1964

The Establishment and Maintenance of Goldfish Cell Lines in Tissue Culture

Helen Mae Kroeker
Loyola University Chicago

THE ESTABLISHMENT AND MAINTENANCE OF GOLDFISH CELL LINES IN TISSUE CULTURE

by

Helen Mae Kroeker

Microbiology Department

Stritch School of Medicine

A Thesis Submitted to the Faculty of the Graduate School of Loyola University in Partial Fulfillment of the Requirements for the Degree of Master of Science

June

1964

LIFE

Helen Mae Kroeker was born in Chicago, Illinois, in 1940.

She was graduated from St. Xavier Academy, Chicago, Illinois, in 1954, from St. Xavier - Mother McAuley High School, Chicago, Illinois, in 1958, and from St. Xavier College, Chicago, Illinois, in 1962, with the degree of Bachelor of Arts.

She began her graduate studies at Loyola University in September, 1962.

ACKNOWLEDGMENT

Sincere gratitude is extended to Doctor Thomas J. Bird who suggested this problem and aided in its fruition.

I also wish to extend thanks to Doctor Frank Frigan for advice in the performance of this work.

TABLES OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION AND PURPOSE

The cultivation of tissues *in vitro* is a relatively young field of science. One of the first attempts at studying the functional properties of cells and how they affect or are affected by their immediate environment was performed by von Reckinghausen (1886) who kept amphibian blood cells alive under various conditions for as long as 35 days.

The first tissue culture experiments were carried on by Roux (1885) who by maintaining the neural plate of a chick embryo in warm saline, proved the closure of the neural tube to be a function of the constituent cells and not the direct effect of mechanical pressure from the adjacent structures.

Harrison (1907) made observations on the living nerve fiber.

Carrel (1914) succeeded in keeping a strain of chick connective tissue cells alive and actively multiplying for 34 years.

Thomson (1914) initiated a different approach to tissue culture along with Strangeways and Fell. This was the start of "organ culture", a technique whose aim it is to maintain small fragments of tissues in a state as close as is possible to their in vivo situation, rather than trying to make cells grow as rapidly as possible.

Maitland and Maitland (1928) developed a simple method for virus multiplication.

Up to this time original methods of tissue culture as fashioned after the meticulous aseptic techniques of Carrel, were extremely tedious. Thus, the labor required to keep cultures free from contamination deterred many biologists from entering the field.

With the stimulus of World War II., the study of chemotherapeutic agents lead to the discovery of antibiotics. The incorporation of antibiotics into tissue culture media lessened the problems of tissue culture to a considerable extent. No longer were the exacting original techniques required for cultural asepsis. There was a resurgence of interest in the field as a practical art.

A great impetus was given to tissue culture as a

field when Enders (1948) *et al.* showed that the polio virus could be cultivated *in vitro* in the absence of nervous tissue.

The contributions of these and others have aided in the development of procedures that have proved and are proving to be advantageous to all fields of experimental biology and medicine. The importance and potential of tissue culture in the areas of morphogenesis, cancer research and virology is far reaching.

Tissues of mammalian, amphibian, and avian origins have been used a great deal for tissue culture studies, but comparatively few observers have used fish tissues.

Since fish are poikilothermic, the development of fish cell lines has many possibilities. In their natural environment they metabolize at relatively low temperatures. If cells of fish origin could be cultivated and stored at low temperatures, they would be useful for cultivating agents which cannot be maintained at the higher temperatures required by some of the mammalian cell lines. They may one day provide an interesting vehicle for viral research.

The purpose of this work was two-fold. The primary objective was to establish a fish cell line in continuous culture from fish cells of varied tissue origins. The second objective was to explore the tolerance of such cells to variations in media, antibiotics, temperature, pH, and the means by which they could be stored.

CHAPTER II

LITERATURE REVIEW

Osowski (1914) observed cellular movement in fragments of tadpoles and trout (Salmo Gairdneri), which were maintained for 24 hours in Ringer's solution. Lewis (1916) studied embryonic tissue maintenance using sterile sea water as a tissue culture medium. Drederer (1921) in studying the behavior of embryonic fish ectoderm cells in tissue culture (from Fundus heteroclitus), used a sea water base and fish bouillon. These cultures were only viable for 10 days. Chlopin (1922) cultured tissues from pike and crusian carp in rabbit plasma, "diluted with homogenous extract".

The above works comprise the rudimentary beginnings of the culturing of fish tissues in vitro. These first attempts involved primarily the maintenance of fish embryos.

The next trend of investigation is seen in the works of such investigators as Grand (1935, 1938, 1941), Gordon (1938, 1941), and Cameron (1935, 1941), who concerned themselves with the morphological and physiological aspects of fish melanomas and leiomyomas.

Schumberger (1949) studying neoplastic goldfish tissue, added embryonic fish extracts to salt solutions enriched with chicken, human cord or fish sera,

Soret and Sanders (1954) succeeded in propagating the virus of Eastern equine encephalomyelitis in fish embryos. Wood (1955) was able to isolate and identify agents which were causative of a mycosis-like granuloma of salmon.

Grützner (1956), of the Robert Koch Institute, Berlin, examined the application of tissue cultures of Lebistes reticulatus (Peters) and Macropodus opercularis (linne) in virology. An attempt was made to demonstrate the lymphocystis virus and the virus causative of carp pox in tissue cultures of tropical fish. Specific diagnostic cell alterations were observed 14-18 days after infection with carp pox in tissue cultures of Lebistes reticulatus.

In the attempts to demonstrate the virus of lymphocystis, morphological cell alterations appeared, however, their specificity requires more extensive examination. They were able, in some instances, to successfully transmit lymphocystis disease <u>in vivo</u>

from viral inoculums grown in these tissue cultures. Electron micrographs of the tumors show what may be the elementary bodies of the virus. More investigative work must be carried out before a positive identification can be made.

Grützner (1958) successfully cultivated the liver and kidney tissues of Tinca vulgaris (a viviparous carp) in vitro. Monolayers developed within 5 - 6 days and could be maintained in a healthy condition for 3 - 4 weeks. The medium employed consisted of SM 199 (synthetic medium 199) supplemented with beef amniotic fluid plus 15-20% calf serum; other media used for culture, but which gave poor growth, were (1) isotonic medium 199 plus 20% calf serum, (2) Lactalbumin-hydrolysate (isotonic) plus 20% calf serum, (3) Lactalbumin-hydrolysate plus isotonic 199, plus 20% calf serum, (4) beef amniotic fluid (isotonic) plus 15-20% calf serum, and (5) beef amniotic fluid plus Lactalbumin-hydrolysate (isotonic) plus 30% calf serum. These cultures were carried through three sub-cultures. The optimal growth was achieved at 20°C.

One of the most fruitful endeavors which involved fish tissue culture was reported by G. Bargen and A. Wessing (1960) of the Bonn University Zoological Institute. They cultivated embryos of Lebistes reticularis (viviparous toothed carp, guppies) for use in studying a virus which leads to the formation of malignant tumors in various tropical fish. Their observations

are based on monolayer cultures and plasma clot cultures. The nutrient medium contained 1 part embryo extract (9 - 10 day old chicken embryo), 4 parts P.C. (phosphate containing) solution and 4 parts human umbilical cord serum or an artificial nutrient medium, (T.C.M. 199, Morgan, Morton and Parker), to which 3% umbilical cord serum is added. The cells were cultivated 2 - 3 weeks without transfer and were maintained through sub-culturing for 6 - 8 weeks. Observations made during the first few days after establishing the cultures, revealed epithilial cells which give way to fibroblastic forms as the cultures increased with age. The following observations of cellular structure were noted:

1. The nucleus is oval or sometimes dumbbell shaped.
2. Nucleoli are sharply defined.
3. Mitochondria can be seen in freshly grown fibroblasts. They were unusually long: a few strands exceed the dimensions of the nucleus and extend from the cell center to the smallest cellular branch.
4. Golgi apparatus is evident after staining with sulfates.
5. A cell wall structure was found that was peculiar to epithelial cells only.

In order to make the observations cited, the fixation and staining process developed by K. E. Wohlfarth-Bottermann (1957) was employed. It involves these basic phases: Fixation of the cells with osmium tetroxide (isotonic) 1% and potassium dichromate 1%. This is followed by exposure to a mixture of 1% phosphotungstic anhydride and uranyl acetate .5% in 70% methanol. After exposure of the cells to the above procedure, the cell population is fixed in a manner in which all of the structural elements of the living cell are preserved and are visible microscopically.

Wolf and Dunbar (1957) cultivated adult teleost tissues in vitro (trout and goldfish). Trout (Salmo gairdneri, Salvelinus fontinalis, and Salmo trutta), was successfully cultured for 65 days at 19°C or lower in 20% serum, 30% synthetic medium 199, 45% Earl's solution and 5% fish embryo extract. Good results were attained with human cord, human homologous sera or bovine amniotic fluid at 20%. Temperatures below 20°C were thought to be essential for growth.

CHAPTER III

MATERIALS AND METHODS

Section A:

Selection of Fish Type and Tissue Types

Previous work with fish tissue culture has centered on trout (Salmo sp.). To a limited extent, goldfish (Carassius auratus) have been studied, however, there is little information concerning their response to their physical and chemical environment. They have not been maintained in continuous culture for an extensive time period. Since so little is known about a fish which is so readily attainable, and one which might have great worth as a tissue culture tool, goldfish were chosen as the species to be used in this investigation.

Three tissues were chosen for culturing; whole fish, fish muscle (from the pectoral region), and fish heart muscle.

Culturing of whole small goldfish was based upon the assumption that a greater initial yield of cells might be obtained

after enzymatic tissue digestion.

Fish muscle and fish heart were chosen respectively since they could be obtained, through aseptic dissection, in a state free from bacterial contamination; a problem which posed difficulty in culturing whole small fish.

Methods of Tissue Preparation

Prior to culturing, the fish were maintained for one week to 10 days in tap water containing the following antibiotics:

Penicillin	100 units/ml
Streptomysin	100 µg/ml
Fungizone	5 µg/ml

Water was changed twice daily.

The general procedure for tissue preparation follows the methods of Parker (1961) and involves the following steps:

1. Tissues were obtained under aseptic conditions.
2. Tissues were cut into discrete fragments.
3. The fragments were washed several times in phosphate buffered saline (prepared according to Kalter, 1963) at a pH of 7.5.
4. The fragments were suspended in 0.25% trypsin (Difco 1:250). This suspension was placed in a trypsinizing flask (Bellco), on a magnetic stirrer for specific, time intervals.

5. The trypsinized cells were harvested and washed, by centrifugation, with Hank's (Cappel) salt solution.

Hank's salt solution is prepared from Cappel 10 x stock solution. The final solution contains the following:

	Expressed in grams/liter
NaCl	8.00
KCl	0.40
$CaCl_2$	0.14
$MgSO_4 \cdot 7H_2O$	0.10
$MgCl_2 \cdot 6H_2O$	0.10
$NaH_2PO_4 \cdot H_2O$	———
$Na_2HPO_4 \cdot 2H_2O$	0.06
KH_2PO_4	0.06
Glucose	1.00
Phenol Red	0.02
$NaHCO_3$	0.35
Gas Phase	Air

(pH $7.2^{\pm}$)

6. The washed cells were suspended in growth medium and counted by means of a Spencer Bright Line hemacytometer.

7. Aliquots of these suspensions were then planted in milk dilution bottles (Bellco).

Section B:

Media, Antibiotics and Supplements for Growth

In establishment of a cell line a major factor is the selection of an efficient growth medium and an efficient maintenance medium. To be effective, a growth medium must stimulate maximum yields of metabolizing cells. To be an effective maintenance media, it must support cells in an actively metabolizing state with limits on the degree of multiplication.

Media used for the work was selected as a result of three experiments:

Experiment I

Small whole goldfish, 2 - 3 inches in length, were trypsinized in .025% trypsin (Difco 1:250), prepared according to the recommendation of Marcus (1956). Trypsinization was carried out for four hours at 10°C. The cells were then washed 2 - 3 times with Hank's solution, suspended in growth medium, counted and inoculated into milk dilution bottles.

The growth medium used consisted of Melnick medium, prepared according to Melnick (1955), with 10% bovine serum and P.S.F. antibiotic solution.

P.S.F. antibiotic solution consists of the following:

Antibiotic	Final Concentration
Penicillin	100 units/ml
Streptomycin	100 µg/ml
Fungizone	5 µg/ml

The freshly inoculated bottles were incubated at 37°C and 20°C.

Samples of media, trypsin, basic salt solution, and serum, were inoculated into Brain Heart Infusion broth (Difco) and Sabouraud's Dextrose agar (Difco) to check for bacterial and for fungal contamination.

Experiment 2

Whole fish cultures were trypsinized, washed, counted and planted according to previously stated methods. This time the growth medium consisted of Melnick medium supplemented with 20% bovine serum and P.S.F. antibiotic solution.

Sterility controls of the media were run according to procedure previously cited.

Experiment 3

As will be seen from the results, excessive contamination was encountered in Experiments 1 and 2. A screening of various media types and various antibiotic types and concentrations was undertaken. An enumeration of media and antibiotics screened is found in Table I, page14.

TABLE I

Code Letter	Base Medium	Antibiotic Type	Concentration
A	Melnick	P.S.F.	4ml/100
B	"	"	3ml/100
C	"	"	2ml/100
D	"	*P.S.F.	1ml/100
E	199(Capell)	Achromycin(Lederle)	1ml/100
F	" "	"	3/4ml/100
G	" "	"	1/3ml/100
H	" "	***Achromycin	1/4ml/100
I	Scherer's(Capell)	"	1/2ml/100
J	"	"	1/4ml/100
K	"	P.S.F.	1ml/100
L	"	"	2ml/100
M	199 (Capell)	**P.S.F.A.	1ml/100
N	"	"	2ml/100

* 1ml P.S.F./100 ml of media ⟶ Final antibictic concentration of
(Penicillin 100 units/ml
(Streptomycin 100 μg/ml
(Fungizone 5 μg/ml

** 1ml P.S.F.A./100 ml media ⟶ Final antibiotic concentration of
(Penicillin 100 units/ml
(Streptomycin 100 μg/ml
(Fungizone 5 μg/ml
(Achromycin 12.5 μg/ml

*** 1/4 ml Achromycin/100 ml media ⟶ Final antibiotic concentration of
(12.5 μg/ml

**** All of the respective media types were supplemented with 20% Bovine Serum

Tissue culture medium MK (monkey kidney) is used as a basal medium to which serum may be added for tissue culture and viral studies. It was described by Melnick (1955) for the culturing of monkey kidney cells for virus propagation and cytopathogenicity studies.

Tissue culture medium Scherer (1955) is a chemically defined basal medium to which serum and other enrichments can be added, as supplements, for use in tissue culture procedures and virus studies. It supports cell proliferation on glass walls of culture vessels, thus providing an excellent means for studying cell susceptibility to virus by direct microscopic examination.

Tissue culture medium 199, prepared according to Morgan, Morton, and Parker (1950), is a chemically defined medium for use in tissue culture and virus studies. It is a nutritive basal medium to which supplementary growth factors may be added for the propagation of tissue cells for morphological, histochemical and physiological studies. It is used for diagnosis, study, detection, titering, and typing of viruses. It has particular advantage in the propagation of poliomyelitis and other viruses. Virus may be serologically differentiated by its use in such techniques as complement fixation, neutralization, hemagglutination-inhibition and hemadsorption tests. An additional use for medium 199 involves its use for preservation of tissues and cells in storage.

Achromycin (Tetracycline Hydrochloride) (Lederle) is a crystalline broad spectrum antibiotic. It is derived from the mold Streptomyces aureofaciens. It is prepared from chlortetracycline by reductive dehalogenation. It is active against a wide range of bacteria and fungi. For this reason it was chosen for use in addition to P.S.F., to increase the range of antibiotic activity.

The procedure for preparation of cells cultured in each respective media types follows the methods of Parker (1961) with the following modifications:

1. Tissues were soaked prior to trypsinization in P.S.F. antibiotic solution for varied time intervals, ranging from 0 - 10 hours.
2. Tissues, after soaking, were trypsinized for for varied time intervals ranging from 1- 10 hours.
3. Following trypsinization, cells were washed, by centrifugation, with Hank's solution.
4. The washed cells were re-suspended in growth medium, counted and planted in milk dilution bottles.
5. Cultures were incubated at 20°C and 37°C.
6. Sterility controls were set up in the usual manner.

Viable monolayer cultures resulting from the initial

cultures were carried on in continuous culture. The procedure employed for the sub-culturing of cells is enumerated below:

1. Bottles containing complete monolayers were incubated 15 minutes at 37° with .025% trypsin, in order to free the cells from the glass.
2. Cells were centrifuged and washed 2 - 3 times in Hank's solution.
3. The cells were re-suspended in growth medium, counted, and inoculated into milk dilution bottles.

Maintenance medium was substituted for growth medium when healthy monolayers were developed and the cells were continuously re-fed with this medium until they were ready to be sub-cultured.

Section C:

Storage of Cells

A problem which poses numerous practical difficulties in the use of tissue culture methods is the storing of cell lines. These stored cells should retain the ability to actively metabolize for use in further sub-culture. A successful long-term process for storage of cell and tissue cultures would eliminate the risks concomitant with continuous growth of a culture over an extended period of time, namely; the increased

possibility of mutation, the possibility of bacterial, fungal and/or viral contamination, and the possible loss of the culture.

One method of storing cells is by means of freezing at extremely low temperatures. The technique used to insure maximum recovery of viable cultures depends upon the particular tissue or cell type.

A method with which the best results have been obtained, and one which is commonly employed today, involves the following steps, (Moline 1964):

1. The cells or tissues to be stored are cooled at a precisely controlled rate. The rate of cooling usually falls within the range of 1°C per minute to 20°C per minute.
2. Protective additives are added to the medium in which the cultures are to be stored. The usual additives are either glycerol or dimethyl sulfoxide in concentrations ranging from 5% to 20%. Glycerol is a hydrophilic and prevents excessive injury to the cells during freezing and thawing. Dimethyl sulfoxide apparently has a vast potential as an additive. It rapidly diffuses into and out of the cell, thus minimizing osmotic shock when the cells are diluted for culturing after thawing.
3. The cells are cooled by use of liquid nitrogen and

stored at liquid nitrogen temperature, -196°C.
For long-term storage temperatures above -100°C allow the formation of ice crystals which is considered as one factor in cell destruction.
Freezing of cells by this method is difficult. Equipment for the constant maintenance of storage temperatures is required.
Thawing of cells must be rapid. A limit of 2 - 3 minutes thawing time has been found to provide optimal survival.

If fish cells could be stored with any degree of simplicity, they would afford a cell line of considerable value. Investigation of the storage potential was undertaken.

With every transfer of cells, 6 ml samples were stored in the freezer at -20°C. The procedure followed was not involved.

Two methods were employed: Half of the sample was suspended in growth medium and placed in the freezer. The other half of the sample was suspended in growth medium, supplemented with 15% glycerol and placed in the freezer. At intervals, the cells were thawed and subcultured.

Section D:

Effects of Temperature and pH

The majority of cell lines in use today require

temperatures of 30° - 37°C for optimal growth. It would be advantageous if a cell line could be established that would have a wider growth temperature range.

Fish are poikilothermic. Fish cell cultures might show optimal growth at temperatures lower than 37°C. Cultures were maintained at temperatures of 10°C, 20°C, and 37°C.

Most mammalian and avian cells are sensitive to changes in the pH of the media. The pH range to which fish cells can be subjected, without loss of viability, was examined.

The media were adjusted to pH ranging from 6 - 8. pH was determined with hydrion paper and verified by pH meter. These media were used to feed the cells for given periods of time. Viability was determined by observation of cell multiplication.

Section E;

Classification of Contaminant

A contaminating microorganism, resistant to P.S.F. antibiotic solution was encountered. The morphological and physiological classification of the bacterium was established.

The morphological data was determined by performing the following:

1. Gram stain according to McClung (1957).
2. Capsule stain according to McClung (1957).
3. Flagella stain according to Leifson (1951).

Diagnostic biochemical tests were run in order to establish

physiological activity of the organism.

Its ability to ferment or oxidize carbohydrates was established for the following sugars; glucose, sucrose, lactose, zylose, mannitol, tetralose, and inositol.

The carbohydrate media was composed of:

Casitone	.1%
Yeast extract	.01%
$(NH_4)_2SO_4$	.05%
Tris Buffer(Difco.)	.05%
Agar	.3%
Phenol red	.001%
Specific sugar	.1%

This medium was formulated by Leifson, (personal communication). The final pH was 6.7.

The ability of the organism to utilize specific amino acids was determined for arginine, lysine, and ornithine. The base medium was iron agar prepared according to the formulation of Edwards and Fife (1961). 10 Grams of the respective amino acid was added to the base.

Citrate medium was prepared according to the formulation of Simmons (1926), at a final pH of 6.7. This test is used to determine the utilization of citrate as the sole source of carbon.

The ability of the organism to produce urease was established by use of the urea test medium prepared according to

the formulation of Rustigan and Stuart (1941). The final pH of the test medium was 6.8.

The agar medium and the reagents for the nitrate reduction test were prepared according to Bailey (1962). The test shows the ability or lack of ability of the organism to reduce nitrates to nitrites or to free nitrogen. The results of a negative nitrate test were confirmed by the addition of a small amount of zinc dust to the medium after incubation. If it were a false negative, the presence of reduced nitrate is revealed by the development of a red color.

The proteolytic capacity of the organism was determined by testing its ability to liquefy gelatin. Nutrient gelatin prepared according to Ewing (1962) was the specific medium used.

There are some microorganisms which possess the ability to deaminate certain amino acids. To find out if the bacterium in question possessed this capacity, the deaminase test following the methods of Ewing (1957) was performed. The specific test medium used was Phenylalanine Agar, and the test reagent was a 10% (w/v) solution of ferric chloride. If phenylalanine has been deaminated to phenylpyruvic acid, a green color develops in the syneresis fluid and in the slant.

CHAPTER IV

RESULTS

Experiments 1 and 2, where medium MK (monkey kidney), supplemented with 10% bovine serum and P.S.F. antibiotic solution was utilized as the growth medium, were unsuccessful. Both of these initial attempts at establishment of fish cell cultures were terminated within 10 - 12 days respectively, due to contamination by a bacterium which was resistant to P.S.F., at the concentrations used.

In both of these experiments, media sterility controls which had been run in Brain Heart infusion broth and on Sabouraud's Dextrose agar remained negative to bacterial or fungal contamination. Thus, the bacterium came from the fish itself and was not introduced from external sources.

Since the contaminant isolated from the cultures

in Experiments 1 and 2 was definitely resistant to P.S.F. at the concentrations of Penicillin 100 units/ml, Streptomycin 100 μg/ml, and Fungizone 5 μ g/ml, not only was there a need for finding a proper growth medium, but also for finding an antibiotic which would eliminate the contaminant common to the fish being used.

The classification of the contaminant from the aspects of its morphological and physiological nature was established. The results of the various determinations will be found in Table I. The organism is a Gram negative polar flagellated rod similar to Aeromonashydrophila.

Experiment 3 entailed the screening of 14 different media types, which were supplemented with various antibiotic mixtures. A listing of media types tested will be noted in Table II. The results of the media and antibiotic screening will be found in Table III.

As can be seen from the results cited in Table III, medias H., J., and M were the most effective. Media H, without Fungizone, was discontinued due to its susceptibility to contamination by mold.

A graphical comparison of the relative value of the three media types will be found in Figure I. Media M , as is illustrated, gave the greatest increase in cell numbers. Media J gave excellent cellular growth, but the increase in cell numbers was more limited than with Media M. These results were to be

expected due to the composition of their basal medias, 199 and Scherer's base respectively.

Cells on these media types survived continuous culture. Since medias J and M were by far more efficient; media M was chosen as the growth medium and media J was chosen as the maintenance medium for further experiments. P.S.F.A. was substituted for the single antibiotic Achromycin in media J. This was done in order to take additional precautions against the possible development of other bacterial or fungal contaminants.

As observed, concomitant with the results of the media screening, it was found that P.S.F. was not effective against contamination unless used at concentrations far in excess of those normally employed. At these high concentrations it was found to be toxic to the fish tissues.

Achromycin was effective. However, it was observed that it was toxic to the fish cells at concentrations in excess of 12.5 μg/ml of growth medium.

To date the cultures derived from small whole goldfish have undergone 28 successful transfers in continuous culture. This is enumerated in Table IV.

After a satisfactory growth and maintenance media were selected, cultures of fish pectoral muscle and fish heart muscle were established. To date the fish muscle cells have undergone 17 transfers. These results are in Table VI. The fish heart

cells have also undergone 18 transfers, as is enumerated in Table V. Sub-culturing beyond 20 passages or over a period of six months is taken as an indication of the establishment of a stable cell line. Preliminary experimentation with the length of exposure to trypsinization and exposure to antibiotic soaking of the tissue fragments prior to trypsinization was performed. For prevention of contamination the most effective period of antibiotic soaking was from 4 to 6 hours of exposure. The best yields of cells were obtained, for the initial establishment of a culture, when the trypsinization process was continued for six hours or more before harvest.

The ability to multiply after storage of the fish tissues was determined. Each time the cells were transferred 6 ml samples were stored at -20°C. A limited number of these stored cells have been thawed and carried in continuous culture. The results so far have been encouraging. Following thawing, cultures of small whole goldfish have undergone transfers to date and are growing satisfactorily. These results are cited in Tables VII and VIII. Cultures of fish pectoral muscle and fish heart muscle have been brought out of storage and are at present growing satisfactorily. These results are in Table IX.

Temperatures of 10°C, 20°C, and 37°C were employed in the cultivation of the various cell cultures. A graphical analysis of the effect of temperatures on the increase in number of cells

of all three types is illustrated in Figure II.

The optimal cell growth or increase was at 20°C; good growth was obtained at 10°C; cells grew at 37°C, but at a very slow rate, and they could not be carried beyond six transfers, without the final loss of the cultures.

The ability of the cells to withstand varied degrees of pH, ranging from 6.0 to 8.0 was determined. It was found that all three types of tissue could withstand this range. The optimum for all tissue types was from 6.8 to 7.2, the best growth being achieved about 7.2. (Table X).

A graph representing the relationship of pH to cell number increase, for all three types of tissues, will be found in Figure III. As seen in the graph the peak of maximal multiplication occurs at pH 7.2.

It was observed that in the initial differentiated cultures, two cell types predominated. The young cultures displayed great numbers of epithelial-like cells. As the cultures underwent sub-culturing the cells began to de-differentiate and the fibroblastic forms began to predominate. (Figures IV through VII.)

CHAPTER V

DISCUSSION

Consideration of all the data reveals certain facts. One problem to consider, concerns the initial establishing of fish cells in culture. In culturing of whole small goldfish, it is difficult to obtain initially sterile tissue preparations. Contamination is introduced from the external body coverings of the fish and from the gastrointestinal tract. This problem was encountered during the first attempts at working with such cultures. One such contaminant predominated in the initial culture experiments. After examining the results of the determination of the morphological and physiological characteristics of the bacterium, a correspondence of the obtained characteristics with those set down for the genus Aeromonas was evident, therefore, the bacterium was designated to be an Aeromonad. A more definitive evaluation places the organism as a

strain of Aeromonas hydrophila. Aeromonas sp. are common to fish both as normal flora and as etiological agents of pathological lesions. The conclusion that the contaminant was introduced by the tissue fragments per se and not from extraneous sources is substantiated by media sterility controls remaining negative and its complete elimination in subsequent studies by use of Achromycin.

Several aspects were considered in arriving at appropriate methods for successful establishment of fish cell lines. A study of exposure of the tissues to antibiotic containing salt solution prior to initial trypsinization was performed and it was observed that prior soaking for 6 hours eliminated contamination.

Trypsinization time was evaluated. Cells harvested after one hour could not be grown in vitro. An interesting phenomenon was that if the cells freed after one hour exposure to trypsin were not removed from the trypsinizing flask, none of the cells (no matter how long the exposure to trypsin) could be grown in vitro. If the first hour's yield was discarded, harvested cells were viable after two hours or more trypsinization. The best yields of cells were obtained after 4 - 6 hours trypsinization. Thus, it is assumed that during the first hour of enzymatic digestion, the fish cells give off some substance which is extremely inhibitory to further cell growth and is toxic to all cells being exposed to them. Either a cell substance is liber-

ated during the first hour of trypsinization, which is inhibitory of growth, or the trypsin itself may combine with liberated substances to become an inhibitory factor.

Experiments were performed to determine if trypsin is inhibitory. The method being to incorporate inactivated trypsin into the culture media. If inhibition is encountered, the next step is a determination of the degree of reversibility. If no inhibition is observed, then a cellular liberation product must have been responsible. The product can be identified by chromatographical analysis.

Incorporation of inactivated trypsin into the media revealed no deleterious effects to cell growth or multiplication. It can, therefore, be concluded that a cellular liberation product was responsible for inhibition.

Figure I reveals medium 199 supplemented with 20% Bovine serum and P.S.F.A. provided an excellent growth medium, because, as per definition, it allowed the maximum cell multiplication. This was media M of the variety of media tested.

Media J, Scherer's medium, supplemented with 20% Bovine serum and 12.5 γg/ml Achromycin supported good cellular growth with emphasis on maintaining cell quality more than quantity.

It has been noted that Achromycin is toxic at concentrations above 12.5 γg/ml media. It was observed that this tetracycline in solution is very acidic. This is most likely attributable to its being commercially prepared as a hydrochloride, which may account for its toxicity.

Whole small goldfish were cultured with ease after a suitable media and antibiotic were determined. Fish pectoral muscle and fish heart muscle do not present the contamination difficultues that are inherent in whole fish cultures, since they are obtained as sterile, when aseptic dissection procedures are followed:

Definite correspondence between the result of all three tissue types cultured can be observed. There is agreement in the several results regarding the influence of such physical factors as pH and temperature on the amount of multiplication for all types of goldfish cells.

It is evident in Figure II that temperature plays an important role in effective culturing techniques. Wolf and Dunbar (1957), believed temperatures below 20° C to be essential for growth of fish cells. In this work it was established that the cells will grow as high as 37° C. The growth rate is slow in comparison to lower temperatures and they did not survive more than seven transfers, but they did grow. At this moment the usefulness of growth at such a temperature is not evident, but such cultures might be fruitful for the study of enzymes active only at such a temperature range, or perhaps may be beneficial for immunological studies and for virological studies.

Considering virological studies, although growth at 37° C is not as good as at lower temperatures, if these fish cells

support viral growth, they may be used for study of some known animal viruses which require temperatures around 37°C. This would enable study of virus adaptability to change of host and environment. For example, Eastern Equine encephalomyelitis has been grown at 19°C in fish embryo cells by Soret and Sanders (1954). This virus is also known to grow in chickens, (normal body temperature of 42°C) and man (normal body temperature 37°C) and insects (normal body temperature 20°- 37°C). Possibly it can be grown in these poikilothermic cells line at temperature of 10°C or less. The transcendence of temperature and species by viruses is as yet not explained. It is known that the common cold virus is grown at 33°C for optimal results. Some Arboviruses and Picornaviruses have been grown in cold-blooded animal cells. Fish cells may yield good growth at 30° - 33°.

This adds value to cell lines of cold-blooded species. These fish cell lines may afford a practical vehicle for viral studies.

In the comparative graph (Figure II) the best growth is achieved at 20°C, and good growth at 10°C. This might be, for by nature fish are cold-blooded and their normal environmental temperature is approximately 20°C. Being cold-blooded they have the ability to adapt to the temperature of their surroundings, thus, they grow well at 10°C. They adapt to growth at 37°C, but it is more incongruous with nature, therefore, it

is poor. Possibly enzyme systems are retarded at 37°C for heat tends to inhibit or destroy enzymes, where cold does not necessarily do so. Their ability to grow at low temperatures may make them valuable as a cell line for studies requiring this temperature range. Growth at 10°C and at 4°- 6°C enables refrigerator storage of such cultures, for they survive at these temperatures without re-feeding for two weeks. A sampling of bottles was maintained at refrigerator temperature. Their metabolism rate slowed but they remain active and healthy. This provides cell lines that minimize maintenance tasks.

Figure III is a graphical evaluation of the effect pH has on fish cell multiplication. Satisfactory cell number increase is attained within an initial range of pH 6.8 - 7.2, the optimal increase occurring at initial pH of 7.2. A marked decrease in multiplication occurs upon exposure to pH 6.0 or pH 8.0. It has been observed that the cells will survive at these limits for two weeks without re-feeding (with media at pH 6.8 - 7.2), but multiplication is almost negligible. The cells are sensitive to these pH limits but are not killed by such exposure. At pH5 and pH 8.5 no growth occurs. Thus, they can be considered as hardy cell lines.

Today a number of disciplines utilize tissue and cell culture techniques for research. The preservation of cells in an unaltered state is a highly desirable prerequisite. The

ability of a cell line to successfully undergo prolonged storage enhances its value as a tool. As previously stated, storage at very low temperatures is an elaborate task requiring special apparatus and the use of culture additives to be successful. In addition the ability of these fish cell lines to survive storage has been investigated. Tables VII and VIII summarize the results obtained for small whole goldfish. Table IX contains the results for fish heart and fish pectoral muscle. These cells will survive storage at -20°C without the use of protective additives. A comparison of the results obtained from cells frozen merely in growth media and cells frozen in growth media containing 15% glycerol show little variation in their ability to be brought back in a healthily metabolizing state and to survive sub-culture. It must be noted here that the data in Tables VII and VIII is based on values obtained at weekly intervals, whereas other data referring to % of cell number increase was based on readings taken at two week intervals. In the original data at readings of two weeks, the percentages in Tables VII and VIII would be increased by approximately 30 - 40%. It can be concluded that no cellular alterations occurred as a result of storage.

Morphologically the cells are small in comparison to some animal cell lines. For example, chick cells are 20 μ in diameter, Hela cells 15 μ and fish cells 10 μ. Cultures of small whole goldfish initially contain cells of both the epithelial and

fibroblastic form. After sub-culture they de-differentiate and and the fibroblastic form is the type surviving. Cultures of fish pectoral muscle and fish heart muscle display the fibroblastic appearance initially and throughout sub-culture.

All three types of fish tissue exhibit a common morphological phenomenon. Young cultures give clearly defined cellular shapes. As they age, microscopically they give the appearance of bone tissue or hyaline cartilage. This characteristic has also been observed by other investigators. Figures IV through VII photographically depict this fibroblastic nature of the fish cells in culture. Work concerning determination of an efficient staining procedure for these fish cells is in progress. This fixative employed for the stained cells in the photographs was Osmium tetroxide 1%. The stain is Delafield's hematoxylin and eosin.

Healthy monolayers develop within 24 - 48 hours. Monolayers become exceedingly thick. Elimination of the serum supplement entirely slows the growth, (monolayers not being complete before 96 hours). However, there is a poor definition of cellular differentiation. Therefore, the serum-free medium is not satisfactory for morphological studies.

Perhaps a serum factor is involved. Studies of incorporation of specific serum fractions, the use of serum ultra filtrates or dialyzed serum may eliminate this complete coalescing of the cells. This is an area of future research.

Monolayers adhere strongly to the glass. If the monolayer becomes extremely thick, the edges begin curling back and a sheet of new cells is laid down in their place immediately. They are very hard to remove from the glass. For transfer a trypsinization time of 20 minutes followed by vigorous agitation to free them from the glass is required. Even after this stringent procedure, many cells are not removed and are lost for transfer. They are very sturdy. After transfer the cells begin to adhere to the glass after 2 - 3 hours.

One side experiment yields an interesting facet regarding cell transfer and the hardiness of the cell lines. There are always some cells remaining on the glass after trypsinization. If the bottles containing remaining monolayer fragments are washed with Hank's salt solution and re-fed, monolayers are again developed within 48 hours. This is supported by the fact that two of the original bottles started in August, have been re-fed after 27 exposures to trypsin and are still actively producing healthy monolayers.

The next phase of this work will involve testing of these cells to determine their usefulness for viral studies. Four viruses will be tested: Sindbis, an arbovirus; Vaccinia, a pox virus; Newcastle's disease virus, a myxo virus; and Poliomyelitus virus, a picornavirus. The objective is to study cytopathogenic effects and other evidence of viral multiplication.

Multiplication will be determined by hemagglutination and neutralization tests.

This work is in progress at the present time; however, due to the scope of such an investigation it will be the subject of future reports and the results are not incorporated as a part of this thesis.

If these goldfish cells are able to support viral growth, they would be valuable cell lines. Tissue culture is always in need of new cell lines for virus research. A line with as flexible a range of survival in terms of physical properties and one which is so readily available, easily established and maintained, has many distinct advantages as a tool for viral and other studies.

CHAPTER VI

SUMMARY

Tissue of Carassius auratus (goldfish) has successfully undergone continuous culture. Three cell lines have been established; whole small goldfish, fish pectoral muscle, and fish heart muscle, respectively.

To date whole fish cultures have been taken through 27 sub-cultures; fish pectoral muscle and fish heart muscle have undergone 18 sub-cultures.

The growth medium consisting of TC medium 199 (Cappel) supplemented with 20% Bovine serum; the maintenance media consisting of TC medium Scherer (Cappel) supplemented with 20% Bovine serum proved satisfactory.

The fish cell lines have been found to grow at temperatures ranging from 4°C - 37°C, the best cell growth and multiplication being observed to occur at 20°C.

Fish cell lines survive a pH range of 6 - 8, the best growth and multiplication occurring at initial pH 7.2.

The cell lines have been observed to withstand a simplified storage process; freezing at -20°C, in growth media without incorporation of protective additives, with no apparent alteration of the cells.

The cell lines are hardy, easily established and maintained, and exhibit potential usefulness, as cell lines for virological studies.

BIBLIOGRAPHY

Bargen, G.v. and Wessing, A., 1959, Untersuchungen Über einen Virusbedingten Tumor bei Fischen. Arch. Virusforschung 9; 521-536.

Cameron, G. and Grand, C.G. and Gordon, M., 1941, Neoplasm Studies. Cancer Research. 1;66.

Cameron, G. and Grand, C.G. and Chambers, R., 1935, Neoplasm Studies. Am. J. of Cancer. 24;36.

Carrel, A., 1914, Present Conditions of a Strain of Connective Tissue Twenty-Eight Months Old. J. Exper. Med. 20;1.

Carrel, A., 1931, The New Cytology. Science. 73;297.

Chain, E., and Others, 1940, Lancet. 2;226.

Chain, E., and others, 1941, Lamcet. 2;177.

Chlopin, N., 1922, Über in vitro Kulturen in dem Embryonalengewebe der Säugetiere. Arch. f. Mikra. Anat., Bonn, XCVI;435-493.

Drederer, P.H., 1921, The Behavior of Cells in Tissue Cultures of Fundus heteroclitus withSpecial Reference to the Ectoderm. Biol. Bull. 41;221.

Duggar, B.M., 1948, Ann. N.Y. Acad. Sci. 51;177.

Ehrlich, P., 1897, Klin. Jahrb. 6;299.

Enders, J.F., Weller, T.H., and Robbins, F.C., 1949, Cultivation of the Lansing Strain of Poliomyelitis Virus in Cultures of Various Human Embryonic Tissues. Science. 109;85.

Finlay, A.C., and others, 1950, Science. 111;85.

Fleming, A., 1929, Brit. J. Exper. Path. 10;226.

Flory, M.E. and Flory, H.W., 1943, Lancet. 1;387.

Flory, H.W. and others, 1949, Antibiotics, London, Oxford University Press.

Gordon, M., and Smith, G.M., 1938, Progressive Growth Stages of Heritable Melanotic Disease in Tissue of Fish from the Day of Birth. Am. J. of Cancer. 24:36.

Gordon, M., 1941, Genetics of Melanomas in Fishes. Cancer Research. 1:656.

Grand, C.G., Chambers, R., and Cameron, G., 1935, Neoplasm Studies. Am. J. of Cancer. 24:36.

Grand, C. G., 1938, Neoplasm Studies. Am. J. of Cancer. 33:394.

Grand, C.G., Gordon, M., and Cameron, G., 1941, Neoplasm Studies. Cancer Research. 1:660.

Grützner, L., 1956, Überprüfung einiger Anwendungsmoglichkeiten der Gewebekulturen von Lebistes reticulatus und Macropodus opercularis in der Virusforschung. Zbl. Bakt., Orig. 165:83-96.

Grützner, L., 1958, Ders,:In vitro-züchtung des Leber und Nierengewebes von Tinca vulgularis Cuv., in Trypsinnierten Einschichtgewebekulturen. Zbt. Bakt., Orig. 173:195-197.

Harrison, R.G., 1907, Observation on the Living Developing Nerve Fiber. Proc. Soc. Exper. Biol. and Med. 4:140.

Kalter, S.S., 1963, Procedure for Roubine Laboratory Diagnosis of Virus and Rickettsial Diseases. Burgess Publishing Company, Minneapolis, Minn.

Leifson, Einar, 1951, Staining, Shape, and Arrangement of Bacterial Flagella. Reprinted from the J. Bact. 62:4.

Lewis, M.R., 1916, Sea Water as a Medium for Tissue Cultures. Anatom.Record. 10:287.

Osowski, H., 1914, Arch. Entwicklungemechen. Organ., 38:547.

Maitland,H.B. and Maitland, M.C., 1928, Cultivation of Vaccinia Virus without Tissue Culture. Lancet. 215:596.

Marcus, P.I., Creciura, S.J. and Puck, T.T., 1956, Clonal Growth in vitro of Epithelial Cells from Normal Human Tissues. J. Exper. Med. 104:615.

Meleney, F.L. and Johnson, B., 1947, J. A. M. A. 133:675.

Melnick, 1955, N. Y. Acad. Sci. 61:755.

McClung, L.S., 1957, General Bacteriology Laboratory Manual, W. B. Saunders Co., Philadelphia and London.

Moline, S. W., 1964, The Low Temperature Preservation of Tissue and Cell Cultures. Linde Cryobiology, Linde Company.

Morgan, J.P., Morton, H.J., and Parker, R.C., 1950, Nutrition of Animal Cells in Tissue Culture. I. Initial Studies on Synthetic Medium. Proc. Soc. Exper. Biol. and Med. 73:1-8.

Parker, R.C., 1961, Methods of Tissue Culture. Paul B. Hoeber, Inc. Medical Division of Harper and Bros.

Paul, John, 1960, Cell and Tissue Culture. The Williams and Wilkins Company, Baltimore.

Roux, W., 1885, Beiträge zür Entwicklumgsmechanik des Embryo. Ztscher. Biol. 21:411.

Scherer, 1953, Am. J. Path. 29 :113.

von Recklinghausen, F.D., 1866, Über die erzeugung von rothen Blutkörperschen. Arch. Mikroskop. Anat. 2:137.

Schumberger, H. G., 1949, Cutaneous Leiomyomas of Goldfish. Am. J. Path. 25:287.

Soret, M.G., and Sanders, M., 1954, In vitro Method for Cultivation of Eastern Equine Encephalomyelitis Virus in Teleost Embryos. Soc. for Exper. Biol. and Med. 87:526.

Strangeways, T.S.P.,and Fell, H.B., 1925-26, Experimental Studies on the Differentiation of Embryonic Tissue Growing in vivo and in vitro. I. The Development of the Undifferentiated Limb-bud (a) When Subcutaneously Grafted into the Post-Embryonic Chick and (b) When Cultivated in vitro. Proc. Roy. Soc. London. s. B. 99:340.

Strangeways, T.S.P. and Fell, H.B., 1926, Experimental Studies on the Differentiation of Embryonic Tissues Growing in vivo and in vitro. LL. The Development of the Isolated Early Embryonic Eye of Fowl when Cultivated in vitro. Proc. Roy. Soc. London. s. B. 100:273.

Thompson, D., 1914, Some Futher Researches on the Cultivation of Tissue *in vitro*. Proc. Roy. Soc. Med. 7:21 (Marcus Beck Lab. Rep.).

Wakeman, S.A., 1944, Proc. Soc. Exper. Biol. and Med. 57:244.

Wolf, K. and Dunbar, C.E., 1957, Cultivation of Adult Teleost Tissue *in vitro*. Proc. Soc. Exper. Biol. and Med. 95:455-458.

Wohlfarth-bottermann, K.E., 1957, Die Kontrastierung Tierischer Zellen und Gewebe im Rahmen Ihrer Elektronemikroskopischen Untersuchung an Ultradünen Schnitten. Naturwissenschaften. 44:287-288.

Wood, E.M., Yasutake, W.T. and Lehman, W.L., 1955, A Mycosis - like Granuloma of Fish. J. of Inf. Diseases. 97:262.

TABLE I

MORPHOLOGICAL AND PHYSIOLOGICAL CLASSIFICATION OF CONTAMINANT

Morphology	1. Gram Negative 2. No Capsule 3. Polar Monotrichous or Polar Mulitrichous Flagella		
	Substance	24 HRS	48 HRS
	Glucose	Fermenter+Acid+Gas	+Acid and Gas
	Lactose	-	-
	Sucrose	+	+
	altose	+	+
	Zylose	-	Alkaline Top,Acid Butt
	Mannitol	+	+
	Tetralose	+	+
	Inositol	-	-
Physiology			
	Citrate	-	+
	Urea	-	-
	Nitrate	-	+
	Gelatin	-	+
	Lysine	-	-
	Argenine	-	+
	Ornithine	-	-
	Deaminase	-	+

TABLE II

TYPES OF MEDIA SCREENED

Code	Base	Serum	Antibiotic	Concentration
A	Melnick	20% Bovine	P. S. F.	4 ml/100
B	"	" "	"	3 ml/100
C	"	" "	"	2 ml/100
D	"	" "	"	1 ml/100
E	199	" "	Achromycin	1 ml/100
F	"	" "	"	3/4 ml/100
G	"	" "	"	1/2 ml/100
H	"	" "	"	1/4 ml/100
I	Scherer	" "	"	1/2 ml/100
J	"	" "	"	1/4 ml/100
K	"	" "	P. S. F.	1 ml/100
L	"	" "	"	2 ml/100
M	199	" "	P. S. F. A.	1 ml/100
N	"	" "	"	1 ml/100

Note: See page for antibiotic concentration conversion factor

TABLE III

RESULTS OF MEDIA SCREENING ON CULTURE OF WHOLE SMALL GOLDFISH

Media Type	Antibiotic Type & Concentration	Average Initial Cell Count	Average Final Cell Counts	No. of Transfers	% Cell Number Increase
A	P.S.F. 4ml/100	250,000/ml	—	0	0
B	" 3ml/100	300,000/ml	—	0	0
C	" 2ml/100	450,000/ml	Contaminated	—	—
D **	" 1ml/100	200,000/ml	"	—	—
E	Achromycin 1ml/100	230,000/ml	—	0	0
F	" 3/4ml/100	200,000/ml	—	0	0
G	" 1/2ml/100	400,000/ml	—	0	0
H **	" 1/4ml/100	450,000/ml	750,000/ml	6	66-2/3% *
I	" 1/2ml/100	300,000/ml	—	0	0
J	" 1/4ml/100	200,000/ml	425,000/ml	23	112%
K	P.S.F. 1ml/100	250,000/ml	Contaminated	—	—
L	" 2ml/100	300,000/ml	"	—	—
M **	P.S.F.A 1ml/100	300,000/ml	752,000/ml	23	150.6%
N	" 2ml/100	450,000/ml	—	0	0

* Cultures wiped out by mold.

(1 ml of P.S.F. (Penicillin 100 units/ml
(in 100 ml of media (Streptomycin 100 µg/ml
((Fungizone 5 µg/ml
(

** Antibiotic conversion factors for concentration per ml media

(1/4 ml Achromycin per 100 ml media (12.5 µg/ml
((Penicillin 100 units/ml (
((Streptomycin 100 µg/ml
(1 ml P.S.F.A. in (Fungizone 5 µg/ml
(100 mls.of media (Achromycin 12.5 µg/ml

1) All media contained 20% Bovine serum
2) pH of Media: 6.8 at time of feeding and transfers
3) All cultures grown at 10°C, 20°C, 37°C,: Data here based on cultures grown at 20°C.

TABLE IV (Part 1)

THE RELATION OF TEMPERATURE TO % OF INCREASE IN CELL NUMBERS FOR WHOLE SMALL GOLDFISH

Temperature	Transfer Number	Average Original Count	Average Final Count	% Increase	Days in Culture
10°	Initial	250,000	-	-	-
20		200,000	-	-	-
37	Inoculum	170,000	-	-	-
10°	#1	150,000	226,000	50.6%	11
20	1	220,000	400,000	81.8%	11
37	1	100,000	123,000	22.0%	11
10°	#2	150,000	223,000	48.6%	18
20	2	210,000	378,000	80.0%	18
37	2	100,000	120,000	20.0%	18
10°	#3	100,000	150,000	50.0%	25
20	3	120,000	227,000	89.2%	25
37	3	200,000	234,000	17.0%	25
10°	#4	110,000	164,000	49.1%	34
20	4	100,000	190,000	90.0%	34
37	4	150,000	177,000	18.0%	34
10°	#5	190,000	283,000	49.0%	41
20	5	100,000	189,000	89.0%	41
37	5	170,000	195,000	14.1%	41
10°	#6	110,000	165,000	50.9%	47
20	6	130,000	240,000	90.7%	47
37	6	100,000	104,000	4.0%	47
10°	#7	120,000	180,000	50.0%	56
20	7	150,000	286,000	90.6%	56
10°	#8	100,000	151,000	51.0%	65
20	8	110,000	208,000	89.0%	65
10°	#9	150,000	222,000	48.0%	74
20	9	100,000	189,000	89.0%	74
10°	#10	110,000	166,000	50.9%	85
20	10	100,000	190,000	90.0%	85
10°	#11	170,000	253,000	48.8%	93
20	11	100,000	190,000	90.0%	93
10°	#12	120,000	182,000	51.6%	99
20	12	150,000	287,000	91.3%	99
10°	#13	100,000	150,000	50.0%	111
20	13	220,000	417,000	89.5%	111
10°	#14	120,000	181,000	50.8%	120
20	14	100,000	190,000	90.0%	120
10°	#15	100,000	151,000	51.0%	128
20	15	180,000	344,000	91.1%	128
10°	#16	100,000	149,000	49.0%	141
20	16	130,000	220,000	90.0%	141

TABLE IV (Part 2)

THE RELATION OF TEMPERATURE TO % OF INCREASE IN CELL NUMBERS FOR WHOLE SMALL GOLDFISH

Temperature	Transfer Number	Average Original Count	Average Final Count	% Increase	Days in Culture
10°	#17	120,000	179,000	49.1%	149
20	17	200,000	382,000	91.0%	149
10°	#18	100,000	151,000	51.0%	161
20	18	190,000	362,000	90.5%	161
10°	#19	110,000	162,000	47.2%	167
20	19	260,000	492,000	89.2%	167
10°	#20	160,000	239,000	49.3%	173
20	20	190,000	361,000	90.0%	173
10°	#21	100,000	150,000	50.0%	184
20	21	130,000	248,000	90.8%	184
10°	#22	100,000	151,000	51.0%	193
20	22	170,000	324,000	90.6%	193
10°	#23	110,000	165,000	50.0%	206
20	23	190,000	363,000	91.1%	206
10°	#24	110,000	165,000	50.0%	214
20	24	155,000	291,000	87.7%	214
10°	#25	100,000	153,000	53.0%	228
20	25	100,000	192,000	92.0%	228
10°	#26	100,000	280,000	47.4%	233
20	26	135,000	253,000	87.4%	233
10°	#27	100,000	148,000	48.0%	241
20	27	170,000	330,000	94.1%	241
10°	#28	100,000	153,000	53.0%	265
20	28	125,000	232,000	85.6%	265

Growth Media : M Maintenance Media : J pH of Media : 6.8

TABLE V (Part 1)

THE RELATION OF TEMPERATURE TO % OF INCREASE IN CELL NUMBERS FOR FISH HEART MUSCLE

Temperature	Transfer Number	Average Original Count	Average Final Count	% Increase	Days in Culture
10°	Initial	250,000	-	-	-
20		250,000	-	-	-
37	Inoculum	250,000	-	-	-
10°	#1	250,000	378,000	51.2%	14
20	1	250,000	450,000	80.0%	14
37	1	200,000	247,000	23.5%	14
10°	#2	100,000	149,000	49.0%	20
20	2	120,000	222,000	85.0%	20
37	2	110,000	138,000	25.4%	20
10°	#3	110,000	169,000	53.6%	26
20	3	100,000	199,000	99.0%	26
37	3	150,000	174,000	16.0%	26
10°	#4	100,000	149,000	49.0%	33
20	4	120,000	225,000	87.5%	33
37	4	120,000	133,000	10.8%	33
10°	#5	150,000	228,000	52.0%	41
20	5	110,000	202,000	83.6%	41
37	5	100,000	113,000	13.0%	41
10°	#6	100,000	150,000	50.0%	47
20	6	170,000	315,000	85.3%	47
37	6	110,000	113,000	2.7%	47
10°	#7	120,000	180,000	50.0%	56
20	7	150,000	285,000	90.0%	56
37	7	110,000	Cells died	-	-
10°	#8	120,000	185,000	54.1%	66
20	8	140,000	251,000	79.3%	66
10°	#9	100,000	153,000	53.0%	75
20	9	160,000	299,000	87.0%	75

Growth Media : M

Maintenance Media : J

pH of Media : 6.8

Initial Antibiotic soak 6 hours

Trypsinization initially 6 hours

TABLE V (part 2)

THE RELATION OF TEMPERATURE TO % OF INCREASE IN CELL NUMBERS FOR FISH HEART

Temperature	Transfer Number	Average Original Count	Average Final Count	% Increase	Days in Culture
10°	#10	150,000	225,000	50.0%	84
20	10	100,000	180,000	80.0%	84
10°	#11	100,000	152,000	52.0%	95
20	11	100,000	194,000	91.0%	95
10°	#12	110,000	163,000	48.1%	104
20	12	170,000	321,000	88.8%	104
10°	#13	100,000	150,000	50.0%	117
20	13	190,000	365,000	92.1%	117
10°	#14	110,000	159,000	43.3%	125
20	14	155,000	290,000	88.6%	125
10°	#15	100,000	153,000	53.0%	139
20	15	160,000	310,000	93.8%	139
10°	#16	120,000	183,000	52.5%	144
20	16	150,000	188,000	25.3%	144
10°	#17	100,000	150,000	50.0%	153
20	17	100,000	192,000	92.0%	153
10°	#18	100,000	160,000	60.0%	167
20	18	175,000	322,000	84.0%	167

Growth Media : M

Maintenance Media : J

pH of Media : 6.8

Initial Antibiotic soak 6 hours

Trupsinization initially 6 hours

TABLE VI (Part 1)

THE RELATION OF TEMPERATURE TO % OF INCREASE IN CELL NUMBERS FOR FISH MUSCLE

Temperature	Transfer Number	Average Original Count	Average Final Count	% Increase	Days in Culture
10°	Initial	200,000	-	-	-
20		200,000	-	-	-
37	Inoculum	200,000	-	-	-
10°	#1	200,000	294,000	42.0%	10
20	1	200,000	396,000	98.0%	10
37	1	200,000	235,000	17.5%	10
10°	#2	150,000	230,000	53.3%	18
20	2	170,000	323,000	90.0%	18
37	2	Cells did not survive second transfer			
10°	#3	100,000	149,000	49.0%	26
20	3	120,000	227,000	89.2%	26
10°	#4	150,000	222,000	41.3%	36
20	4	170,000	320,000	88.1%	36
10°	#5	100,000	150,000	50.0%	45
20	5	110,000	208,000	89.9%	45
10°	#6	100,000	149,000	49.0%	57
20	6	130,000	239,000	83.1%	57
10°	#7	150,000	225,000	50.0%	67
20	7	110,000	208,000	89.1%	67
10°	#8	160,000	240,000	50.0%	76
20	8	100,000	192,000	92.0%	76
10°	#9	110,000	159,000	53.6%	86
20	9	150,000	286,000	92.0%	86

Growth Media : M

Maintenance Media : J

pH of Media : 6.8

Antibiotic soak for 4 hours prior to trypsinization

Trypsinization initially lasted 4 hours

TABLE VI (Part 2)

THE RELATION OF TEMPERATURE TO % OF INCREASE IN CELL NUMBERS

FOR FISH MUSCLE

Temperature	Transfer Number	Average Original Count	Average Final Count	% Increase	Days in Culture
10°	#10	140,000	210,000	50.0%	95
20	10	150,000	284,000	89.3%	95
10°	#11	100,000	150,000	50.0%	106
20	11	155,000	290,000	87.1%	106
10°	#12	137,000	204,000	48.9%	115
20	12	180,000	342,000	90.0%	115
10°	#13	120,000	182,000	51.7%	128
20	13	149,000	284,000	90.6%	128
10°	#14	130,000	200,000	53.8%	136
20°	14	100,000	193,000	93.0%	136
10°	#15	150,000	222,000	48.0%	141
20°	15	110,000	205,000	86.3%	141
10°	#16	100,000	152,000	52.0%	149
20°	16	200,000	390,000	95.0%	149
10°	#17	130,000	179,000	37.7%	163
20	17	100,000	207,000	107.0%	163

Growth Media : M

Maintenance Media : J

pH of Media : 6.8

Antibiotic soak for 4 hours prior to trypsinization

Trypsinization initially lasted 4 hours

TABLE VII

STORAGE RESULTS FOR TISSUES OF SMALL WHOLE GOLDFISH

Time in Storage	Count Put In Storage	Initial Count After Storage	Final Count After Storage	% Increase
2 Mos.	260,000	150,000	210,000	40.0%
2 Mos.	190,000	100,000	158,000	58.0%
3 Mos.	250,000	120,000	191,000	59.1%

Cells were stored at -20°C in Media M

They were brought back on Media M at pH 6.8

TABLE VIII

STORAGE RESULTS FOR TISSUE OF SMALL WHOLE GOLDFISH

Time in Storage	Count Put In Storage	Initial Count After Storage	Final Count After Storage	% Increase
2 Mos.	280,000	140,000	201,000	43.5%
2 Mos.	210,000	110,000	168,000	52.7%
3 Mos.	290,000	170,000	274,000	61.1%

These cells were stored on Media M supplemented with 15% Glycerol

They were brought back on Media M at pH 6.8

TABLE IX

STORAGE RESULTS FOR FISH HEART AND FISH MUSCLE CELLS

Tissue Type	Time in Storage	Count Put In Storage	Initial Count After Storage	Final Count After Storage	% Increase
Fish Heart	5 Mos.	149,000	100,000	222,000	122%
Fish Muscle	4 Mos.	294,000	220,000	395,000	80%

Cells were stored at -20°C in Media M

Cells were brought back on Media M

Counts are average of 5 transfers

Fish Heart	5 Mos.	149,000	80,000	222,000	150%
Fish Muscle	4 Mos.	294,000	220,000	402,000	82.7%

Cells were stored at -20°C in Media M supplemented with 15% Glycerol

Cells were brought back on Media M

Counts are average of 5 transfers

TABLE X (Part 1)

EFFECT OF pH VARIANCE ON % OF CELL NUMBER INCREASE

Tissue Type	Temperature	% of Cell Number Increase at pH Range 6.0	6.8	7.0	7.2	8.0	Number of Transfers
Whole Fish	10°	14.7	49.0	51.6	28.1	7.3	4
	20°	23.0	89.0	89.5	91.1	5.0	4
Fish Muscle	10°	10.9	47.0	49.3	46.0	4.6	4
	20°	17.0	90.0	91.8	90.3	0	4
Fish Heart	10°	8.0	47.7	50.0	47.0	0	4
	20°	11.6	87.0	89.3	87.7	0	4

All transfers were two weeks apart

TABLE X (Part 2)

Cell Counts from which the % of increase in No. of cells was calculated

Whole Fish	pH 6.0 Initial	Final	% Incr.	pH 6.8 Initial	Final	% Incr.	pH 7.0 Initial	Final	% Incr.
10°	150,000	172,000	14.7%	100,000	149,000	49.0%	120,000	182,000	51.6%
20°	100,000	123,000	23.0%	100,000	189,000	89.0%	220,000	417,000	89.5%
Fish Muscle									
10°	110,000	122,000	10.9%	115,000	169,000	47.0%	150,000	224,000	49.3%
20°	100,000	117,000	17.0%	140,000	266,000	90.0%	110,000	211,000	91.8%
Fish Heart									
10°	165,000	178,000	8.0%	170,000	251,000	47.7%	100,000	150,000	50%
20°	120,000	134,000	11.6%	130,000	243,000	87.0%	140,000	265,000	89.3%

Whole Fish	ph 7.2 Initial	Final	% Incr.	pH 8.0 Initial	Final	% Incr.
10°	110,000	141,000	28.1%	150,000	161,000	7.3%
20°	180,000	344,000	91.1%	160,000	168,000	5.0%
Fish Muscle						
10°	115,000	168,000	46.0%	125,000	183,000	4.6%
20°	145,000	276,000	90.3%	110,000	-	0
Fish Heart						
10°	100,000	147,000	47.0%	155,000	-	0
20°	130,000	244,000	87.7%	140,000	-	0

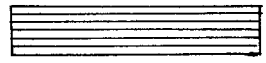
Counts are average values of four transfers

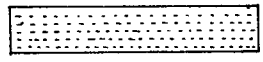
KEY FOR INTERPRETATION

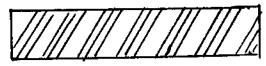
OF FIGURE I

EFFECTS OF VARICUS MEDIA TYPES ON

CELL MULTIPLICATION *

MEDIA TYPE H 

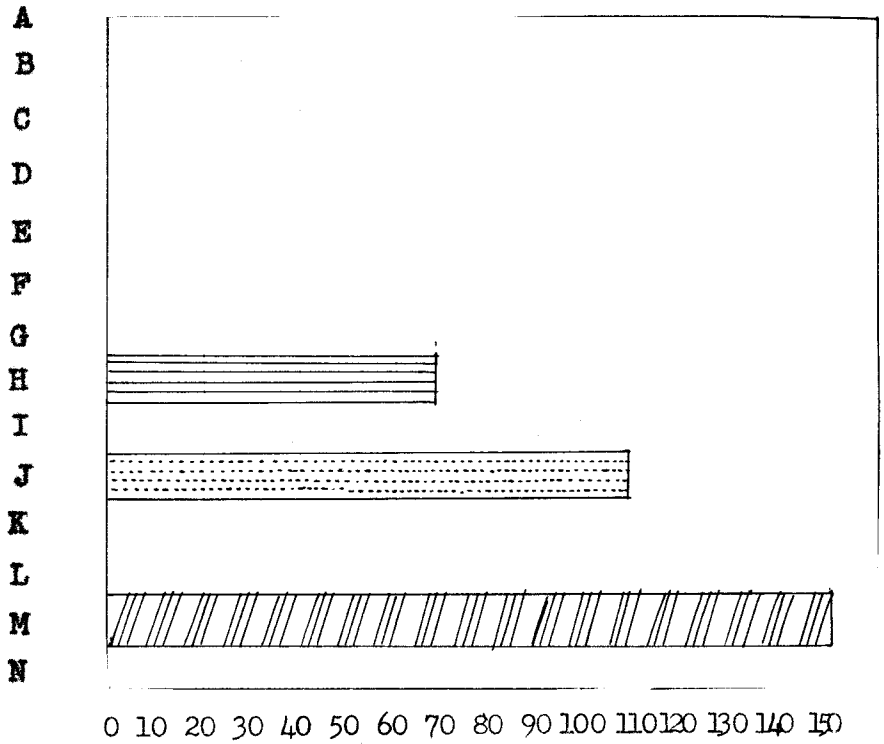
MEDIA TYPE J 

MEDIA TYPE M 

* From data presented in Table III

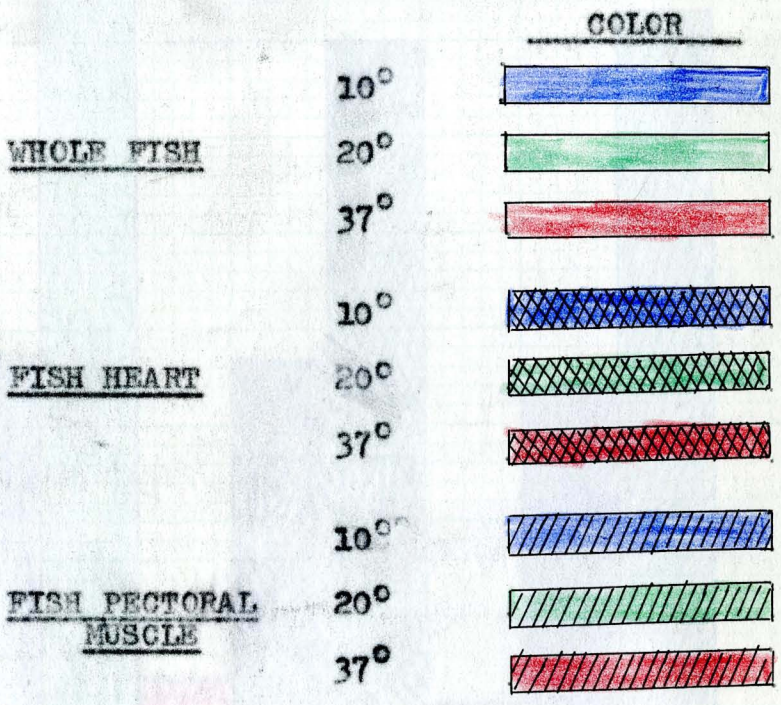
F I G U R E I

EFFECTS OF VARIOUS MEDIA TYPES ON CELL MULTIPLICATION



KEY FOR INTERPRETATION OF FIGURE II

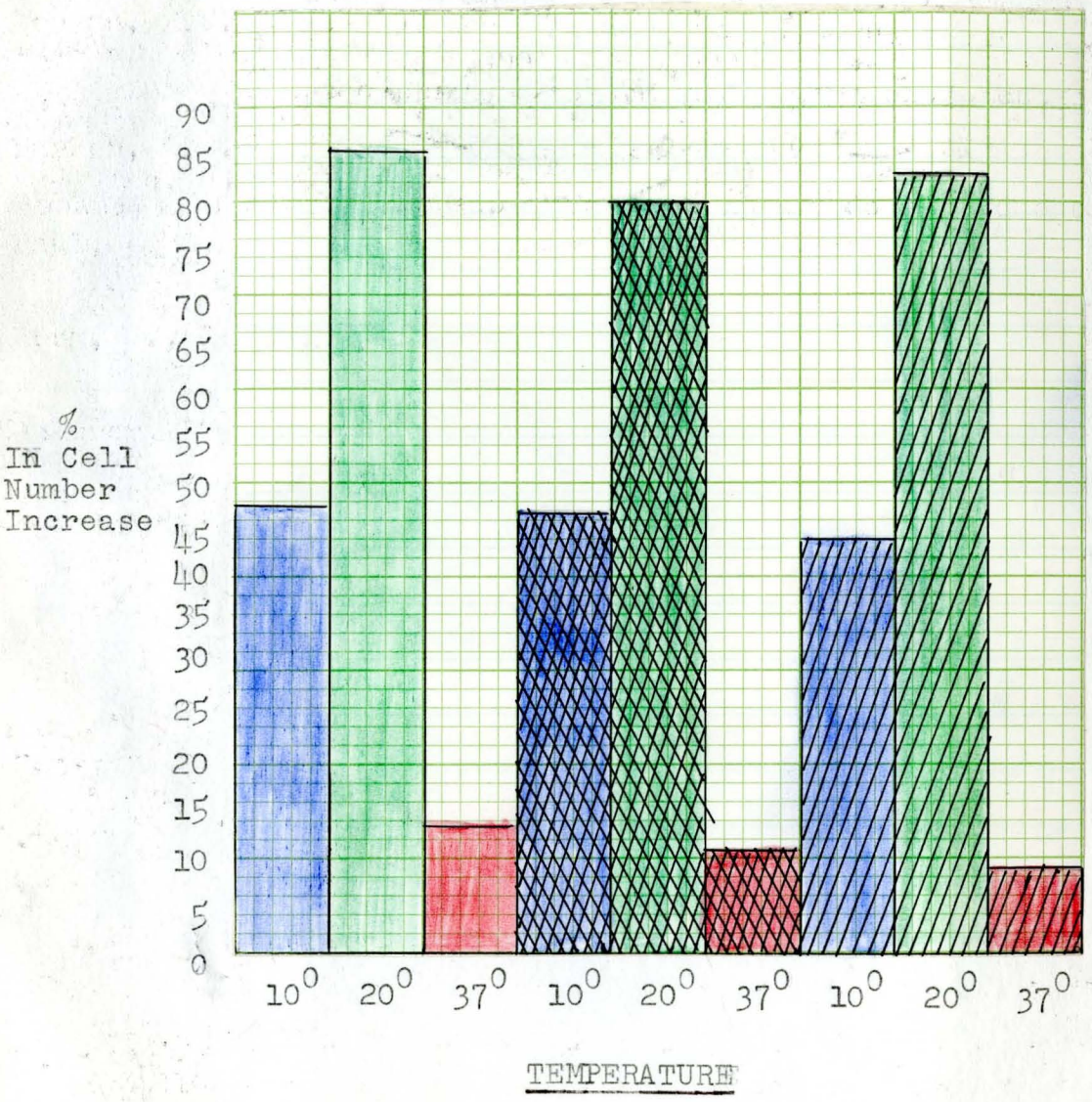
EFFECT OF TEMPERATURE ON CELL MULTIPLICATION *



* From data presented in Tables IV, V, VI

FIGURE II

EFFECT OF TEMPERATURE ON CELL MULTIPLICATION



KEY FOR INTERPRETATION OF FIGURE III

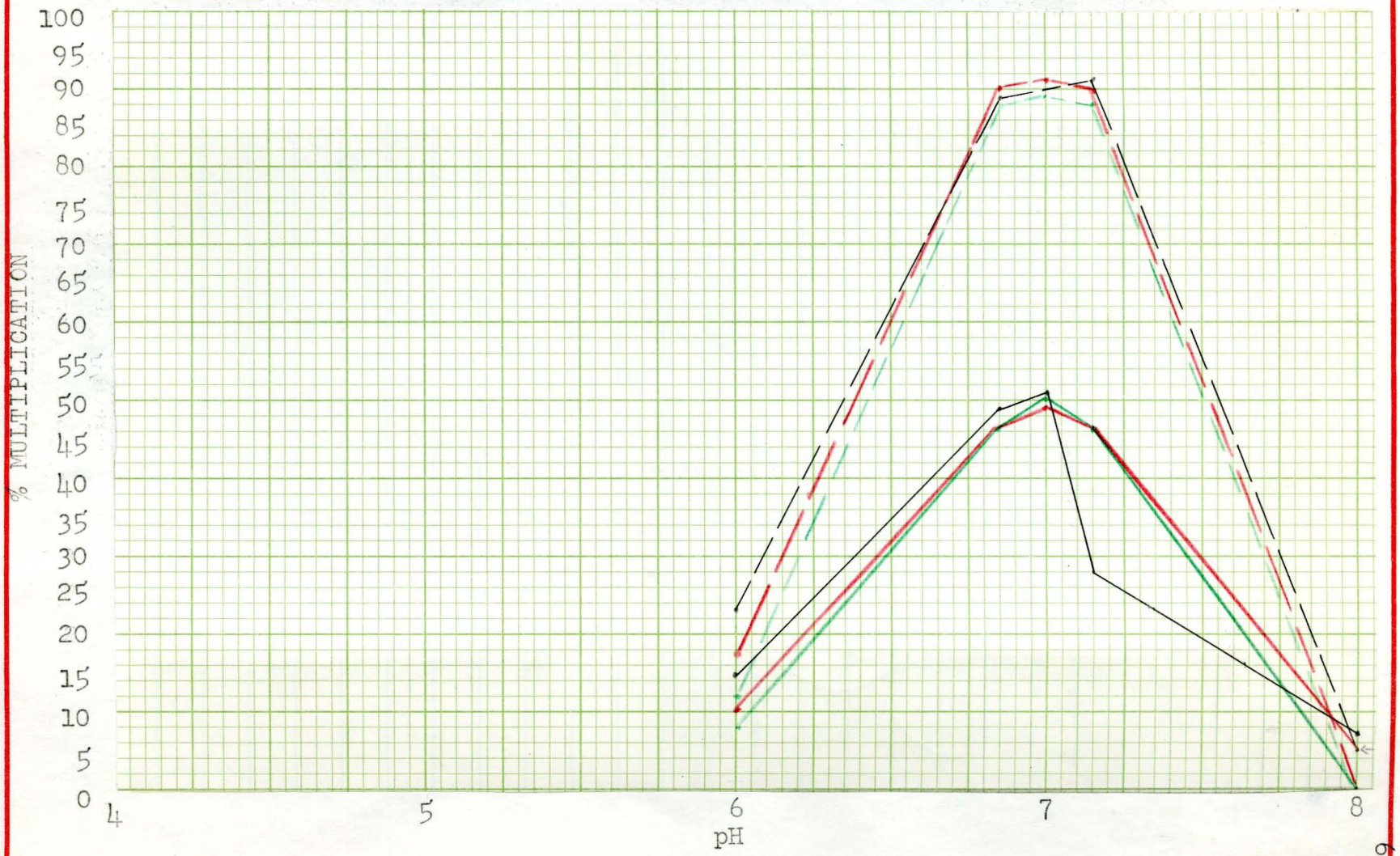
EFFECT OF VARIANCE OF PH ON CELL MULTIPLICATION *

		COLOR
WHOLE FISH	10°	———
	20°	— —
FISH PECTORAL MUSCLE	10°	———
	20°	— —
FISH HEART	10°	———
	20°	— —

* From data presented in Table VIII

FIGURE III

EFFECT OF VARIANCE OF pH ON CELL MULTIPLICATION



LEGEND FOR PHOTOGRAPHS

Tissue Type: Fish Heart Cells

Fixative: Osmium Tetroxide

Stain: Hematoxylin - Eosine

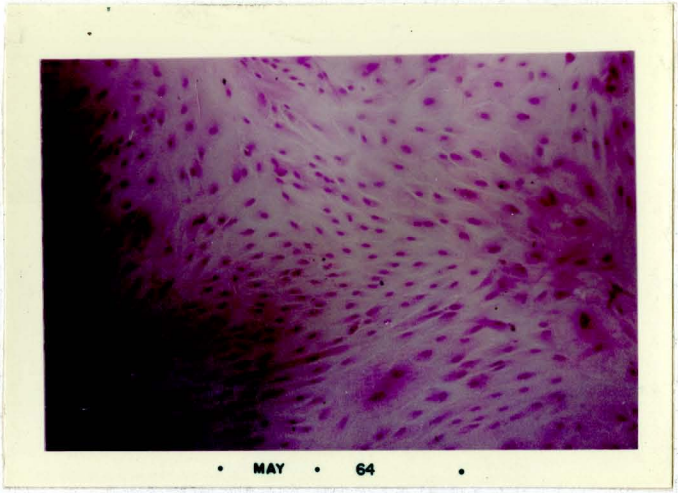
Figure IV: Low power 10X

Figure V: High Dry 43X

Figure VI: High Dry 43X

Figure VII: Oil Immersion 97X

FIGURE IV



MAGNIFICATION: 10 X

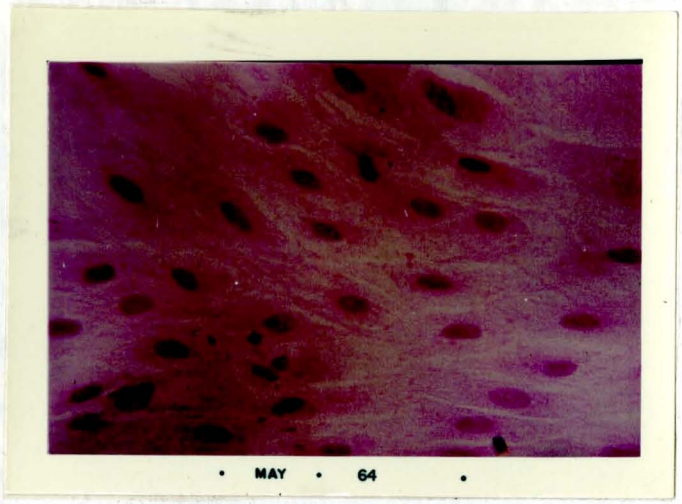
FILM: Kodacolor X

FIGURE V

MAGNIFICATION: 43 X

FILM: Kodacolor X

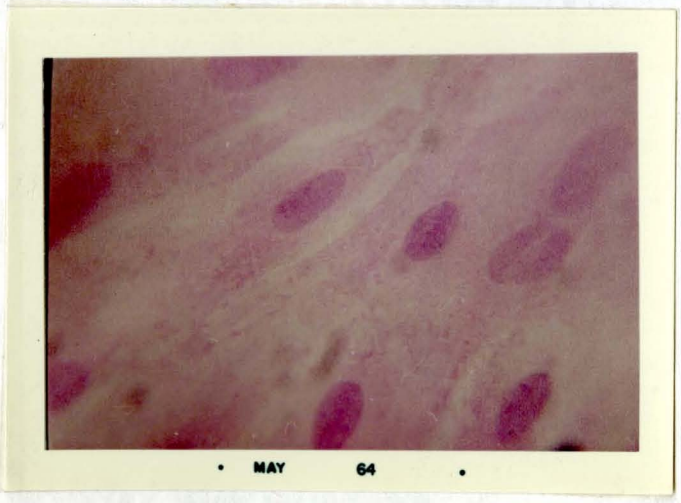
FIGURE VI



MAGNIFICATION: 43 X

FILM: Kodacolor X

FIGURE VII



MAGNIFICATION: 97 X

FILM: Kodacolor X

APPROVAL SHEET

The thesis submitted by Helen Mae Kroeker has been read and approved by three members of the Department of Microbiology.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference tocontent, form, and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

May 26 1964
Date

Thomas J. Bird
Signature of Advisor