The 0.25 percent trypsin solution was stored frozen at -20° C.; however, the other enzymes were made fresh and used the same day, as the

effect of any form of storage on these compounds was not known. The procedure followed in these tests was the same as that outlined for the preparation of primary cultures by enzyme dispersion. The entire process was carried out at 15° C. Tissue was excised from the animals, minced, weighed, placed in a standard trypsinizing flask and washed in Hanks' BSS. The test enzymes were all adjusted to pH 7.3 to 7.4 and added to the flask containing the tissue and magnetic mixing bar. The ratio of tissue to enzyme solution was approximately 1:40. A 15-minute pre-enzyme treatment was given and the fluid discarded. Fresh enzyme was added and the tissue agitated slowly by means of a magnetic stirrer. Samples were removed at 15-minute intervals for one hour. The four samples were counted in the hemocytometer and the cell concentration in the enzyme solution was determined for each time interval. Care was taken to count only nuclei with attached cytoplasm. None of the preparations were cultured in these experiments as the main purpose was to determine cell liberation from the tissue mass. Trypsin was tested with six tissues excised from sockeye and chinook salmon. The enzyme produced suspensions of cells and small clumps of cells suitable for planting from embryonic, fin, kidney, air bladder, liver and gill tissue of both species (Table 4). The concentration of cells obtained from each tissue of both sockeye and chinook increased with time of exposure to the enzyme. Kidney cells

Table 4. Results of cell counts from fish tissues treated with various enzymes at 15° C. to obtain dispersed material for the preparation of primary cultures.

Fish	Enzyme	Enzyme conc. %	Tissue	No. of expts.	Cells per ml of enzyme solution determined at [1] 15 min.	30 min.	45 min.	60 min.
Sockeye salmon	Trypsin	0.25	Embryonic	2	190,000	400,000	766,000	1,149,000
			Fin	3	485,000	804,000	1,034,000	1,236,000
			Kidney	4	1,019,000	1,868,000	2,309,000	2,985,000
			Air bladder	3	314,000	528,000	730,000	1,073,000
			Liver	5	329,000	744,000	901,000	982,000
			Gill	3	283,000	716,000	887,000	1,311,000
Chinook salmon	Trypsin	0.25	Embryonic	3	215,000	301,000	754,000	1,298,000
			Fin	1	627,000	864,000	1,107,000	1,344,000
			Kidney	1	882,000	1,281,000	1,515,000	2,004,000
			Air bladder	1	285,000	504,000	735,000	933,000
			Liver	1	324,000	738,000	882,000	981,000
			Gill	1	234,000	552,000	834,000	1,245,000
Sockeye salmon	Collagenase	0.20	Kidney	2	2,257,000	2,675,000	3,565,000	2,410,000
		0.10			2,219,000	1,695,000	2,186,000	1,766,000
		0.05			1,463,000	1,243,000	1,590,000	1,282,000
Sockeye salmon	Hyaluroni-dase	0.20	Kidney	2	665,000	1,016,000	1,212,000	1,542,000
		0.10			218,000	335,000	336,000	429,000
		0.05			202,000	311,000	339,000	718,000

(continued next page)

Table 4 (continued)

Fish	Enzyme	Enzyme conc. %	Tissue	No. of expts.	Cells per ml of enzyme solution determined at [1] 15 min.	30 min.	45 min.	60 min.
Sockeye salmon	Pancrease	1.00	Kidney	1	168,000	99,000	102,000	330,000
		0.50			120,000	51,000	99,000	103,000
		0.10			188,000	547,000	673,000	1,733,000
		0.05			191,000	270,000	389,000	456,000
Sockeye salmon	Papain	0.10	Kidney	2	668,000	2,548,000	1,615,000	2,404,000
		0.05			587,000	1,038,000	1,218,000	1,300,000
		0.025			373,000	560,000	1,128,000	1,002,000
Steelhead trout	RDE	0.20	Embryonic	1	320,000	503,000	279,000	243,000
		0.10			197,000	413,000	481,000	427,000
		0.05			165,000	674,000	333,000	247,000

[1] Cell counts are mean values where more than one experiment was conducted.

were released in large numbers after only 15 minutes of agitation in the presence of the trypsin solution.

Collagenase has been used for the preparation of other animal tissues for culture and was tested at three concentrations with sockeeye salmon kidney tissue (19, p. 64). This enzyme gave rise to large numbers of free cells at all concentrations and intervals examined (Table 4). Results were not consistent but did indicate collagenase is very active on this tissue.

Hyaluronidase was also tested at the same concentrations as collagenase (0.2, 0.1, and 0.05 percent). Kidney tissue from sockeye salmon was again used in this experiment. Cell yields were not as great with this enzyme as those obtained with collagenase. The 0.2 percent concentration produced the highest cell counts and results indicated an increase in cell concentration with time. Enzyme concentrations of 0.1 and 0.05 percent produced similar cell concentrations at 15, 30, and 45 minutes; however, the 60-minute sample indicated the 0.05 percent hyaluronidase preparation produced a higher cell yield than did the 0.1 percent solution.

Pancrease and papain each gave suitable yields of whole cells. Pancrease was erratic as the cell counts did not necessarily increase with higher concentrations of the enzyme. All four enzyme concentrations tested appeared to be active for the tissue. Papain produced excellent suspensions of cells at all concentrations tested. The activity of the

enzyme appears to generally increase with time and concentration. Papain has been employed for the preparation of chick embryo and bovine embryonic kidney tissue for cultivation (20, p. 56-58).

The receptor-destroying enzyme (RDE) was tested on steelhead embryonic cells. This agent at all concentrations tested was extremely destructive. Cell counts did not correspond with either concentration of enzyme or time of exposure (Table 4). The suspension was laden with nuclei; however, whole cells were not numerous indicating the destructive nature of this agent, apparently directed toward the cytoplasmic membrane.

Determination of the Toxicity of Four Enzymes

Papain (0.1 percent), collagenase (0.05 percent) and hyaluronidase (0.2 percent) were used for the preparation of primary cultures of steelhead trout embryonic cells employing the standard technique already described in detail. In addition, primary cultures were also established with trypsin (0.25 percent) for comparison with the other three enzymes. The purpose of this experiment was to determine which of these enzymes would provide the best preparation of cells for primary cultivation. The concentration of enzymes used was determined from data collected in the experiments dealing with cell liberation from fish tissues by these compounds. The pH of all enzyme solutions was adjusted to 7.3 to 7.4. This experiment was done in duplicate and care was taken to

maintain identical test conditions. All cultures were prepared in the same manner; the only difference was the enzyme used to separate the cells from the tissue mass. After the standard 30-minute enzyme treatment the cells were harvested and the enzyme solutions removed from the separate lots of cells by centrifugation. Each group of cells were then resuspended in Eagle's basal medium with 20 percent agamma calf serum. The concentration of cells was determined for the four preparations and the volume of medium containing the cells adjusted to give equal concentrations of cells in the test groups in order to obtain a uniform inoculum. The cell inoculum used for all four preparations in both experiments was 800,000 cells per ml of medium. Roller tubes (12 tubes per group) were inoculated with the cell suspensions prepared by the four enzymes. Cultures were incubated at 18° C. The seeded cultures were placed on their sides in a stationary roller drum for 24 hours to allow the cells to become attached to the glass walls of the roller tubes. At the end of this period the nutrient fluid was changed on all cultures and the roller drum was placed in motion. This revolving rack turning at a rate of about 12 revolutions per hour kept the attached cells bathed in the nutrient fluid. This was the procedure followed throughout this work whenever roller tubes were used for the preparation of primary or subcultured monolayer cultures. Four tubes from each group were removed and the cells counted at zero, one and two weeks. Results of these two

experiments and the mean values of both have been shown (Table 5).

The cell cultures prepared in trypsin were significantly different from the cultures prepared by the other enzymes throughout the experiment. Base line counts for the trypsin group were very high in the first experiment. This may have been the result of improper determination of the cell inoculum for this test group. Examination of the means of the two experiments shown in Table 5 indicate the papain and hyaluronidase preparations had fewer cells by the end of the experiments than were present at the base line or zero time. Collagenase showed a slight increase in cells at the end of the two-week test compared with the number of cells present in these cultures at zero time. The trypsin treated group of cultures increased in growth after one week reaching over a million cells by the end of two weeks.

Pancrease and RDE were not included in these experiments; however, qualitative tests with these enzymes indicated that RDE is very toxic to steelhead trout embryonic cells. The enzyme concentration tested was 0.1 percent. Pancrease was also tested at a concentration of 0.1 percent and did produce viable primary cultures of steelhead embryonic cells. It was not considered equal to or better than trypsin but it is believed the enzyme warrants further investigation.

Although the enzyme studies were not exhaustive and the same type of cells could not be used in all experiments, it

Table 5. Results of experiments to determine the toxicity of four enzymes for steelhead trout embryonic tissue prepared for primary cultivation in Eagle's basal medium with 20 percent agamma calf serum and incubated at 18° C.

Enzyme	Conc. tested %	Incubation time in weeks	Number of cells per individual culture tube				Mean no. cells per four culture tubes
			1	2	3	4	
Experiment No. TC-138							
Trypsin	0.25	0	626,000	495,000	565,000	643,300	582,300
		1	865,000	742,300	662,000	577,300	711,800
		2	1,445,000	1,336,000	2,186,000	1,466,700	1,608,700
Papain	0.1	0	45,000	60,000	11,700	98,300	53,800
		1	6,000	37,300	6,700	12,700	15,700
		2	37,300	20,000	9,300	6,700	17,800
Collagenase	0.05	0	169,300	56,700	63,300	72,700	90,500
		1	17,300	495,000	28,300	31,700	143,000
		2	251,700	35,000	25,000	135,000	111,700
Hyaluronidase	0.2	0	102,700	100,700	14,700	42,700	65,000
		1	12,700	27,300	15,000	14,000	17,300
		2	29,300	27,300	22,300	9,300	22,100
Experiment No. TC-139							
Trypsin	0.25	0	356,000	179,300	176,700	410,700	280,700
		1	172,300	110,000	227,300	137,300	161,700
		2	711,700	715,000	621,700	595,000	660,900

(continued next page)

Table 5 (continued)

Enzyme	Conc. tested %	Incubation time in weeks	Number of cells per individual culture tube				Mean no. cells per four culture tubes
			1	2	3	4	
Papain	0.1	0	11,700	13,300	10,000	56,000	22,800
		1	12,700	8,300	20,000	6,700	11,900
		2	79,300	17,300	10,700	16,700	31,000
Collagenase	0.05	0	20,000	29,300	114,300	60,000	56,100
		1	24,000	41,000	43,300	29,300	34,400
		2	68,300	63,300	36,000	47,300	53,700
Hyaluronidase	0.2	0	66,000	21,700	42,700	37,300	41,900
		1	14,000	10,700	16,700	7,300	12,200
		2	29,300	20,000	29,300	41,700	30,100

Average of two experiments, No. TC-138 and No. TC-139

Enzyme	Conc. tested %	Mean number of cells per eight culture tubes incubated for		
		0 week	1 week	2 weeks
Trypsin	0.25	431,500	436,800	1,134,800
Papain	0.1	38,300	13,800	24,400
Collagenase	0.05	73,300	88,700	82,700
Hyaluronidase	0.2	53,400	14,800	26,100

appears that the following conclusions are justified:

(1) All of the proteolytic enzymes gave rather comparable degrees of dispersion of salmon kidney tissue except pancrease, which appeared inferior to the rest. The action of collagenase seemed to be more rapid than the others. Hyaluronidase, which acts upon the polysaccharide cementing substance of animal tissues, also produced satisfactory dispersion.

(2) In cultures of embryonic steelhead cells prepared by dispersion with trypsin, papain, collagenase or hyaluronidase, only those treated with trypsin were able to multiply and produce a monolayer sheet during a two-week incubation period. The other three enzymes appear to have been more toxic to the cells as indicated by the small percentage of cells in the inoculum which adhered to the glass of the culture tubes. Those which did adhere were apparently unable to multiply.

Optimum Concentration of Cell Inoculum for Primary Cultures

Repeated failures to obtain growth in trypsinized primary cultures of salmon tissues led to a study of the importance of cell concentration in the inoculum. This proved to be an extremely critical factor, and the information obtained made possible the successful preparation of primary cultures of embryonic cells at will. The optimum concentration of cell inoculum for seeding primary cultures of embryonic cells in Leighton tubes was determined by means of two qualitative experiments covering a range of 156 thousand to 10 million

cells per ml of culture medium. The experiments were conducted in the same manner employing coho embryonic cells grown in LY medium with 20 percent human serum and incubated at 18° C. The cells were prepared from embryos by routine enzyme dispersion of tissue with trypsin (0.25 percent). The range of cell concentrations tested and a summary of the results obtained in the experiments at one and two weeks are given in Table 6. In the two experiments, five Leighton tubes were prepared for each individual inoculum tested. The volume of cell suspension inoculated was 1 ml in each case. These tubes were then examined microscopically for the presence of growth, the amount of which was graded as 0, +1, +2, +3, and +4. These values represent the relative amounts of growth present in tubes inoculated with the various concentrations of cell suspensions. Results indicated that inocula of 156, 312, and 625 thousand cells per ml of medium (and per tube) were not sufficient to produce the desired growth in the tubes. The two lower concentrations gave no visible growth whatever. A concentration of ten million cells per ml of culture medium appeared to be too high; cell debris was very heavy and the cultures failed to form a continuous sheet of cells. Cultures inoculated with 2.5 and 5 million cells per ml of nutrient fluid gave rise to heavy growth within one week; however, extraneous materials and cell debris were present. The cultures inoculated with 1.25 million cells per ml of medium appeared to be more satisfactory than any of the others examined. The cells were

Table 6. Results of qualitative experiments to determine the optimum concentration of cells for inoculation of primary cultures of coho salmon embryonic cells cultured in lactalbumin hydrolysate-yeast extract medium containing 20 percent human serum and incubated at 18° C.

Number of cells inoculated per ml of medium and per Leighton tube	Relative growth after incubation for	
	1 week	2 weeks [1]
10,000,000	+3	+3
5,000,000	+4	+4
2,500,000	+4	+4
1,250,000	+3	+4
625,000	+1	+1
312,000	0	0
156,000	0	0

[1] +1, +2, and +3 indicate increasing amounts of growth and +4 designates a confluent cell sheet. Zero indicates no growth.

initially scattered over the glass surface and could be observed to advance, forming a continuous sheet of cells during the period between one and two weeks. These observations seemed to indicate that if rapid heavy growth is required, the 2.5 million cell inoculum should be selected; however, when this type of growth pattern is not needed the preferred inoculum is considered to be 1.25 million cells per ml of medium as it requires fewer cells yet produces satisfactory cultures. Subsequent utilization of these data for the

preparation of primary cultures of embryonic cells from various species of salmon and the steelhead trout grown in this and other media have indicated the optimum concentrations for inoculation were apparently very similar for all of these trypsinized cells. The inoculum adopted for the routine preparation of primary cultures of embryonic cells in this laboratory has been 1 to 1.5 million cells per ml of medium.

Optimum Concentration of Cell Inoculum for Subcultures

Initially, subcultured cells were planted at a concentration of 300,000 cells per ml of medium. Growth experiments performed with these cultures indicated a lag phase of approximately one week before an increase in the cell population occurred. Two experiments were carried out to observe the effects of three inocula representing three different cell concentrations on the growth of subcultured cells. The purpose of these experiments was to determine the proper cell inoculum and in addition attempt to reduce the initial growth lag encountered when cultures were planted at 300,000 cells per ml of medium. Coho salmon embryonic cells which were three subcultures (transfers) removed from the primary culture were used in these tests. The cells were removed from the stock culture with trypsin as already described and prepared for cultivation in Eagle's basal medium with 20 percent agamma calf serum. The three concentrations tested were three, six, and nine hundred thousand cells per ml of culture medium.

There were 16 roller tubes planted for each concentration tested, which allowed four tubes to be removed from each group at zero, one-half, one, and two week intervals for growth measurement. The incubation temperature was 18° C. The nutrient fluid was changed after 24 hours incubation and at five day intervals after this initial feeding until the experiment was terminated.

Results of both experiments indicated that the increased concentrations of cells in the inocula produced significantly greater amounts of growth throughout the course of both experiments, as would be expected (Table 7). It was interesting to note that for each concentration tested only about one-third of the cells became attached to the glass after planting, as determined by the base line count (zero time). Increased cell inocula also had an effect on cell survival at planting. Examination of the average base line counts of both experiments indicate 6.5 percent more cells were recovered from cultures planted at 900,000 cells per ml of medium than those planted at 600,000 cells per ml. There was a 4.5 percent better recovery of cells planted at 600,000 cells than those seeded at 300,000 cells per ml of medium. The overall difference in cell survival between inocula of nine and three hundred thousand cells per ml of medium was 11 percent.

The two higher inocula reduced the growth lag from one week to approximately one-half week. The average values of

Table 7. The effects of cell inoculum on the growth of coho salmon embryonic cells sub-cultured in Eagle's basal medium containing 20 percent agamma calf serum at 18° C.

Concentration of inoculum; cells per ml of medium and per culture tube	Incubation time in weeks	Number of cells per individual culture tube				Mean no. cells per four culture tubes
		1	2	3	4	
Experiment No. TC-130						
300,000	0[1]	67,700	91,700	76,000	68,300	75,900
	1/2	81,700	58,300	58,300	47,300	61,400
	1	226,700	184,300	165,000	125,000	175,300
	2	440,000	375,000	511,700	324,000	412,700
600,000	0	222,700	253,300	201,700	222,700	225,100
	1/2	245,000	219,300	287,300	206,700	238,300
	1	723,300	679,300	690,000	830,000	730,700
	2	1,090,000	890,700	1,201,700	922,700	1,028,800
900,000	0	392,700	421,000	360,000	421,700	398,800
	1/2	297,300	476,700	247,700	415,000	359,200
	1	1,181,700	1,223,300	1,092,700	976,700	1,118,400
	2	1,713,300	1,610,000	1,749,300	1,627,300	1,675,200
Experiment No. TC-133						
300,000	0	83,300	75,000	93,300	64,300	79,000
	1/2	77,300	90,700	45,000	84,000	74,300
	1	93,300	83,300	125,000	87,300	97,200
	2	227,300	240,000	194,000	175,000	209,100
600,000	0	152,700	146,000	184,300	146,000	157,300
	1/2	197,300	205,000	256,700	196,700	214,500

(continued next page)

Table 7 (continued)

Concentration of inoculum; cells per ml of medium and per culture tube	Incubation time in weeks	Number of cells per individual culture tube				Mean no. cells per four culture tubes
		1	2	3	4	
600,000	1	365,000	417,300	428,300	435,000	411,400
	2	710,700	661,700	709,300	629,300	677,800
900,000	0	212,700	248,000	271,000	293,000	256,000
	1/2	306,000	338,300	405,000	381,700	357,800
	1	643,300	594,000	673,300	606,700	629,300
	2	854,000	970,000	884,000	1,256,000	991,000

Average of two experiments, No. TC-130 and No. TC-133

Concentration of inoculum; cells per ml of medium and per culture tube	Mean number of cells per eight culture tubes incubated for			
	0 week	1/2 week	1 week	2 weeks
300,000	77,400	67,800	136,200	310,900
600,000	191,200	226,400	571,000	853,300
900,000	327,400	358,500	873,800	1,333,100

[1] Cell counts shown for the zero time period were actually made 18 to 24 hours after the cultures were prepared. They indicate the numbers of cells from the inoculum which have adhered to the glass surface and are thus potentially able to grow.

both experiments were calculated for the three treatments at each interval examined over the two-week period in which the tests were conducted (Table 7). A plot of this data afforded better comparison of the effect of the three cell concentrations on growth (Figure 16). The 900,000 cell inoculum had a higher base line count and a rapid increase in growth after one-half week. The 300,000 cell inoculum showed much less increase in growth and a prolonged growth lag was evident. The 600,000 cell inoculum was intermediate between these two and in general followed the same curve as did the highest inoculum tested (Figure 16).

The results seem to indicate that both the 600,000 and 900,000 cell inoculum produced rapidly growing subcultures which possessed a reduced growth lag or a more rapid growth rate between the one-half and one week interval. However, the smaller inoculum has the advantage over the higher concentration in that it requires only two-thirds as many cells to prepare. In view of these facts it was concluded that a cell concentration of 600,000 per ml was the best level for inoculation of subcultures.

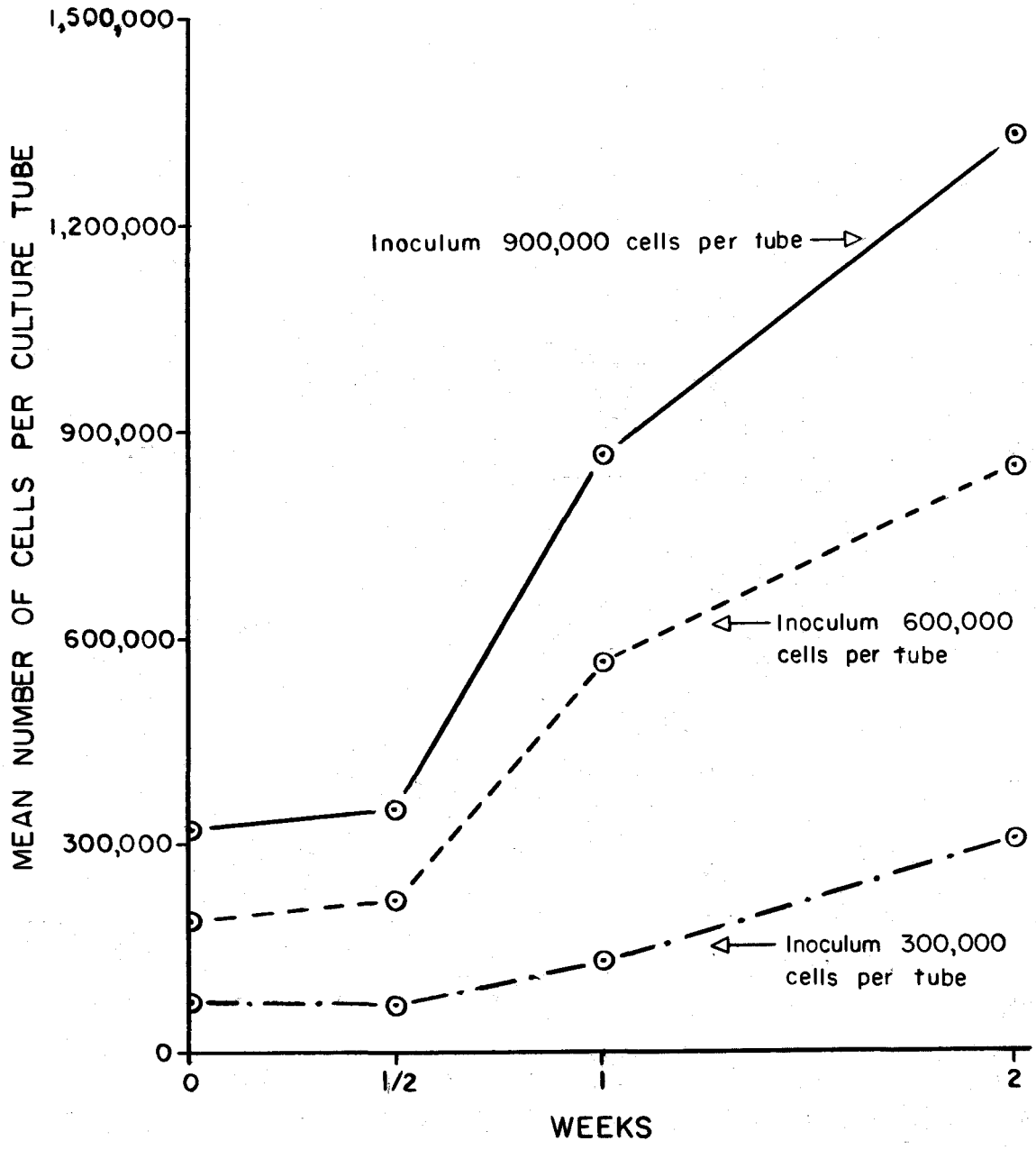

Figure 16. Effects of cell inoculum on the growth of coho salmon embryonic cells subcultured in Eagle's basal medium containing 20 percent agamma calf serum and incubated at 18° C.

Factors Influencing the Growth of Cell Cultures

Comparison of the Growth of Coho Salmon Embryonic Cells Cultured in Four Different Media

The effects of four different media, Eagle's basal medium, LY, YELP, and Hanks'-YE, were compared to determine their growth promoting properties for coho salmon embryonic cells. The complete formulation of these media has already been indicated. Cells employed in this experiment were obtained by the routine use of stock cultures and had been transferred twice since their original isolation in primary culture. These cells were harvested and resuspended in the individual media at a concentration of 300,000 cells per ml of each nutrient fluid. A group of 20 roller tubes was prepared for each of the four media, and all tubes in one group received 1 ml of the same medium, containing the above mentioned cell inoculum. They were all incubated at 18° C. in a revolving rack. This number of roller tubes allowed samples of four tubes to be removed and counted for each test medium at intervals of zero, one, two, three, and four weeks.

Examination of the results from the first experiment revealed the Eagle's basal medium cultures contained slightly but significantly more cells than either YELP or Hanks'-YE

media at the base line determination (Table 8).[4] After one week of incubation the Eagle's BM group was slightly ahead of all the other three preparations; however, the four experimental groups were still in the lag phase of their repective growth curves indicating that cell division had not yet begun. Growth of the cultures began between the first and second week as indicated by the increase in cell populations determined at the end of the second week of incubation. At this time there was no significant difference in growth between cells cultured in Eagle's BM and YELP or between those grown in YELP and Hanks'-YE.[5] The Eagle's BM cultures however had significantly more growth than did those in Hanks'-YE or LY. Cultures grown in the YELP and those in Hanks'-YE produced greater amounts of growth than did those in the LY medium and the values were found to be significantly different. Cell growth was greatly increased at the third and fourth week observations. During this period the test preparation separated into two groups. Eagle's BM and YELP cultures showed no growth differences from each other but did contain significantly more cells than did

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[4] The least significant difference in the means of four replicate cultures with counts in the range of 10,000 to 99,999 cells was found to be >25,950 cells. (See section on statistical methods.)

[5] The least significant difference in means of four replicate cultures in the range of 100,000 to 499,999 cells was found to be >64,540 cells.

Table 8. Comparison of the growth of coho salmon embryonic cells cultured at 18° C. in four different media containing 20 percent agamma calf serum.

Media tested[1]	Incubation time in weeks	Number of cells per individual culture tube				Mean no. cells per four culture tubes
		1	2	3	4	
Experiment No. TC-125						
Hanks'-YE	0	70,000	85,000	89,300	91,000	83,800
	1	113,300	86,000	80,000	114,000	98,300
	2	342,700	380,700	345,000	326,700	348,800
	3	514,000	506,000	415,000	480,700	478,900
	4	860,700	712,700	860,000	852,700	824,000
Eagle's BM	0	143,300	112,700	103,300	136,700	124,000
	1	166,700	99,700	134,000	95,000	123,900
	2	459,300	380,000	424,000	442,700	426,500
	3	511,700	800,700	711,700	751,700	693,500
	4	1,022,300	1,226,700	1,019,300	1,419,300	1,172,000
LY	0	94,000	96,700	104,000	130,700	106,300
	1	68,300	88,700	121,700	82,300	90,300
	2	249,300	370,000	258,300	167,300	261,200
	3	281,700	430,700	652,700	463,300	457,100
	4	1,041,700	670,700	935,000	792,700	860,100
YELP	0	81,700	86,000	121,700	95,000	96,100
	1	100,700	78,300	89,300	81,700	87,500
	2	456,700	421,700	344,000	420,700	410,800
	3	718,300	649,300	633,700	702,700	674,700
	4	1,407,300	1,327,300	1,140,700	1,079,300	1,238,700

(continued next page)

Table 8 (continued)

Media tested[1]	Incubation time in weeks	Number of cells per individual culture tube				Mean no. cells per four culture tubes
		1	2	3	4	
Experiment No. TC-127						
Hanks'-YE	0	98,300	91,700	71,700	100,000	90,400
	1	47,700	116,700	96,700	124,300	96,300
	2	492,700	429,300	410,000	311,700	413,400
	3	670,700	752,700	751,700	641,700	704,200
	4	885,000	816,700	890,000	783,300	843,800
Eagle's BM	0	109,300	97,700	106,000	101,700	103,700
	1	110,000	164,300	194,300	253,300	180,500
	2	397,700	333,300	380,000	375,000	371,500
	3	863,300	951,700	821,700	772,700	852,100
	4	878,300	887,300	1,053,300	890,000	927,200
LY	0	69,300	81,000	121,700	106,000	94,500
	1	95,000	76,000	97,700	127,700	99,100
	2	411,700	418,300	356,000	369,300	388,800
	3	526,700	506,700	550,700	668,300	588,100
	4	810,700	961,700	690,000	845,000	826,900
YELP	0	90,000	98,300	83,300	95,000	91,600
	1	79,300	92,700	150,000	131,000	113,200
	2	433,300	286,700	388,300	411,000	379,800
	3	782,700	781,700	787,300	793,300	786,200
	4	855,700	1,016,700	825,000	958,300	913,700

(continued next page)

Table 8 (continued)

Average of two experiments, No. TC-125 and No. TC-127

Media tested	Mean number of cells per eight culture tubes incubated for				
	0 week	1 week	2 weeks	3 weeks	4 weeks
Hanks'-YE	87,100	97,300	381,100	591,600	833,900
Eagle's BM	113,800	157,200	399,000	772,800	1,049,100
LY	100,400	94,700	325,000	522,600	843,500
YELP	93,800	100,400	395,300	730,400	1,076,200

[1] Antibiotics were employed at concentrations of 100 units penicillin, 100 micrograms streptomycin and 25 units Mycostatin per ml of each medium tested.

the LY or Hanks'-YE cultures. Cell growth in the latter two media preparations were not significantly different from each other at the third or fourth week observation.

This experiment was later repeated in the same manner as above for comparative purposes. Results of the second experiment indicated the same general growth trends for cell cultured in the four test media (Table 8, Exp. TC-127). The relative amounts of growth obtained in the four media were not identical to those in the first experiment during the first two weeks. However, the Eagle's BM and YELP again produced more growth at the third and fourth week observations than the Hanks'-YE or LY media. This was in general agreement with the growth pattern observed in the first experiment.

Data from the two experiments were combined and the average value determined for the individual media tested at each of the five intervals where growth was measured. These values represent the mean number of cells in eight culture tubes (Table 8). When these data were plotted graphically it could be seen that the Eagle's BM and YELP produced more cell growth after the second week of incubation than did the LY or Hanks'-YE (Figure 17).

There appears to be little doubt that all four preparations tested are capable of producing healthy growing cultures of coho salmon embryonic cells. The data show however that Eagle's BM and YELP produced a larger number of cells over a three or four week period than the other two nutrient fluids

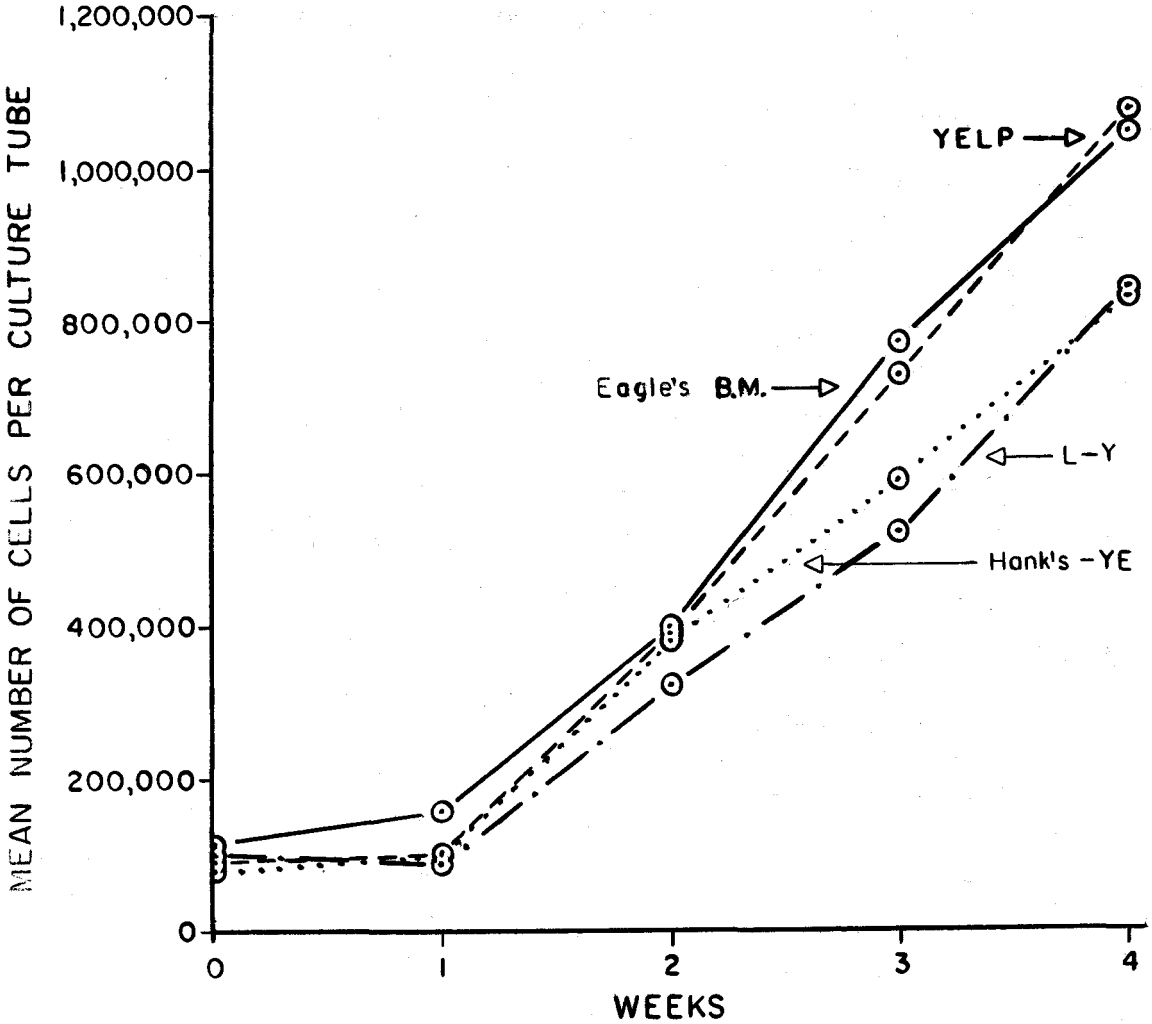

Figure 17. Comparison of the growth of coho salmon embryonic cells cultured at 18° C. in four different media containing 20 percent agamma calf serum.

tested.

Examination of the composition of Eagle's BM and YELP in the materials and supplies section of this report shows that both contained a greater variety of nutrients than did the LY or Hanks'-YE media. In addition to this, Eagle's BM and YELP were similar in that both employed Eagle's amino acids and vitamins in their formulation. This may have accounted for the fact that Eagle's BM and YELP were more growth stimulatory than LY or Hanks'-YE and that they produced approximately equal amounts of cell growth over the four-week experiment.

Comparison of Eagle's Basal Medium to Eagle's Minimum Essential Medium for Growth of Steelhead Embryonic Cells

Steelhead trout embryonic cells were grown in Eagle's basal medium and Eagle's minimum essential medium in order to determine which of these preparations would best stimulate growth. The formula for these media have already been shown in detail under the materials and supplies section of this report. Twenty percent agamma calf serum was incorporated in each of the media. The cells used in this experiment were obtained from the routine use of stock cultures and had been transferred once since prepared in primary culture. They were resuspended in each medium to give a concentration (cell inoculum) of 600,000 cells per ml of nutrient fluid and planted in 1 ml volumes in roller tubes, which were incubated at 18° C. Fluid changes were made 24 hours after initiating

the experiment and at five day intervals thereafter until completion of the tests. The total number of cells was determined in four roller tubes of each media at zero, one-half, one, and two week intervals. This experiment was subsequently repeated utilizing the same procedure.

Results of the two experiments were virtually the same. The Eagle's MEM was more growth stimulatory than was the Eagle's BM (Table 9). In the first experiment the base line count was not significantly different between the two groups; however, significant differences in growth were present at each of the three remaining observation periods and the superiority of the MEM increased in magnitude with time. The second experiment showed significantly higher cell counts in the MEM cultures at all four intervals measured. The two experiments have been combined by determining average cell counts at each interval (Table 9). These results indicate that Eagle's MEM produced larger amounts of cell growth than did the Eagle's BM at one-half, one, and two week intervals (Figure 18). At the latter period the MEM gave about twice as much growth as did the basal medium. The Eagle's MEM is an improved preparation developed since the Eagle's BM was devised. This newer medium contains twice the concentration of essential amino acids plus the non-essential amino acids. In addition, inositol and sodium pyruvate were present.

Table 9. Comparison of the growth of steelhead trout embryonic cells cultured at 18° C. in Eagle's basal medium and Eagle's minimum essential medium containing 20 percent agamma calf serum.

Media tested[1]	Incubation time in weeks	Number of cells per individual culture tube				Mean no.[2] cells per four culture tubes
		1	2	3	4	
Experiment No. TC-145						
Eagle's BM	0	225,000	150,000	245,000	162,700	195,700
	1/2	249,300	168,300	221,700	250,700	222,500
	1	150,700	333,300	323,300	296,700	276,000
	2	465,000	437,300	248,300	366,000	379,200
Eagle's MEM	0	186,700	167,300	193,300	179,300	181,600
	1/2	333,300	340,000	216,000	261,700	287,800
	1	619,300	570,000	339,300	330,000	464,600
	2	786,700	583,300	550,000	541,700	615,400
Experiment No. TC-146						
Eagle's BM	0	183,300	188,300	170,700	191,700	183,500
	1/2	253,300	216,000	157,300	223,300	212,700
	1	226,700	309,300	189,300	221,700	236,700
	2	331,700	488,300	315,000	360,300	373,800
Eagle's MEM	0	277,300	434,000	279,300	202,700	298,300
	1/2	416,000	228,300	246,000	243,300	283,400
	1	370,000	350,700	411,000	384,300	379,000
	2	1,026,700	801,700	900,000	781,700	877,300

(continued next page)

Table 9 (continued)

Average of two experiments, No. TC-145 and No. TC-146

Media tested	Mean number of cells per eight culture tubes incubated for			
	0 week	1/2 week	1 week	2 weeks
Eagle's BM	189,600	217,600	256,400	376,500
Eagle's MEM	240,000	285,600	421,800	746,400

[1] Antibiotic concentrations were 100 units penicillin, 100 micrograms streptomycin and 25 units Mycostatin per ml of each medium.

[2] Least significant difference for mean cell counts in range of 100,000 to 499,999 cells was found to be >64,540.

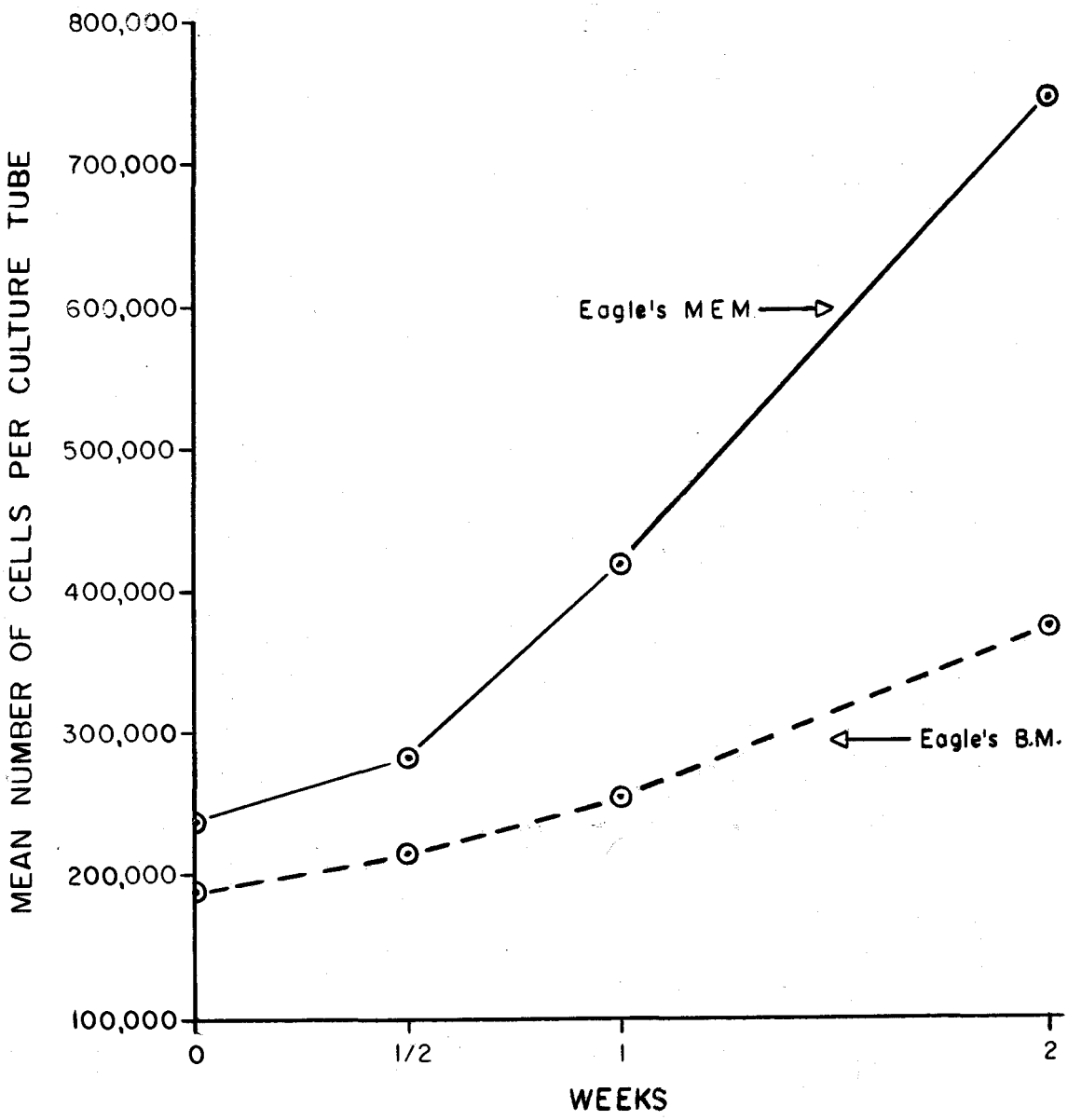

Figure 18. Comparison of the growth of steelhead trout embryonic cells cultured at 18° C. in Eagle's basal medium and Eagle's minimum essential medium containing 20 percent agamma calf serum.

Lack of Growth Stimulation for Cells Grown in Medium Containing 25 Percent of the Same Fluid Removed from an Actively Growing Culture

During a visit to this laboratory, Dr. R. C. Parker suggested an experiment to determine if the growth of salmon cells could be enhanced by compounding the fresh medium in such a way that it contained 25 percent of the same medium removed from an actively growing culture. This practice has been employed more or less empirically by other workers using various animal cell cultures. The purpose of such a test was to determine if growth stimulatory factors were present in the fluid portion of cultures which were in the logarithmic growth phase and therefore undergoing rapid mitosis and cellular development.

The experiment was conducted using coho salmon embryonic cells obtained by means of the routine use of stock cultures. These cultures had been transferred twice prior to use in this experiment. The cells were resuspended in Eagle's basal medium supplemented with 20 percent agamma calf serum and in the same preparation mixed with 25 percent of this nutrient fluid from an actively growing culture of coho salmon embryonic cells. The cell inoculum was 300,000 per ml of each medium. Nutrient fluids were changed on both sets of cultures 24 hours after initiating the experiment and at four day intervals thereafter until termination of the test. At each fluid change 40 ml of each medium were prepared. The Eagle's BM containing the fluid from the growing cultures was made by

combining 30 ml of fresh Eagle's medium with 10 ml of the fluid from the growing cultures. The other group of cultures received Eagle's BM compound in the normal manner at each fluid change. The cultures were planted in roller tubes (20 for each test group) and the total cells contained in each of four tubes determined for both test groups at intervals of zero, one-half, one, two, and three weeks. Incubation temperature for this experiment was 18° C. This experiment was repeated at a later date following the same procedure described above.

Results of both experiments indicated no stimulation in cell growth was obtained by the addition of fluid from actively growing cultures (Table 10). No significant differences were found between the two test groups in either experiment at any of the four intervals examined. The two experiments were averaged and the growth curves plotted. These two growth curves (control and experimental) were found to be almost identical (Figure 19). The results failed to show the presence of any growth stimulating factors in nutrient fluid from growing cultures. They did however demonstrate that the techniques employed for the preparation and enumeration of these cultures could produce similar results when identical conditions were maintained between experiments.

Table 10. Lack of growth factors for coho salmon embryonic cells in Eagle's basal medium removed from cultures in active growth phase.

Media tested [1,2]	Incubation time in weeks	Number of cells per individual culture tube				Mean no. cells per four culture tubes
		1	2	3	4	
Experiment No. TC-131						
Eagle's BM, fresh	0	67,300	74,000	50,700	66,000	64,500
	1/2	145,000	109,300	153,300	134,000	133,200
	1	219,300	194,000	204,000	176,700	198,500
	2	213,300	265,000	231,000	258,700	242,000
	3	529,300	478,300	528,300	---	512,300
Eagle's BM with 25% medium from active growth phase	0	43,300	80,700	69,300	71,700	66,200
	1/2	110,000	127,300	110,700	116,000	113,500
	1	204,000	197,300	209,300	157,300	192,000
	2	211,700	174,300	313,300	211,000	227,600
	3	558,300	490,000	523,300	525,000	536,900
Experiment No. TC-132						
Eagle's BM, fresh	0	60,700	32,700	29,300	69,300	48,000
	1/2	94,300	98,300	39,300	90,000	80,500
	1	97,300	92,700	25,000	96,700	77,900
	2	190,700	247,300	169,000	191,700	199,700
	3	334,000	350,700	336,700	382,700	326,000

(continued next page)

Table 10 (continued)

Media tested[1,2]	Incubation time in weeks	Number of cells per individual culture tube				Mean no. cells per four culture tubes
		1	2	3	4	
Eagle's BM with 25% medium from active growth phase	0	63,300	47,300	51,700	44,000	51,600
	1/2	99,300	74,300	93,300	83,300	87,600
	1	71,700	74,000	95,000	59,300	75,000
	2	265,000	160,700	137,300	175,000	184,500
	3	329,300	350,000	363,300	---	347,500

Average of two experiments, No. TC-131 and No. TC-132

Media tested	Mean number of cells per eight culture tubes incubated for				
	0 week	1/2 week	1 week	2 weeks	3 weeks
Eagle's BM, fresh	56,200	106,800	138,200	220,800	419,200
Eagle's BM with 25% medium from active growth phase	58,900	100,600	133,500	206,000	442,200

[1] All test media contained 20 percent agamma calf serum.

[2] Antibiotic concentrations in each medium were 100 units penicillin, 100 micrograms streptomycin and 25 units Mycostatin per ml.

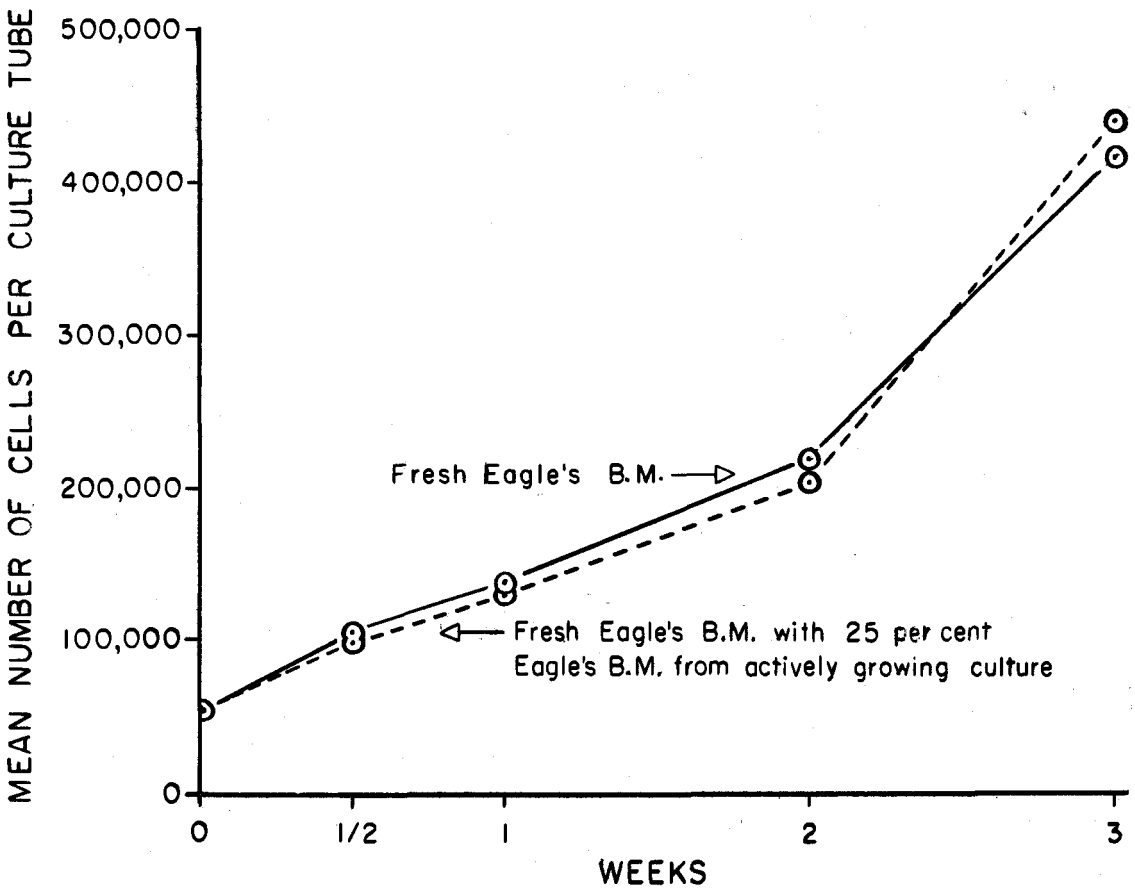

Figure 19. Lack of growth factors for coho salmon embryonic cells in Eagle's basal medium removed from cultures in active growth phase.

Comparison of the Effect of Various Antibiotic Concentrations on Cell Growth

In the materials and supplies section of this report the levels of antibiotic used for the control of microbial contamination in cell cultures were discussed. Two qualitative experiments were conducted employing steelhead embryonic cells prepared from stock cultures in the usual way. Cells were grown in LY medium containing 20 percent human serum and incubated at 18° C. Experimental concentrations of antibiotics were added to separate lots of this medium as follows:

Penicillin - 100 units per ml of fluid
Streptomycin - 100 micrograms per ml of fluid
Mycostatin - 0 units per ml of fluid

Penicillin - 100 units per ml of fluid
Streptomycin - 100 micrograms per ml of fluid
Mycostatin - 25 units per ml of fluid

Penicillin - 1000 units per ml of fluid
Streptomycin - 1000 micrograms per ml of fluid
Mycostatin - 0 units per ml of fluid

Penicillin - 1000 units per ml of fluid
Streptomycin - 1000 micrograms per ml of fluid
Mycostatin - 100 units per ml of fluid

The cells, which had been twice transferred prior to use in this experiment, were suspended in the various media at 300,000 per ml and planted in roller tubes. Twelve tube cultures were prepared for each of the four test concentrations of antibiotics. The nutrient fluid was changed after 24 hours incubation. New fluid containing the same concentration of antibiotics was then added every five days after this initial change. Culture tubes from the various experimental groups were compared for relative growth by microscopic examination over a three-week period.

No gross difference in the amount of cell growth or appearance of the cells was observed between any of the experimental groups containing different concentrations of antibiotics. This seemed to indicate that even the maximum concentrations of these antibiotics tested showed no evidence of toxicity detectable in a qualitative test of this kind.

The Effect of Vitamin B_{12} on the Growth of Steelhead Trout Embryonic Cells

Vitamin B_{12} has been shown to be beneficial for growth of Strain L mouse cells (10, p. 87-94). A preliminary experiment was designed to test the effects of this vitamin on the growth of steelhead trout embryonic cells at three different concentrations. The vitamin B_{12} (Nutritional Biochemicals Corporation) was added to Eagle's basal medium containing 20 percent agamma calf serum at levels of 0.0 (control), 0.5, 1.0, and

2.0 mg per liter. A stock solution of the vitamin (10X) was made up in Earle's BSS, filter sterilized, dispensed in tubes, and stored frozen at -20° C. This solution was then added to Eagle's BM by volume to give the required concentration of vitamin. Steelhead embryonic cells were obtained by the routine use of stock cultures and had received one transfer since their original preparation in primary culture. The cell inoculum contained in each preparation was 600,000 per ml of fluid. These suspensions were added to roller tubes (16 tubes per experimental group) in the usual 1 ml volumes and incubated at 18° C. Cell counts in the cultures were determined at zero, one-half, one, and two weeks. The fluid was changed 24 hours after the experiments started and at five day intervals thereafter until the test was terminated. Results indicate that at the concentrations tested vitamin B_{12} not only failed to stimulate growth of these cells but inhibited cellular development (Table 11). The base line counts for all test groups were very close; however, at the one-half week determination growth in the control group was significantly higher than in the three preparations containing the vitamin. This difference in growth continued to increase so that after two weeks the control cultures contained nearly twice as many cells as the vitamin cultures. At this point also the cultures containing 2 mg of vitamin B_{12} per liter had significantly smaller cell counts than those containing 1.5 or 1 mg per liter. The data, when plotted as growth curves showed clearly that the control

Table 11. The lack of growth stimulation by vitamin B_{12} for steelhead trout embryonic cells cultured at 18° C. in Eagle's basal medium with 20 percent agamma calf serum.

Concentration of vitamin B_{12} tested mg per liter	Incubation time in weeks	Number of cells per individual culture tube				Mean no. cells per four culture tubes
		1	2	3	4	
Experiment No. TC-144						
0.0	0	188,300	229,300	175,000	195,000	196,900
	1/2	468,300	539,300	484,000	513,300	501,200
	1	511,700	635,000	370,700	710,000	556,600
	2	1,044,000	1,091,700	846,700	846,700	957,200
0.5	0	149,300	240,700	184,000	173,300	186,300
	1/2	309,300	327,300	176,000	372,700	296,100
	1	274,000	454,000	416,700	260,700	351,400
	2	582,700	403,300	596,700	522,700	526,400
1.0	0	214,000	202,700	166,700	176,000	189,900
	1/2	325,000	276,000	329,300	503,300	358,400
	1	265,000	346,700	367,300	313,300	323,100
	2	564,000	563,300	592,700	506,000	556,500
2.0	0	245,000	206,000	190,700	156,000	199,700
	1/2	294,000	503,300	361,700	294,000	363,300
	1	344,000	256,700	376,000	140,700	279,400
	2	345,000	716,000	350,700	400,700	453,100

Antibiotics were added to each medium to give a concentration of 100 units penicillin, 100 micrograms of streptomycin and 25 units Mycostatin per ml.

group grew better than did the cells which received vitamin B_{12} and that the cultures grown in the highest concentration of the vitamin produced the least amount of cells by the end of the experiment (Figure 20).

This experiment was not repeated as the results indicated no growth stimulation was obtained by the addition of vitamin B_{12} at these concentrations. However, it cannot be concluded from this single experiment that this factor is not required by these cells. For a thorough study of this question experiments with lower vitamin concentrations should be carried out. It is, of course, entirely possible that the embryonic steelhead cells can synthesize required amounts of this vitamin.

The Effect of Oxaloacetic Acid on Cell Growth

Dr. R. C. Parker also mentioned, during his visit to this laboratory, that oxaloacetic acid had been found to be very beneficial as a growth promoting compound in certain animal cell cultures. He recommended the testing of this material as a possible growth stimulant for salmon and steelhead cells. The concentration of oxaloacetic acid employed in Parker's laboratory was 5 millimolar. An experiment was carried out to determine if a growth stimulatory effect could be observed in cultures of coho salmon embryonic cells grown in Eagle's basal medium (20 percent agamma calf serum) with oxaloacetic acid added. Media were prepared containing concentrations of 0.0 (control), 2.5, 5.0, and 10.0 mM oxaloacetic acid. Coho salmon

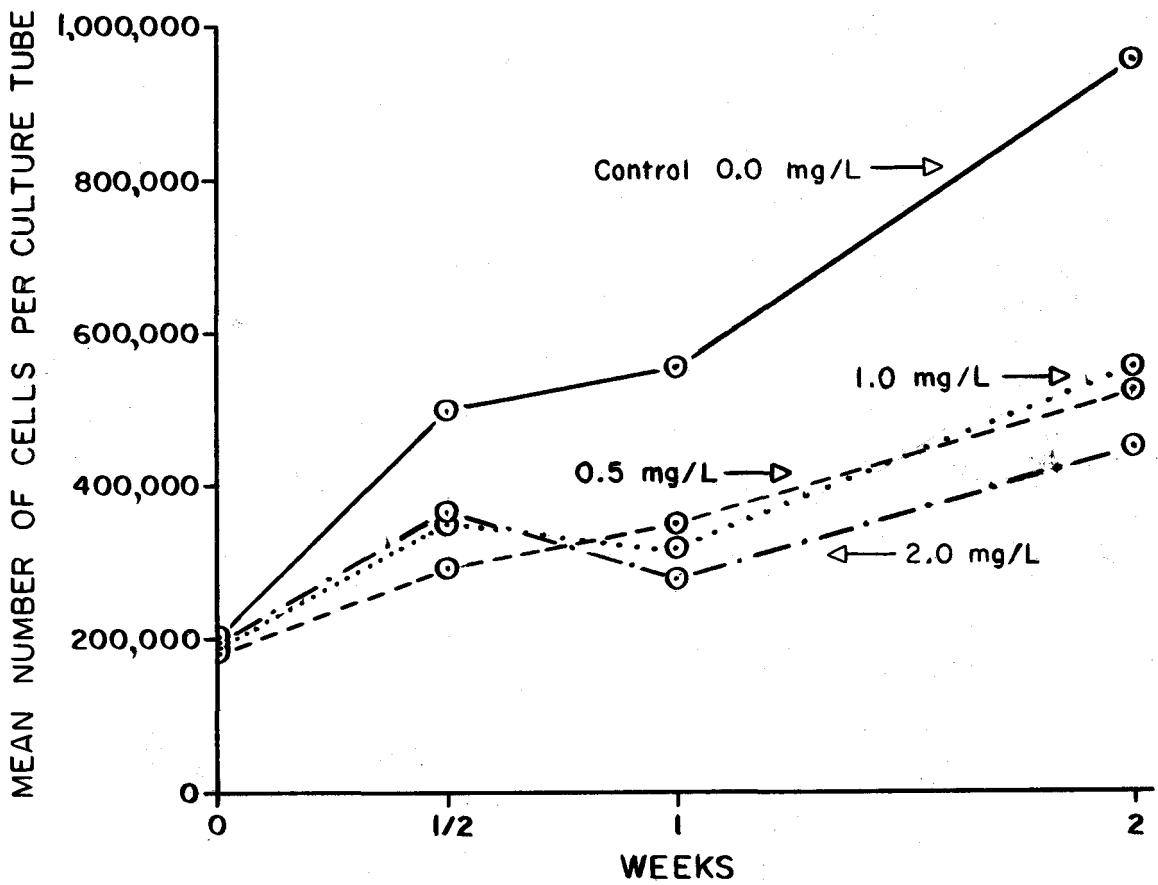

Figure 20. The lack of growth stimulation by vitamin B_{12} for steelhead trout embryonic cells cultured at 18° C. in Eagle's basal medium with 20 percent agamma calf serum.

cells were obtained by means of the routine use of stock cultures and resuspended in each of the four media to give a concentration of 600,000 cells per ml. These cells had been transferred twice prior to use in this experiment. Twenty roller tubes were planted with cells suspended in one of the test media, and similar groups were prepared for each of the other media. This allowed four sample tubes to be removed at intervals of zero, one-half, one, and two weeks for growth determination. Fluid was changed 24 hours after starting the experiment and at four day intervals thereafter until termination of the tests.

Results of the first experiment indicated no significant difference in number of cells between the cultures at the base line or one-half week determination. However, the cultures grown in Eagle's BM containing the 10 mM concentration of oxaloactic acid had significantly less growth than the controls, and the other cultures with less oxaloacetic acid after incubation for one week (Table 12, Exp. TC-136). At the end of the experiment the control group had a significantly higher mean cell population than did any of the groups which contained oxaloacetic acid. Cultures with the 10 mM concentration had significantly less growth than did those with the 5.0 or 2.5 mM levels. In descending order of magnitude based on amount of cell growth were the controls, 2.5 mM, 5.0 mM, and last the 10.0 mM oxaloacetic acid cultures. The conclusion indicated

Table 12. The inhibitory effect of oxaloacetic acid on the growth of coho salmon embryonic cells cultured at 18° C. in Eagle's basal medium with 20 percent agamma calf serum.

Concentration of oxaloacetic acid tested mM per liter	Incubation time in weeks	Number of cells per individual culture tube				Mean no.[1] cells per four culture tubes
		1	2	3	4	
Experiment No. TC-136						
0.0	0	249,300	276,700	206,700	169,300	225,500
	1/2	226,700	289,300	207,300	252,700	244,000
	1	360,000	485,000	480,000	406,700	432,700
	2	670,700	974,300	890,000	769,300	826,100
2.5	0	250,700	238,300	210,000	185,000	221,000
	1/2	252,700	254,000	230,000	199,300	234,000
	1	454,000	440,000	446,700	366,000	426,700
	2	736,300	688,300	676,000	717,300	711,200
5.0	0	254,000	220,000	204,000	191,700	217,400
	1/2	279,300	192,700	178,300	267,300	229,400
	1	446,000	468,300	316,700	245,000	396,000
	2	686,700	709,300	695,000	528,300	654,800
10.0	0	184,000	242,700	168,300	181,700	194,200
	1/2	236,000	196,000	169,300	186,700	197,000
	1	302,700	222,700	286,700	158,300	242,600
	2	232,700	396,000	314,000	279,300	310,000

(continued next page)

Table 12 (continued)

Concentration of oxaloacetic acid tested mM per liter	Incubation time in weeks	Number of cells per individual culture tube				Mean no.[1] cells per four culture tubes
		1	2	3	4	
Experiment No. TC-141						
0.0	0	150,000	118,300	130,700	110,700	127,800
	1/2	110,000	96,000	138,300	140,700	121,000
	1	250,700	222,700	200,700	167,300	210,400
	2	265,000	354,000	235,000	308,300	290,600
2.5	0	91,700	60,700	96,700	116,000	91,400
	1/2	80,700	100,000	81,700	80,000	85,800
	1	125,000	140,000	109,300	100,700	118,800
	2	203,300	196,000	265,000	182,700	211,800
5.0	0	83,300	142,700	120,000	137,300	120,800
	1/2	106,000	67,300	87,300	94,000	88,500
	1	128,300	82,700	138,300	90,700	110,000
	2	169,300	157,700	125,000	142,700	146,900
10.0	0	124,000	98,300	96,000	73,300	97,900
	1/2	68,300	86,000	87,300	71,700	78,300
	1	105,000	110,000	94,000	114,000	105,800
	2	117,300	160,700	164,000	95,000	134,300

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Table 12 (continued)

Average of two experiments, No. TC-136 and No. TC-141

Concentration of oxaloacetic acid tested mM per liter	Mean number of cells per eight culture tubes incubated for			
	0 week	1/2 week	1 week	2 weeks
0.0	176,600	182,500	321,600	558,400
2.5	156,200	159,200	272,800	461,500
5.0	169,100	159,000	253,000	400,800
10.0	146,000	137,600	174,200	222,200

[1] Least significant difference for mean cell counts in the range of 100,000 to 499,999 cells was found to be >64,540.

by this experiment was that the addition of this compound inhibited cell growth and that this effect increased with concentration.

A second experiment was conducted in the same manner as the first to attempt confirmation of these results. Here again the controls which contained no oxaloacetic acid grew significantly better than did the test cultures containing the acid at the one-half, one, and two week counts. Cultures with the 2.5 and 5.0 mM concentrations of oxaloacetic acid were intermediate in cell growth and the 10.0 mM concentration as before produced the least amount of growth (Table 12, Exp. TC-141).

The results of both of these experiments have been combined and the average values determined for the individual media preparations at each point where cell growth was measured. The combined results are shown to illustrate the inhibitory effect of this compound on the growth of coho salmon embryonic cells (Figure 21).

The Effect of Fish, Human, and Horse Sera on Growth of Steelhead Embryonic Cells

All media preparations examined during the course of this work required the addition of animal sera to produce growth of salmon and steelhead cells. The purpose of this preliminary experiment was to determine growth differences of steelhead embryonic cells prepared in primary cultures by standard methods already described and grown in lots of LY medium

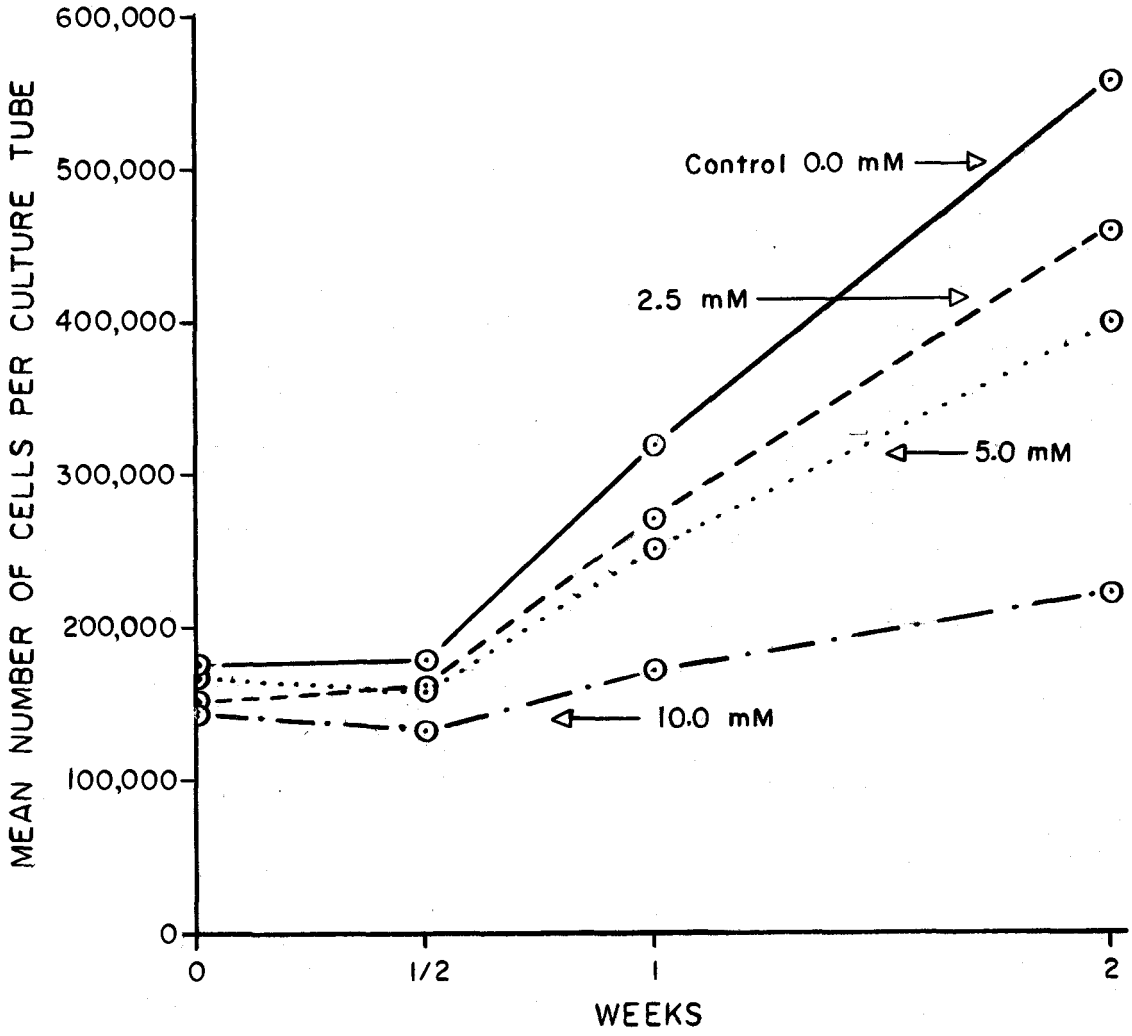

Figure 21. The inhibitory effect of 2.5, 5.0, and 10.0 mM concentrations of oxaloacetic acid on the growth of coho salmon embryonic cells cultured at 18° C. in Eagle's basal medium with 20 percent agamma calf serum.

supplemented with fish, horse, or human serum at concentrations of 10, 20, and 40 percent. The trypsinized cells were harvested, separated into nine equal portions, and resuspended in the media to be tested. The concentration of cells in each nutrient fluid was 1.5 million per ml. These cells were planted in a volume of 1 ml per roller tube and incubated at 18° C. The first fluid change was made 24 hours after initiation of the experiment and others at five day intervals until termination of the tests. Four tubes from each set of cultures were removed and the total number of cells determined at intervals of zero, one, and three weeks.

Several qualitative tests performed with fragment or explant cultures prior to this experiment indicated that homologous fish serum did not enhance cell growth and was in fact toxic. In this experiment steelhead serum failed to support growth over the three-week test period at any of the concentrations examined (Table 13). The cultures containing 10 percent fish serum appeared more nearly normal and the cells lasted longer than did those in the 20 and 40 percent concentrations. The reason for this unexpected toxicity is unknown.

LY medium containing human serum at the 10 percent level did not support cell growth. Cultures grown in medium containing 20 percent human serum underwent a one week lag in growth; however, the cell population had increased over 2.5 fold between the one and three week observations. The highest

Table 13. Results of an experiment to compare the effects of three animal sera added to lactalbumin hydrolysate-yeast extract medium at concentrations of 10, 20, and 40 percent on the growth of primary cultures of steelhead trout embryonic cells incubated at 18° C.

Serum	Conc. %	Incubation time in weeks	Number of cells per individual culture tube				Mean no. cells per four culture tubes
			1	2	3	4	
Steelhead trout	10	0	1,700	6,700	1,700	0	3,300
		1	5,000	1,700	10,000	6,700	5,900
		3	0	0	0	0	0
	20	0	20,000	29,300	11,000	8,300	17,200
		1	0	0	0	1,600	400
		3	0	0	0	0	0
	40	0	22,000	7,000	4,000	3,300	9,000
		1	0	0	0	0	0
		3	0	0	0	0	0
Human	10	0	10,000	10,300	12,700	10,300	10,700
		1	20,000	20,000	22,000	26,000	22,000
		3	5,000	1,700	700	6,700	3,500
	20	0	81,700	86,000	107,700	88,000	90,800
		1	90,000	54,000	140,000	98,000	95,600
		3	285,000	210,000	257,000	182,000	233,500

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Table 13 (continued)

Serum	Conc. %	Incubation time in weeks	Number of cells per individual culture tube				Mean no. cells per four culture tubes
			1	2	3	4	
Human	40	0	153,000	136,000	52,000	63,000	103,500
		1	356,700	332,000	296,700	426,700	354,000
		3	290,000	244,000	64,300	193,000	197,800
Horse	10	0	6,000	9,300	21,600	6,700	10,900
		1	10,000	5,700	6,700	8,300	7,700
		3	0	0	0	0	0
	20	0	30,700	16,300	57,000	28,000	33,000
		1	80,000	80,000	30,000	66,700	64,200
		3	43,000	23,000	37,700	43,000	36,700
	40	0	63,000	---	7,300	10,000	26,700
		1	5,000	1,900	0	3,300	3,400
		3	15,000	23,000	38,300	13,300	22,400

Antibiotics were added to each medium at concentrations of 100 units penicillin, 100 micrograms streptomycin and 25 units Mycostatin per ml.

concentration of human serum tested (40 percent) showed an increase in cells of over threefold between the base line and one week growth determination. There was, however, a sharp decline in cell numbers by the end of the experiment (Table 13). Thus the 40 percent serum content appeared to reduce the lag phase significantly, and resulted in more growth at one week than was found at three weeks in the medium with 20 percent serum. However, maximum growth was apparently reached before the third week, and the cultures began to deteriorate.

Horse serum did not support satisfactory growth of these cultures at any of the three concentrations tested. However, this serum did not appear acutely toxic as the cells in the medium containing 20 or 40 percent serum maintained a reasonably normal microscopic appearance throughout the three-week test period. Slight growth occurred during the first week in the cultures containing 20 percent serum but those containing 10 percent or 40 percent serum showed no growth at any time.

It has been concluded from these tests that none of the three sera tested at the 10 percent level in LY medium supported the growth of primary cell cultures of steelhead trout embryonic cells. Homologous fish serum was toxic for the cells at all concentrations examined in this experiment. Horse serum appeared non-toxic but failed to support growth. Cells grown in the presence of 20 and 40 percent human serum contained in LY medium showed significant but only moderate

growth, as indicated by increases of two- to threefold in cell numbers. It was, however, distinctly superior to the other two sera for these cells and conditions.

The Effect of Human and Calf Serum With and Without Gamma Globulin on Cell Growth

The purpose of these experiments was to compare the growth of chinook salmon embryonic cells cultured in Eagle's BM containing 20 percent human and calf serum with and without gamma globulin. They were part of a continued effort to obtain a serum that when added to the medium would yield the maximum growth of cells. These four serum preparations were obtained from Hyland Laboratories and had been pretested for toxicity to known animal cell lines. Chinook embryonic cells were obtained from stock cultures which had not been transferred prior to use in these experiments. Four lots of Eagle's BM were prepared each containing 20 percent of one of the four sera to be tested; the cells were resuspended in each to give a concentration of 300,000 per ml.

These media containing cells at the concentration mentioned above were added to roller tubes (1 ml per tube) and incubated at 18° C. Twenty tubes were prepared for each test medium to allow for the removal of four tubes at the zero, one, two, three, and four weeks growth periods. The first fluid change was made 24 hours after incubation was initiated; subsequent changes were made at five-day intervals until the experiment

was completed.

The results revealed no significant differences in numbers of cells between any of the media at the base line or zero determination (Table 14). Cultures in the medium containing agamma calf serum showed significantly higher cell counts than the other three groups at the one week determination. This difference continued and by the second week the medium had produced over five times the cell growth found in the cultures containing plain calf or human serum and about three times that in cultures containing agamma human serum. The third week determination indicated that cultures in the human serum without gamma globulin had dropped significantly below those in the calf and human serum groups, both of which had increased four- to fivefold over the inoculum. Cultures in the agamma calf serum medium, however, had reached a far higher level at this point, representing an increase of almost fourteenfold over the inoculum. Growth determination made at four weeks indicated a leveling off of growth in the agamma calf serum containing cultures; however, they remained far above all the other groups in cell count. The calf serum cultures had significantly more growth than did the human serum group. There was no difference between the latter cultures and those grown in the medium containing agamma human serum.

Two more experiments were performed to confirm the results obtained here. All three experiments indicated that agamma calf serum gave significantly more growth over the four-week

Table 14. The effect of 20 percent human and calf serum with and without gamma globulin in Eagle's basal medium on the growth of chinook salmon embryonic cell cultures incubated at 18° C.

Serum	Incubation time in weeks	Number of cells per individual culture tube				Mean no.[1,2] cells per four culture tubes
		1	2	3	4	
Experiment No. TC-108						
Calf	0	59,300	51,700	43,300	55,300	52,000
	1	48,300	44,300	46,000	28,300	41,700
	2	84,300	99,300	72,700	73,300	82,400
	3	241,700	331,700	328,300	150,000	262,900
	4	224,300	152,700	270,000	231,000	219,500
Agamma calf	0	57,700	55,000	49,300	94,300	64,100
	1	117,700	78,300	89,300	90,000	93,800
	2	481,700	501,300	434,000	348,300	443,800
	3	756,000	930,000	787,300	926,700	850,000
	4	751,700	921,000	758,300	661,000	773,000
Human	0	56,000	42,700	55,000	39,300	48,300
	1	58,300	31,700	42,700	35,000	41,900
	2	142,700	40,000	50,000	108,300	85,200
	3	371,300	102,700	136,000	237,700	212,000
	4	68,300	99,300	116,000	273,300	139,200
Agamma human	0	47,700	46,700	39,300	50,000	45,900
	1	30,700	20,000	30,700	19,000	25,100

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Table 14 (continued)

Serum	Incubation time in weeks	Number of cells per individual culture tube				Mean no.[1,2] cells per four culture tubes
		1	2	3	4	
Agamma human	2	154,300	134,300	174,300	156,700	154,900
	3	37,700	36,000	71,700	46,000	47,800
	4	152,700	111,000	120,000	75,000	114,700
Experiment No. TC-109						
Calf	0	42,700	61,700	40,000	48,300	48,200
	1	60,000	79,300	46,000	67,700	63,300
	2	130,000	103,300	84,300	124,000	110,400
	3	215,000	454,300	305,000	192,700	291,700
	4	220,000	342,700	208,300	253,300	256,100
Agamma calf	0	60,000	41,000	38,000	28,300	41,800
	1	107,000	110,000	109,000	126,000	113,000
	2	224,300	182,700	300,000	293,300	250,100
	3	431,700	394,300	359,000	284,300	367,300
	4	344,000	460,000	409,300	436,000	412,300
Human	0	36,000	43,300	43,300	34,300	39,200
	1	74,300	136,700	67,700	131,000	102,400
	2	246,700	228,300	262,700	186,000	230,900
	3	224,000	376,000	233,300	275,000	277,000
	4	160,000	336,000	155,000	----	217,000

(continued next page)

Table 14 (continued)

Serum	Incubation time in weeks	Number of cells per individual culture tube				Mean no.[1,2] cells per four culture tubes
		1	2	3	4	
Agamma human	0	47,700	26,000	38,300	28,300	35,100
	1	157,700	213,300	195,000	39,000	151,200
	2	50,700	22,700	91,700	105,000	67,500
	3	21,700	503,000	82,700	81,000	169,600
	4	213,300	94,000	260,700	120,000	172,000
Experiment No. TC-110						
Calf	0	55,000	36,700	91,700	83,300	66,700
	1	111,000	86,700	91,000	77,700	94,600
	2	141,000	124,300	160,700	137,700	140,800
	3	211,700	215,000	230,000	166,000	205,700
	4	484,300	350,000	517,700	---	450,700
Agamma calf	0	77,700	159,300	127,700	112,700	119,400
	1	167,700	171,700	174,300	141,700	163,800
	2	303,700	235,700	282,300	297,700	279,900
	3	493,000	629,300	411,000	495,000	507,100
	4	930,000	893,300	1,257,700	621,300	925,600
Human	0	99,300	106,700	120,000	143,300	117,300
	1	136,700	118,700	109,300	121,000	121,400
	2	222,700	94,300	111,700	325,000	251,200
	3	349,300	89,300	---	94,300	177,600
	4	83,300	172,700	291,700	306,000	213,600

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Table 14 (continued)

Serum	Incubation time in weeks	Number of cells per individual culture tube 1	2	3	4	Mean no.[1,2] cells per four culture tubes
Agamma human	0	113,300	108,300	116,000	117,700	113,800
	1	43,300	96,700	146,000	181,700	116,900
	2	326,700	284,000	196,700	222,700	257,500
	3	542,700	136,000	16,700	103,300	199,800
	4	243,300	54,300	92,600	---	97,500

Average of three experiments, No. TC-108, TC-109, and TC-110

Serum	Mean number of cells per twelve culture tubes incubated for 0 week	1 week	2 weeks	3 weeks	4 weeks
Calf	55,600	66,500	111,200	253,400	308,800
Agamma calf	75,100	123,500	324,600	574,800	703,600
Human	68,300	88,600	189,100	222,200	189,900
Agamma human	64,900	97,800	160,000	139,000	128,100

[1] Least significant difference for mean cell counts in the range of 10,000 to 99,999 cells was found to be >25,950.

[2] Least significant difference for mean cell counts in the range of 100,000 to 499,999 cells was found to be >64,540.

period than did the other three test media (Table 14). In each case the calf serum produced more growth by the end of the experiment than did the media containing human or agamma human serum. The cultures grown in Eagle's BM containing agamma human serum had produced less growth by the fourth week determination than had any of the other culture preparations. There was also more growth variation encountered with cultures grown in this serum. The reason for this variation is not known.

The three experiments were summarized by averaging the values for each growth measurement (Table 14). The values obtained represent the mean numbers of cells present in 12 culture tubes. Figure 22 illustrates the effects these sera had on the growth of chinook salmon embryonic cells.

The Effect of a One and Three Percent CO_2 Atmosphere on Cell Growth

Several experiments were performed to determine the effects of a CO_2 atmosphere on growth of salmon cells. These experiments were carried out in a vacuum desiccator as no CO_2 incubator was available. Coho salmon embryonic cells were obtained from stock cultures and resuspended in Eagle's basal medium containing 20 percent agamma calf serum at a concentration of 300,000 cells per ml. Cells were planted in Leighton tubes rather than roller tubes. Cultures which were to be incubated in a CO_2 atmosphere were stoppered with gauze-wrapped cotton plugs to allow the gaseous mixture access to the culture

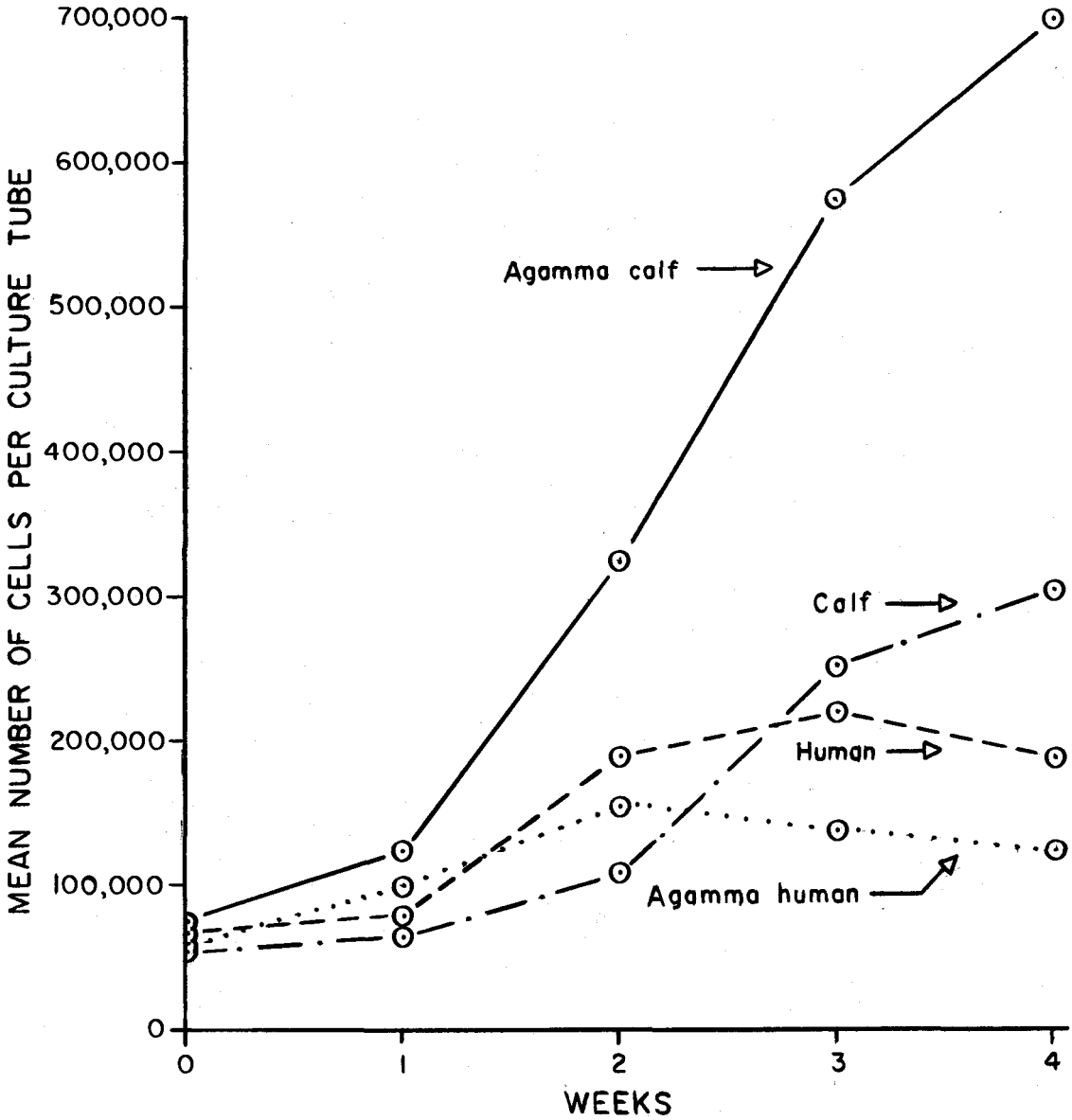

Figure 22. The effects of 20 percent human and calf serum, with and without gamma globulin in Eagle's basal medium, on the growth of chinook salmon embryonic cell cultures incubated at 18° C.

medium while the control group received the usual rubber stoppers. Control cultures were placed in Leighton tube racks and incubated in the normal manner at 18° C. The cultures which received added CO_2 were placed in a vacuum desiccator and the pressure reduced the desired amount within the container by means of a vacuum pump and manometer. Pressure was returned to normal by the addition of 99.9 percent CO_2. The desiccator containing the cultures was then placed in an 18° C. incubator. Fluid changes were made after 24 hours incubation and at two-day intervals thereafter until completion of the experiment. The pH of the medium removed from the cultures was determined at each fluid change. The CO_2 atmosphere was also adjusted to the proper level each time the medium was changed on the cells.

Initial experiments were carried out employing CO_2 atmospheres of five and eight percent. Both these levels were unsatisfactory, however, as they caused a sharp decrease in the pH of the medium which became toxic to the cells. CO_2 levels of one and three percent did not produce this decline in the pH of the medium and were therefore selected for testing.

The first experiment conducted with cells cultured in the one and three percent CO_2 atmosphere indicated that both were inhibitory for cell growth when compared to the control group which had been grown in the normal manner in air. The base line determination indicated no significant difference between the control and one percent CO_2 test group; however, the

cultures incubated in the three percent CO_2 atmosphere had significantly smaller cell counts than controls (Table 15). At the one and two week observations the control cultures remained significantly higher in cell count than the three percent CO_2 cultures; they were also higher than the cultures in one percent CO_2, but not significantly so. During this same period the one percent CO_2 cultures also grew significantly better than did the three percent CO_2 group. Third and fourth week examinations indicated the controls had produced much more growth than either of the CO_2 cultures and that the same relationship existed between the one and three percent test groups. In summary, the control cultures in air showed the most cell growth at each interval where measurements were made during the four-week test period. Cells grown in a one percent CO_2 atmosphere were intermediate and those cultured in a three percent CO_2 atmosphere produced the least amount of cell growth.

This experiment was repeated, and again the controls grew better than either of the groups of cultures grown in the presence of CO_2. The one percent CO_2 preparation in this experiment followed more closely the growth pattern of the three percent CO_2 group. This caused the controls to become significantly higher in cell count than the other test groups at the one week observation and this difference remained through the fourth week of experimentation (Table 15, Exp. TC-135). Both experiments were therefore in general agreement and indicated

Table 15. The inhibitory effect of a one and three percent CO_2 atmosphere on the growth of coho salmon embryonic cells cultured at 18° C. in Eagle's basal medium with 20 percent agamma calf serum.

Concentration of CO_2 added	Incubation time in weeks	Number of cells per individual culture tube				Mean no. cells per four culture tubes
		1	2	3	4	
Experiment No. TC-134						
No CO_2 added	0	124,000	111,700	121,700	176,000	133,400
	1	192,700	184,300	133,300	142,300	163,200
	2	278,300	324,000	348,300	297,300	312,000
	3	427,300	505,000	432,700	361,700	431,700
	4	632,700	540,700	550,700	564,000	572,000
1% CO_2	0	111,700	110,700	98,300	139,300	115,000
	1	154,300	68,300	123,300	140,000	112,500
	2	226,000	280,700	296,000	192,700	248,900
	3	321,700	270,700	327,300	349,300	317,300
	4	378,300	462,700	359,300	308,300	377,200
3% CO_2	0	78,300	106,700	102,700	87,300	93,800
	1	89,300	73,300	88,300	67,300	79,600
	2	200,700	174,000	115,000	109,300	149,800
	3	252,000	127,300	99,300	185,000	165,600
	4	210,700	153,300	61,700	82,700	124,800

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Table 15 (continued)

Concentration of CO_2 added	Incubation time in weeks	Number of cells per individual culture tube				Mean no. cells per four culture tubes
		1	2	3	4	
Experiment No. TC-135						
No CO_2 added	0	64,000	61,700	87,300	53,300	66,600
	1	106,700	110,700	123,300	115,000	113,900
	2	203,300	210,700	162,700	212,700	197,400
	3	331,700	392,700	244,000	413,300	345,400
	4	634,000	646,000	516,000	591,700	596,900
1% CO_2	0	60,700	21,700	30,000	60,700	43,300
	1	98,300	65,000	68,300	33,300	66,200
	2	124,000	107,300	53,300	111,700	99,100
	3	200,300	273,300	244,000	141,700	214,800
	4	291,700	180,700	293,300	149,300	253,800
3% CO_2	0	46,000	56,700	68,300	44,000	53,800
	1	60,700	65,700	65,000	87,300	69,500
	2	84,000	78,300	87,300	120,700	92,300
	3	120,700	200,000	132,700	134,000	146,900
	4	86,000	169,300	194,000	182,700	158,000

(continued next page)

Table 15 (continued)

Average of two experiments, No. TC-134 and No. TC-135

Concentration of CO_2 added	Mean number of cells per eight culture tubes incubated for				
	0 week	1 week	2 weeks	3 weeks	4 weeks
No CO_2 added	100,000	138,600	254,700	388,600	584,800
1% CO_2	79,200	89,400	174,000	266,000	315,500
3% CO_2	73,800	74,600	121,000	156,200	141,400

that both one and three percent CO_2 concentrations in the atmosphere were inhibitory for the growth of coho salmon embryonic cells and the degree of inhibition increased as the CO_2 tension increased. Results of both experiments were averaged and the mean cell count of eight culture tubes for each set of experimental conditions plotted to illustrate the inhibitory effect of CO_2 on cell growth (Figure 23).

It has already been mentioned that the nutrient fluid from all cultures in both experiments were changed at two-day intervals. The pH of the fluid removed from these cultures was determined at each fluid change to ensure that the addition of CO_2 from the atmosphere did not reduce the pH of the medium to a level which would become toxic. The following data indicates the minimum-maximum (range) and mean pH values reached in individual cultures of each group. This was determined by measuring the pH of the medium at each 48-hour fluid change over the four-week test period.

First experiment	pH Range	Mean pH
Control cultures	7.46 to 7.85 $\pm$ 0.05	7.62 $\pm$ 0.05
Cultures in one percent CO_2	7.65 to 7.87 " "	7.70 " "
Cultures in three percent CO_2	7.22 to 7.60 " "	7.38 " "

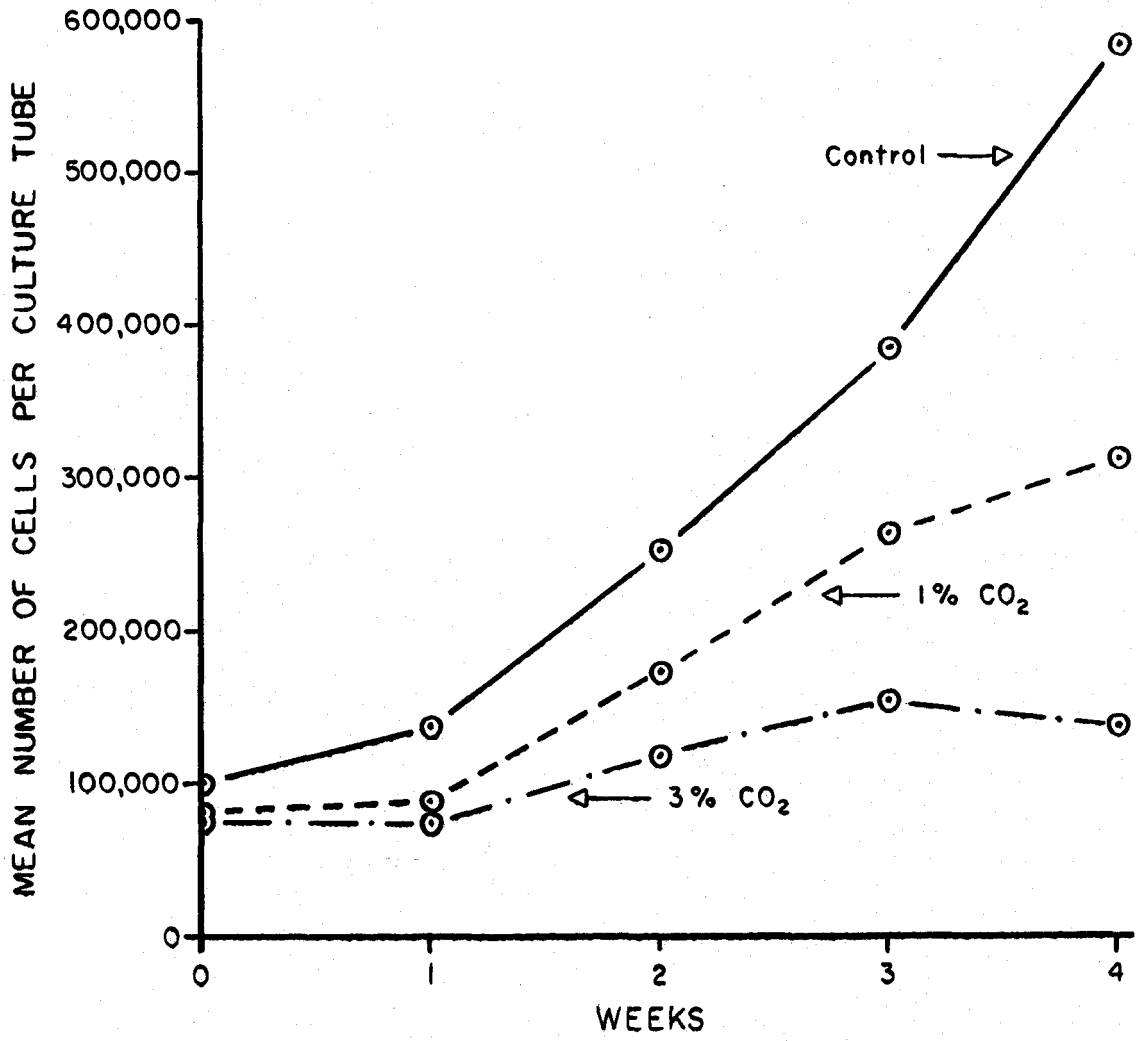

Figure 23. The inhibitory effect of a 1 and 3 percent CO_2 atmosphere on the growth of coho salmon embryonic cells cultured at 18° C. in Eagle's basal medium with 20 percent agamma calf serum.

Second experiment	pH Range	Mean pH
Control cultures	7.50 to 7.85 ± 0.05	7.58 ± 0.05
Cultures in one percent CO_2	7.53 to 7.86 " "	7.70 " "
Cultures in three percent CO_2	7.23 to 7.50 " "	7.42 " "

pH values of 7.0 to 8.0 are not believed to be inhibitory to the growth of coho salmon embryonic cells. All the values shown above were within this range and would be most unlikely to cause the observed inhibition of growth in the presence of one and three percent CO_2. Still more convincing is the fact that the range of pH values found in the cultures exposed to one percent CO_2 was very close to the comparable range in control cultures. Yet growth was inhibited significantly in the CO_2 group. It was therefore concluded that this inhibition was not due to pH differences.

The Effect of Temperature on Cell Growth

The purpose of these experiments was to examine the effect of five different incubation temperatures on the growth of coho salmon embryonic cells and to determine within what range the optimum lay. Temperatures employed were 4, 13, 18, 23, and 35° C. The incubation temperature of 18° C. was used for the cultivation of cells throughout the work described in this thesis and was employed here as a control group of cultures to be compared for growth with cultures incubated at the

experimental temperatures. Incubators were selected and the temperatures pre-set at the desired temperature. Temperature variation in each incubator used to culture the test groups was approximately ± 0.5° C. Steelhead embryonic cells used in these experiments were obtained from stock cultures and resuspended in Eagle's basal medium containing 20 percent agamma calf serum. These cells had been transferred once since their original isolation in primary culture. The concentration of cell inoculum contained in this medium was 300,000 per ml. Sixteen roller tubes were inoculated for each of the five experimental groups. This allowed four tubes to be removed for cell enumeration from each of the experimental temperature groups at zero, one-half, one, and two week intervals. The medium was changed on all cultures after 24 hours incubation and at four day intervals thereafter until completion of the experiments.

Results indicated that cultures incubated at 18° and 23° C. were the only ones which produced growth (Table 16, Exp. TC-147). The cell growth obtained at these two temperatures was almost identical. Cells cultured at 13° C. were barely able to maintain the cell concentration present at the base line count. Although the incubation temperature of 4° C. was not lethal, the cell concentration at the end of the experiment was less than at the base line determination. Thirty-five degrees centigrade was found to be lethal.

Table 16. The effects of temperature on the growth of steelhead trout embryonic cells cultured in Eagle's basal medium with 20 percent agamma calf serum.

Temperature	Incubation time in weeks	Number of cells per individual culture tube				Mean no. cells per four culture tubes
		1	2	3	4	
Experiment No. TC-147						
4° C.	0	76,700	46,700	45,700	36,700	51,500
	1/2	26,700	31,700	41,700	42,700	35,700
	1	6,700	3,300	26,000	24,000	15,000
	2	29,300	30,000	24,300	29,300	28,200
13° C.	0	96,700	63,300	64,000	119,300	85,800
	1/2	82,700	79,300	80,000	83,300	81,300
	1	94,000	49,300	93,300	77,300	78,500
	2	88,300	66,000	87,300	66,000	76,900
18° C.	0	148,300	151,700	129,300	131,700	140,200
	1/2	88,300	111,000	131,700	156,000	121,800
	1	277,300	114,000	194,000	191,700	194,300
	2	498,300	628,300	579,300	437,700	535,900
23° C.	0	76,000	66,700	123,300	89,300	88,800
	1/2	146,700	125,000	106,700	79,300	114,400
	1	199,300	182,700	171,700	176,700	182,600
	2	609,300	432,700	597,700	496,700	534,000
35° C.	0	13,300	18,300	42,700	23,300	24,400

(continued next page)

Table 16 (continued)

Temperature	Incubation time in weeks	Number of cells per individual culture tube				Mean no. cells per four culture tubes
		1	2	3	4	
35° C.	1/2	0	0	0	0	0
	1	0	0	0	0	0
	2	0	0	0	0	0
Experiment No. TC-148						
4° C.	0	65,000	64,300	55,000	54,300	59,700
	1/2	73,300	52,700	58,300	36,700	55,300
	1	38,300	46,700	23,300	31,700	35,100
	2	33,000	40,000	37,700	41,700	38,100
13° C.	0	89,300	89,300	64,000	120,000	90,700
	1/2	94,300	86,700	72,700	79,300	83,300
	1	102,700	122,700	86,700	117,300	107,300
	2	81,000	87,700	66,700	100,000	83,800
18° C.	0	108,000	88,300	100,000	136,000	108,100
	1/2	86,000	102,700	159,300	126,700	118,700
	1	256,300	189,300	232,700	246,700	231,200
	2	618,300	745,000	679,300	765,000	701,900
23° C.	0	46,700	66,700	67,300	64,000	66,200
	1/2	102,700	131,000	100,000	74,300	102,000
	1	266,700	246,000	284,300	295,000	273,000
	2	574,300	714,300	698,300	695,300	670,500

(continued next page)

Table 16 (continued)

Temperature	Incubation time in weeks	Number of cells per individual culture tube				Mean no. cells per four culture tubes
		1	2	3	4	
35° C.	0	10,000	20,000	17,300	14,000	17,100
	1/2	0	0	0	0	0
	1	0	0	0	0	0
	2	0	0	0	0	0

Average of two experiments, No. TC-147 and No. TC-148

Temperature	Mean number of cells per eight culture tubes incubated for			
	0 weeks	1/2 week	1 week	2 weeks
4° C.	55,600	45,500	25,100	33,200
13° C.	88,200	82,300	92,900	80,400
18° C.	124,200	120,200	212,800	618,900
23° C.	77,500	108,200	227,800	602,200
35° C.	20,800	0	0	0

Statistically, the cultures at 18° C. showed significantly higher cell counts than all the other test groups at the base line determination (Table 16). There was no difference in cell numbers between the 23° C. and 13° C. groups at this observation; however, both of these groups were significantly greater than the 4° and 35° C. groups. There was also a significant difference between cells incubated at 4° and 35° C. A base line count has been indicated for cultures incubated at 35° C., but the cells were believed to be already dead. This test group had reduced the cell count to zero by the half-week period and was subsequently discontinued.

At the one-half week determination there was no significant difference in the growth of cultures incubated at 18° and 23° C. These were, however, significantly greater than values obtained from cultures incubated at 4° and 13° C. There was also a significantly greater number of cells present in cultures incubated at 13° C. compared with those exposed to the 4° C. temperature. One and two week determinations maintained this same relationship; however, separation of the groups increased greatly as the cells incubated at 18° and 23° C. entered the logarithmic growth phase, producing larger numbers of cells at each of the two intervals.

A second experiment was performed to attempt confirmation of these observations. Results were almost identical. The 37° C. temperature was lethal while those at 4° and 13° C.

failed to produce growth (Table 16, Exp. TC-148). Again the cells incubated at 18° and 23° C. produced large numbers of cells and almost identical growth curves.

The data of the two experiments were averaged together and the resulting growth curves plotted (Figure 24). Each point on this curve represents the mean number of cells in eight culture tubes.

The plotted data show clearly that steelhead embryonic cells cultured in Eagle's BM produced more growth at 18° and 23° C. than at any of the other temperatures tested. It should be mentioned that these cells had been in stock cultures incubated at 18° C. for approximately six weeks. They could have become adapted to this environment which might have influenced the results.

Experimental work dealing with this subject has continued since the termination of this portion of the research. Results of these later experiments have revealed that the incubation temperature of 23° C. is at least equal to or perhaps better than 18° C. for the growth of salmonid cell cultures. Cultures incubated at 25° C., however, have produced less growth than those exposed to 18° C. There is also information indicating that 28° C. is lethal for these cell cultures.

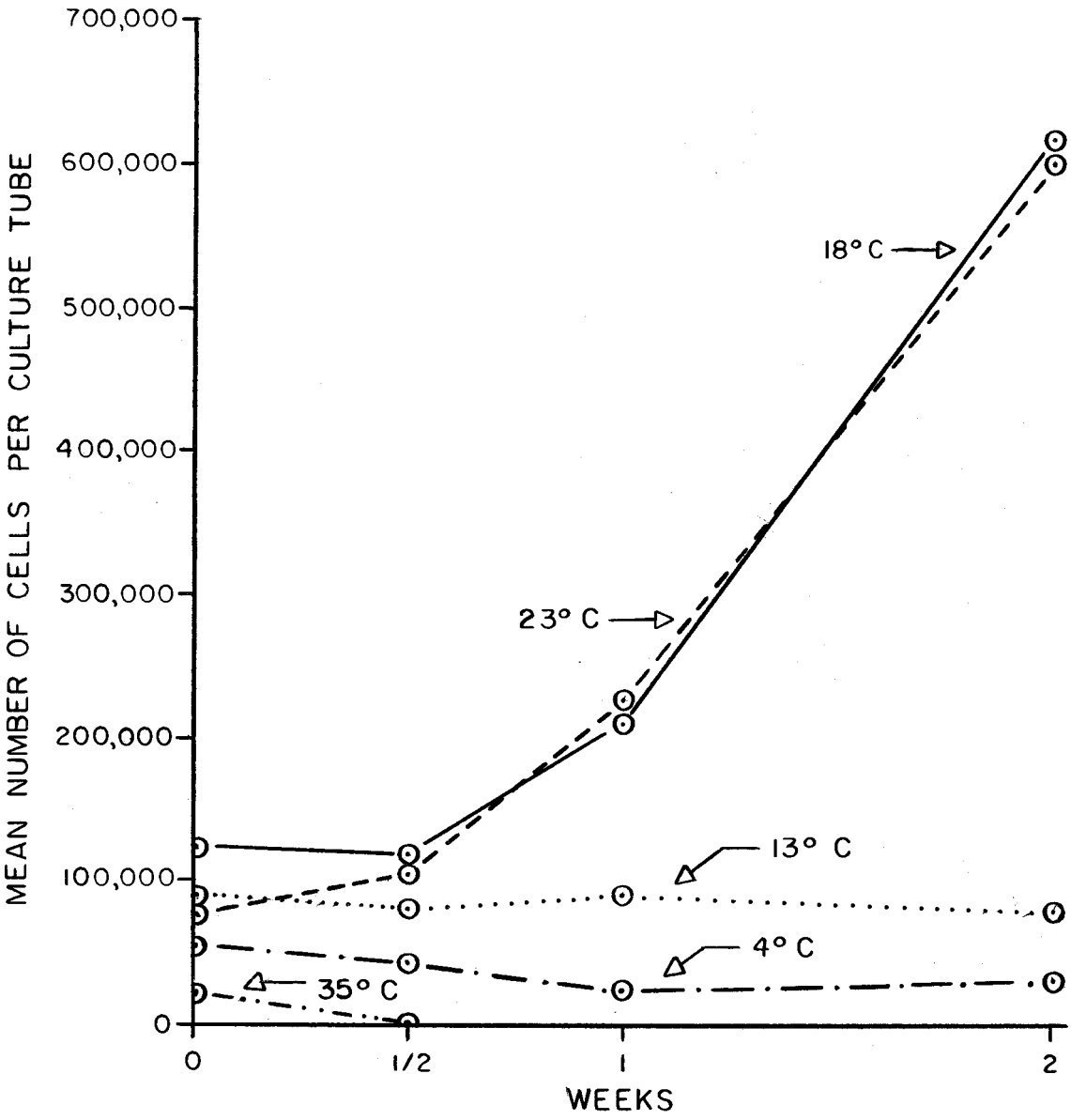

Figure 24. The effects of temperature on the growth of steelhead embryonic cells cultured in Eagle's basal medium with 20 percent agamma calf serum.

Observations on the Changes in pH of Eagle's MEM with 20 Percent Agamma Calf Serum in Cultures of Chinook Salmon Embryonic Cells

The purpose of these experiments was to determine changes in the pH of Eagle's MEM (with 20 percent agamma calf serum) in cultures of chinook salmon embryonic cells at various stages of growth. Cells prepared from stock cultures which had been transferred twice prior to use were resuspended in the test medium at a concentration of 600,000 per ml, and inoculated into roller tubes. One ml of medium containing the cells was introduced into each of 50 roller tubes. The cultures were incubated at 18° C. throughout the 28-day observation period. Four tubes were removed at intervals of zero, one, two, three, and four weeks for growth determination. The nutrient fluids from two of these four tubes employed for cell enumeration were used for pH determination. Observations were obtained at intervals where no cell count was made by removing the fluid from two tubes for pH measurement. Cultures used for these determinations were discarded. In both cases the pH was determined on each of the two tubes and a mean pH value calculated. The nutrient fluid was changed on all remaining cultures at intervals of 2, 6, 12, 16, and 27 days. The new fluid introduced had been adjusted to pH 7.3 $\pm$ 0.05.

Results of the first experiment revealed that within 10 hours after planting the cells the pH of the medium had risen from 7.3 to 8.12 (Table 17). When fresh fluid at pH 7.3 was

Table 17. Observations of pH changes in Eagle's minimum essential medium with 20 percent agamma calf serum as the nutrient fluid for actively growing cultures of chinook salmon embryonic cells

Experiment No. TC-154

Growth in cells[1] per ml of medium	Time	Tube 1 pH	Tube 2 pH	Mean pH
306,100	0	7.30	7.30	7.30
	10 hours	8.04	8.20	8.12
	24 hours	8.00	8.03	8.02
	*2 days	7.80	7.88	7.84
---	3 "	--	--	--
	4 "	7.70	7.70	7.70
	5 "	7.60	7.60	7.60
	*6 "	7.50	7.50	7.50
650,700	7 "	7.70	7.70	7.70
	8 "	7.50	7.50	7.50
	9 "	7.50	7.50	7.50
	10 "	7.43	7.42	7.42
	11 "	7.26	7.26	7.26
	*12 "	--	--	--
1,244,300	14 "	--	--	--
	15 "	7.28	7.28	7.28
	*16 "	--	--	--
1,364,400	21 "	--	--	--
	*27 "	--	--	--
2,354,200	28 "	7.16	7.20	7.18

(continued next page)

Table 17. (continued)

Experiment No. TC-156

Growth in cells[1] per ml of medium	Time	Tube 1 pH	Tube 2 pH	Mean pH
531,600	0	7.30	7.30	7.30
	10 hours	8.00	8.00	8.00
	24 hours	7.90	7.90	7.90
	*2 days	7.76	7.78	7.77
730,400	3 "	7.75	7.75	7.75
	4 "	7.71	7.75	7.73
	*6 "	7.43	7.43	7.43
1,198,200	7 "	7.65	7.63	7.64
	8 "	7.45	7.45	7.45
	*10 "	7.22	7.22	7.22
	11 "	7.55	7.55	7.55
1,289,000	14 "	7.12	7.20	7.16
	*16 "	--	--	--
	17 "	7.63	7.63	7.63
	20 "	7.33	7.31	7.32
1,524,500	21 "	--	--	--
	*24 "	7.13	7.25	7.19
1,376,000	28 "	7.00	6.98	6.99

[1]Mean number of cells in four culture tubes.

*Nutrient fluid changed in cultures. The pH of new fluid was adjusted to 7.3 $\pm$ 0.05. pH determinations indicated for these intervals were taken from the old fluid removed.

added on the second and sixth day, the pH again increased to about 7.7. Fluid added on the 12th day did not show this pH rise, nor did fluids added later. At termination of the experiment the pH of the fluid was found to be 7.18. Growth in the cultures was excellent, increasing from 306,100 at the base line count to over two million cells per culture by the end of the experiment.

In a second experiment carried out in the same manner, there again was an increase in alkalinity of the medium after planting the cultures, and pH values remained above the adjusted 7.3 through the first 11 days (Table 17, Exp. TC-156). pH values below 7.3 were not obtained until after 20 days incubation. There was also indication in this experiment of an increase in the pH of the medium 24 hours after a fluid change. This was noted one day after the medium had been changed on the 6th, 10th, and 16th day in this experiment but not after the change on the 27th day. Growth was also satisfactory in this test, increasing from 531,600 at the base line count to over a million cells per ml of medium during the 28 days of incubation.

It was concluded from these results that there is an initial increase in pH of the nutrient fluid of the culture from 7.3 to about 8.0 followed by a very slow decline reaching approximately 7.0 (neutrality) in about 28 days. These high pH values were maintained in spite of the fact that fluid

changes were made with medium adjusted to pH 7.3. It is also of interest to note that in several cases when the medium was changed an increase in pH occurred 24 hours thereafter.

The importance of these findings were believed to be:

1. The pH levels never dropped below 7.0 even after four weeks of incubation. This was important in culture maintenance, allowing long intervals between fluid changes. It is a characteristic of these salmon cells, but not of HeLa cells, and suggests some important difference in metabolism worthy of further investigation.
2. The pH rise after medium renewal is also of interest and obviously must have a metabolic basis. This also deserves further study.

Estimation of the Mean Cell Division Time for Coho Salmon Embryonic Cells

It was desired to obtain an estimate of the mean cell division time for coho salmon embryonic cells from various cultures grown under the same conditions. Sixteen observations were obtained from several experiments in which cells had been planted from subcultures at a concentration of 300,000 per ml in Eagle's BM with 20 percent agamma calf serum and incubated at 18° C. When individual graphs indicating the increase in total number of cells per culture tube with time were plotted, portions of each curve showed where growth was occurring at a

constant rate. The time between two points showing a twofold increase in cells was considered one generation time. Variation in the time required to double the number of cells in a culture under identical experimental conditions ranged from 89.76 to 280.80 hours (Table 18). The mean cell division time was estimated to be 182.6 hours. This would seem to indicate a rather slow division rate for coho salmon embryonic cells when cultured under these conditions. It is possible that the cell division time might be shorter at a temperature of about 23° C. and in a better nutrient medium such as Eagle's MEM.

Cell Line Studies

Throughout this research cell cultures of various tissues have been maintained for study under conditions of continuous cultivation. This was done in an attempt to establish a stable cell line from the tissues of a salmonid fish. The advantages of a cell line are obvious. There is no longer the need to return to the intact animal as a cell source because these cells have become adapted to continuous cultivation and may be grown, transferred and multiplied under laboratory conditions in a cell culture system indefinitely. The RG-2 rainbow trout gonad cells represent a very successful effort along this line (52, p. 1065-1066).

Cultures were prepared by the enzyme dispersion method and planted in milk dilution bottles. A total of 22 cultures

Table 18. Estimate of the mean cell division time for coho salmon embryonic cells inoculated at 300,000 per ml of Eagle's basal medium with 20 percent agamma calf serum and incubated at 18° C.

Observation number	Increase in cell population (per ml of medium)		Total cell increase	Time required to produce increase (hours)
	From	To		
1	200,000	400,000	200,000	143.28
2	100,000	200,000	100,000	148.80
3	200,000	400,000	200,000	162.96
4	400,000	800,000	400,000	140.40
5	200,000	400,000	200,000	106.80
6	400,000	800,000	400,000	224.64
7	200,000	400,000	200,000	207.84
8	150,000	300,000	150,000	168.48
9	200,000	400,000	200,000	89.76
10	400,000	800,000	400,000	168.48
11	300,000	600,000	300,000	151.68
12	600,000	1,200,000	600,000	169.59
13	200,000	400,000	200,000	280.80
14	200,000	400,000	200,000	210.96
15	200,000	400,000	200,000	202.08
16	200,000	400,000	200,000	247.20
			Total time =	2923.68
			Mean cell division time =	182.6

were established and studied under conditions of continuous cultivation. Seventeen have now been terminated as a result of death caused by contamination, toxicity, or inability of the cells to maintain themselves in continuous culture (Table 19). Cells with culture numbers less than 90 were grown in LY medium with 20 percent human serum and those with numbers greater than 90 were cultured in Eagle's basal medium with 20 percent agamma calf serum. Transfers of these cultures were made when microscopic inspection indicated growth was heavy enough to warrant such action. The number of cultures were expanded or reduced depending on the amount of cells harvested from a given group. Transfers (subcultures) were accomplished by the addition of 0.25 percent trypsin to all bottles comprising a particular culture. After the cells were freed from the glass they were pooled and centrifuged (600 rpm for 15 minutes) and the trypsin solution removed. The cells were then resuspended in the nutrient fluid to give cell concentrations of 300,000 to 600,000 per ml and replanted in bottles. All cultures were incubated at 18° C. Embryonic cells of salmon, trout and steelhead trout grew best under these conditions. The hepatoma cells obtained from diseased rainbow trout also grew well. Cultures now terminated which did not become victims of contaminating microorganisms or chemical toxicity produced interesting cellular changes prior to death. These cultures, particularly TC-70, 87, 107, 98, 60, and 95, seemed initially

Table 19. Results of unsuccessful attempts to establish a stable cell line from fish tissues

Species	Tissue	Culture number	No. of transfers	Age of culture in days at death
Coho salmon	Embryonic	TC-70	13	107
Coho salmon	Embryonic	TC-86A	8	60
Coho salmon	Embryonic	TC 87	13	365
Chinook salmon	Tumor	TC-6	1	84
Chinook salmon	Embryonic	TC-107	4	164
Sockeye salmon	Embryonic	TC-12	3	76
Sockeye salmon	Kidney	TC-19	1	49
Sockeye salmon	Gonad	TC-24	2	61
Steelhead trout	Embryonic	TC-5A	1	70
Steelhead trout	Embryonic	TC-98	3	250
Rainbow trout	Embryonic	TC-86B	3	66
Rainbow trout	Hepatoma	TC-35	4	86
Rainbow trout	Hepatoma	TC-41	1	18
Rainbow trout	Hepatoma	TC-46	2	16
Rainbow trout	Hepatoma	TC-52	2	67
Rainbow trout	Hepatoma	TC-60	4	129
Rainbow trout	Hepatoma	TC-95	2	156

quite normal. They did, however, enter a critical period in which the cells became enlarged, vacuolated and granular. Cell division appeared to halt and death soon followed.

Five culture series are still viable after 4 to 13 transfers and results thus far have been most encouraging (Table 20). The cells were prepared for primary culture and subsequently transferred (subcultured) in the same manner as the first group of cultures already discussed. The one important change has been the substitution of Eagle's minimum essential medium with 20 percent agamma calf serum for Eagle's basal medium containing this serum. The cultivation of these cells will be continued to determine if a stable cell line can be obtained from one or more of the groups. Two of the five groups of cultures are over a year old and there is indication they can survive transfer and expansion. The interval between transfers has varied; however, when possible the cells were subcultured each month. If cell growth became abundant enough prior to one month, transfers were made sooner.

Cultivation of Rainbow Trout Hepatoma Cells

In 1960 a routine disease examination of live rainbow trout shipped from Idaho to California revealed that large numbers of these fish had nodular tumors of the liver. The condition referred to as hepatoma or hepatic carcinoma was subsequently found in stocks of hatchery-reared trout over most of the

Table 20. Results of experiments presently in progress directed toward development of a stable cell line from fish tissues

Species	Tissue	Culture number	No. of transfers	Age days [1]
Coho salmon	Embryonic	TC-119	13	383
Chinook salmon	Embryonic	TC-114	13	405
Chinook salmon	Embryonic	TC-152	6	167
Steelhead trout	Embryonic	TC-137	8	267
Rainbow trout	Hepatoma	TC-149	5	212

[1] Number of days since culture series was started as of March 13, 1964.

United States. Because trout are utilized as food by humans and because of the wide spread interest in all forms of cancer, this high incidence of hepatoma in these animals has received a great deal of attention. Rucker et al. have discussed the events which transpired as a result of the discovery of this disease and has described the pathology (35, p. 3-7). Wood and Larsen also described the pathology and in addition commented on the geographical distribution, incidence and possible etiology of hepatoma in rainbow trout (55, p. 471-479). The factors which seem most responsible for this condition have been discussed by Snieszko. It is his opinion that results obtained thus far indicate nutrition, genetic make-up, and metabolism are the most likely factors influencing the occurrence of hepatoma in

trout (39, p. 145-149).

It is also interesting to note that this same disease has been reported in rainbow trout hatcheries located in northern Italy (5, p. 524 and 537). A recent report by Jackson and Wolf indicates the condition may be caused by the addition of cottonseed meal to the diet (45, p. 676-678). These authors suggest that a carcinogen is contained in cottonseed meal and is either some portion of the meal itself or has been introduced during cultivation or processing. There appears to be no infectious agent involved and it seems to be the considered opinion of most investigators that diet or diet plus other environmental factors are responsible for hepatoma in hatchery-reared trout.

The interest in hepatoma shown by this present investigation was only concerned with the tumors as a source of cells for cultivation. The Roaring River trout hatchery operated by the Oregon State Game Commission furnished adult two- and three-year-old rainbow trout which had obvious tumors. The cells from these tumors were examined in tissue spreads, histological sections, ultra-thin sections for the electron microscope, and under conditions of cultivation in tubes, bottles, and Rose chambers. Normal trout liver tissue was also examined for comparison with the abnormal material.

Tissue spreads were examined in the phase microscope. Liver cells from healthy trout were normal in appearance

(Figure 25). Some fatty degeneration was noted; however, this is commonly found in hatchery-fed trout. Hepatoma cells were often laden with vacuoles. This was the most obvious difference noted. In one advanced tumor examined, several groups of odd-shaped cells were observed. These cells were greatly distended and a fibrous material could be seen in the cytoplasm (Figure 26). Two experienced cytologists independently expressed the opinion that these filaments represented endoplamic reticulum, but similar filaments were not seen in the normal liver cells. Only one tumor examined by this method revealed these unusual appearing cells.

In sections stained with hematoxylin-eosin the tumors appeared as densely staining nodular areas (Figure 27). Mitotic figures were not as numerous as might be expected in cancerous tissue. Vacuoles were observed in all histological sections prepared from this tissue (Figure 28). These same vacuoles were also present in ultra-thin sections of the tumor examined in the electron microscope. The cause and content of these vacuoles were not determined.

Cultivation of the hepatoma cells was accomplished by both the fragment (explant) and enzyme dispersion methods (Tables 2 and 3). The fragment method for the primary culture, followed by treatment with trypsin (0.25 percent), for transfer to subcultures, was also used. Repeated attempts to culture normal liver cells of rainbow trout by all these methods failed.

Figure 25. Tissue spread of normal liver cells from rainbow trout prepared for examination with the phase microscope. Liver cells are suspended in Hanks' BSS. (X 533)

Figure 26. Tissue spread prepared from hepatoma tissue of a rainbow trout and examined with the phase microscope. Cells were suspended in Hanks' BSS. Note the distended cytoplasm containing the spiral filaments. (X 533)

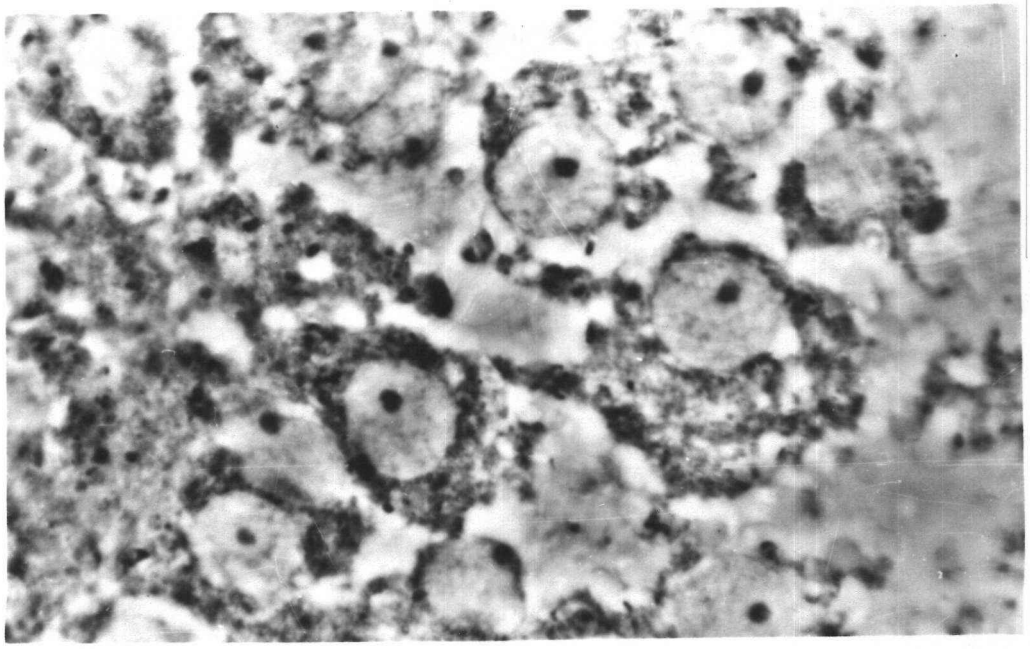

Figure 25.

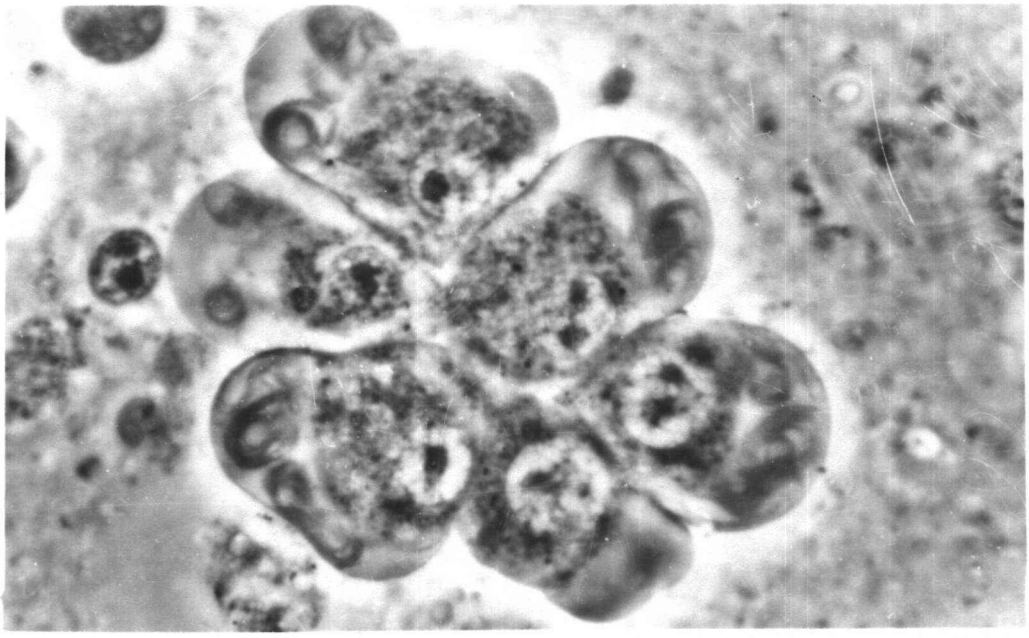

Figure 26.

Figure 27. Section of rainbow trout liver bearing a hepatoma. The tumor can be seen as the dark staining area across the lower portion of the photograph. Note the fibrous tissue invasion in the center of the field. Normal tissue can be identified as the light staining area at the top of the field. The tissue was stained with hematoxylin-eosin dye. (X 133)

Figure 28. Section of hepatoma tissue excised from the liver of a rainbow trout. The vacuoles present in this section of tissue are characteristic of those observed in most of the tumors examined. Tissue was stained by the same method indicated above. (X 533)

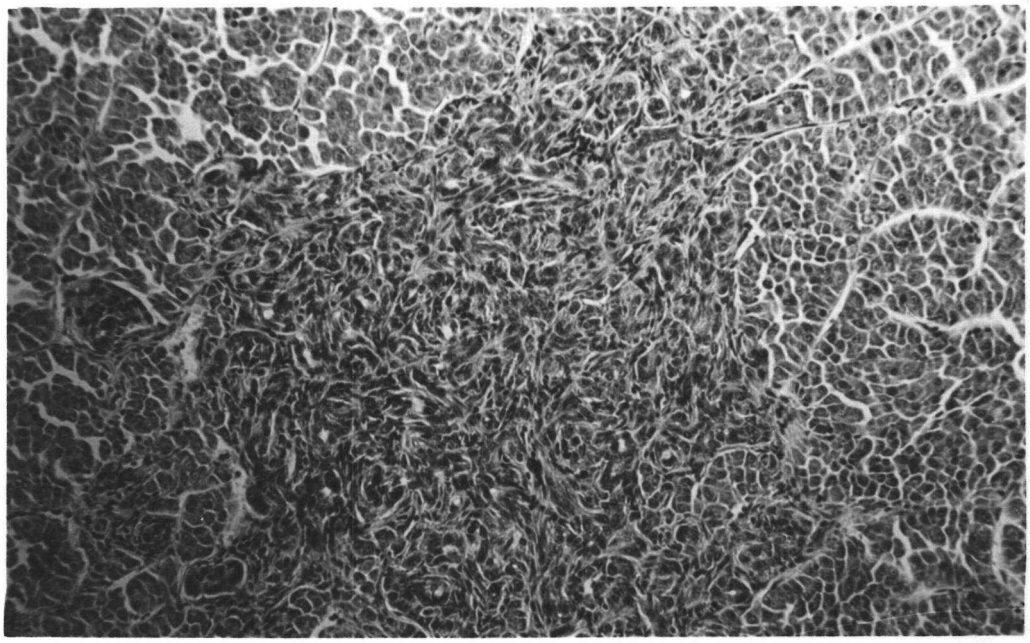

Figure 27.

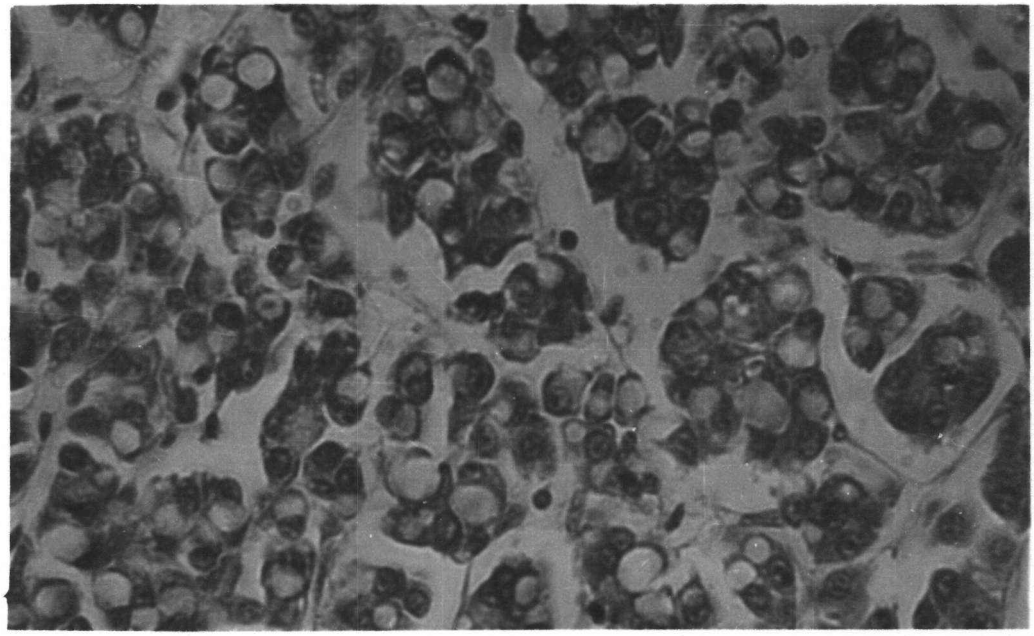

Figure 28.

The cultures prepared by the enzyme dispersion method were planted at one million cells per ml of nutrient fluid. The primary cultures required approximately one month before new cells appeared. This initial lag phase in growth was also present in the explant cultures. When primary cultures were transferred in subculture, however, they reacted much the same as described for the stock cultures of embryonic cells. New cells were observed in subculture after approximately one week of incubation. The inoculum for the subcultures was generally 600,000 cells per ml of nutrient fluid. Hepatoma cultures were composed of large epithelial-like cells; however, fibroblastic cells were also observed. Mitotic figures were evident in subcultures after one week of cultivation. In one culture incubated for two months, mitotic activity was still in progress. Cell division seemed most active between 14 and 21 days after planting when incubated at 18° C. in Eagle's basal medium with 20 percent agamma calf serum.

Figures 29, 30, and 31 indicate hepatoma cells in the process of mitosis. This culture was prepared on a coverslip in a Leighton tube and incubated for 21 days. The coverslip with cells attached was then removed and stained by the May-Grünwald-Giemsa method. The prophase, anaphase and telophase are represented in these figures. The various steps of mitosis were also observed in Rose chambers containing living tumor cells. It was also of interest to note the bizarre nuclei

Figure 29. Twenty-one day old coverslip culture of hepatoma cells from rainbow trout stained by the May-Grünwald-Giemsa method. These cells were prepared for cultivation by the enzyme dispersion method and have been subcultured twice in Eagle's basal medium with 20 percent agamma calf serum. The two cells shown are in the process of division and have entered the prophase. (X 1,200)

Figure 30. The trout hepatoma cells shown here are from the same culture indicated above and have been treated in the same manner. The large cell in the center of the field is undergoing mitosis and is in the anaphase. (X 1,200)

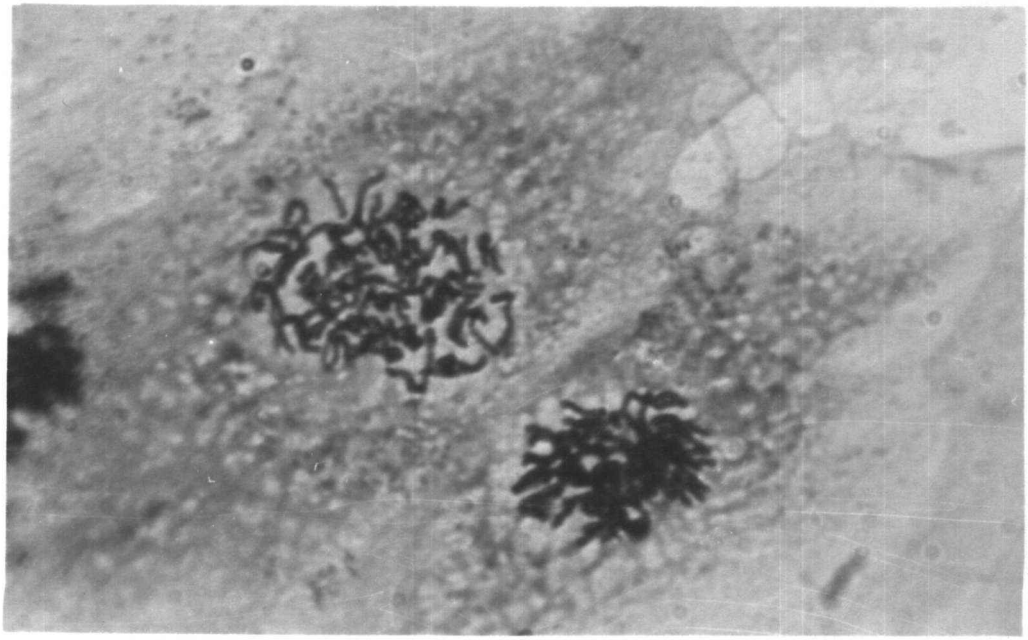

Figure 29.

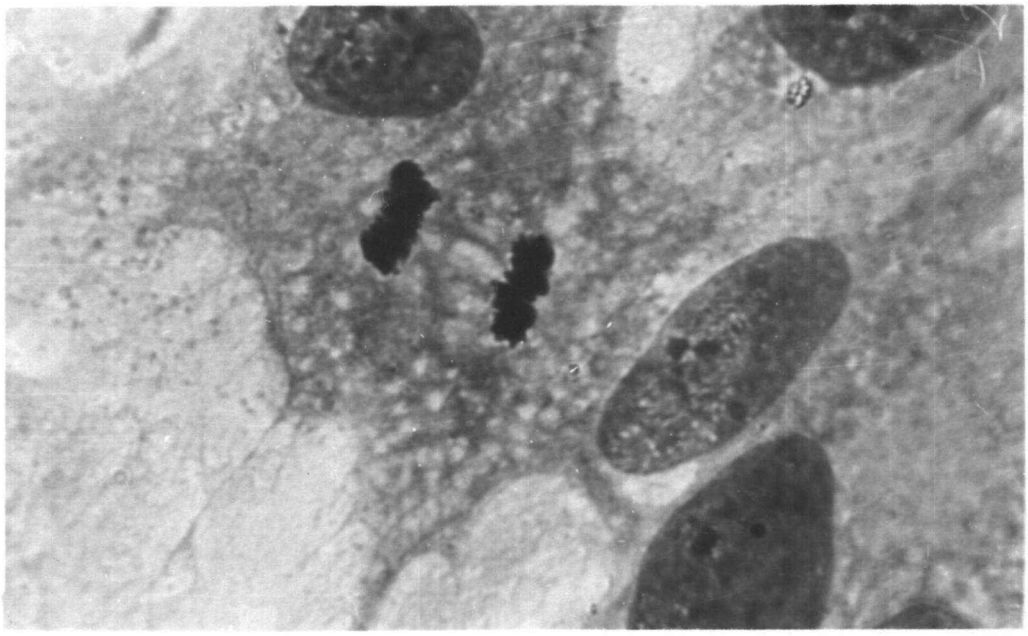

Figure 30.

Figure 31. Trout hepatoma cells from the same culture shown in Figures 29 and 30. The cell in the center has almost completed mitosis and is in the late telophase. (X 1,200)

Figure 32. Trout hepatoma cell from the same culture mentioned above with multiple nuclei and connecting filaments.
(X 1,200)

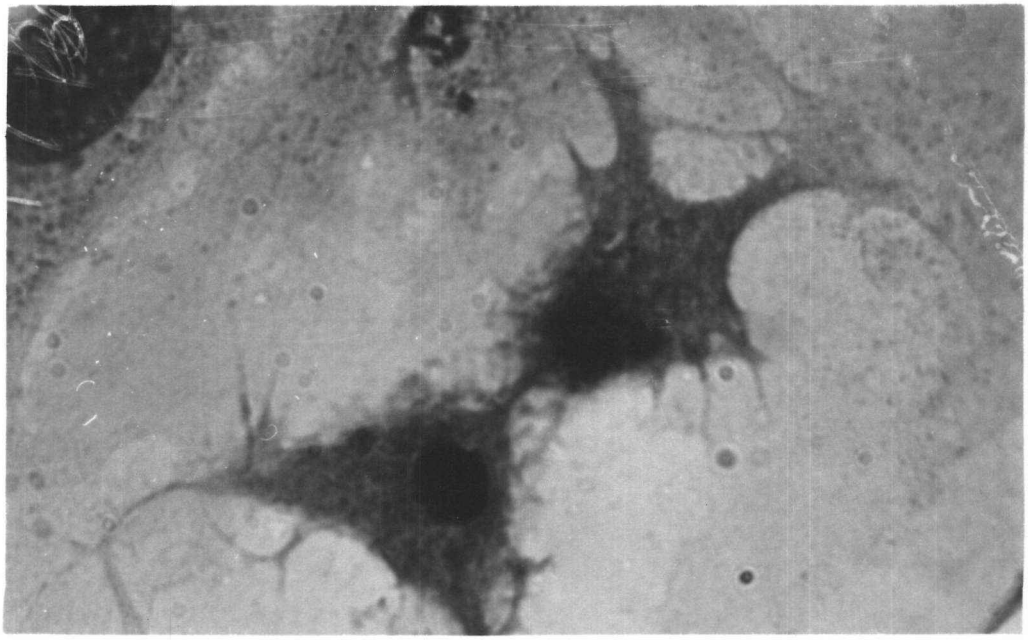

Figure 31.

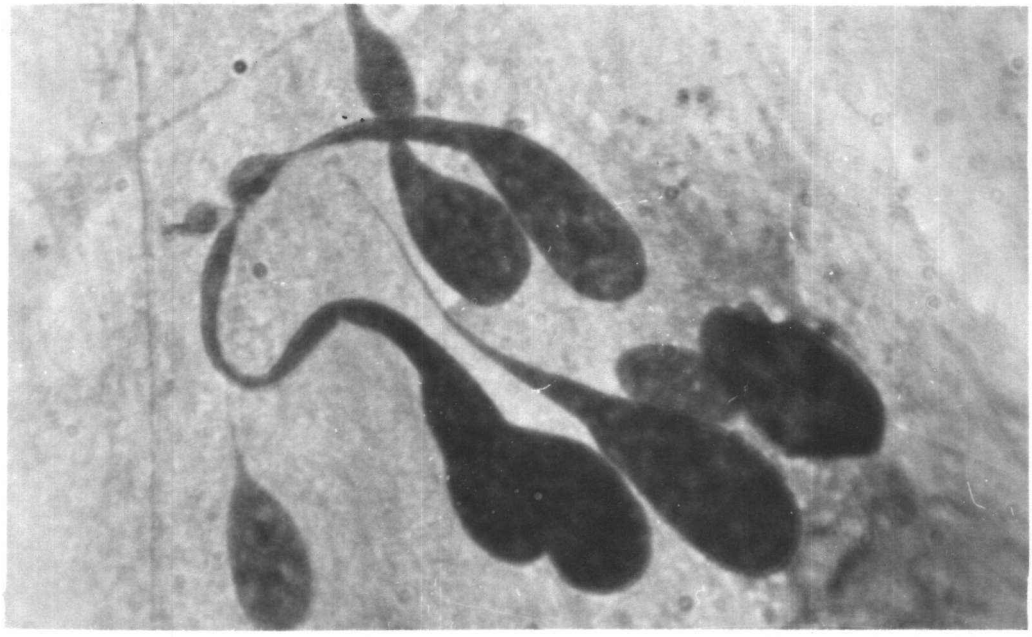

Figure 32.

found in many cells in these stained cultures. Multinucleated cells were present in most cultures and in addition there were odd-shaped nuclei which consisted of a series of lobes often held together by fine filaments (Figure 32). Experiments designed to test the ability of these cells to grow under conditions of continuous cultivation are discussed under that heading in this report. Seven cultures of hepatoma cells have been studied in this series of experiments (Tables 19 and 20). One of these cultures (TC-149) is still viable and has been transferred five times during 212 days of incubation.

DISCUSSION

Virtually all procedures employed in the field of fish pathology have been adapted from techniques used in human and veterinary medicine. It has been the role of the fish pathologist to take these procedures and modify, alter, or improve them so that they may be employed in the study of fish diseases. Techniques for *in vitro* cultivation of cells and tissues of fish are no exception. As already mentioned, information concerning the cultivation of fish cells was very limited when compared to that available for other animals. Interest in cultivation of fish cells has, however, increased. This interest has been stimulated by the need for laboratory methods applicable to the study of viral agents infectious for fishes (46, p. 249-256) (44, p. 1-36).

Results of experiments in this report indicate the relative value of different methods for the *in vitro* cultivation of cells from tissues of salmonid fishes and the importance of various factors influencing their survival and growth in cell culture systems.

The fragment or explant technique and the method of enzyme dispersion for preparation of monolayer cultures as developed for this work have been described in detail. The enzyme dispersion technique was the method of choice for reasons already stated; however, further effort will be required before this technique is completely satisfactory for all tissues. The

complete inability to produce viable cultures of normal liver cells and the problem of obtaining dependable cultures of kidney, gill, and air bladder cells indicate the need for further study. This technique does appear satisfactory for the preparation of primary cultures of embryonic, fin, and hepatoma cells. Low temperature (4° C.) trypsinization lasting approximately eight to ten hours has been used successfully in certain laboratories for the preparation of primary cultures of fish and monkey kidney tissue (53, p. 1890-1891) (1, p. 575-577). This modification of the enzyme dispersion method was tested several times without any indication of improvement for the preparation of primary cultures from tissues of salmon and steelhead trout. The fragment method followed by enzyme treatment was successfully utilized for the preparation of several cultures of trout hepatoma cells.

The long term incubation of stock cultures has been a very helpful tool in this research project. Embryonic tissues were an excellent source of cells for cultivation, producing more and faster growth than any of the other tissues examined. The fact that embryos were available for only a short time each year limited their use until it was observed that these cells could be held in large numbers for long periods of time without an unreasonable amount of care. This was one of the most important practical developments arising from this investigation. It makes possible both examination with viruses, and

metabolic studies of the cells.

Trypsin (0.25 percent) was the most useful enzyme examined during the course of this work. Excellent cell suspensions were obtained by the treatment of embryonic, fin, and hepatoma tissue with this enzyme. These cell suspensions when planted in primary culture produced viable cultures which could be utilized for experimentation. Trypsin was also employed for the transfer of these primary cell cultures in subsequent subculture.

Determination of the proper cell inoculum for trypsinized primary cultures was one of the more important observations made. Cultures which do not receive approximately one to one and a half million cells per ml of medium either fail to survive at all or grew so slowly as to be of no value. The cell density was found to be most important in order to ensure growth. Until this was learned, progress of the research was slow. It was only after the optimum cell inoculum was determined that it became possible to establish the stock culture system as a source of cells for experimentation. Cells inoculated at this concentration appeared to attach to the glass within a few hours after planting and most had stretched out over the surface after one or two days. There appeared to be a definite relationship between the concentration of cell inoculum and the ability of cells to survive and grow in culture. The presence of larger numbers of cells, at least

to a point, was apparently stimulatory for a certain proportion of the cells in the culture.

The importance of cell inoculum for subcultures was also examined. Culture tubes with 300,000 cells per ml in Eagle's BM experienced a one-week growth lag. This was repeatedly observed in various experiments where this concentration of cells was employed. The cells attached to the glass within a few hours after planting and by two days most of them had spread out and exhibited processes. These cells nevertheless did not start to divide until after one week of incubation. They could be generally expected to enter the logarithmic growth phase between the first and second week of incubation.

Cultures seeded with 600,000 cells per ml of medium also underwent an initial growth lag; however, it was reduced to one-half week. These cells began active growth between one-half and one week. There was also indication that the growth rate was more rapid under these conditions than in the cultures inoculated with 300,000 cells per ml of medium.

When the concentration of the inoculum was increased to 900,000 cells per ml of medium there was still a one-half week growth lag, but this was followed by a faster growth rate than with the smaller inocula.

Results of these experiments show that the lag phase is reduced and that growth is stimulated by increasing the cell concentration in the inoculum. There was also some reason to

believe that cell survival was enhanced at the higher planting levels. A cell concentration of 600,000 per ml of medium was considered adequate for planting subcultures. While the lag and growth rate were about the same as that obtained with the higher inoculum, the preparation required fewer cells.

All of these findings suggest that some growth factor is supplied by the cells in the inoculum and that enough of this factor to reduce the lag to a minimum is only supplied by an inoculum of 600,000 cells or more per tube, or by 1.0 to 1.5 million cells from trypsinized primary tissue.

The four media compared to determine their ability to produce growth of salmon cells were all found to induce growth, forming suitable cell cultures. The Eagle's BM and YELP gave best results in both experiments. Eagle's BM has been used in stock cultures and for routine preparation of other cultures in the laboratory with good results. It has now been replaced with Eagle's MEM as a result of experimentation which indicated the latter medium is a superior nutrient fluid for these cells. The new and improved Eagle's MEM contains inositol and sodium pyruvate not present in Eagle's BM, and in addition higher levels of the essential amino acids. Non-essential amino acids were also present in this medium. All five media used in these experiments were developed for the <u>in vitro</u> cultivation of warm-blooded animal cells. It was interesting to note that these media were

capable of producing cell growth from tissues of cold-blooded animals indicating the nutrient requirements of these cells must be similar to those of warm-blooded animals.

None of the media examined here were chemically defined. Blood serum was required in order to ensure growth of the cultured cells. Any additional work with these cells should certainly include experiments directed toward development of a chemically defined growth medium. Critical nutrition studies await the solution of this problem.

Antibiotics were not found to be toxic to these cells at concentrations as high as 1000 units of penicillin, 1000 micrograms of streptomycin, and 100 units of Mycostatin. This was decidedly helpful as it was impossible to excise certain organs aseptically. The embryonic tissues used were probably always contaminated with bacteria and fungi. To obtain this tissue it was necessary to rupture the shell allowing the embryo to pass through the break. No suitable method was found to remove microorganisms from the outer surface of the eggs without creating a toxic situation for the tissue. The planted tissue therefore was unavoidably contaminated and without the assistance of antibiotics none would have survived. Cultures in which contaminants were not controlled by the antibiotics were discarded.

Vitamin B_{12} was not included in the commercially prepared Eagle's BM. It was thought that this compound might be

stimulatory for cell growth and was therefore tested at concentrations of 0.5, 1.0, and 2.0 mg per liter of Eagle's BM. It not only failed to enhance cell growth but was inhibitory. Growth differences between the control and test cultures were reduced far enough to be statistically significant. To determine what factors were involved, the experiment should be repeated and other concentrations examined. It is possible that the concentrations tested were too high and that reduced levels of the vitamin in this medium would give different results. No explanation for the reduced cell growth as a result of exposure to the vitamin is available.

Oxaloacetic acid was also tested at three different concentrations (2.5, 5.0, and 10.0 mM) in Eagle's basal medium. Parker felt that the addition of this compound at the 5.0 mM level to media was very beneficial for the growth of warm-blooded animal cells. This four-carbon unit plays an important role in the Kreb's cycle and it was assumed that its addition would stimulate energy production and therefore create a more favorable growth condition. This was not true as the addition of oxaloacetic acid was inhibitory to the cell cultures. While this could be attributed to increased CO_2 production, believed to be inhibitory as a result of tests with CO_2 in the atmosphere of incubating cultures, the pH of the medium did not indicate that more CO_2 was being liberated into the nutrient fluid. However, it should be pointed out that one percent

CO_2 in the atmosphere caused inhibition of cell growth without obvious alteration of the pH of the medium.

Homologous fish serum was found to be toxic for fish cells. This was demonstrated several times in steelhead trout embryonic cell cultures and was also observed in cultures of chinook salmon embryonic cells. The reason is not known; however, one possibility may be the source of serum. In order to obtain enough serum to conduct these experiments it was necessary to remove blood from mature adult salmon and steelhead. The blood chemistry of these animals may be altered by the physical and physiological stress placed on them as a result of migration and maturation. Horse serum, although not toxic, was not satisfactory as a serum supplement in the medium of the cell cultures because growth was inadequate.

Human serum was used extensively during the early part of this work and was found to be beneficial for cell cultures as long as it was employed in any of the media at the 20 percent level. Agamma calf serum was found superior as a supplement, especially in Eagle's MEM. Such supplemented media were favorable for the cultivation of embryonic cells from Pacific salmon and steelhead trout. Calf serum also was used with success in several cultures, but was inferior to the agamma calf serum. The reason for this difference was not determined, however, there may have been some toxic material present in the gamma globulin fraction of the calf serum which was growth

inhibitory.

It has generally been considered beneficial to culture warm-blooded animal cells in a CO_2 atmosphere of three to eight percent primarily to assist in pH control (7.3-7.4) of media not strongly buffered and for the stimulation of growth for certain cells. Initial experiments with the fish cell cultures employed CO_2 levels of five and eight percent, which have been routinely used for the culture of warm-blooded animal cells incubated at 35-37° C. These levels were totally unsatisfactory for culture of fish cells, which were incubated at 18° C. At this temperature CO_2 is more soluble than at 35° C. and consequently a sharp decrease in pH of the medium was encountered. Although levels of one and three percent CO_2 did not create an unfavorable pH, inhibition of chinook salmon embryonic cell growth was consistently observed. It was considered that the CO_2 used in these tests might have contained impurities which were causing the reduced cell growth. However, with a new tank of CO_2 (99.9 percent pure) the same inhibitory effect was observed using coho salmon embryonic cells. This inhibition represents an interesting metabolic problem deserving investigation in further studies of this type.

Incubation temperatures of 18° and 23° C. were found to be most satisfactory for cell growth. Temperatures of 28° and 37° C. proved to be lethal for salmon and steelhead embryonic

cells. Four and 13° C. were not lethal; however, they failed to promote growth. There appeared to be a lack of agreement between the temperature favoring the growth of the intact animal (approximately 10-15° C.) and that which is optimum for growth of the animal cells in tissue culture. Because the cells used to prepare the cultures had been stored for some time at 18° C., the optimum *in vitro* temperature could have resulted from adaptation to this temperature. To study the question of adaptation future experiments should employ cultures pre-exposed to test temperatures. It would also appear desirable to study temperatures intermediate between 18° and 25° C. at increments of 1°.

The pH of cultures of chinook salmon embryonic cells in Eagle's minimum essential medium was observed to increase from 7.3 to approximately 8.0 shortly after planting. The pH of the fluid remained on the alkaline side throughout most of the experiment; values near neutrality were found only after 24 to 28 days incubation. This was in spite of periodic fluid changes in which the pH of the new medium had been adjusted to 7.3. It was also observed in several cases that the pH of the medium increased 24 hours after a fluid change.

It is generally believed that the initial increase in pH is a result of loss of CO_2 from the medium, coupled with inactivity of the cells. This does not, however, explain the continued presence of alkaline pH values while active cell

growth was in progress, nor the fact that these cultures never produced acid conditions in the fluid. Possibily some alkaline material liberated into the medium by the cells as a result of normal metabolism could cause pH changes such as those observed. The pulse in alkalinity detected after several of the fluid changes may have been induced by the release of an alkaline end product following the rapid utilization of some added nutrient. This is another metabolic property of these cell cultures deserving investigation in future studies.

Regardless of the cause of this reaction the effect appeared to be an advantage for the cultures as they were not exposed to hydrogen ion toxicity. The pH pattern exhibited by these cells appeared to be an important factor in the long term incubation of stock cultures. These cultures were maintained at 18° C. with a minimum amount of care, requiring fluid changes at two to three week intervals and transfers every four to six weeks.

The development of cell cultures which could be carried out under conditions of continuous cultivation was one of the initial objectives of this program. Until recently proper conditions for continuous propagation of cells has not been available. Results have been more encouraging since the change to Eagle's MEM with 20 percent agamma calf serum. Development of a stable cell line from any one of the cultures indicated in Table 19 would be an asset to further cell culture studies as well as for virus research. This is a

distinct possibility in view of the fact that two culture series have now been carried through over 12 transfers during a period of about one year. Cells from hepatoma tissue have been watched with interest in this regard. A cell line of abnormal or cancerous cells would afford interesting material for study. More effort has been put into the studies over the past several months. The cell inoculum has been more precisely determined and the progress of the cultures observed and followed closely. An increase in the incubation temperature from 18° to 23° C. is being considered.

Hepatoma tissues from rainbow trout were employed in several studies, the most important of which involved continuous cultivation of these cells. Observations believed to be of major interest have been the heavy vacuolation of the cells, the formation of the odd-shaped nuclei in culture, the prolonged lag in growth of primary cultures, and the distended fiber-containing cells found in one tumor. The studies have not been quantitative in nature. Cultivation and experimentation with hepatoma cells were considered so complex as to require a separate investigation.

SUMMARY AND CONCLUSIONS

1. The fragment or explant technique applied to flask or tube cultures afforded a convenient and reliable method for the cultivation of a variety of tissues from salmonid fishes. Such cultures have been used in tissue culture studies and it is believed they could be employed in certain types of virus investigations.

2. The enzyme dispersion technique using trypsin has been developed for use in this work for certain kinds of tissue. Embryonic tissue of Pacific salmon and steelhead trout was routinely prepared for cultivation employing this method. Excellent cultures of hepatoma tissue have been established by the enzyme dispersion technique. This method was more advantageous than the fragment method, and most of the experiments reported in this thesis were carried out with cultures prepared in this way.

3. The enzymes collagenase, papain, hyaluronidase, pancrease and RDE were compared with trypsin to determine their ability to liberate cells from embryonic tissue for the preparations of primary cell cultures. All but pancrease gave high cell yields. Collagenase, papain and hyaluronidase were also compared with trypsin to determine their toxicity for cells prepared and planted in primary culture. All the test enzymes appeared to be more toxic than trypsin. Qualitative tests indicated RDE was very toxic for steelhead embryonic

cells. Pancrease, tested in the same manner as RDE, did give rise to viable cultures after cells had been separated from the tissue and planted in primary culture by means of this enzyme. Trypsin then was the only satisfactory enzyme studied for cell dispersion and subsequent growth of the dispersed cells.

4. The optimum cell concentration in the inoculum for primary culture of embryonic cells was found to be from one to one and a half million cells per ml of medium. This planting level also appears to be adequate for seeding hepatoma and fin cells in primary culture. This proved to be a very critical factor in the preparation of such cultures. The discovery of and adherence to this optimum concentration made possible the preparation of successful cultures routinely, where prior to that time results were irregular and often unsuccessful.

5. The effect of three concentrations of cell inocula for the preparation of subcultures was examined. Cells planted at 300,000 per ml exhibited a growth lag of approximately one week. Cultures receiving 600,000 and 900,000 cells per ml of medium retained a lag phase; however, it was reduced to one-half week. Cell survival and growth rate were also increased by elevating the concentration of cell inoculum. An inoculum containing 600,000 cells per ml was found to be optimum for seeding subcultures.

6. Eagle's BM, YELP, LY, and Hanks'-YE media, each containing 20 percent agamma calf serum, produced excellent

growth of embryonic cells. The Eagle's BM and YELP, however, gave about one-fourth more growth over a four-week period than did the other two media.

7. The improved Eagle's MEM was compared with Eagle's BM to determine which of these preparations were best able to stimulate growth of embryonic cell cultures. Both media were supplemented with 20 percent agamma calf serum. The Eagle's MEM was significantly more stimulatory for growth of the cultures than was the Eagle's BM. This was by far the best nutrient medium of any that were tested and was selected as the standard medium for all cultures of these cells.

8. The addition of 25 percent by volume Eagle's BM medium from an actively growing culture to 75 percent by volume of this same medium to be used for initiating new cultures did not stimulate growth of the cells.

9. Antibiotics were used routinely throughout this work. Qualitative experiments indicated concentrations as high as 1000 units of penicillin, 1000 micrograms of streptomycin, and 100 units of Mycostatin per ml of culture medium showed no detectable evidence of toxicity. Prolonged exposure of cultures to 100 units of penicillin, 100 micrograms of streptomycin and 25 units of Mycostatin per ml of culture medium also gave no indication of a toxic effect.

10. Vitamin B_{12} introduced into Eagle's BM with 20 percent agamma calf serum at concentrations of 0.5, 1.0, and

2.0 milligrams per liter not only failed to stimulate but was found to be inhibitory for the growth of steelhead embryonic cells.

11. Oxaloacetic acid failed to enhance growth of coho salmon embryonic cells when added to Eagle's basal medium containing 20 percent agamma calf serum. Concentrations of oxaloacetic acid tested were 2.5, 5.0, and 10.0 mM. All three exhibited an inhibitory effect on the growth of these cells.

12. Homologous fish serum was found to be toxic for steelhead trout embryonic cells at concentrations of 10, 20, and 40 percent in LY medium. Horse serum was examined under the same conditions as the fish serum and while not toxic, failed to support growth. Human serum in LY medium produced growth of these cells when added at the 20 and 40 percent levels; however, the 10 percent concentration failed to initiate cell growth.

13. Twenty percent agamma calf serum in Eagle's BM produced significantly better growth of chinook salmon embryonic cells than did human, agamma human, or calf serum added at the same concentration in this medium. This was definitely the best serum component of any that were studied and was adopted for all culture work with these cells.

14. The growth of coho salmon embryonic cells was inhibited by incubation in an atmosphere of one and three percent CO_2. The inhibition was not a pH effect. It was the

exact opposite of the stimulation of growth by CO_2 reported for some mammalian cells.

15. Steelhead trout embryonic cells were found to grow best, and almost equally well at 18° and 23° C. Thirty-five and 28° C. were lethal for these cultures. Four and 13° C. failed to permit cell growth. There is indication that 25° C. may be inhibitory but not lethal.

16. The pH of Eagle's MEM used as the nutrient fluid in cultures of chinook salmon embryonic cells has been observed to increase from the adjusted level of 7.3 to about 8.0 shortly after planting the cells. This rise in pH was followed by a slow decline as growth increased and over two weeks of incubation was required to reach pH values below 7.3. There was also indication of an increase in pH of this medium 24 hours after a fluid change was made. The alkaline pH maintained in the medium of these cultures over long periods of time was believed to be associated with their ability to withstand extended periods of incubation without frequent fluid changes.

17. A number of cell cultures have been examined under conditions of continuous cultivation. Five of these preparations were still viable at the time of writing and have been maintained for varying periods of time. Two of them have been carried through 13 transfers or subcultures over a period of a little over one year.

18. Cells obtained from the hepatic carcinoma of rainbow trout have been cultured and studied during the course of this work.

19. The mean cell division time for coho salmon embryonic cells inoculated at 300,000 cells per ml of Eagle's BM with 20 percent agamma calf serum and incubated at 18° C. has been estimated to be 182.6 hours.

20. Determination of methods for the preparation of explant and enzyme dispersed cell cultures and the factors influencing their growth has given reliable techniques for the in vitro cultivation of cells from tissues of Pacific salmon and steelhead trout. In addition, other important factors made available by this work were the optimum concentration of cell inoculum for primary and subcultures, proper medium with serum supplement for cell growth and the optimum temperature range for incubation. The development of the stock culture technique whereby large numbers of cultures may be stored and used as required for experimental purposes has been most important. The results described in this thesis are at present being employed for the study of the biological and physical properties of the sockeye salmon virus and for studies regarding the physiology of fish cells under conditions of cultivation.

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