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Title: CHARACTERISTICS OF TWO SALMONID FISH CELL LINES: GLYCOLYTIC RATES, CO_2 REQUIREMENTS AND SUSCEPTIBILITY TO REOVIRUS AND REOVIRUS-LIKE AGENTS

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The research presented in this thesis is concerned with the characterization of two salmonid cell lines, CSE 119 derived from embryos of coho salmon (Oncorhynchus kisutch) and STE 137 derived from embryos of steelhead trout (Salmo gairdneri). The rates of glucose utilization and lactic acid production during the most active growth phase of each cell line were determined and the results were compared to data from parallel experiments using a human embryonic cell line. The salmonid cells were grown at 23° C and the human cells at 35° C. All cells were grown in Eagle's minimal essential medium supplemented with 20% dialyzed newborn agamma calf serum. The $Q^{O_2}_{CO_2}$ values (equivalent microliters CO_2 produced per mg dry weight of cells per hour) were calculated for each cell type, and values of 7.97 and 4.50 were obtained for CSE 119 and STE 137, respectively. These values were somewhat lower than

the value of 13.19 calculated for the human cells.

In all three cell lines studied, the rates of glucose utilized and lactic acid produced per cell decreased over the growth periods observed. The decreases in the salmonid cells were most likely related to a concomitant increase in population density. In addition, it was shown that the very gradual fall in pH observed during the growth of the salmonid cells is apparently due in part, at least, to the smaller amount of lactic acid produced by these cells.

During the course of the studies with the salmonid cell lines, an investigation of the CO_2 requirements of these cells was undertaken. For these experiments, Tris buffer was used to replace the bicarbonate in the medium and 20% dialyzed serum was again used. Results from the salmonid cells grown at 18° C were compared to parallel experiments using HeLa cells grown at 35° C. It was shown that growth under 2% (salmonid) and 3% (HeLa) atmospheric CO_2 was comparable to growth in stoppered cultures for CSE 119, STE 137 and the HeLa cells. The salmonid cells showed good growth in cultures that were open to the air (0.03% CO_2), but little or no growth occurred in the HeLa cells under the same conditions.

To determine if CO_2 was actually required for growth of the salmonid cells, CO_2 free cultures were prepared using Conway microdiffusion dishes in which the cells were grown in the center well and a 10% solution of KOH was added to the outer well. The cultures

were sealed with high vacuum grease. Under these conditions, both salmonid cell lines demonstrated a growth requirement for CO_2 comparable to that shown by the HeLa cells. Attempts were made to use oxalacetate to substitute for CO_2 in these cultures. Oxalacetate partially substituted for CO_2 in the HeLa cell cultures, but little or no growth occurred in the salmonid cell cultures under the same conditions.

As part of a continuing effort to determine the viral susceptibility of salmonid cell lines, the coho cells (CSE 119) and the steelhead cells (STE 137) were tested for their susceptibility to Reovirus types 1 and 3, Infectious Pancreatic Necrosis (IPN) virus and Wound Tumor virus (WTV). Neither salmonid cell line nor HeLa cells were shown to produce infectious virus when inoculated with either reovirus type at 26 ° C. The salmonid cells would not tolerate 30 ° C for even short periods of time. Good replication of both reovirus types occurred in HeLa cells at 35 ° C.

IPN virus replicated well in both salmonid cell lines at 18 ° C. Phase contrast studies of IPN infected salmonid cells showed an early webbing of the cytoplasm, followed by a rounding up of the cells, shrinkage of the nuclei and a heavy margination of nuclear chromatin.

Because of its similarity to the reoviruses and its ability to replicate in an insect cell line, attempts were made to infect the salmonid cells with WTV at 23 ° C. No indications of WTV inclusion bodies or of a cytopathic effect were found in either salmonid cell line.

Characteristics of Two Salmonid Fish Cell Lines: Glycolytic Rates, CO_2 Requirements and Susceptibility to Reovirus and Reovirus-like Agents

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INTRODUCTION AND LITERATURE REVIEW

Introduction

The research presented in this thesis is concerned with the characterization of two cell lines derived from embryonic tissue of coho salmon (Oncorhynchus kisutch) and steelhead trout (Salmo gairdneri). First attempts to establish cell lines derived from fish met with only limited success (Wolf and Dunbar, 1957). Later, Wolf et al. (1960a) adapted the cell culture techniques used for in vitro culture of mammalian cells to fish cells. Using these methods, Wolf and Quimby (1962) established the first fish cell line, RTG-2, from rainbow trout gonad cells. Since that time a number of fish cell lines have been established (Clem, Moewus and Sigel, 1961; Gravell and Malsberger, 1965; Pfitzner, 1965) including the two cell lines used in these studies (Fryer, Yusha and Pilcher, 1965).

Work on the characterization of the two fish cell lines was divided into three sections. The first section dealt with growth and glycolysis, the second, with CO_2 requirements and the last, with susceptibility of the cells to Reovirus, Infectious Pancreatic Necrosis virus and Wound Tumor virus, human, fish and plant viruses,

respectively. The results of these studies are compared to similar work with homoiothermic cell cultures.

Growth and Glycolysis

There have been a great number of studies on glucose utilization and lactic acid production in cell cultures derived from homoiothermic animals, but similar data on cell cultures from poikilothermic vertebrates is meager. Cells grown *in vitro* appear to degrade glucose to either CO_2 or lactic acid. The conversion to lactic acid, called glycolysis, appears to predominate in most cell culture systems.

Several factors have been found which influence growth and glycolysis of cells *in vitro*. In 1958, Zwartouw and Westwood published extensive studies on some of the factors influencing the growth and glycolysis of cells in tissue culture. Using four established cell lines derived from mammalian tissues, they found glucose utilization to be influenced by glucose concentration, population density, hydrogen ion concentration, oxygen concentration, CO_2 concentration and serum concentration.

The effect of glucose concentration has been noted by several researchers. Wilson, Jackson and Brues (1942) reported that the rate of glucose utilization was increased by increasing the glucose concentration in the medium. Eagle *et al.* (1958) related the rate of

sugar metabolized to the amount available. Graff et al. (1965) stated that cells in culture convert glucose in excess of their low threshold requirement to lactic acid, the magnitude of glycolysis depending on the concentration of glucose.

The pH of the medium has been shown to influence glycolysis. Zwartouw and Westwood (1958) reported that the glycolytic rate of cells was progressively increased as the pH of the medium was made more alkaline. This result was confirmed by Broda et al. (1959) using chicken mesenchyme tissue.

Other factors influencing glycolysis have also been noted. Wu (1959), studying serum effects, found a shift in the limiting enzyme of glycolysis with cells cultured in different sera. Sanford and Westfall (1969) reported an inverse relationship between inoculum size and glycolytic rate.

It is obvious that any discussion of glycolysis must take into account a great number of factors which will be determined by the type of medium used and the method of cultivation. Any comparisons of glycolytic rates must be made with these factors in mind.

One of the earliest papers dealing with glycolysis in tissue cultures was published by Wilson et al. in 1942. Working with chick embryo tissue, they reported that lactic acid production was related in rate and amount to glucose utilization. Westfall, Peppers and Earle (1955), working with HeLa cells, found they used 2, 485

milligrams of glucose and produced an excess of 500 milligrams of lactic acid in a 24 day period. During this time, the cell number increased from 1.0×10^7 to 2.6×10^9. Since these early reports on glycolysis in tissue and cell culture, glycolytic rates have been reported for Earle's strain L cells (Phillips and Feldhaus, 1956), human embryonic and malignant cells (Leslie, Fulton and Sinclair, 1957), human cells of malignant and normal origin (Green, Henle and Deinhardt, 1958), human cell cultures (Eagle et al., 1958), L strain fibroblasts (Munyon and Merchant, 1959), mammalian cell strains (Bryant, Schilling and Earle, 1958), mouse lymphoblasts (Bailey, Gey and Gey, 1959), HeLa cells (Wu, 1959), malignant and non-malignant lymphoblasts (Homburg et al., 1961), human diploid cells (Cristofalo and Kritchevsky, 1965), and murine tumor producing clones (Sanford and Westfall, 1969). Because of the diverse methods of reporting data on glycolytic rates and because of the many methods used in these studies, comparisons of actual data are difficult and of a somewhat limited value.

A phenomenon of interest that appears to be universal in studies on glycolysis is the conclusion that at early stages of cell growth (i.e., low population densities) the rate of glycolysis is greatly increased and that the rate decreases as the population increases. Early workers found that as the cells in a culture multiply, the rate of glucose utilization per cell decreases (Wilson et al., 1942; Fulton, Sinclair

and Leslie, 1956; Zwartouw and Westwood, 1958). To explain this this effect, Leslie et al. (1957) hypothesized that lactic and keto acids leaked out of the cells until the external concentrations approach those normally existing inside the cell in vivo. If the amount of glucose required to provide for this leakage is subtracted from the total utilized, there is a very high correlation between the remainder and the average amount of tissue per tube.

Whitfield and Rixon (1961), in their studies of the effects of dilution of L mouse cells in suspension cultures, related high glycolytic rates upon dilution to the CO_2 concentration. In older, undiluted cultures, in which there was a relatively high CO_2 concentration, the CO_2 reacted with a fraction of the phospho-enol-pyruvate produced from glucose to form oxalacetate. In cells confronted with low CO_2 tension, resulting from dilution, more of the phospho-enol-pyruvate would be converted to pyruvate and ultimately to lactate.

Graff et al. (1965) concluded that initial high glycolytic rates were the result of an excess supply of glucose. They explained that cells in culture actually require very little glucose either for proliferation or maintenance--much less than they can or do consume when it is offered. Cells in culture convert glucose in excess of their low threshold requirement to lactic acid, the magnitude of glycolysis depending on the concentration of glucose. They reported that when glucose concentration is minimal, the cells, in fact, become

consumers rather than producers of lactic acid. Leslie et al. (1957) had also reported that lactic acid is utilized from the medium when the cell population is large and glucose concentration is reduced to 40% or less of the original values.

Sanford and Westfall (1969) summarized possible mechanisms for the observed increased glycolysis at low cell densities. They stated that this increased glycolysis may be caused by a decrease in CO_2 concentration (Whitfield and Rixon, 1961), increased pH (Paul, 1959; Broda et al., 1961), leakage of metabolites (Zwartouw and Westwood, 1958), increased amount of glucose available per cell (Jones and Bonting, 1956) or increased cell volume (Munyon and Merchant, 1959). Kruse and Miedema (1965) felt that with carefully controlled pH and glucose concentration in perfused animal cell cultures, they were able to show a direct correlation between rates of glucose uptake and population density.

There seems to be some question as to whether the glycolytic rate of cells grown in vitro is representative of the cell type or whether it is an artifact of the cell culture technique. Green et al. (1958) were unable to correlate glycolytic rates with regard to the source of the cells studied and hypothesized that cells adapted to tissue culture conditions adopt a common type of glucose metabolism which would tend to obscure differences in glycolysis which may have existed initially. Eagle et al. (1958) suggested that the amount of

glucose metabolized and the amount of lactic acid formed is related primarily to the rate at which the substrates become available to the cell rather than the cell type. Cristofalo and Kritchevsky (1965) felt that their studies supported the suggestion that the glycolytic rate cannot be used as a criterion for alterations in cells since, metabolically, cells grown in culture resemble each other more than they resemble their respective tissues of origin.

Carbon Dioxide Requirements

The second phase of the research on the two fish cell lines was a study of the CO_2 requirements of the fish cells *in vitro*. Werkman, (Werkman and Wilson, 1951) hypothesized that all forms of life assimilated CO_2 and that this assimilation was an essential physiological function providing for the synthesis of indispensable metabolic intermediates.

Information on CO_2 requirements of fish cell cultures is lacking. However, work done with mammalian cells provides some background material. Harris (1954), working with explants from 12-15 day chick hearts, showed that bicarbonate was essential for growth and maintenance of the cells within a physiological pH range of 7.2-7.8. Proliferation in bicarbonate-free media could be restored if the external pH value was adjusted to 8.5-9.5 at the time of culturing. He related the bicarbonate requirement to the maintenance of appropriate

intracellular pH values.

Geyer and Chang (1958) found that human conjunctival and HeLa cells multiplied rapidly in the presence of bicarbonate whereas, in its absence, a decrease in the number of cells was found. Working with the same cell types in the absence of CO_2, Geyer and Neimark (1958) found multiplication could be restored by the addition of normal cell extract. Swim and Parker (1958) demonstrated that strains of fibroblasts derived from human, mouse and rabbit tissues failed to proliferate at a significant rate when cultured in a medium without bicarbonate in unstoppered flasks. However, sufficient CO_2 was produced by the cells to promote growth in the same bicarbonate free medium when stoppered flasks were employed.

Carbon dioxide has been shown to affect Coxsackie virus replication. Chang (1961) reported that survival of primary amnion cells under conditions of CO_2 depletion did not significantly differ from survival of cells under normal conditions in that in both cases cell numbers dropped to about half the original value in 14 days. However, synthesis of Coxsackie virus, Group B, type 1 was suppressed under these same conditions of CO_2 depletion. Buthala (1963) related the size of plaques produced by Coxsackie A-21 virus to CO_2 concentration.

Oxalacetate has been shown to substitute for bicarbonate in a number of cases. Gwatkin and Siminovitch (1960) showed that

oxalacetic acid replaced the CO_2-bicarbonate system for L and HeLa cells but not for monkey kidney cells. Kieler (1960) reported that oxalacetate made Yoshida ascites tumor cells independent of CO_2. Kelley, Adamson and Vail (1960) demonstrated growth of three neoplastic lines of human cells in liquid suspension culture in free exchange with the air when oxalacetate was substituted for bicarbonate. Phillips and Feldhaus (1956) suggested a mechanism for oxalacetate substitution for bicarbonate. They theorized that pyruvate in the presence of CO_2 could be converted to oxalacetate. Whitfield and Rixon (1961) proposed that phospho-enol-pyruvate reacted with CO_2 to form oxalacetate in L mouse cells.

In a search for a non-bicarbonate buffer for use in cell cultures, Swim (1961) found that when cultures were initiated in stoppered flasks with a large inoculum, growth in Tris buffered medium was comparable to that obtained in bicarbonate buffered medium. Martin (1964) found that Tris-HCl buffers had relatively low toxicity for human diploid fibroblasts. However, small amounts of bicarbonate appeared to be essential for growth.

Chang, Leipins and Margolish (1961) in studies on the CO_2 requirements of HeLa and conjunctival cells in Tris-buffered medium containing labeled bicarbonate found most of the activity associated with the purines and pyrimidines of the cells. They found that a combination of ribosides and oxalacetate provided conditions as good

or better than CO_2 for multiplication of CO_2-depleted conjunctival cells. Runyan and Geyer (1967) reported that ribonuceosides, alone or in combination with oxalacetate, would only partially substitute for CO_2 in strain L mouse fibroblasts and HeLa cell cultures. Deoxyribonucleosides, a simple purine and pyrimidine combination, or extracts from normal cells would also only partially substitute for CO_2 in strain L mouse fibroblasts.

It appears that most work to this date supports Werkman's original hypothesis that all forms of life assimilate CO_2 and that this assimilation is an essential physiological function.

Viral Susceptibility of Poikilothermic Cell Cultures

The susceptibility of poikilothermic cell cultures to various viruses of both poikilothermic and homoiothermic origin is of considerable interest. Perhaps the most important use of such poikilothermic cell cultures is as a tool for the propagation and study of viruses. Information concerning the range of susceptible cell types to viral agents is useful in studies of viral specificity, classification and may give some insights into the evolutionary aspects of viruses.

A number of cell cultures of poikilothermic origin have been shown to support the replication of viruses of homoiothermic cells. One of the earliest papers in this area (Sanders and Soret, 1954) reported the propagation of Eastern equine encephalomyelitis virus

at 23-25° C in embryo cultures of a viviparous fish, a species of Gambusia. Since that time a number of viruses have been reported to replicate in a variety of poikilothermic cell types. The following includes some of these listed by virus, susceptible cell type and temperature of susceptibility: Newcastle and parainfluenze I viruses in tortoise kidney cell culture at 37° C (Fauconnier and Pachopos, 1962); mumps, vaccinia and herpes simplex viruses in tortoise kidney cell cultures at 33° C or 37° C (Fauconnier, 1963); parainfluenza I virus in tortoise kidney cell cultures at 37° C (Shindarov, 1962); Venezuelan equine encephalitis and Eastern equine encephalitis viruses in a cell line derived from rainbow trout gonads (RTG) at 22° C (Officer, 1964); ECHO-11, Mahoney strain of type 1 polio virus and type 3 adenovirus in a cell line derived from the fathead minnow at 34° C (Gravell and Malsberger, 1965); vaccinia virus in lizard liver cells at 37° C (Shindarov and Tonev, 1965); tick-borne encephalitis and Western equine encephalomyelitis viruses in cell cultures derived from the slow-worm at 25° C (Somogyiová and Řeháček, 1965); group A and B arboviruses in cell cultures from Koch ticks at 27° C (Řeháček, 1965); parainfluenza type 1 and Sindbis viruses in tortoise kidney cells at 37° C (Falcoff and Fauconnier, 1965); vaccinia, herpes simplex, pseudorabies and vesicular stomatitis viruses in cell cultures from turtle heart at 36° C (Clark and Karzon, 1967); Western equine encephalitis virus in cell lines derived from chinook salmon and

steelhead trout at 26° C (Nims, 1968); vaccinia, fowl pox, Newcastle, mumps, herpes simplex, Sindbis, vesicular stomatitis and rabies viruses in a cell line derived from the fatnead minnow at 36° C (Solis and Mora, 1970); and representatives of the pox, herpes, myxo, rhabdo and arbovirus groups in reptile cell lines at 36° C (Clark, Cohen and Karzon, 1970). The criteria used to show viral replication in each of the above listed cases varied a great deal and the validity of each report should be judged independently.

Several viruses of homoiothermic origin have been reported not to replicate in certain poikilothermic cell types. Among these are the following: parainfluenza types 2 and 3 and Reovirus type 1 in tortoise kidney cell cultures at 33° C or 37° C (Fauconnier, 1963); polio, vaccinia, vesicular stomatitis, Newcastle disease and pseudorabies viruses in cell cultures from Koch ticks at 27° C (Řehářek, 1965); Reovirus type 1 in turtle heart cell culture at 36° C (Clark and Karzon, 1967); Newcastle disease virus in cell lines derived from chinook salmon and steelhead trout at 23° C or 26° C (Nims, 1968).

Any attempts to infect poikilothermic cell cultures with homoiothermic viruses must take into account the temperature optimum for both cells and viruses. Clark and Karzon (1968) studied the effect of incubation temperature on the growth of mammalian and amphibian viruses in cultures of mammalian and reptilian origin. They found

that despite the different temperature optima of the host cells, the mammalian viruses replicated at maximum rates at 36° C and the amphibian viruses replicated at maximum rates at 23° C in each host type. At the intermediate temperature of 30° C, near maximal rates of replication of both amphibian and mammalian viruses were usually observed. Nims (1968) reported replication of Western equine encephalitis virus was questionable at 23° C, but it multiplied well at 26° C in two cell lines derived from salmonid fish.

The research presented in this thesis included studies on the susceptibility of the steelhead trout and coho salmon cell lines to Reovirus types 1 and 3, to Infectious Pancreatic Necrosis (IPN) virus and to Wound Tumor virus (WTV).

Reovirus (Human)

The ubiquitous nature of reoviruses and antibody to them, together with a possible relation to a plant (WTV) and a fish (IPN) virus makes them of particular interest in the study of the viral susceptibility of poikilothermic cells. The term "reovirus" was proposed in 1959 by Albert Sabin as a group name for a number of viruses then classified as being identical with or related to ECHO type 10 virus.

Reoviruses are ether resistant, icosahedral viruses, 60 to 75 mμ in diameter which contain double stranded ribonucleic acid (Rosen, 1968). Although there is disagreement about the exact number of

sub-units, it seems that the figure of 92 capsomeres suggested by Vasquez and Tournier (1962) is most likely to be correct (Stanley, 1967).

Reoviruses from man and lower animals can be grouped by hemagglutination-inhibition techniques into three serotypes (Rosen, 1960). Reovirus types 1, 2 and 3 share a common complement-fixing antigen (Sabin, 1959). Hemagglutination-inhibition tests reveal antigenic relationships among all three types, but types 1 and 2 appear to be more closely related to each other than either is to type 3 (Rosen, 1968). All strains of Reovirus types 1, 2 and 3 possess the property of agglutinating human erythrocytes (Rosen, 1968). Strains of type 3, but not those of types 1 and 2, agglutinate bovine erythrocytes (Eggers, Gomatos and Tamm, 1962).

Reoviruses have an exceptionally wide host range and type 3 has even been isolated from mosquitoes (Parker, Baker and Stanley, 1965). Antibody to reoviruses has even been demonstrated in fish. Of nine trout sera tested for hemagglutination-inhibition activity to Reoviruses types 1, 2 and 3, none inhibited types 1 and 2, but seven inhibited Reovirus type 3. No neutralizing activity for Reovirus type 3 was detected in the seven sera showing hemagglutination-inhibition activity (Stanley et al., 1964).

Perinuclear cytoplasmic inclusions which often completely surround the nucleus are typical of reovirus infection. One method

of demonstrating this inclusion is with acridine orange staining. Rhim, Jordan and Mayor (1962), working with type 1 reovirus stained with acridine orange, reported a pale green cytoplasmic inclusion surrounding the nucleus and often extending throughout the cytoplasm in long threads. Gomatos et al. (1962) found the same type of inclusions in Reovirus type 3 infected L cells stained with acridine orange.

Infectious Pancreatic Necrosis Virus (Fish)

Infectious Pancreatic Necrosis (IPN) virus has been shown to have a morphology similar to the reoviruses. IPN virus has been shown to be the etiological agent of an acute, virulent and highly fatal disease occurring in very young eastern brook trout (Wolf et al., 1960b). Since that time IPN virus has been isolated from a number of cold water fish including coho salmon fingerlings (Wolf and Pettijohn, 1970).

In 1960, Wolf (Wolf et al., 1960b) described the viral nature of IPN in trout. The agent of IPN demonstrated a high degree of specificity for trout tissue. They were unable to demonstrate a cytopathic effect in a number of mammalian cell types at either 19° C or 37° C. Attempts with brown trout and with bluegill caudal fin epithelium also failed to produce a cytopathic effect. Gravell and Malsberger (1965) reported the replication of IPN virus at 23° C in a fathead minnow (Pimephales promelas) cell line. Beasley, Sigel and

Clem (1966) reported the replication of IPN virus at 20° C in a cell line derived from grunt fin tissue of the blue striped grunt (Haemulon sciurus).

Malsberger and Cerini (1963) first described IPN virus as ether stable. They reported the lack of the capacity to hemagglutinate or hemadsorb to cells. Cerini and Malsberger (1965) using RTG-2 cells, further characterized IPN virus. They reported particles of 18.5 mμ mean diameter (having hexagonal profiles and few, probably 12, capsomeres. More recently, Moss and Gravell (1969) made an extensive study of IPN virus. They used virus prepared in RTG cells as well as virus prepared from visceral homogenates of infected rainbow trout. Virus particles were found exclusively in the cytoplasm of infected cells. The virions were hexagonal in profile and approximately 55 mμ in diameter. Negative stains of virus particles revealed unenveloped icosahedra, approximately 65 mμ in diameter with probably 92 capsomeres. They found the virus to morphologically resemble members of the reovirus group. They also report that preliminary data indicates that IPN virions contain double-stranded ribonucleic acid.

Wound Tumor Virus (Plant)

A plant virus, Wound Tumor virus (WTV), has been shown to have a morphology similar to the reoviruses. WTV measures 60 mμ

in diameter and has icosahedral symmetry with 92 capsomeres (Bils and Hall, 1962). The genetic material of WTV is double-stranded ribonucleic acid.

Black (1945) reported that 43 species of plants in 20 families could be infected by WTV. Inclusion bodies, spherical in shape and varying in size from less than one and up to four microns in diameter occur in the cytoplasm of tumor cells from plants that had been infected with WTV (Littau and Black, 1952). Chiu and Black (1967) reported that nine cell lines derived from leafhopper (Agallia constricta) embryos were susceptible to WTV at 24° C. By the direct method of fluorescent antibody staining, infected cells fluoresced and discrete stained spots appeared in the cytoplasm, frequently in perinuclear locations.

Chiu, Reddy and Black (1966) reported the serial passage of WTV in leafhopper (A. constricta) cell culture at 24° C. Although there was definite viral replication through the seven virus passages, WTV appeared to produce no cytopathic effects on the cultured leafhopper cells. Gomatos and Tamm (1963) reported that inoculation of WTV onto monolayers of monkey kidney cells or mouse fibroblasts and passage serially three times over a 41 day period produced no evident cytopathic effects on the monolayers.

Because of the many similarities between agents of the reovirus groups and WTV, a number of attempts have been made to show

a serological relationship between these viruses. Streissle and Maramorosch (1963) reported a complement-fixing antigen common to both reoviruses and Wound Tumor virus. However, since that time a number of investigators using a variety of techniques have been unable to show any serological realtionship between reoviruses and Wound Tumor virus (Gomatos and Tamm, 1963; Gamez, Black and MacLeod, 1967; Streissle, Rosen and Tokumitsu, 1968).

MATERIALS AND METHODS

Glassware and Rubber Stoppers

All glassware was immersed in water immediately after use. It was held in water until it could be washed. All washing was done by hand and consisted of a preliminary scrubbing using Microsolv (Microbiological Associates) followed by soaking in the detergent for at least an hour. This was followed by a second wash, ten rinses in tap water and five rinses in deionized, distilled water. Pipettes were cleaned by soaking in Microsolv followed by tap and deionized, distilled water rinses. White latex rubber stoppers were cleaned in the same manner as glassware. New rubber was boiled consecutively in 0.5 N NaOH, 4% HCl and Microsolv, then washed as usual.

All glassware was sterilized either by dry heat overnight at 200 °C or by autoclaving 20 minutes at 121 °C. Rubber stoppers were autoclaved for 30 minutes.

Cell Lines

Cell lines CSE 119, STE 137, CHSE 214, human embryonic and HeLa were used in these studies. All cell lines were maintained by serial passage from the time of their initiation or purchase to the time the experiments were conducted. Cell line CSE 119 was derived

from embryos of coho salmon in February, 1963. Cell line STE 137 was derived from embryos of steelhead trout in June, 1963. Cell line CHSE 214 was derived from embryos of chinook salmon in October, 1964. All salmonid cell lines were initiated by the method of Fryer et al. (1965). They are all heteroploid. Cells from cell lines CSE 119 and CHSE 214 are fibroblast-like while those from cell line STE 137 tend to be more epithelial-like.

The human embryonic cells were diploid, fibroblast-like, and derived from three to twelve week old human embryos. They were obtained from Flow Laboratories, Rockville, Maryland. HeLa cells were obtained from Microbiological Associates, Bethesda, Maryland.

The fish cell lines were maintained at 18 °C or 23 °C and transferred at one to three week intervals depending on their growth rate. The human cell lines were maintained at 35 °C and were transferred weekly.

Each time cell cultures were transferred, the cell suspensions were checked for sterility by inoculating into thioglycollate broth (Difco). Periodically the cells were tested for mycoplasma contamination by inoculation into M-13 Mycoplasma broth (Stevens, 1969).

Reoviruses

Reoviruses types 1 and 3 were obtained through the National

Institute of Health, Bethesda, Maryland, and represent Lang and Abney strains, respectively. Stock virus of both strains was prepared in HeLa cells at 35° C. Virus was released by two cycles of freezing at -60° C and thawing at 25° C of HeLa cells seven to nine days after infection. The cell suspension was then centrifuged at 2,000 r.c.f. (relative centrifugal force-gravities) for 20 minutes. The supernatant fluid was dispensed and frozen at -60° C until used.

Titrations of stock virus showed $10^{5.5}$ $TCID_{50}$ per 0.5 ml for Reovirus type 1 and $10^{5.75}$ $TCID_{50}$ per 0.5 ml for Reovirus type 3. Identification of both reovirus types was confirmed by hemagglutination-inhibition tests according to the method of Rosen (1960).

Infectious Pancreatic Necrosis Virus

Infectious Pancreatic Necrosis (IPN) virus was obtained from Dr. William Wingfield (California Department of Fish and Game). The viral material was first treated with 20% ether at 4° C for 24 hours to remove mycoplasmal contamination. It was then plaque purified in cell line CHSE 214 by the method of McCain (1970). Stock virus was prepared in cell line CHSE 214 at 18° C using the same methods used for preparation of reoviruses. Virus was released by two cycles of freezing and thawing of CHSE 214 cells three days after infection. The cell suspension was then centrifuged at 2,000 r.c.f. for 20 minutes. The supernatant stock virus was

dispensed and frozen at -60 ° C until used.

Titration of stock IPN virus showed $10^{8.24}$ $TCID_{50}$ per 0.5 ml. Identification of stock IPN virus was confirmed by virus neutralization using goat anti-IPN virus serum from Microbiological Associates.

Wound Tumor Virus

Wound Tumor virus (WTV) was obtained from Dr. L. M. Black in the form of infected sweet clover tissue. Two methods were used for stock virus preparation.

For the first preparation, several prominent stem tumors were removed and their surfaces were sterilized by immersion in 70% ethanol for three to five minutes. This was followed by five washes in sterile distilled water. The tissue was then ground in an aqueous solution of 0.1 M glycine, 0.01 M $MgCl_2$ using a mortar and pestle and 0.5 grams of sterile alundum. This crude extract was then centrifuged at 2,000 r.c.f. for 10 minutes. The supernatant was mixed with an equal volume of cell culture medium containing 10% newborn agamma calf serum, dispensed and frozen at -60 ° C until used.

The second preparation was made with several root tumors. The tissue was first treated with 70% ethanol and then rinsed in culture medium. It was homogenized in a VirTis "23" homogenizer at full speed for three minutes. This material was then centrifuged

at 2,000 r.c.f. for 20 minutes. The supernatant was mixed with an equal volume of culture medium containing 5% serum and this was used without further dilution to infect the salmonid cells.

Both types of preparation of stock WTV were contaminated. Three different methods were used to control the contamination. Stock WTV was passed through a Millipore filter with a 0.22μ pore size. Another method included treating stock virus with 20% ether at 4° C for 24 hours. Finally, some of the stock virus was placed on the cells in the presence of penicillin at 500 units per ml and streptomycin at 500 μg per ml.

Culture Medium

Eagle's minimal essential medium (MEM) (Eagle, 1959) prepared using either Hank's (Merchant, Kahn and Murphy, 1964) or Earle's salts (Earle, 1943) and supplemented with various percents of either dialyzed or untreated newborn agamma calf serum was used for all cell cultures. Medium prepared with Earle's salts will be designated E-MEM and that prepared with Hank's salts designated H-MEM. All medium was supplemented with newborn agamma calf serum from Hyland Laboratories, Los Angeles, California. In some cases the serum was dialyzed in the cold against 10 volumes of either Earle's or Hank's salt solution for 24 hours with one change of salt solution at 12 hours. Serum was used at either a 5%, 10% or

20% level depending on the nature of the experiment. Unless otherwise stated, all medium contained penicillin at 100 units per ml and streptomycin at 100 μg per ml.

Both Earle's and Hank's salt solutions were sterilized by autoclaving. The essential amino acids, nonessential amino acids, sodium pyruvate (not used in glycolysis work), vitamin mixture and glutamine were obtained as sterile concentrated solutions from Microbiological Associates, Bethesda, Maryland. The sodium bicarbonate and Tris buffer (used in the CO_2 work) were sterilized by autoclaving. The penicillin and streptomycin were sterilized by millipore filtration. Finally, the dialyzed serum was sterilized through a Seitz filter.

Frequent sterility checks using thioglycollate broth (Difco) were made on both components and complete medium.

Cell Transfers

To maintain the stock cultures of the cell lines used in this work, they were periodically transferred. Experimental cultures were also prepared by the same transfer technique. After the old medium was removed, the cells were detached from the glass using a solution of 0.2% trypsin (Difco) in GKN (8 gm NaCl, 4 gm KCl and 1 gm glucose per liter). Phenol red was included as a pH indicator. The trypsin was sterilized by Seitz filtration. Scraping with a rubber

policeman was sometimes required to completely remove the cells. When the cells were free of the glass, the suspension was centrifuged at 225 r.c.f. for 10 minutes. The trypsin was then decanted and the cells were resuspended in culture medium. Cells were then enumerated, diluted to give the proper concentration and planted in sterile culture glassware.

Cell Counting

Cells were enumerated by the nuclei counting method of Sanford and Earle (1951). The cells were first removed from the glass using trypsin supplemented by scraping with a rubber policeman when necessary. In some cases the cells were dispersed in the trypsin by repeated trituration through a pipette and prepared for immediate counting. At other times, after the cells were freed from the glass, they were first centrifuged at 500 r.c.f. for 10 minutes. The trypsin was then decanted and the cells were resuspended in culture medium for counting by trituration through a pipette.

In either case the cells were diluted in culture medium to give a countable concentration. One part cell suspension was then added to two parts crystal violet-citric acid dye and counted in a hemocytometer. Counts were made either in duplicate or quadruplicate.

Glucose and Lactic Acid Determination

The first step in both determinations involved the removal of protein from the culture medium. The following steps were used for blanks, standards and samples:

(1) one ml of the culture fluid was placed in a large glass conical centrifuge tube and diluted with nine ml of distilled water;

(2) five ml of 2% zinc sulfate were added to the tube;

(3) five ml of 1.8% barium hydroxide were added to the tube;

(4) the material was mixed and centrifuged at 2,000 r.c.f. for 30 minutes;

(5) aliquots of the supernatant fluid were set aside for the glucose and lactic acid determinations.

Glucose concentration was determined using Glucostat from Worthington Biochemical Corp., Freehold, New Jersey. The reagent was prepared by dissolving the contents of the chromogen vial and the Glucostat vial separately in distilled water. These were then mixed and distilled water added to a final volume of 80 ml. The following steps were then used on the protein free solutions:

(1) eight ml of the Glucostat reagent were added to two ml. of protein free fluid in a test tube;

(2) the reaction was allowed to proceed at room temperature

for 10 minutes;

(3) the reaction was stopped by adding one drop of 4 M HCl to each tube;

(4) the optical densities of all samples and standards were read against the blank at a 400 mμ wave length using a Beckman model B spectrophotometer;

(5) the glucose concentration was calculated using the known concentrations in the standards.

Lactic acid was determined using the Barker and Summerson method (1941) with several modifications by Hullin and Noble (1953). The following steps were used on the protein free solutions:

(1) one ml. of 20% cupric sulfate was added to two ml. of protein free fluid;

(2) the volume was brought to 10 ml with distilled water;

(3) one gram of calcium hydroxide was added to each tube, it was covered with parafilm, agitated and allowed to set at room temperature for one hour;

(4) the tubes were centrifuged at 2,000 r.c.f. for 20 minutes and a one ml aliquot of supernatant fluid was removed and placed in glass stoppered test tubes;

(5) one drop of 12% cupric sulfate was added to each tube;

(6) the tubes were placed in an ice bath and 6 ml of concentrated sulfuric acid were added to each;

(7) the tubes with glass stoppers in place were then heated in a water bath at 60° C for 30 minutes;

(8) the tubes were then cooled in an ice bath and two drops of p-phenylphenol reagent was added to each tube;

(9) the tubes with glass stoppers in place were agitated and placed in a 30° C water bath for 30 minutes;

(10) the tubes were then placed in a boiling water bath for 90 seconds;

(11) the optical densities of the blank, samples and standards were read against a distilled water blank at 560 mμ wave length using a Beckman model B spectrophotometer;

(12) the blank value was subtracted from the standard and the sample values and the amount of lactic acid in each sample was calculated from the known concentrations in the standards.

Dry Weight Determinations

Mean dry weight per cell was determined for cell lines CSE 119, STE 137 and the human embryonic cells. First the old medium was removed and the cell monolayer was washed with phosphate buffered saline (PBS). The cells were then suspended in the PBS by scraping from the glass with a rubber policeman followed by repeated trituration through a pipette. A small aliquot was set aside for

counting and five ml, of the cell suspension were placed in a pre-weighed bottle. The suspension was then dried in a vacuum over phosphorous pentoxide in a desiccator to constant weight. The weight of the cells minus the calculated weight contributed by the PBS was determined and the mean dry weight per cell was calculated using the previously made cell count.

Mean Cell Volume

The mean cell volume for cell line CSE 119 was determined at days 2, 4, 6, 8 and 10 during its growth period. All the steps in the procedure were carried out at approximately 18° C so as to maintain the cells in as nearly a normal state as possible. Monolayers were first washed with a trypsin-versene solution (0.15 gm trypsin, 0.02 gm versene, 0.8 gm NaCl, 0.04 gm KCl, 0.06 gm $NaHCO_3$, phenol red and 100 ml. of water). The monolayers were then treated with the trypsin-versene solution for 20 minutes to remove the cells. The cells which had separated from the glass were centrifuged at 225 r.c.f. for 10 minutes. The trypsin versene solution was decanted and the cells were resuspended in culture medium and transferred with washings to a 25 ml volumetric flask. The volume was brought to the mark with culture medium. Van Allen Thrombocytocrit tubes were then filled with the cell suspension and an aliquot of cell suspension was used to count the cells. The Van Allen tubes were

then centrifuged first at low speed followed by 30 minutes at 2, 000 r. c. f. The packed volume was measured and using the cell counting data, the mean cell volume was calculated.

Glycolysis Experiments

Rates of glucose utilization and lactic acid production were determined for cell lines CSE 119, STE 137 and the human embryonic cells. For each experiment, 150 ml Pyrex milk dilution bottles were inoculated with an eight ml volume of cell suspension containing from 300, 000 to 500, 000 cells per ml in the case of the fish cell lines. The inoculum for the human cell strain contained 200, 000 to 300, 000 cells per ml of medium. The medium used was E-MEM containing 20% dialyzed serum. Fish cells were incubated at 23 ° C. After two days incubation of CSE 119 cultures, four bottles were removed from the incubator and the culture medium pooled. The pH was measured immediately and the pooled fluid was frozen for later chemical analysis. The cell monolayer was removed from each bottle by exposure to a known volume of 0. 2% trypsin and gentle scraping. The cells in the four bottles were dispersed by repeated trituration through a pipette. The suspensions plus rinsings were pooled, adjusted to a known volume and counted. Four separate counts were made on each suspension, involving a total of at least 400 cells, and the mean value for the total cell population in the four bottles was

calculated. Groups of four bottles were removed from the incubator after 4, 6 and 10 days incubation, and the above process was repeated at each interval. After six days, the culture fluid was removed and replaced with fresh medium.

In the case of cell line STE 137, which had a slower growth rate than CSE 119, groups of culture bottles were removed for analysis after incubation periods of 2, 6, 10, 14 and 18 days. The nutrient medium was changed at the 10 day interval. The human embryonic cells, which had the most rapid growth rate, were incubated at 35° C. Groups of cultures were removed for analysis after incubation periods of 2.5, 4.0, 5.5 and 7.0 days, and the medium was changed after each 1.5 day interval.

The frozen culture medium was melted, the protein was removed and the protein free solution analyzed for glucose and lactic acid. The amounts of glucose utilized and lactic acid produced per cell over the growth intervals employed for each cell line were calculated.

Growth at Different CO_2 Levels

Growth of cell lines CSE 119, STE 137 and HeLa cells was studied at different atmospheric CO_2 levels. Eight ml of cell suspension was initially inoculated into 150 ml Pyrex milk dilution bottles and sealed with rubber stoppers. CSE 119 cells were inoculated at

approximately 400,000 cells per ml, STE 137 at approximately 300,000 cells per ml and HeLa cells at approximately 200,000 cells per ml. The medium used during the initial growth period was H-MEM with 20% serum. The fish cells were incubated at 18° C and the human cells at 35° C. At day two for HeLa cells, day three for CSE 119 and day four for STE 137, two bottles were taken from the incubator and the culture medium was removed. The cells were then detached from the glass using 0.2% trypsin and gentle scraping. The cell suspensions together with rinsings from each bottle were added to two tubes and centrifuged at 500 r.c.f. for 10 minutes. The cells in each tube were then resuspended in a known amount of culture medium and duplicate counts were made for each culture. The number of cells in each original bottle was calculated and the mean value obtained.

The culture medium on the remaining bottles was removed and replaced with H-MEM with 20% dialyzed serum. The bicarbonate in this medium was replaced with 5 mM Tris (Tris (hydroxymethyl) aminomethane) buffer. The bottles were then divided into four groups to be placed under conditions of increased atmospheric CO_2, reduced CO_2, stoppered and open cultures. The cells were reincubated under these conditions.

The four different CO_2 tensions were achieved by placing the cells under each of the following conditions:

(1) stoppered--These were regular latex stoppered bottles. The CO_2 level in the cultures was determined by the level of CO_2 produced by the cells.

(2) open--These were bottle cultures in which the latex stopper was replaced by gauze wrapped cotton plugs. They were incubated, sealed only with the cotton plugs allowing free gas exchange with the atmosphere. The CO_2 level was determined by that of the air, approximately 0.03%.

(3) increased CO_2--These were bottle cultures in which the latex stopper was replaced by a gauze wr apped cotton plug and they were placed in a sealed plexiglass incubator. The pressure in the incubator was reduced the desired amount as measured by a mercury manometer. The pressure was then returned to atmospheric by the addition of CO_2 gas. In the case of HeLa cells a 3% CO_2 level was used. A 2% CO_2 level was used for the fish cells due to the greater solubility of CO_2 at the lower incubation temperature used for these cells.

(4) reduced CO_2--These were bottle cultures in which the latex stoppers were replaced by stoppers into which a glass tube had been inserted containing a strip of filter paper (Geyer and Chang, 1958). After sealing the bottles

with these special stoppers, the filter paper was soaked with a 10% solution of KOH by injecting the solution through the rubber stopper onto the filter paper. This trap absorbed CO_2 produced by the cells maintaining a reduced CO_2 atmosphere in the cultures.

At various intervals, two bottles of cells under each of the four conditions were removed and the cells enumerated in the same manner as the initial counts. At the same intervals, the medium was changed on the remaining bottles. The intervals for the HeLa cells were days 3, 4, and 5 for CSE 119 days 5, 7 and 9 and for STE 137 days 8, 12 and 16. During the course of the experiments the pH was maintained between 7.6 and 7.0 by the addition of sterile 0.25% NaOH.

CO_2 Free and Oxalacetate Enriched Conditions

Growth of cell lines CSE 119, STE 137 and HeLa cells was studied under CO_2 free conditions, alone and with the addition of 2 mM oxalacetate. Cells used in these experiments were first washed in H-MEM with 20% dialyzed serum in which Tris buffer had been substituted for the bicarbonate. Three ml of cell suspension was then planted in the center well of Conway microdiffusion dishes using the same medium. Cultures of HeLa cells were initiated by using an inoculum of 100,000 to 200,000 cells per ml and the fish cells were initiated at 200,000 to 500,000 cells per ml. Five ml of 10%

KOH were added to the outer well of each dish to absorb any CO_2 produced by the cells. The cultures were then sealed with silicone high vacuum grease and incubated at 35 ° C for the HeLa cells and 18 ° C for the fish cells. Control cultures were prepared in a similar fashion except the KOH and grease seal were omitted. The control cultures were covered with petri dish tops and incubated under 2% CO_2 for the fish cells and 3% CO_2 for the HeLa cells. Oxalacetate supplemented cultures were prepared in the same manner as the CO_2 free cultures and 2 mM oxalacetate as the sodium salt was added to the culture medium. During these experiments the medium was not changed on any of the cultures so that no CO_2 could be admitted to the sealed Conway microdiffusion dishes.

Two cultures each under conditions of 2% CO_2 (fish) or 3% CO_2 (human), CO_2 free and CO_2 free plus 2 mM oxalacetate were removed from the incubator at days 2, 4, 6 and 8. The cells were detached from the glass using trypsin and scraping. The cells plus rinsings were centrifuged at 500 r. c. f. for 10 minutes, the trypsin was decanted, and the cells were resuspended in culture medium. Duplicate counts were made on each of the two cultures for each condition and the mean cell number was calculated from the four counts. The pH of all cultures was maintained between 7. 0 and 7. 9 by the addition of sterile 0. 25% NaOH where necessary.

Hemagglutination and Hemagglutination-inhibition

Hemagglutination and hemagglutination-inhibition tests were performed on the reoviruses using the methods of Rosen (1960). Antisera prepared in chickens against Reovirus type 1 (Lang strain) and Reovirus type 3 (Abney strain) was obtained from Grand Island Biological Company, Grand Island, New York. The antisera was kaolin and human O red blood cell treated for removal of nonspecific hemagglutinins.

Human red blood cells, type O, were drawn and mixed with an equal portion of Alsever's solution at pH 6.1. The cells were then washed in dextrose-gelatin-veranol buffer (Clark, and Casals, 1958) and made up to 0.75% in the same solution.

Virus Neutralization

A neutralization test was performed on the stock IPN virus. Goat anti-IPN virus serum was obtained from Microbiological Associates, Bethesda, Maryland. The maintenance medium used in the test was H-MEM with 5% newborn agamma calf serum. The antiserum was diluted 1:10 with the maintenance medium and mixed with equal parts of 10^{-5}, 10^{-6}, and 10^{-7} dilutions of stock virus. This was incubated for 30 minutes at 20°C. At the end of this time, 0.5 ml of each mixture was added to each of three Leighton tubes

containing monolayers of cell line CHSE 214. The same dilutions of stock virus mixed with equal volumes of maintenance medium were added to another set of Leighton tubes at the same time. Two sets of control tubes were prepared containing 0.5 ml of the maintenance medium alone on one set and 0.5 ml of the diluted antiserum on the other set. All the Leighton tubes were then incubated at 18°C. After two hours adsorption time, 0.5 ml of maintenance medium was added to all cultures and they were reincubated at 18°C. The cultures were observed and scored for CPE at days 1, 2 and 3.

Acridine Orange Staining

Cells to be stained with acridine orange were grown on cover slips in Leighton tubes. The cover slips were removed and rinsed once in Hank's salt solution. The cells were then fixed in acid-alcohol (two parts absolute ethanol to one part glacial acetic acid) for five minutes. This was followed by two rinses of two minutes each in McIlvaine's buffer at pH 3.8 (Cruickshank, 1965). The cells were then stained for four minutes in a solution of 0.01% acridine orange prepared in the same buffer. This was followed by two more rinses in buffer for two minutes each. Finally, the cover slips were mounted in buffer and sealed with clear nail polish (Cutex).

The stained acridine orange preparations were observed and photographed using a mercury arc light source attached to a Leitz

research microscope. A blue filter was used on the light source and the microscope was set up for darkfield optics. Barrier filters (Blau Abs., 2.5 mm OGI) were used in the ocular for observation and on the camera for photography.

Virus Infectivity Titrations

All infectivity titrations were done using Leighton tubes. The cells were grown in H-MEM with 10% serum. For virus dilutions, adsorption and incubation, the serum level was dropped to 5%. This will be called maintenance medium.

The reoviruses were titrated in HeLa cells and IPN virus was titrated in cell line CHSE 214. In both cases the basic technique was the same. First, the old medium was removed from monolayer cultures of the cells to be used for the titration. The stock virus or tissue culture fluid suspected of containing virus was diluted in maintenance medium and for each dilution 0.5 ml was added to each of three replicate cultures. These cultures were then incubated at the appropriate temperature for two hours to allow time for viral adsorption. At the end of this time, an additional 0.5 ml of maintenance medium was added to each culture and they were reincubated until they were read.

By the fourth day all the dilutions containing IPN virus showed a strong cytopathic effect and were scored accordingly by microscopic

observation.

In the case of the reoviruses a different technique had to be employed. Since it was difficult to distinguish between normal cell degeneration and viral CPE, all the cells were stained with acridine orange. Infected cells had easily distinguishable cytoplasmic viral inclusions which appeared green. Each coverslip for each replicate of each dilution was scanned for 10 minutes before it was scored as negative. For both the reoviruses and IPN virus, virus concentration in $TCID_{50}$ per 0.5 ml was calculated by the Reed-Muench formula.

Serial Passage of Viruses in Fish Cells

Attempts were made at serial passage of the reoviruses, IPN virus and WTV in cell lines CSE 119 and STE 137. With slight variations, the methods used were essentially the same for the three viruses. Stock virus was first diluted and titrated. A 150 ml Pyrex milk dilution bottle containing a monolayer of fish cells was rinsed once with maintenance medium and 0.8 ml of the appropriate virus dilution was added to the bottle. The reoviruses were incubated at 26° C, WTV at 23° C and IPN virus at 18° C for two hours allowing for viral adsorption. At the end of this time, 7.2 ml of maintenance medium was added to the bottles and they were reincubated. After 3.5 days for IPN virus and seven days for the reoviruses and WTV, the bottles were removed and placed in the -60° C freezer. A single

freeze-thaw cycle was used to strip the remaining cells from the glass. The fluid plus cells were then removed from the bottles and homogenized in a VirTis "23" homogenizer at full speed for 30 seconds. The homogenized suspensions were then centrifuged at 2,000 r.c.f. for 20 minutes. The supernatant fluid was diluted with maintenance medium, titrated and 0.8 ml of an appropriate dilution was adsorbed to a bottle of fish cells for the next passage. Because it was not possible to titrate the WTV, passage material was used to infect the cells in Leighton tubes with cover slips. At various periods after infection the cells were stained using acridine orange and observed for viral inclusions. Serial passages were continued for all the viruses until at least three and as many as eight passages were completed.

Rose Chambers

The cytopathic effect of IPN virus on both CSE 119 and STE 137 cells was observed and photographed in Rose chambers. The Rose chambers were loosely assembled and autoclaved. After drying and cooling, they were tightened and the cells, suspended in H-MEM with 10% serum, were injected into the chambers. After monolayers had formed, the old medium was removed and a 1:1000 dilution of stock IPN virus in maintenance medium was placed on the cells. Maintenance medium without virus was placed on control cultures.

The chambers were then incubated at 18° C and were observed and photographed daily using phase optics.

Photography

All photography was done using a Leica camera on a Leitz research microscope. Color photomicrographs of acridine orange stained cells were made using high speed Ektachrome (ASA 160, Kodak) with 30 second exposure times. Phase photomicrographs were made using Panatomic X (ASA 32, Kodak) with exposure times of 1.0 seconds.

RESULTS

Glycolysis

This research on two salmonid cell lines was divided into three sections: growth and glycolysis, CO_2 requirements and viral susceptibility. Results from the section on growth and glycolysis will be presented first.

Glucose Analysis

An experiment was run to see if Glucostat, a glucose test reagent from Worthington Biochemical Corporation, could be used to quantitatively test for glucose in minimal essential medium (MEM). Figures 1 and 2 show the results of a comparison using distilled water and MEM as the suspending fluid in the glucose analysis. Both in distilled water (Figure 1) and in MEM (Figure 2) the optical density readings were directly proportional to the glucose concentration over the range tested (0.5 to 2.0 mg glucose). It therefore appeared that Glucostat could be used for the determination of glucose concentration in our medium.

Lactic Acid Analysis

Several experiments were run to see if the Barker and Summerson (1941) determination could be used to quantitatively measure lactic

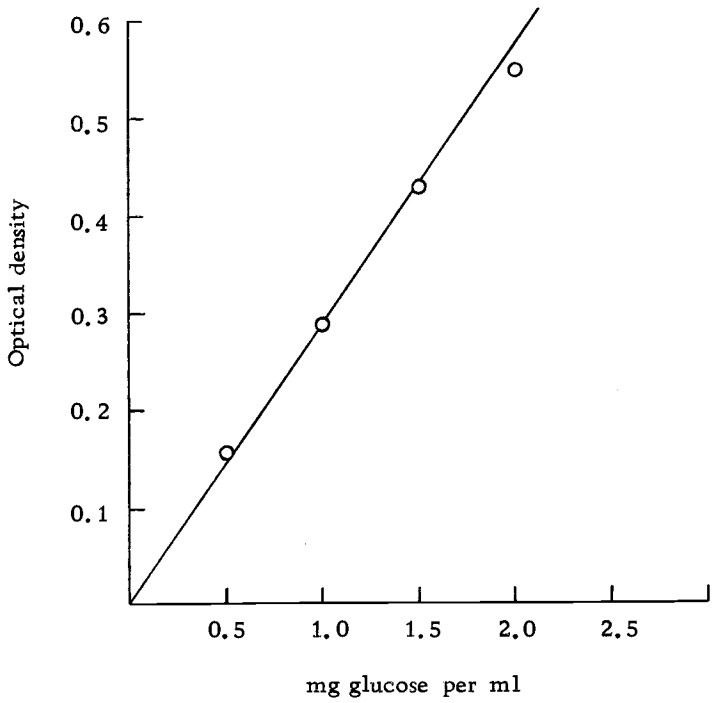

Figure 1. Quantitative determination of glucose in distilled water using Glucostat.

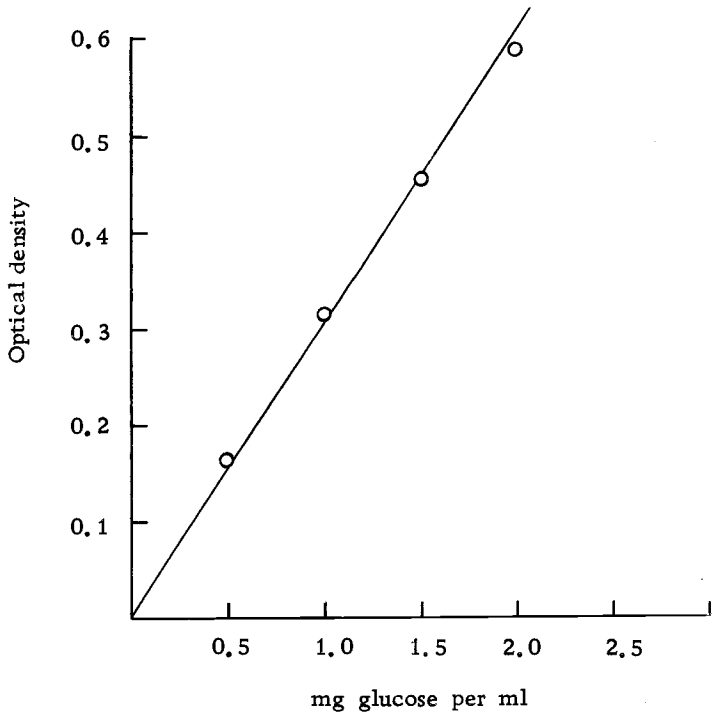

Figure 2. Quantitative determination of glucose in minimal essential medium using Glucostat.

acid in MEM. Several modifications of the method by Hullin and Noble (1953) were included in the procedure. Figure 3 shows the results of a comparison of the analysis using distilled water and MEM as the suspending fluid. In both cases the optical density readings were directly proportional to the lactic acid concentration over the range tested (3 to 20 μg lactic acid). However, the readings in MEM were slightly lower than those in distilled water. This effect could have been due to a slight shift in the peak of the absorption spectrum when dissolving the lactic acid in MEM and could thereby necessitate the use of a different wavelength in reading the optical densities. Therefore, the absorption spectrum in MEM was compared to that found with distilled water using a Cary model 11 recording spectrophotometer. The results can be seen in Figure 4. An identical absorption peak was found in each case at both 10 and 20 μg of lactic acid. In both cases this peak was very close to the 560 mμ wavelength recommended for the analysis. Since the absorption peaks were identical and the optical density was directly proportional to the lactic acid concentration over the tested range, it appeared that the Barker and Summerson determination with the Hullin and Noble modifications could be used for the determination of lactic acid in our medium.

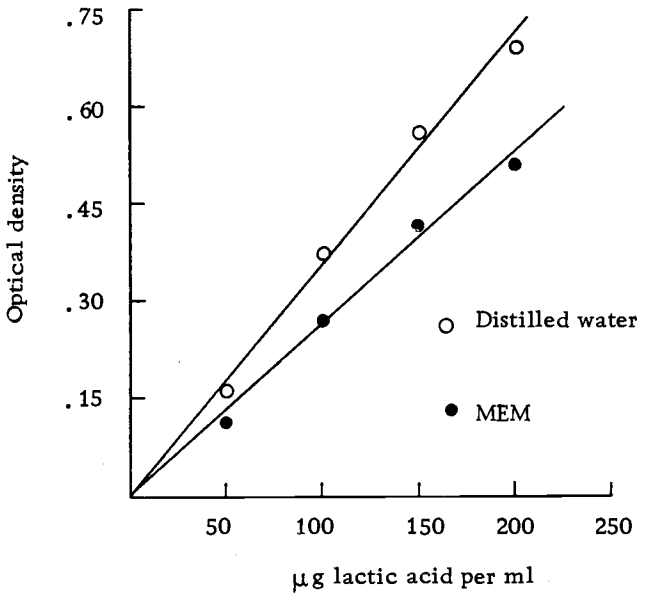

Figure 3. Quantitative determination of lactic acid in distilled water and in minimal essential medium using the Barker and Summerson determination (Hullin and Noble modifications).

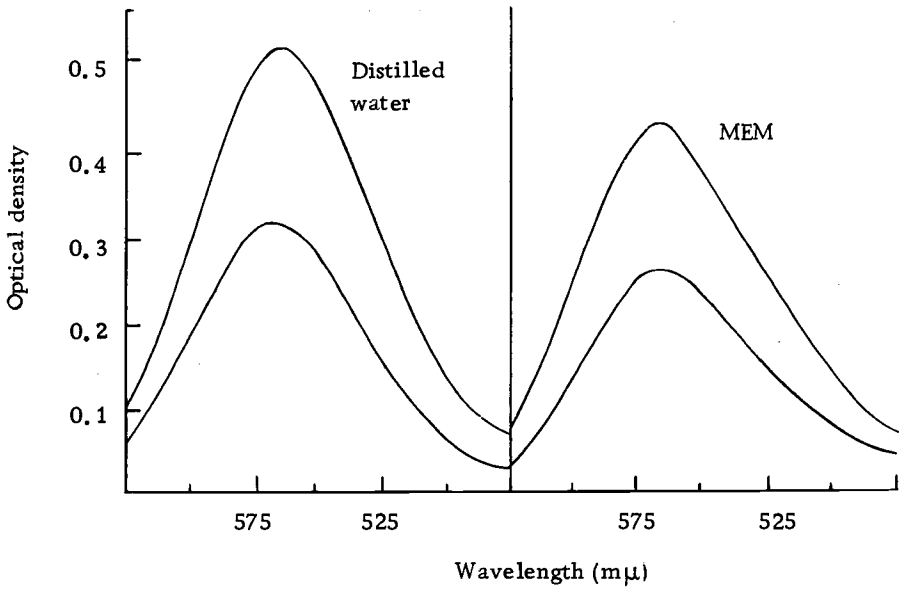

Figure 4. Comparison of absorption spectra of lactic acid determination using 10 and 20 μg lactic acid per ml in distilled water and in minimal essential medium.

Lactic Acid in Serum

At the start of the glycolysis experiments, it was found that the serum contained enough lactic acid to interfere with lactic acid production by the cells. When lactic acid levels in the medium were high, cells grown *in vitro* did not produce lactic acid at nearly as high a rate as in medium that did not contain lactic acid. Four different sera were tested for their lactic acid content. The results can be seen in Table 1. All four sera contained lactic acid in excess of 250 μg per ml. Therefore, the lactic acid had to be removed if serum was to be used in the medium for the glycolysis experiments.

Serum Dialysis

Since commercial sera contained an excessive amount of lactic acid, the serum used in the experiments on glycolysis was dialyzed against Earle's balanced salt solution at 4-6° C for 24 hours. The results of the dialysis can be seen in Table 2. All the detectable lactic acid was removed by the dialysis procedure. Therefore, dialyzed newborn agamma calf serum was used for all glycolysis experiments.

Growth of Cell Lines

A number of experiments were run to determine the optimal serum concentration, interval between medium changes and growth

Table 1. Lactic acid content of four calf sera.

Sera	μg lactic acid per ml
Gamma globulin free newborn calf[a]	288
Fetal calf[a]	345
Gamma globulin free calf[a]	261
Agamma newborn calf[b]	306

[a]Grand Island Biological Company, Grand Island, New York

[b]Hyland Laboratories, Los Angeles, California

Table 2. Results of the dialysis of newborn agamma calf serum.

	serum before dialysis	serum after dialysis
μg lactic acid per ml.	401	no detectable lactic acid
duplicate μg lactic acid per ml.	444	no detectable lactic acid

period for each of the cell lines used in the glycolysis studies. Because the best growth occurred at a serum concentration of 20% for the fish cell lines, all medium used contained this level of serum. The optimal interval between medium changes and the optimal growth period differed for all three cell lines. For CSE 119 it was found that a growth period of 10 days with a medium change at the sixth day was best; for STE 137, a growth period of 18 days with a medium change on the tenth day was best; and for the human embryonic cell line a growth period of seven days with a medium change after each 1.5 day interval was optimal. A comparison of the growth rates of the three cell lines using the culture methods described above can be seen in Figure 5. The curves represent the mean rates obtained from three separate experiments with each cell line. The cell generation times measured during the period of most rapid growth were 122 hours for STE 137, 50 hours for CSE 119 and 45 hours for the human embryonic cells.

Rates of Glucose Utilization and Lactic Acid Production

The rates of glucose utilization and lactic acid production for cell line CSE 119, cell line STE 137 and human embryonic cells during four 2 day, 4 day and 1.5 day intervals, respectively, were determined. The results can be seen in Figures 6, 7 and 8. The cell numbers indicated are the means of the cell counts at the beginning

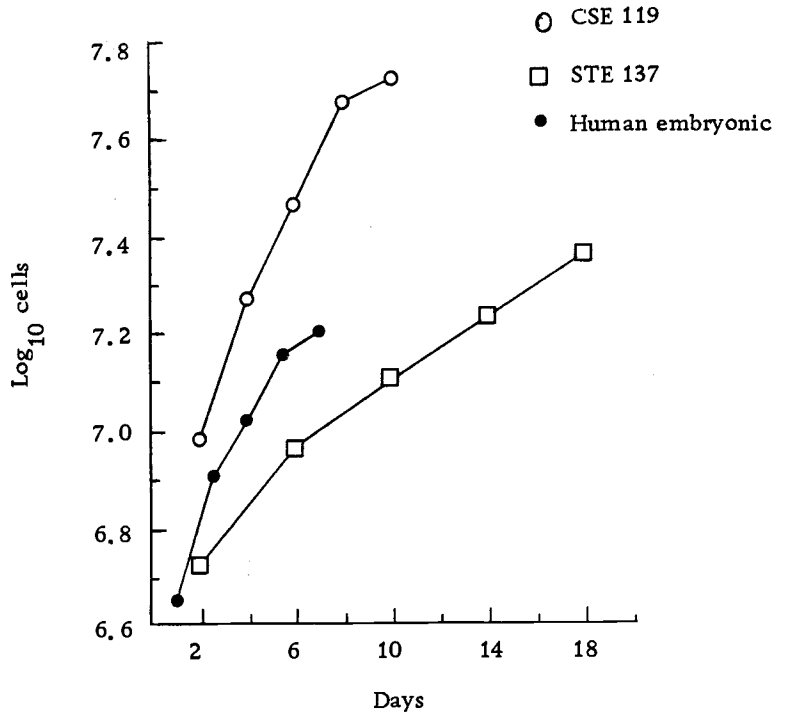

Figure 5. Growth rates of two salmonid fish cell lines at 23°C and a human cell line at 35°C in Eagle's minimal essential medium with 20% dialyzed newborn agamma calf serum.

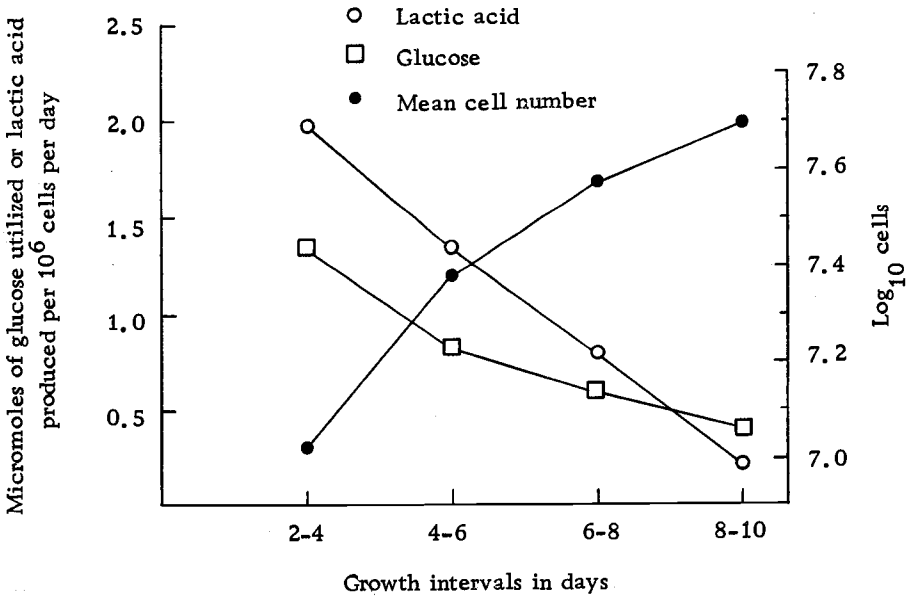

Figure 6. Rates of glucose utilized and lactic acid produced per 10^6 cells per day in cultures of the coho salmon cell line, CSE 119.

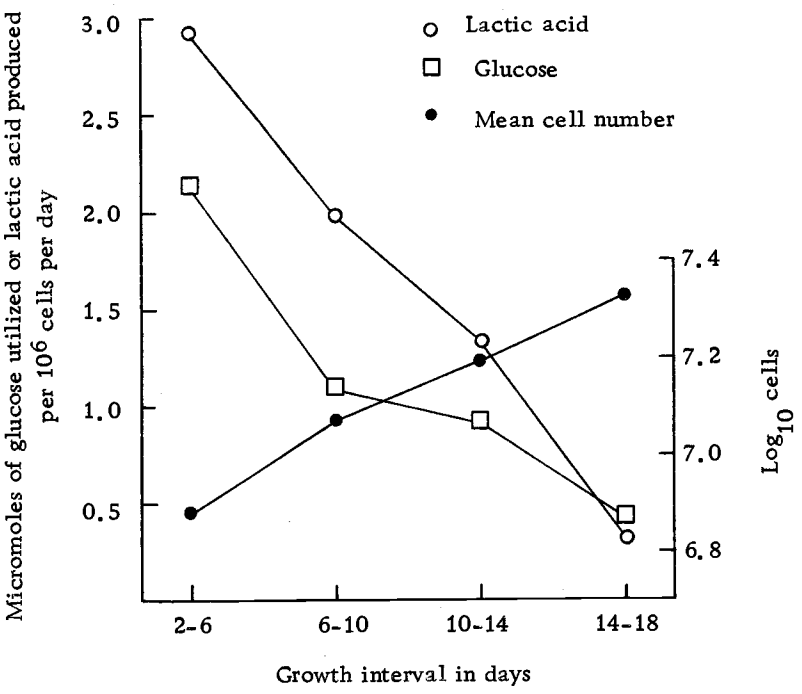

Figure 7. Rates of glucose utilized and lactic acid produced per 10^6 cells per day in cultures of steelhead trout cell line, STE 137.

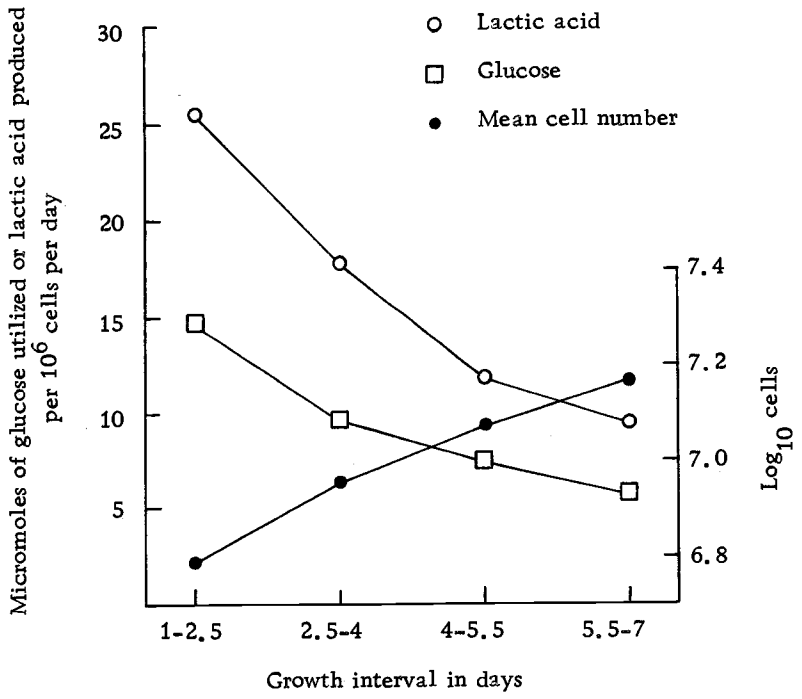

Figure 8. Rates of glucose utilized and lactic acid produced per 10^6 cells per day in cultures of human embryonic cells.

and end of each measurement interval. These curves also represent the mean values obtained from three replicate experiments.

The rates of glucose utilization and lactic acid production measured in cultures of CSE 119 during four 2-day intervals in a 10 day growth period are shown in Figure 6. It is apparent that the rates of glucose utilization and lactic acid production expressed in terms of μmoles per 10^6 cells per day were highest in the first 2-day interval, when the growth rate was maximal. Thereafter, they decreased steadily through the remainder of the experimental period. The molar ratio of lactic acid produced to glucose utilized indicates that the proportion of glucose appearing as lactic acid was about 74%, 80% and 66%, respectively, during the first three measurement intervals. This proportion dropped to about 26% during the 8 to 10-day period. In all the glycolysis experiments no attempt was made to maintain a constant pH, but the range of pH variation was limited by changing the culture medium at the appropriate intervals. In experiments with CSE 119, the greatest pH variation involved a drop from an initial value of 7.5 or 7.6 to 7.1 or 7.2

The rates of glucose utilization and lactic acid production measured in cultures of STE 137 during four 4-day intervals in an 18 day growth period are shown in Figure 7. The same steady decrease in the rates of glucose consumption and lactic acid formation observed with CSE 119 was also evident in STE 137. Both rates are

seen to be somewhat higher during the first measurement interval than those found with CSE 119. The proportion of glucose utilized appearing as lactic acid varied from about 66% in the first 4-day interval to about 90% and 74%, respectively, in the second and third intervals. It fell to less than 20% in the 14 to 18-day period. Again, the pH variation involved a drop from an initial value of 7.5 or 7.6 to 7.1 or 7.2

In order to compare the data obtained from the two fish cell lines with a mammalian cell line of comparable origin, glucose utilization and lactic acid production were studied in cultures derived from human embryos. Because of their more rapid rate of growth, an experimental growth period of seven days with four measurement intervals of 1.5 days each were employed. The data obtained with these cells is shown in Figure 8. The general course of the glucose and lactic acid curves is similar to those found with the two fish cell lines. However, the actual numbers of μmoles of glucose consumed and of lactic acid formed per 10^6 cells per day were 7 to 10-fold higher than for the fish cells. The proportion of glucose used appearing as lactic acid was about 86%, 89%, 78% and 84%, respectively, for the first, second, third and fourth measurement intervals. These values were somewhat higher than most of those observed with the fish cell lines and the sharp fall noted with the latter toward the end of the experimental growth period did not occur. The pH

range in the human embryonic cell cultures varied from the initial value of 7.6 or 7.7 down to about 6.8 at the end of the 7-day incubation period, despite changes of the culture medium at each 1.5 day interval.

The results of the glycolysis experiments with the three cell lines can be seen summarized in Table 3. It is apparent that on the basis of rates per 10^6 cells per day the human embryonic cells utilized much more glucose and produced much more lactic acid than either of the salmonid fish cell lines.

Dry Weight Measurements

Comparisons of metabolic rates on the basis of cell numbers are commonly employed, but because of the variation in cell volume and weight between different cell cultures, it is desirable to determine such rates in terms of another frame of reference. The dry weight of cells has been most frequently used as a basis for comparing the data of different investigators. For this reason the dry weights of the three cell types employed in the glycolysis studies were determined. The results can be seen in Table 4. Dry weights were determined from cultures incubated from 10-12 days for CSE 119, from cultures incubated from 15-20 days for STE 137 and from cultures incubated from 10-12 days for the human embryonic cells.

Table 3. Comparison of glycolysis in two salmonid fish cell lines and human embryonic cells.

Cell line	Glucose utilized[a] (μmoles/10^6 cells/day)	Lactic acid produced[a] (μmoles/10^6 cells/day)
CSE 119[b]	1.33	1.96
STE 137[b]	2.14	2.83
Human embryonic[c]	14.74	25.45

[a]All values refer to mean rates from three experiments, during the first portion of the experimental growth period (1.5-4.0 days).

[b]Cells grown at 23°C.

[c]Cells grown at 35°C.

Table 4. Determination of the dry weight of the salmonid fish and human embryonic cells.

Cell line	Dry weight (μg x 10^{-4} per cell)				
	Exp. 1	Exp. 2	Exp. 3	Exp. 4	mean value
CSE 119[a]	2.38	2.39	2.45	1.93	2.29
STE 137[b]	5.62	6.09	6.28	5.49	5.87
Human[c] embryonic	19.77	18.83	16.73	16.81	18.03

[a]From cultures incubated 10-12 days at 23°C.

[b]From cultures incubated 15-20 days at 23°C.

[c]From cultures incubated 10-12 days at 35°C.

Glucose Utilization and Lactic Acid Production Calculated on a Dry Weight Basis

Using the dry weight values for the three cell lines studied, the rates of glucose utilization and lactic acid production in μmoles per mg dry weight per day were calculated. The results can be seen in Tables 5, 6 and 7. For purposes of comparison, the data during the first portion of the experimental growth period (day 2-4 for CSE 119, day 2-6 for STE 137 and day 1-2.5 for the human embryonic cell line) is summarized in Table 8. It is evident that when compared on a dry weight basis, the metabolic activity of CSE 119 appears greater than that of STE 137, whereas the reverse is true on the basis of cell numbers (i.e., Table 3). Furthermore, the differences between the fish cells and the human embryonic cells are much smaller on the weight basis, but the activity of the human cells is still considerably greater.

Because many reported values for aerobic glycolysis are given in terms of manometric data, the $Q^{O_2}_{CO_2}$ values were calculated from the μmoles of lactic acid produced per day. This calculation is based on the assumption that when cells produce lactic acid aerobically in a medium containing bicarbonate, neutralization of the acid results in the decomposition of an equivalent amount of bicarbonate to produce gaseous CO_2. These values for the three cell lines are also

Table 5. Calculation of glucose utilized and lactic acid produced in cell line CSE 119 on a dry weight basis.

Exp. no.	Time period days	Mean cells x 10^6	Mean pH	µmoles lactate per 10^6 cells per day	µmoles glucose per 10^6 cells per day	µmoles lactate per mg dry wt. per day	µmoles glucose per mg dry wt. per day
35	2-4	17.44	7.38	2.64	1.66		
36	2-4	13.10	7.50	2.18	1.06		
37	2-4	10.83	7.30	1.11	1.25		
mean	2-4			1.96	1.33	8.56	5.81
35	4-6	33.00	7.18	0.66	0.65		
36	4-6	20.00	7.30	1.30	0.68		
37	4-6	18.50	7.25	2.00	1.13		
mean	4-6			1.32	0.82	5.76	3.58
35	6-8	48.40	7.25	0.56	0.37		
36	6-8	27.30	7.23	0.95	0.80		
37	6-8	36.90	7.23	0.80	0.60		
mean	6-8			0.77	0.59	3.36	2.58
35	8-10	61.40	7.20	0.08	0.33		
36	8-10	38.90	7.15	0.38	0.45		
37	8-10	45.80	7.23	0.13	0.40		
mean	8-10			0.20	0.39	0.87	1.70

Table 6. Calculation of glucose utilized and lactic acid produced in cell line STE 137 on a dry weight basis.

Exp. no.	Time period days	Mean cells x 10^6	Mean pH	µmoles lactate per 10^6 cells per day	µmoles glucose per 10^6 cells per day	µmoles lactate per mg dry wt. per day	µmoles glucose per mg dry wt. per day
38	2-6	7.00	7.33	2.97	1.78		
41	2-6	8.37	7.38	3.41	2.74		
50	2-6	7.93	7.55	2.12	1.89		
mean	2-6			2.83	2.14	4.82	3.65
38	6-10	11.20	7.13	2.27	0.80		
41	6-10	11.91	7.18	1.93	1.17		
50	6-10	12.50	7.25	1.79	1.34		
mean	6-10			1.99	1.10	3.39	1.87
38	10-14	13.90	7.20	1.61	0.72		
41	10-14	18.00	7.35	1.71	1.41		
50	10-14	16.10	7.29	0.73	0.61		
mean	10-14			1.35	0.91	2.30	1.55
38	14-18	15.30	7.15	0.70	0.40		
41	14-18	25.80	7.25	0.17	0.38		
50	14-18	22.70	7.20	0.15	0.49		
mean	14-18			0.34	0.42	0.58	0.72

Table 7. Calculation of glucose utilized and lactic acid produced in the human embryonic cell line on a dry weight basis.

Exp. no.	Time period days	Mean cells x 10^6	Mean pH	µmoles lactate per 10^6 cells per day	µmoles glucose per 10^6 cells per day	µmoles lactate per mg dry wt. per day	µmoles glucose per mg dry wt. per day
46	1-2.5	5.58	7.45	23.37	14.93		
47	1-2.5	4.17	7.45	31.87	18.78		
52	1-2.5	8.70	7.30	21.37	10.65		
mean	1-2.5			25.45	14.74	14.13	8.18
46	2.5-4	8.40	7.10	18.34	9.79		
47	2.5-4	6.60	7.10	22.47	12.08		
52	2.5-4	12.40	7.13	12.67	8.01		
mean	2.5-4			17.83	9.96	9.91	5.53
46	4-5.5	12.00	7.30	12.22	6.61		
47	4-5.5	8.60	7.12	12.27	9.43		
52	4-5.5	15.60	7.23	11.13	6.41		
mean	4-5.5			11.87	7.48	6.59	4.16
46	5.5-7	14.70	7.00	8.42	4.36		
47	5.5-7	9.90	7.10	10.63	7.71		
52	5.5-7	19.50	7.25	9.77	5.23		
mean	5.5-7			9.61	5.77	5.34	3.21

Table 8. Comparison of glycolysis on a dry weight basis in two salmonid fish cell lines and human embryonic cells.

Cell line	Glucose utilized[a] (μmoles/mg of dry wt/day)	Lactic acid produced[a] (μmoles /mg of dry wt/day)	$^{O_2}Q_{CO_2}$[d]
CSE 119[b]	5.81	8.56	7.97
STE 137[b]	3.65	4.82	4.50
Human[c] embryonic	8.18	14.13	13.19

[a] All values refer to mean rates from three experiments, during the first portion of the experimental growth period (1.5-4.0 days).

[b] Cells grown at 23°C.

[c] Cells grown at 35°C.

[d] $^{O_2}Q_{CO_2}$ = equivalent microliters CO_2 produced per mg dry weight of cells per hour.

Table 9. Variation in mean cell volume of cell line CSE 119 during a 10 day growth period.

Day	Cells per ml	mean cell volume (μ^3)
2	4.0×10^5	1704
4	6.6×10^5	1561
6	8.4×10^5	1806
8	11.4×10^5	1914
10	17.2×10^5	1707

indicated in Table 8. The $Q^{O_2}_{CO_2}$ values are given in equivalent microliters CO_2 produced per mg dry weight of cells per hour.

Mean Cell Volume

Munyon and Merchant (1959) suggested that the mean cell volume decreased progressively with the age of cultures and that this decrease could explain the decreasing rates of glucose utilization found over a period of cell growth. To determine whether this was occurring in the fish cell lines an experiment was run with the coho salmon cell line (CSE 119). The mean cell volume was determined over a 10 day growth period using Van Allen Thrombocytocrit tubes. The results can be seen in Table 9. It is evident that the mean cell volume did not decrease with the age of the culture over the 10 day period measured and that decreasing cell volume cannot account for the decreasing rates of glucose utilization or lactic acid production found in the growth of these salmonid fish cells.

Correlation Between Lactic Acid Production and pH Drop

It has been observed that growth of salmonid cell lines in vitro is not accompanied by the same rapid pH drop as has been noted with many mammalian cell lines. The lower rates of lactic acid production may account for this and for this reason the effect of lactic acid on the pH was investigated. The results can be seen in Table 10. In

Table 10. Comparison of lactic acid production and pH drop in two salmonid fish cell lines and a human embryonic cell line.

	Experimental pH drop					artificial pH drop			
Cell line	Interval (days)	pH start	pH finish	Δ pH	lactic[a] prod.	lactic[b] added	pH start	pH finish	Δ pH
CSE 119	2-6	7.60	7.20	0.40	304	300	7.60	7.00	0.60
CSE 119	6-10	7.30	7.20	0.10	177	180	7.30	6.91	0.39
STE 137	2-10	7.50	7.10	0.40	580	580	7.50	6.55	0.95
STE 137	10-18	7.20	7.10	0.10	272	270	7.20	6.71	0.49
Human	1-2.5	7.70	7.20	0.50	561	560	7.70	6.78	0.92
Human	2.5-4	7.35	6.90	0.45	649	650	7.30	6.36	0.94
Human	4-5.5	7.50	7.10	0.40	618	620	7.50	6.51	0.99
Human	5.5-7	7.20	6.85	0.35	523	520	7.20	6.40	0.80

[a] μg lactic acid per ml of culture medium.

[b] μg lactic acid per ml added as calculated from dilution of reagent lactic acid. Values from actual lactic acid determinations of the diluted materials were in close agreement with these figures.

every case the actual experimental pH drop was smaller than that produced by adding a similar amount of lactic acid to the complete medium containing 20% dialyzed serum. In the case of CSE 119, the experimental drop over an 8 day period was 0.5 pH units with the total production of 481 μg lactic acid per ml of culture medium. With STE 137 the experimental drop over a 16 day period was again 0.5 pH units with a total production of 852 μg lactic acid per ml of culture medium. With the human embryonic cell line, the experimental drop over a six day period was 1.7 pH units with a total production of 2351 μg lactic acid per ml of culture fluid. These results can be seen summarized on a per day basis in Table 11. The data suggests a correlation between pH drop and lactic acid production. It therefore appears that the lower rate of lactic acid production in the fish cells could in part account for the long periods these cells can be held with very little pH change.

Carbon Dioxide Requirements

The second part of the research involved a study of the CO_2 requirements of the salmonid fish cell lines.

Statistical Analysis

In order to show differences in CO_2 requirements between the two salmonid fish cell lines and HeLa cells, it was necessary to

Table 11. Summary of pH drop and lactic acid production per day with cell lines CSE 119, STE 137 and human embryonic cells.

cell line	pH drop in pH units per day	μg lactic acid produced per ml. of culture medium per day
CSE 119[a]	0.06	60.1
STE 137[b]	0.03	53.3
human embryonic[c]	0.28	391.8

[a] over an 8 day growth period at 23°C.

[b] over a 16 day growth period at 23°C.

[c] over a 6 day growth period at 35°C.

calculate the degree to which growth under the various conditions of CO_2 tension must differ to be significant. Calculations were done using duplicate counts from each of three replicate experimental runs under the four conditions of CO_2 tension used in the experiments. The least significant difference was based on a comparison of the three replicate experiments done under identical conditions.

In order to compare data from both low and high counts at the same time, the data from 40 groups of replicate experimental runs at days 2-16 was "transformed" into $\log_{10}$ values. These are shown in Table 12. The deviations from the mean of the $\log_{10}$ of each experimental replicate are shown in Table 13. The final calculations of the least significant difference are shown in Table 14 and gave a value of 0.1414. This meant that the difference in the $\log_{10}$ of cell counts between two cultures must be greater than 0.1414 to be significant.

Growth at Four Different CO_2 Levels

Growth of the coho salmon (CSE 119) cell line and the steelhead trout (STE 137) cell line was compared to growth of HeLa cells at four different CO_2 levels. The CO_2 levels were produced by the following methods:

(1) Stoppered--rubber stoppered cultures, the level of CO_2

Table 12. Log_{10} cell numbers for 40 groups of replicate observations of growth in CO_2 experiments.

Observation number	Log mean number of cells per culture from duplicate observations in each of three replicate experiments			mean log of three replicates
1	6.4314	6.4829	6.3324	6.4200
2	6.5527	6.6075	6.7033	6.6263
3	6.6053	6.7520	6.7536	6.7093
4	6.7050	6.9186	6.8513	6.8338
5	6.6232	6.5563	6.5966	6.5933
6	6.7466	6.6857	6.6767	6.7042
7	6.8035	6.8675	6.7202	6.8014
8	6.4983	6.3617	6.5211	6.4669
9	6.4654	6.6355	6.5786	6.5658
10	6.3692	6.7050	6.5502	6.5623
11	6.5145	6.4654	6.4183	6.4683
12	6.5159	6.4065	6.5378	6.4914
13	6.4099	6.6821	6.6180	6.5843
14	6.7202	6.7033	6.7110	6.7118
15	7.0531	7.0043	6.9469	7.0043
16	7.2279	7.1875	7.1399	7.1847
17	7.4518	7.3711	7.3617	7.3962
18	6.7723	6.7723	6.7110	6.7528
19	7.0607	7.0294	6.9685	7.0212
20	7.3010	7.2672	7.2330	7.2672
21	7.4065	7.5024	7.4594	7.4579
22	6.9340	6.7789	6.7789	6.8370
23	7.1644	6.9165	6.9708	7.0334
24	7.0934	7.0719	7.1553	7.1072
25	6.8774	6.6767	6.6964	6.7604
26	6.9320	6.6812	6.6955	6.7860
27	7.0414	6.5955	6.7903	6.8476
28	6.3522	6.3979	6.4548	6.4031
29	6.9420	6.8663	6.8432	6.8859
30	7.2201	7.1847	7.0212	7.1492
31	7.1987	7.3979	7.2672	7.2967
32	6.9031	6.7627	6.8482	6.8420
33	7.1584	7.0453	6.9717	7.0645
34	7.2625	7.3284	7.2900	7.2945
35	6.8500	6.8657	6.8921	6.8698
36	7.0569	7.0934	7.0645	7.0719
37	7.1847	7.2380	7.1732	7.1987
38	6.8325	6.7126	6.9090	6.8254
39	7.0294	6.9528	7.0334	7.0043
40	7.1072	7.0414	7.2014	7.1206

Table 13. Deviations from the $\log_{10}$ mean for 40 groups of replicate observations of growth in CO_2 experiments.

Observation number	Deviation from log mean		
1	0.0114	0.0629	0.0876
2	0.0736	0.0188	0.0770
3	0.1040	0.0427	0.0443
4	0.1288	0.0848	0.0175
5	0.0299	0.0370	0.0033
6	0.0424	0.0185	0.0275
7	0.0021	0.0661	0.0812
8	0.0314	0.1052	0.0542
9	0.1004	0.0697	0.0128
10	0.1931	0.1427	0.0121
11	0.0462	0.0029	0.0500
12	0.0245	0.0849	0.0464
13	0.1744	0.0978	0.0337
14	0.0084	0.0085	0.0008
15	0.0488	0.0000	0.0574
16	0.0432	0.0028	0.0448
17	0.0556	0.0251	0.0345
18	0.0195	0.0195	0.0418
19	0.0395	0.0082	0.0527
20	0.0338	0.0000	0.0342
21	0.0514	0.0445	0.0015
22	0.0970	0.0581	0.0581
23	0.1310	0.1169	0.0626
24	0.0138	0.0353	0.0481
25	0.1170	0.0837	0.0640
26	0.1460	0.1048	0.0905
27	0.1938	0.2521	0.0573
28	0.0509	0.0052	0.0517
29	0.0561	0.0196	0.0427
30	0.0709	0.0355	0.1280
31	0.0980	0.1012	0.0295
32	0.0611	0.0793	0.0062
33	0.0939	0.0192	0.0928
34	0.0320	0.0339	0.0045
35	0.0199	0.0041	0.0223
36	0.0150	0.0215	0.0074
37	0.0140	0.0648	0.0255
38	0.0071	0.1128	0.0836
39	0.0251	0.0515	0.0291
40	0.0134	0.0792	0.0808

Table 14. Calculation of the least significant difference from the deviations found in 40 groups of replicate observations of growth in CO_2 experiments.

Combined sum of squares of the deviations = 0.6023

Divisor (sum of degrees of freedom) = (3-1) × 40 = 80

Variance = $\frac{0.6023}{80}$ = 0.0075

Estimated standard deviation = $\sqrt{0.0075}$ = 0.0866

Standard error of difference between means =

$$\pm \sqrt{\left(\frac{0.0866}{3}\right)^2 \times 2} = \frac{0.0866}{1.732} \times 1.414 = 0.0707$$

In Fisher's Table of t with 80 degrees of freedom, and P = 0.05

$$t = 2.0$$

Least significant difference = 0.0707 × 2.0 (t value) = > 0.1414

Example: For the cell number to be considered significantly lower than a count of 4,210,000 ($\log_{10}$ = 6.6243), the second count would have to be 3,040,000 ($\log_{10}$ = 6.4829) or less.

determined by the amount produced by the cells themselves

(2) Open--cotton plugged cultures open to the air, the level of CO_2 determined by the amount in the air, approximately 0.03%

(3) Increased CO_2--cotton plugged cultures in a CO_2 incubator containing 3% atmospheric CO_2 in the case of the HeLa cells and 2% CO_2 in the case of the salmonid fish cells

(4) Reduced CO_2--cultures with special CO_2 traps containing 10% KOH.

All cells were grown during an initial period in stoppered 150 ml Pyrex milk dilution bottles before being put under the experimental conditions so that they would be at maximum growth at the time the conditions of CO_2 tension were applied. For HeLa cells this was two days, for CSE 119 this was three days and for STE 137, it was four days. The fish cells were grown at 18° C and the HeLa cells at 35° C. After the initial growth period, the medium was removed from the cultures and replaced with bicarbonate free medium. The cultures were then reincubated under the four conditions of CO_2 tension and observed for growth. The results can be seen in Figures 9, 10, and 11. Results for all three cell lines represent mean values from three independent experimental runs. Significant differences

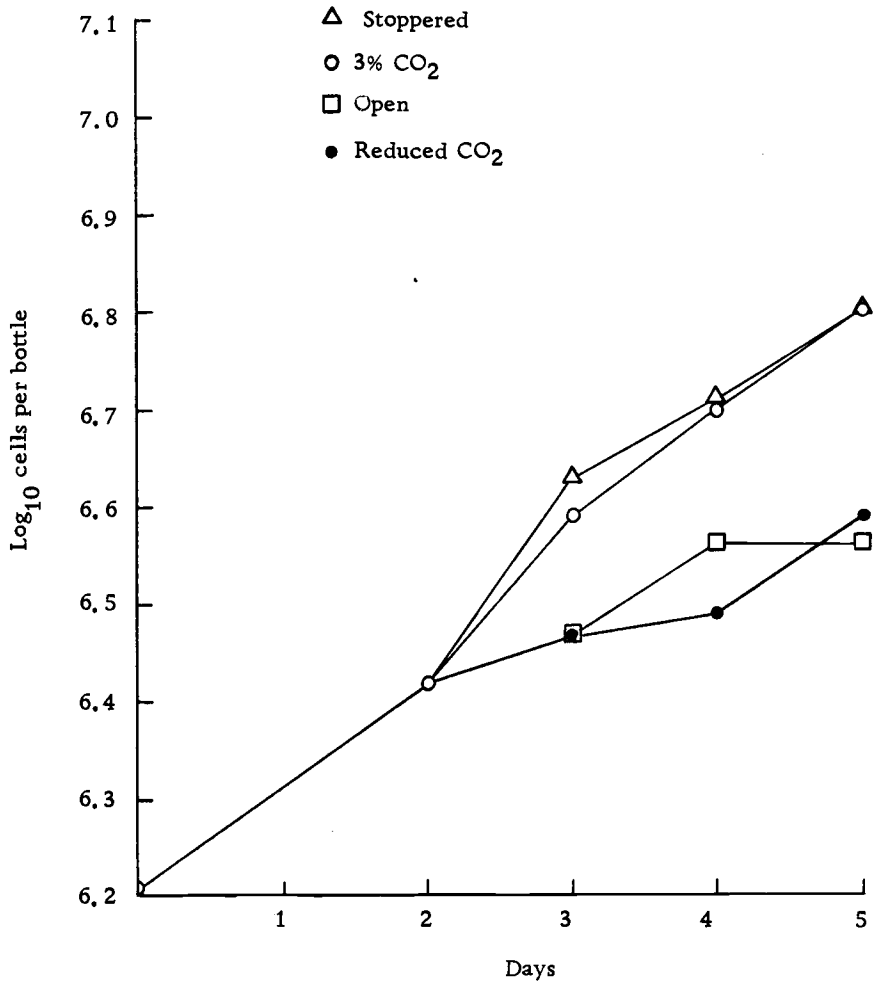

Figure 9. Growth of HeLa cells at 35°C in stoppered cultures, cultures open to the air (0.03% CO_2), cultures in 3% atmospheric CO_2, and in cultures with reduced CO_2 (CO_2 removed by KOH traps).

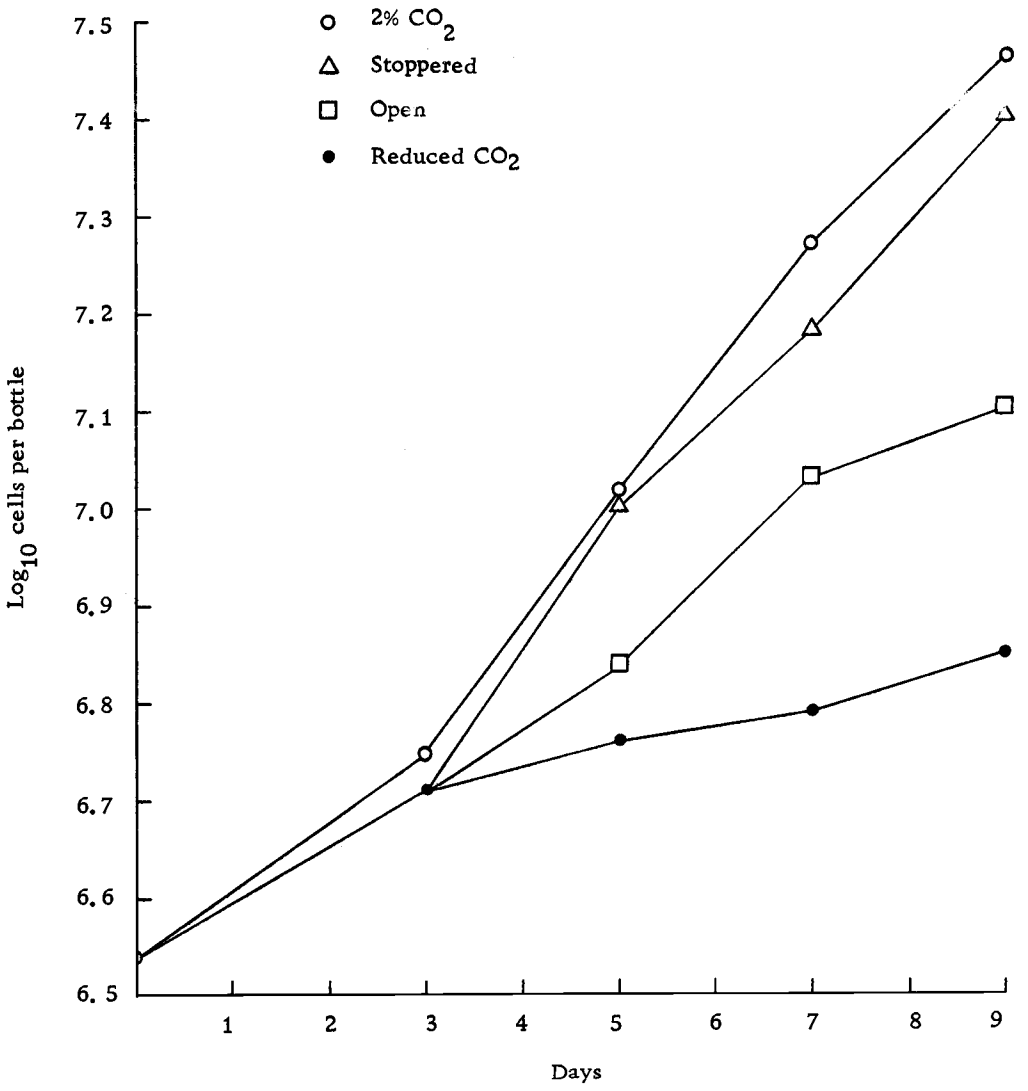

Figure 10. Growth of CSE 119 cells at 18°C in stoppered cultures, cultures open to the air (0.03% CO_2), cultures in 2% atmospheric CO_2, and in cultures with reduced CO_2 (CO_2 removed by KOH traps).

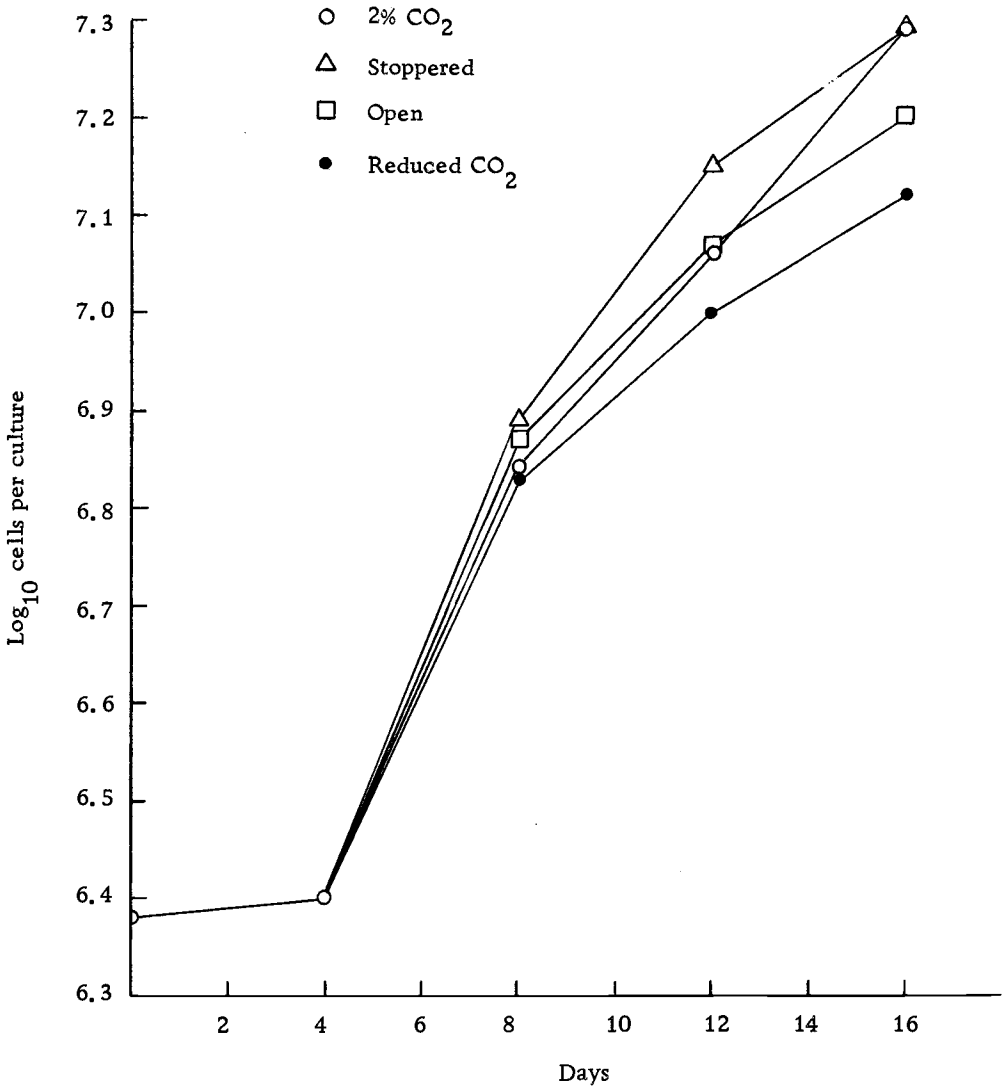

Figure 11. Growth of STE 137 cells at 18°C in stoppered cultures, cultures open to the air (0.03% CO_2), cultures in 2% atmospheric CO_2 and in cultures with reduced CO_2 (CO_2 removed by KOH traps).

were judged on the basis of the calculated least significant difference (0.1414) on a $\log_{10}$ scale.

In Figure 9 it can be seen that growth of HeLa cells was significantly repressed under conditions of reduced CO_2 (CO_2 removed by traps) and open cultures. Growth in 3% CO_2 did not significantly differ from the growth in stoppered cultures.

In Figure 10, it can be seen that growth of the coho salmon cell line was significantly repressed in both the open and reduced cultures. However, the cells in the open cultures grew significantly better than those in the reduced CO_2 cultures. Growth in 2% CO_2 did not significantly differ from the growth in the stoppered cultures.

The steelhead trout cell line showed the least dependence on CO_2 of the three cell lines as can be seen in Figure 11. Only the reduced CO_2 cultures showed a significantly lower growth rate than the stoppered cultures but even under conditions of reduced CO_2 the cells grew well. Neither the open nor the 2% CO_2 cultures showed a significant difference in growth from the stoppered cultures.

Absolute Requirement for CO_2

For the most part, animal cells grown *in vitro* have been shown to require CO_2 as an essential nutrient. Because of the seemingly low requirement for CO_2 shown by the salmonid fish cells, experiments were conducted to determine if CO_2 was actually essential for

growth of these cells. Carbon dioxide free conditions were obtained by growing the cells in the center well of Conway microdiffusion dishes with 10% KOH in the outer well to trap any CO_2 produced by the cells. Growth under these CO_2 free conditions was compared to growth under 2% and 3% atmospheric CO_2. The results can be seen in Figure 12. All three cell lines grew well in the bicarbonate free medium when placed in 2% CO_2 (fish) and 3% CO_2 (HeLa). However, little or no growth occurred in the same medium in CO_2 free cultures. These results have been summarized in Table 15. Both salmonid cell lines demonstrated a growth requirement for CO_2 comparable to that shown for HeLa cells.

Oxalacetate Substitution for CO_2

Oxalacetate has been reported to partially substitute for CO_2 in the growth of HeLa cells (Runyan and Geyer, 1967). Since the CO_2 requirements of the salmonid fish cell cultures seemed different from those of the HeLa cell cultures, the ability of oxalacetate to substitute for CO_2 in all three cell lines was tested. The results can be seen in Figure 13. None of the cell lines grew in the bicarbonate free medium in the CO_2 free Conway microdiffusion dishes. When the medium was supplemented with 2 mM oxalacetate only the HeLa cells showed a growth response. All three cell lines grew well when supplemented with CO_2. The results have been summarized in Table 16.

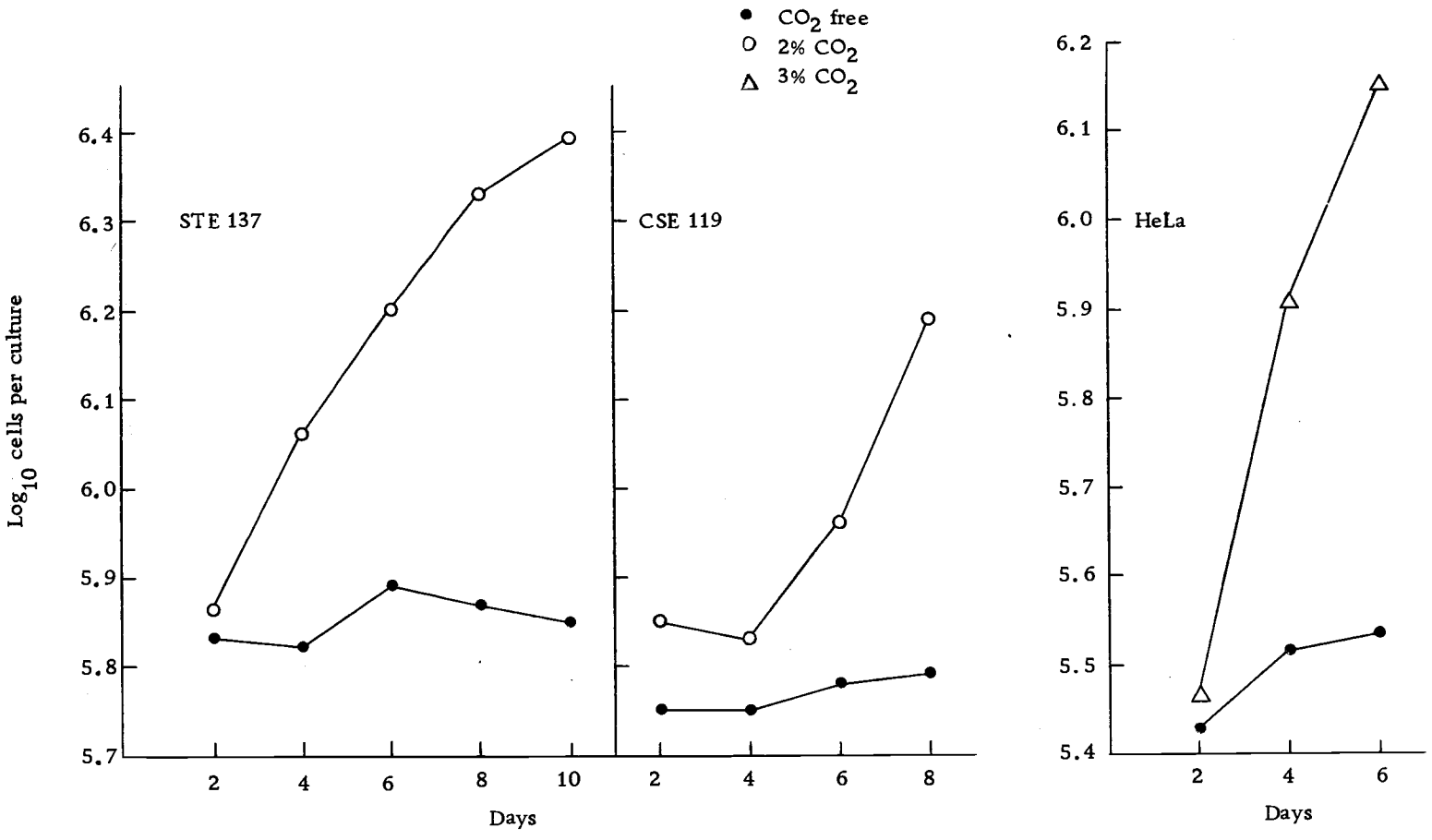

Figure 12. Growth of CSE 119, STE 137, and HeLa cells in bicarbonate free medium under 2% atmospheric CO_2 (fish) and 3% atmospheric CO_2 (HeLa), and under CO_2 free conditions in Conway microdiffusion dishes with a KOH trap.

Table 15. Growth of HeLa cells and two salmonid cell lines in CO_2 free and CO_2 containing atmospheres.

Cell Type	Culture Condition[d]		Cells x 10^5/culture at Day 2	Day 8
HeLa[a]	CO_2 free[c]	Exp. 1	3.1	3.6
	3% CO_2	Exp. 1	3.8	36.0
CSE 119[b]	CO_2 free	Exp. 1	5.6	6.1
		Exp. 2	11.3	10.2
	2% CO_2	Exp. 1	7.1	15.6
		Exp. 2	8.8	33.8
STE 137[b]	CO_2 free	Exp. 1	6.6	7.4
		Exp. 2	4.9	7.4
	2% CO_2	Exp. 1	7.2	21.0
		Exp. 2	5.4	25.8

[a]Cells grown at 35°C.

[b]Cells grown at 18°C.

[c]Cells grown in Conway microdiffusion dishes with KOH trap.

[d]All cells were grown in bicarbonate free medium with Tris buffer.

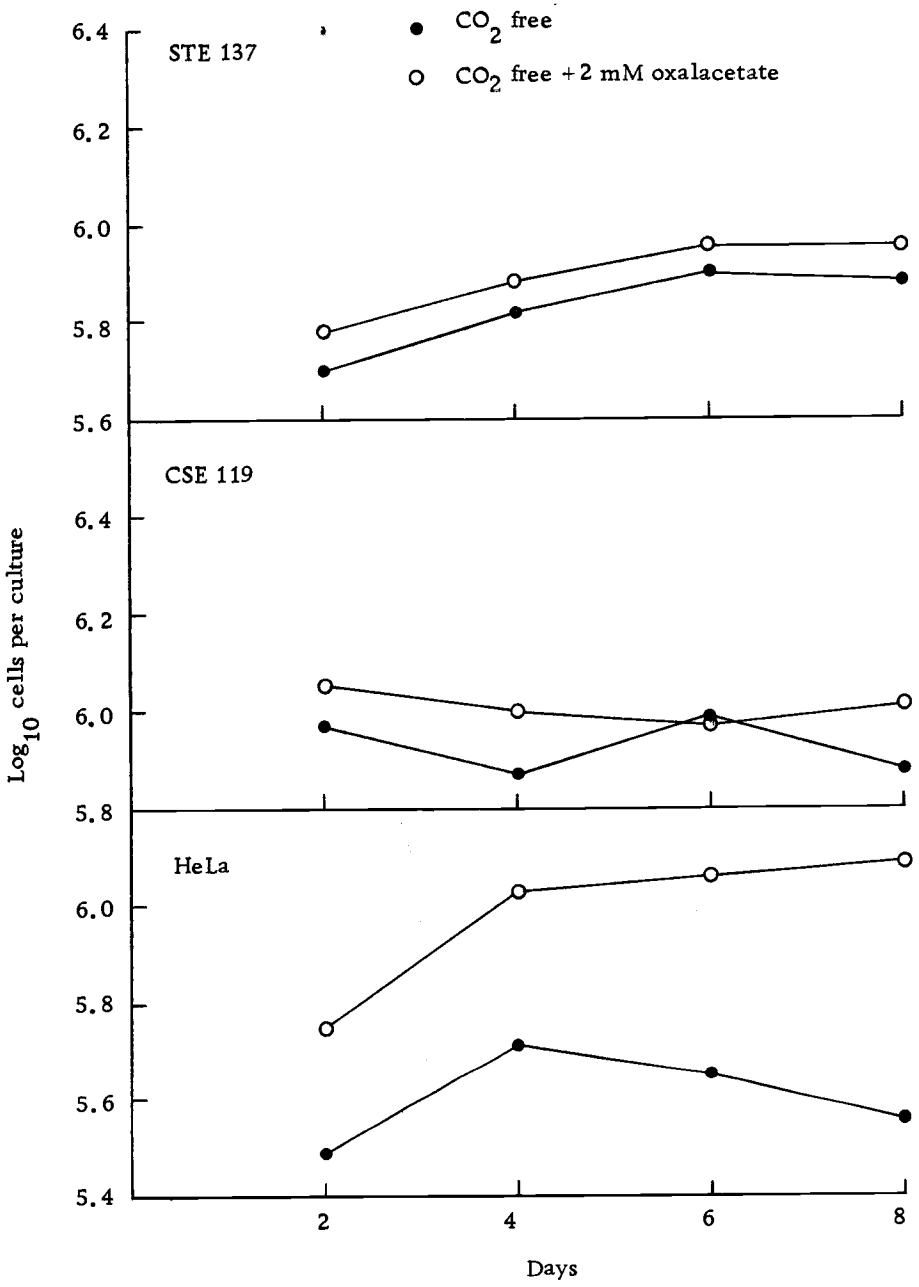

Figure 13. Growth of CSE 119, STE 137 and HeLa cells in bicarbonate free medium in CO_2 free Conway microdiffusion dishes with KOH traps and under the same conditions in medium supplemented with oxalacetate.

Table 16. Growth of HeLa cells and two salmonid cell lines in CO_2 free and CO_2 containing atmospheres in medium supplemented with oxalacetate.

Cell Type	Cultural Conditions[d]			
	CO_2 free[e] + 2 mM oxalacetate		CO_2[c] + 1 mM oxalacetate	
	Cells x 10^5/culture at Day 2	Day 8	Cells x 10^5/culture at Day 2	Day 8
HeLa[a]	5.5	12.4	3.8	36.0
CSE 119[b]	9.2	7.4	8.8	33.8
STE 137[b]	6.0	8.9	5.4	25.8

[a]Cells grown at 35°C.

[b]Cells grown at 18°C.

[c]HeLa cells grown under 3% CO_2 and fish cells grown under 2% CO_2.

[d]Tris buffer was substituted for bicarbonate in all culture media.

[e]Cells grown in Conway microdiffusion dishes with KOH trap.

As had been reported, oxalacetate partially substituted for CO_2 in the HeLa cell cultures, but little or no growth occurred in the salmonid fish cell cultures under the same conditions.

Viral Susceptibility

Studies of the viral susceptibility of salmonid fish cell lines were initiated by Nims (1968). In a furtherance of this effort, the two salmonid fish cell lines were tested for their susceptibility to Reovirus types 1 and 3, Infectious Pancreatic Necrosis virus and Wound Tumor virus.

Reovirus Types 1 and 3

The identity of the stock viruses used in these experiments was confirmed by hemagglutination-inhibition tests. The results can be seen in Table 17. Type 1 antiserum inhibited hemagglutination by stock type 1 virus through the 1:1024 dilution. Type 3 antiserum inhibited hemagglutination by the stock type 3 virus through the 1:512 dilution. Control serum failed to inhibit hemagglutination by either type 1 or type 3 virus.

Virus infectivity titrations of both Reovirus types 1 and 3 were carried out in HeLa cells. Infected cells were identified by the presence of cytoplasmic viral inclusions demonstrated by acridine orange staining. Figure 14 shows a typical green viral inclusion in a HeLa cell. Inclusions of the same type were produced by both Reovirus

Table 17. Identification of Reovirus types 1 and 3 through hemagglutination-inhibition.

Test system[b]	antiserum dilutions[a] 1:4	1:8	1:16	1:32	1:64	1:128	1:512	1:1024	1:2048	1:4096	1:8192
Type 1 virus[c] and type 1 antiserum[d]	-	-	-	-	-	-	-	-	+	+	+
Type 3 virus[c] and type 3 antiserum[d]	-	-	-	-	-	-	-	+	+	+	+
Type 1 virus and control serum[e]	+	+	+	+							
Type 3 virus and control serum[e]	+	+	+	+							
Type 1 virus titration				+	+	+	-				
Type 3 virus titration			+	+	-	-					

[a](-) indicated no hemagglutination; (+) indicated hemagglutination.

[b]Human O erythrocytes used diluted 0.75% in dextrose-veronal-gelatin buffer.

[c]Reovirus types 1 and 3 stock virus.

[d]Reovirus antisera was obtained from G. I. B Co. and was kaolin and human O RBC treated.

[e]Control serum was normal fetal calf serum.

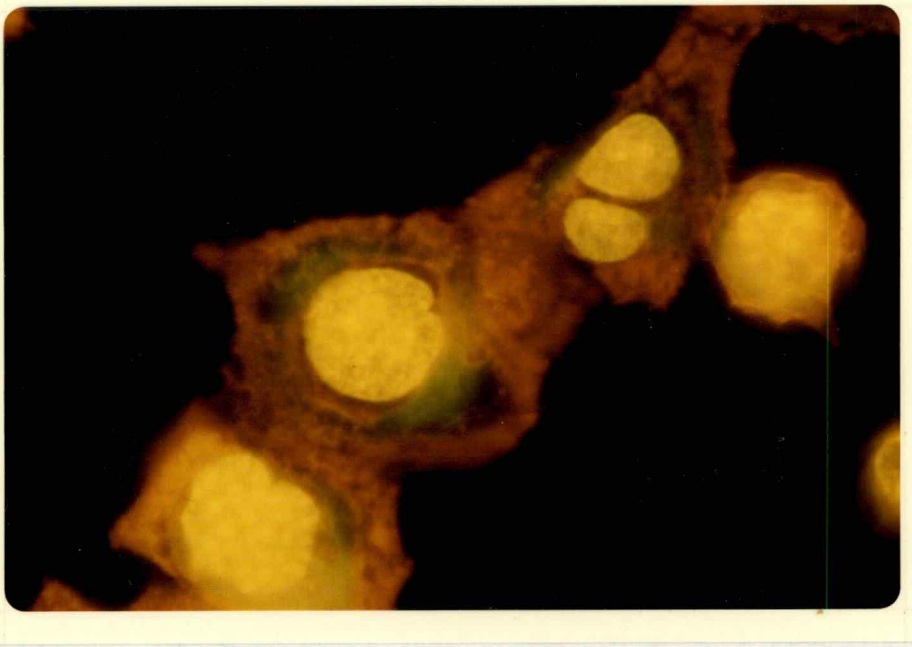

Figure 14. Photomicrograph of acridine orange stain of Reovirus type 3 infected HeLa cells at 35° C, day 4 post-infection.

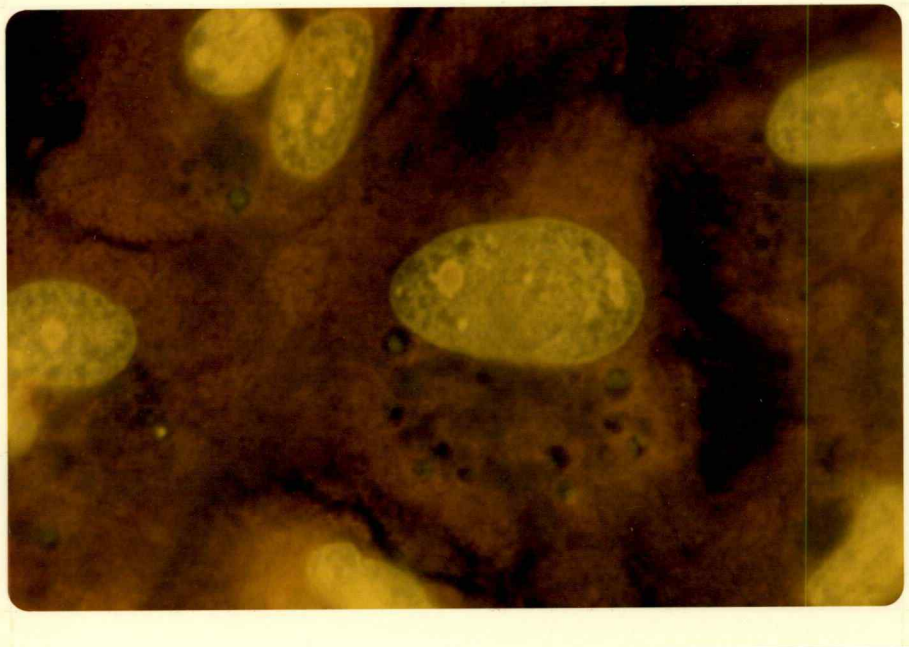

Figure 15. Photomicrograph of acridine orange stain of Reovirus type 3 infected STE 137 cells at 26° C, day 3 post-infection.

types 1 and 3. Their large size and perinuclear location were very characteristic and easily recognized.

Attempts to demonstrate reovirus inclusions in both salmonid fish cell lines at 26 ° C using the acridine orange staining technique yielded questionable results. Reovirus infected cells of both fish cell lines would show round or oval shaped green cytoplasmic inclusions as can be seen in Figure 15 but this type of inclusion could sometimes be seen in control cultures. The frequency of such inclusions seemed higher in the steelhead trout (STE 137) cell line than in the coho salmon (CSE 119) cell line. In both fish cell lines, the frequency of inclusions in the infected cells seemed higher than in control cells.

Serial passages of both Reovirus types 1 and 3 in both fish cell lines at 26 ° C were attempted and the results can be seen in Table 18. Neither Reovirus type 1 nor type 3 could be passed in either fish cell line as measured by both infectivity and hemagglutination titrations. In all four cases, transfers were continued through five passages but no adaptation of the virus to the cells was detected.

Since the reovirus strains used were both of mammalian origin, it was possible that the lower incubation temperature of 26 ° C may have been responsible for the failure of the virus to replicate in the fish cells. Therefore, HeLa cells were infected with both Reovirus types 1 and 3 and incubated at 26 ° C. The results of acridine orange

Table 18. Summary of reovirus serial passages in two salmonid cell lines.

Cell line[a]	Virus[b]	Infectivity, [c] $TCID_{50}$/0.5 ml					Hemagglutination titration[d]				
		Calc. Inoc.	Titer Inoc.	1st Pass Day 7	3rd Pass Day 7	5th Pass Day 7	Stock Virus	1st Pass Day 7	3rd Pass Day 7	5th Pass Day 7	Total[g] dilution
CSE 119	Reo 1	$10^{2.5}$	-	$<10^{1}$[e]	$<10^{1}$	-	1:128	<1:2[f]	<1:2	-	10^{4}
CSE 119	Reo 3	$10^{2.75}$	-	$10^{1.75}$	$<10^{1}$	$<10^{1}$	1:32	<1:2	<1:2	<1:2	10^{8}
STE 137	Reo 1	$10^{2.5}$	$10^{1.24}$	$<10^{1}$	$<10^{1}$	-	1:128	<1:2	<1:2	-	10^{4}
STE 137	Reo 3	$10^{2.75}$	$10^{2.24}$	$10^{1.75}$	$<10^{1}$	$<10^{1}$	1:32	<1:2	<1:2	<1:2	10^{8}

[a] All cells infected at 26°C.

[b] Stock virus prepared in HeLa cells at 35°C.

[c] Infectivity titrations carried out in HeLa cells at 35°C.

[d] Hemagglutination titration method of Rosen.

[e] $<10^{1}$ = no infected cells observed in HeLa cell titration of 10^{1} dilution of passage material.

[f] <1:2 = no hemagglutination detected in 1:2 dilution of passage material.

[g] Total dilution = combined passage dilutions of original inoculum of 1st passage.

staining of infected and control HeLa cells can be seen in Figures 17, 18, and 19. Both viral types produced typical green inclusions in the HeLa cells at this temperature. However, the inclusions lacked the intensity of those produced at 35° C. The control cells showed definite signs of degeneration at 26° C.

Finally, an attempt was made to pass Reovirus type 3 at 26° C in HeLa cells. Titration of the inoculum showed that $10^{1.75}$ $TCID_{50}$ per 0.5 ml was adsorbed to the cells. The virus infected cells were incubated at 26° C for seven days at which time the virus in homogenates of the cells and culture fluid was again titrated. Only $10^{1.75}$ $TCID_{50}$ per 0.5 ml were detected and no hemagglutinating activity was found in the passage material. It therefore appeared that although the characteristic large perinuclear viral inclusions were formed in HeLa cells at 26° C, little or no infectious virus was formed.

An attempt was made to pass the reoviruses in the salmonid fish cells at 30° C. After one night incubation both infected and control cells were completely destroyed due to the elevated temperature.

Infectious Pancreatic Necrosis Virus

Identification of the stock Infectious Pancreatic Necrosis (IPN) virus was confirmed by viral neutralization as can be seen in Table 19. At least 10^{2} $TCID_{50}$ of IPN virus was neutralized by a 1:10

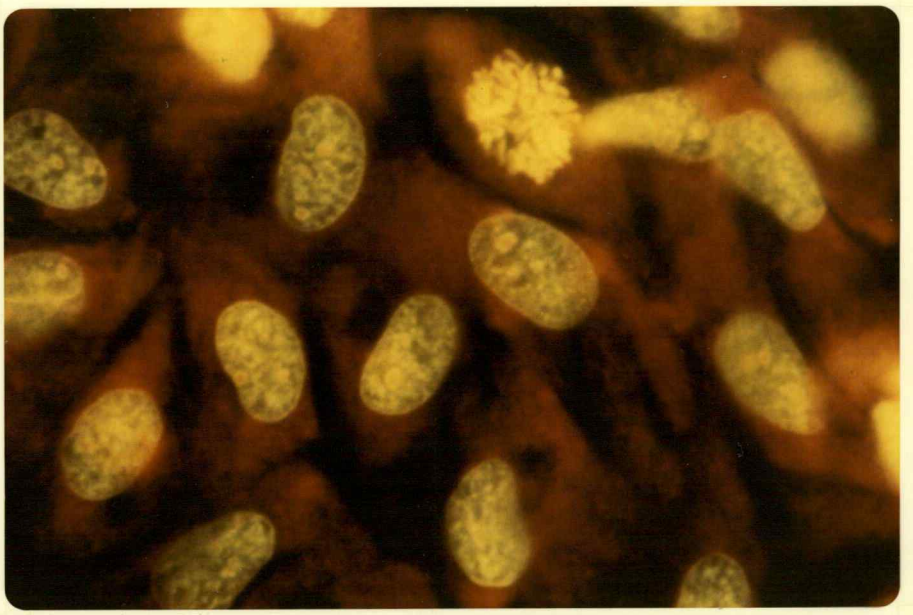

Figure 16. Photomicrograph of acridine orange stain of control STE 137 cells at 26° C, same age as cells in Figure 15.

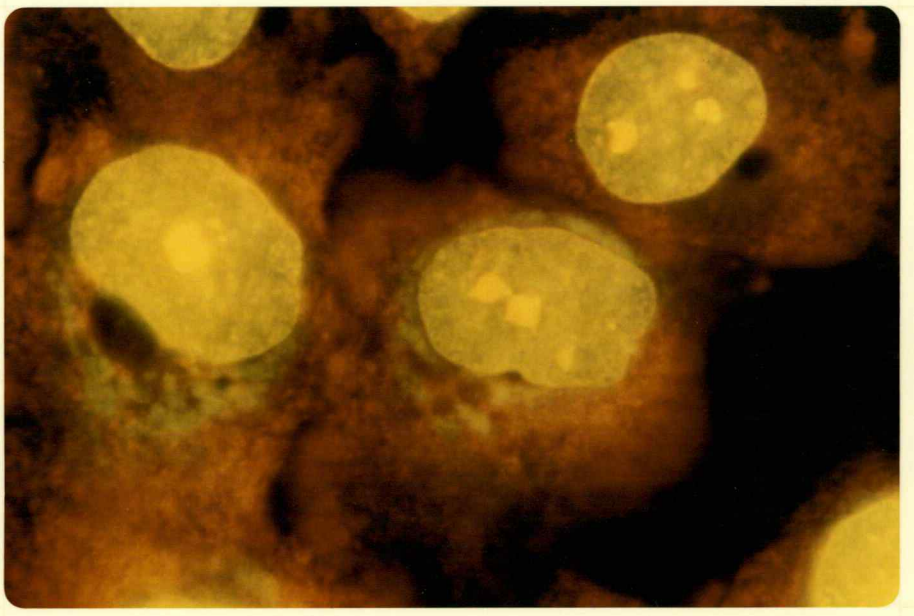

Figure 17. Photomicrograph of acridine orange stain of Reovirus type 1 infected HeLa cells at 26° C, day 4 post-infection.

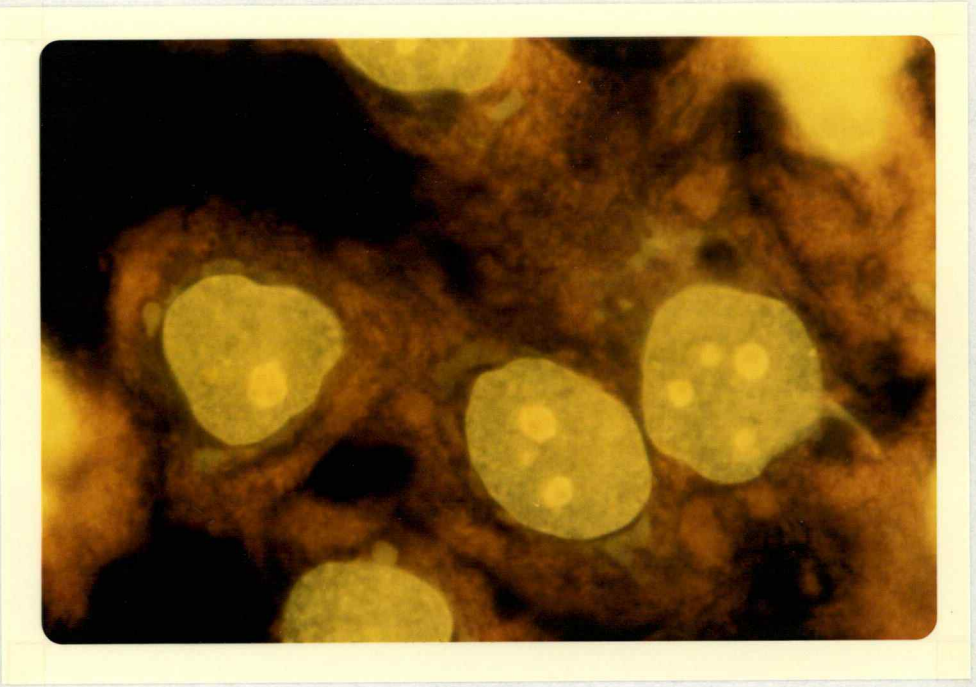

Figure 18. Photomicrograph of acridine orange stain of Reovirus type 3 infected HeLa cells at 26° C, day 4 post-infection.

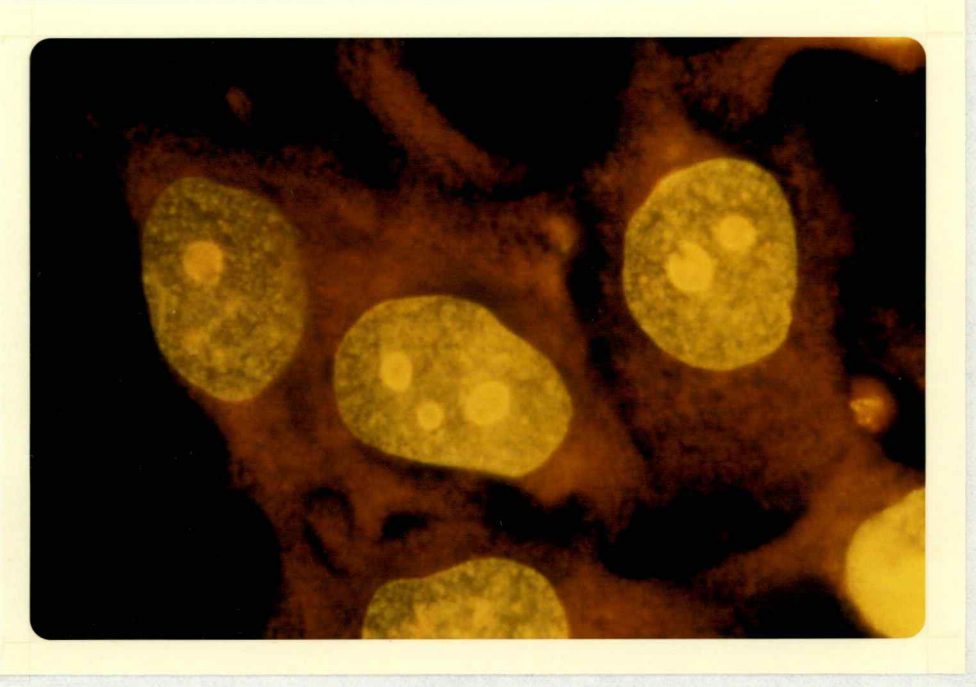

Figure 19. Photomicrograph of acridine orange stain of control HeLa cells at 26° C, same age as cells in Figures 17 and 18.

Table 19. Serum neutralization test on Infectious Pancreatic Necrosis (IPN) virus.

Dilution of stock virus[b]	Stock virus Tubes 1	2	3	virus + anti-IPN serum[a] Experiment 1 Tubes 1	2	3	virus + anti-IPN serum[a] Experiment 2 Tubes 1	2	3
2×10^5	+	+	+	+	-	+	+	-	-
2×10^6	+	+	+	-	-	-	-	-	-
2×10^7	+	+	+	-	-	-	-	-	-

Controls	Tubes 1	2	3
Cells	-	-	-
Cells + anti-IPN serum	-	-	-

[a] 1:10 dilution of goat anti-IPN serum from Microbiological Associates, Bethesda, Maryland.

[b] Stock virus, $10^{8.24}$ $TCID_{50}$/0.5 ml.

[c] (+) indicated viral CPE at three days post infection.

dilution of IPN-antiserum. This indicates a definite serological relationship between the virus used in our experiments and the ATCC strain of IPN virus against which the antiserum was prepared.

Serial passages of IPN virus in the coho salmon (CSE 119) cell line and the steelhead trout (STE 137) cell line at 18° C were attempted and the results can be seen in Table 20. Good replication of the virus was shown in both salmonid cell lines on the initial passage. Also, the virus was successfully carried for five passages and again good replication was shown in both cell lines during the fifth passage.

Since IPN virus has been shown to have many of the characteristics of the reovirus group (Moss and Gravell, 1969), infected cells of both salmonid cell lines were stained with acridine orange. The results can be seen in Figures 20 and 21. Green inclusions were not detected in either cell line at 24 or 36 hours after infection. Both cell lines showed considerable cell degeneration by 36 hours with rounding up of the cells and shrinkage of the nuclei (Figure 21).

A phase contrast study of IPN infection of both salmonid cell lines was made and the results can be seen in Figures 22-29. An early webbing of the cytoplasm was seen in both cell lines (Figures 23 and 25). This was followed by a rounding up of the cells and shrinkage of the nuclei (Figures 27 and 29). Finally a heavy margination of nuclear chromatin was observed. By 36 hours the cell

Table 20. Summary of Infectious Pancreatic Necrosis virus serial passages in two salmonid fish cell lines.

Cell line[a]	Infectivity titer,[b] $TCID_{50}$/0.5 ml				Total[c] Dilution
	Inoculum 1st Pass	1st Pass Titration Day 3.5	Inoculum 5th Pass	5th Pass Titration Day 3.5	
CSE 119	$10^{3.5}$	$10^{9.24}$	$10^{4.5}$	$10^{9.75}$	10^{13}
STE 137	$10^{3.5}$	$10^{9.5}$	$10^{2.75}$	$10^{9.24}$	10^{14}

[a] All cells infected at 18°C.

[b] Infectivity titrations carried out in cell line CHSE 214 at 18°C.

[c] Total dilution = combined passage dilutions of 1st passage inoculum.

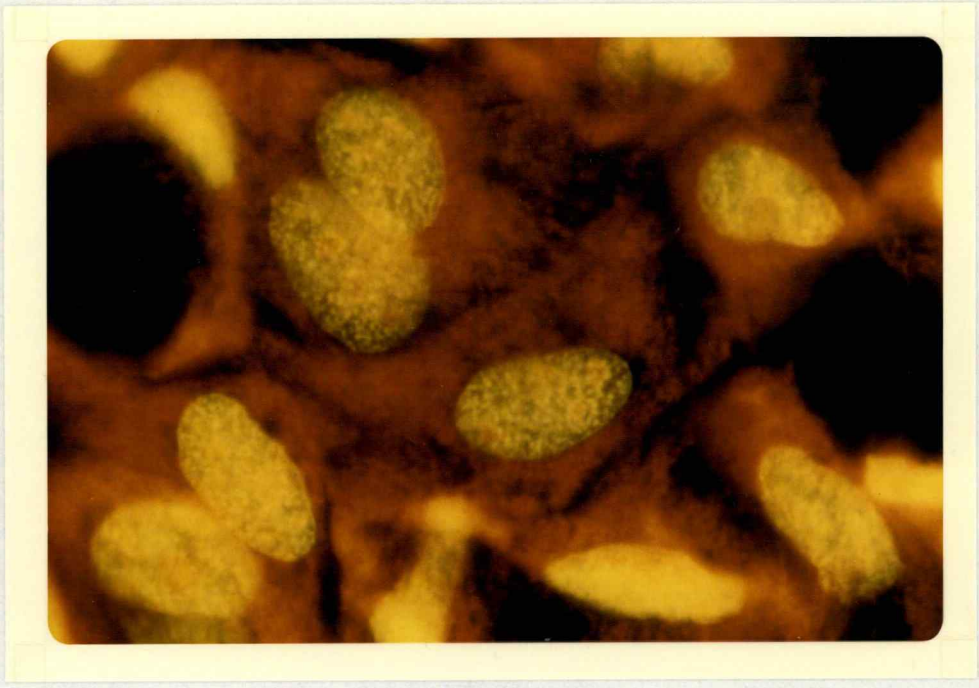

Figure 20. Photomicrograph of acridine orange stain of control STE 137 cells at 18° C, same age as cells in Figure 21.

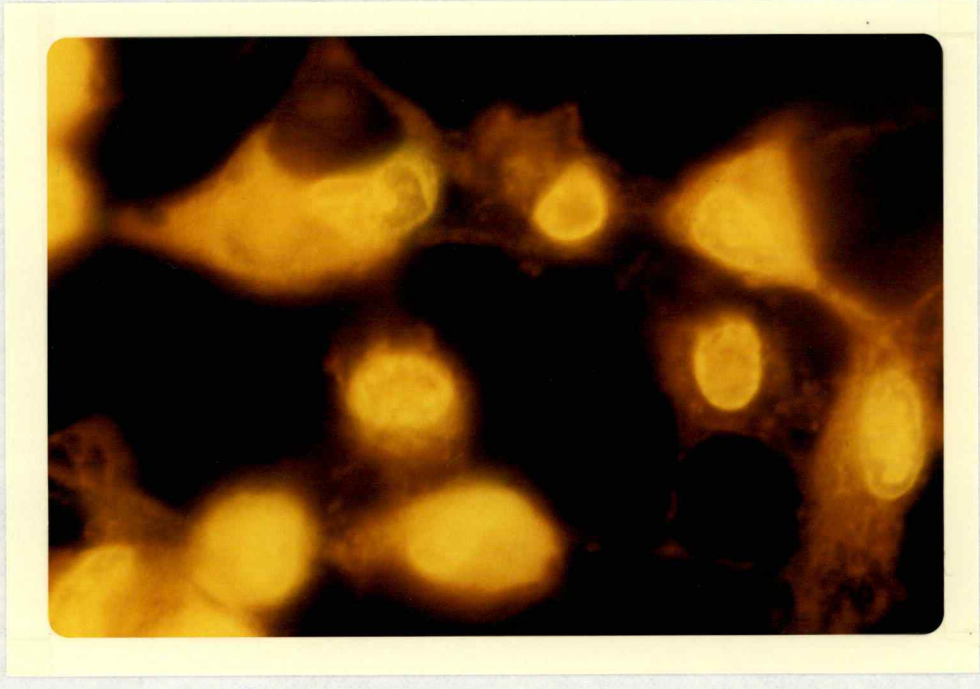

Figure 21. Photomicrograph of acridine orange stain of IPN virus infected STE 137 cells at 18° C, 36 hours post-infection.

Figure 22. Phase contrast photomicrograph of control CSE 119 cells, same age as cells in Figure 23.

Figure 23. Phase contrast photomicrograph of IPN virus infected CSE 119 cells at 18 hours post-infection.

Figure 24. Phase contrast photomicrograph of control STE 137 cells, same age as cells in Figure 25.

Figure 25. Phase contrast photomicrograph of IPN virus infected STE 137 cells at 18 hours post-infection.

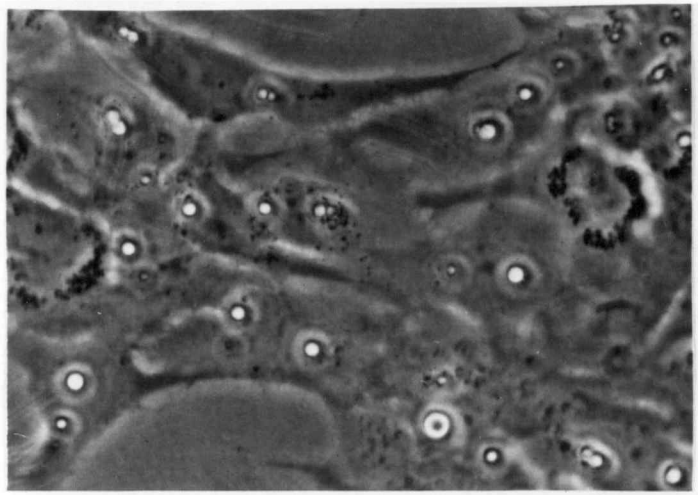

Figure 22

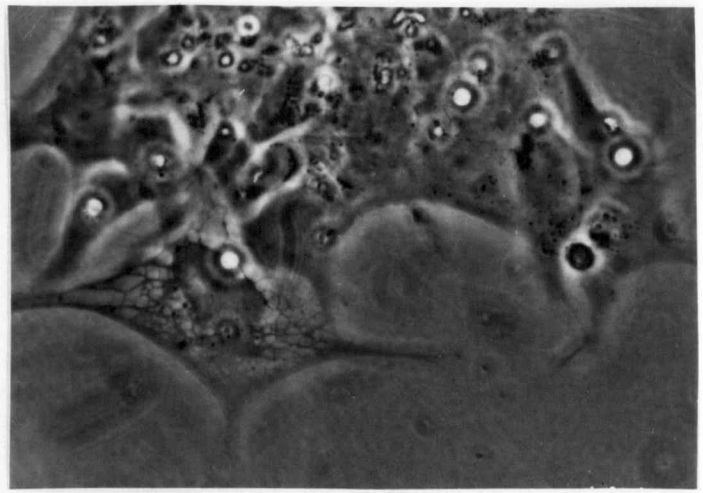

Figure 23

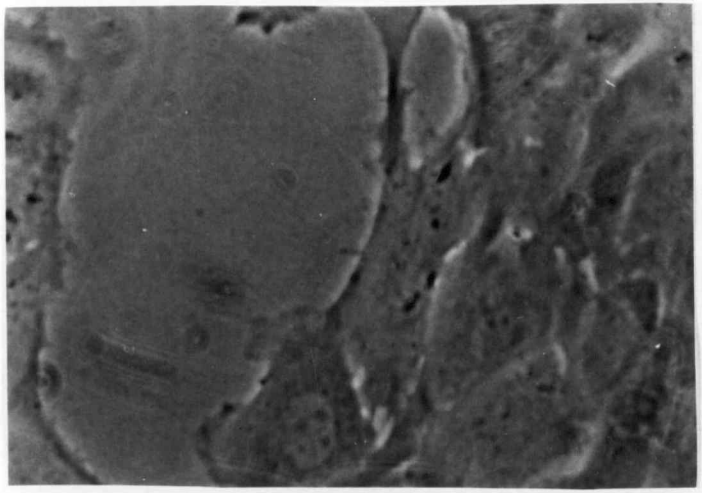

Figure 24

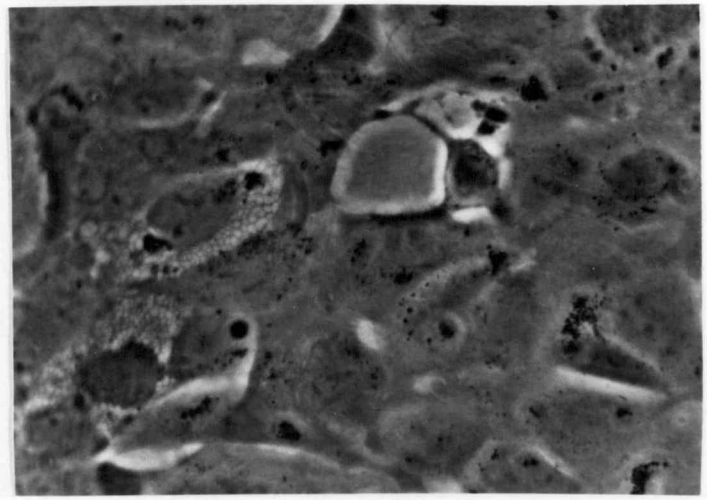

Figure 25

Figure 26. Phase contrast photomicrograph of control CSE 119 cells, same age as cells in Figure 27.

Figure 27. Phase contrast photomicrograph of IPN virus infected CSE 119 cells at 36 hours post-infection.

Figure 28. Phase contrast photomicrograph of control STE 137 cells, same age as cells in Figure 29.

Figure 29. Phase contrast photomicrograph of IPN virus infected STE 137 cells at 36 hours post-infection.

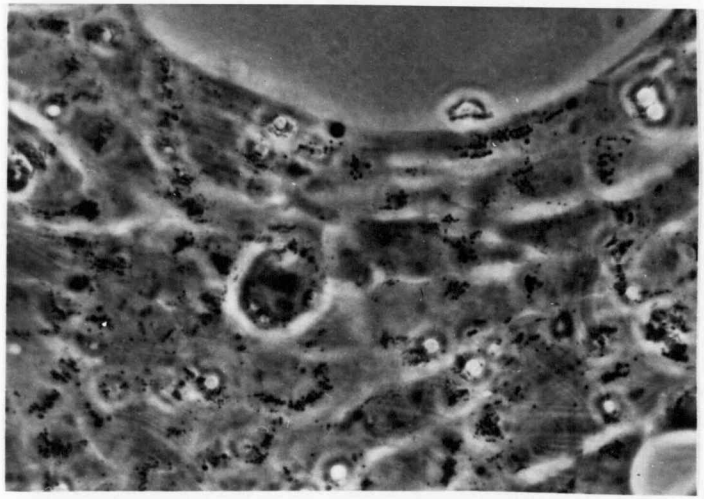

Figure 26

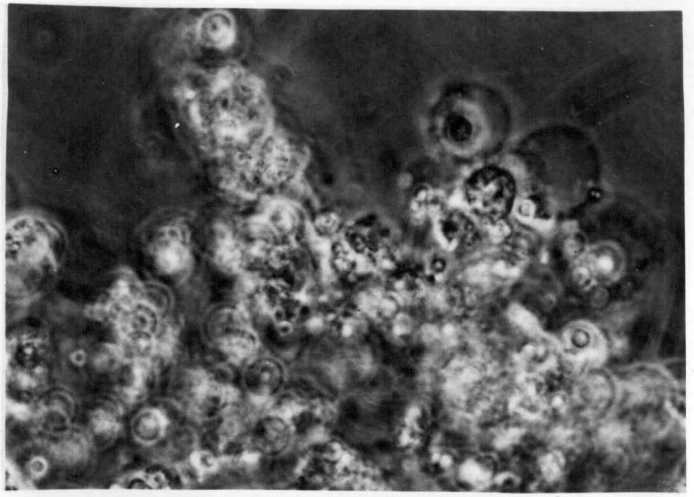

Figure 27

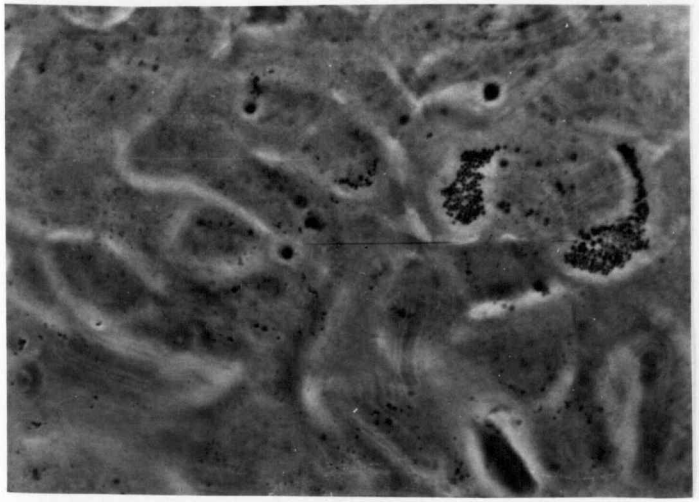

Figure 28

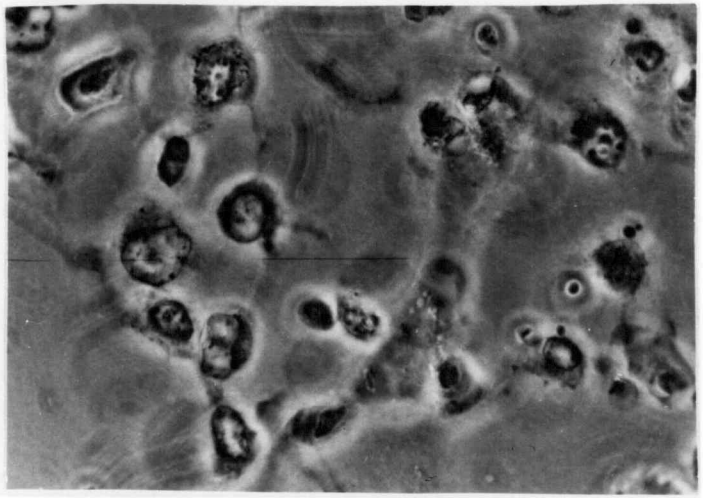

Figure 29

monolayer had been almost completely destroyed.

Wound Tumor Virus

Since an insect cell line has been shown to be susceptible to Wound Tumor virus (WTV) at 24° C (Chiu, Reddy and Black, 1966), a number of attempts were made to infect both salmonid cell lines with the virus. Infectious material was provided by L. M. Black in the form of tumorous plant tissue. The tumor tissue was prepared by either grinding using a mortar and pestle or homogenization. Contamination was controlled by either Millipore filtration, ether treatment or the use of high antibiotic levels. Infected cells were stained at the first and at subsequent passages using acridine orange. Again control cultures contained some green inclusions. From time to time it appeared that there was a greater frequency of such inclusions in WTV infected cells. However, such observations were not consistent and it must be concluded that under experimental conditions used in this research, no conclusive evidence of replication of WTV in either salmonid cell line was demonstrated.

DISCUSSION AND CONCLUSIONS

Poikilothermic cell lines can be of great value both in studies of cell metabolism and in studies of viruses that infect them. The purpose of this research was to initiate studies on poikilothermic cell metabolism and to continue work establishing the role these cell lines may play in virus research. The two cell lines studied were a coho salmon cell line (CSE 119) and a steelhead trout cell line (STE 137).

Glycolysis

The glycolysis studies involved a comparison of the *in vitro* rates of glucose utilization and lactic acid production by salmonid fish cells with similar data from mammalian cells of comparable origin. A survey of reported data on similar studies using homoiothermic cells showed that a wide range of cultural conditions had been employed in these studies and that these cultural conditions can affect the glycolytic rates. Therefore in the studies reported here, parallel data was collected on a human embryonic cell line grown under the same cultural conditions used for measurements on the fish cells. The one difference in conditions was that it was necessary to grow the fish cells at 23 ° C and the human cells at 35 ° C.

Preliminary experiments showed that the newborn agamma calf

serum which was added to the medium contained a significant amount of lactic acid and that this level of lactic acid appeared to limit lactic acid production in the growing cells. Because of this, all serum was dialyzed to remove lactic acid before use in the glycolysis experiments. In addition, observed differences in growth rates required longer growth periods for the fish cells and more frequent medium changes for the human cells.

Comparisons of glycolytic rate data can be made on a per day basis, on a per cell per day basis or on a per milligram dry weight per day basis. Comparisons on a per day basis do not take into account the relative cell numbers. Comparisons on a per day per cell basis do not take into account the relative differences in the size of the cells. Since a larger cell would be expected to use more glucose and produce more lactic acid than a small cell and since the human embryonic cells were shown to be almost eight times larger than the coho salmon cells on a dry weight basis, significant comparisons must be made on a per day per mg dry weight basis. When compared on this basis, the coho salmon cells (CSE 119) consumed 71% as much glucose and produced 61% as much lactic acid per day per mg dry weight as the human cells. The steelhead trout cells (STE 137) consumed 45% as much glucose and produced 34% as much lactic acid per day per mg dry weight as the human cells.

The Q values (equivalent microliters CO_2 produced per mg dry

weight per hour) for aerobic glycolysis were calculated for the two fish cell lines and the human embryonic cells. The values for the fish cells were lower than those calculated for the human cells. The Q value for the coho salmon cells (CSE 119) was 7.97 and for the steelhead trout cells (STE 137) it was 4.50. These values are lower than the Q value of 13.19 found with the human embryonic cells and are also lower than values of 11.2 (Cristofalo and Kritchevsky, 1965) and 9.5 (Graff and McCarty, 1958) reported for human and mouse cells respectively. This would tend to indicate a lower level of aerobic glycolysis in the fish cell lines than in mammalian cells.

Perhaps of more significance is the fact that the proportion of glucose utilized that appeared as lactic acid was somewhat lower in the fish cell lines than in the human cells. The molar ratio of lactic acid produced from a mole of glucose was 1.47:1 in the case of CSE 119, 1.32:1 in the case of STE 137 and 1.73:1 for the human embryonic cells. Cristofalo and Kritchevsky (1965) found that the lactate to glucose ratio for the human cell line they studied was very close to the theoretical 2:1. This suggests that in the fish cells, a greater percent of the glucose was being metabolized by a pathway other than glycolysis than was true in cultures of human cells or that some of the lactic acid was being oxidized. Further experimentation would be required to determine what pathways might be involved.

The gradual fall in pH which occurs in the fish cell cultures is

in contrast to the rapid fall characteristic of most mammalian cell lines. This difference is apparently due in part at least to the smaller amount of lactic acid produced by the salmonid cells.

It was observed that steadily decreasing rates of glucose utilization and lactic acid production occurred over the growth periods studied for all three cell lines. This same effect has been observed in virtually every study that has been reported for glycolysis of animal cells in vitro. It has been suggested that this decrease could be due to decreasing amounts of glucose available per cell (Dewey and Green, 1959), an increase in CO_2 concentration (Whitfield and Rixon, 1961), decreased pH (Paul, 1959) (Broda et al., 1961), leakage of metabolites (Zwartouw and Westwood, 1958), or an increase in cell volume (Munyon and Merchant, 1959).

In the case of the salmonid fish cells, increasing cell volume does not appear to account for the decreasing per cell rates since volume measurements showed little or no change over a ten day growth period with CSE 119. It also seems unlikely that the effect could be due to a pH change since the pH was maintained between 7.6 and 7.1 for the fish cells over the growth periods studied.

It seems most likely that the decreasing rates of glucose utilization and lactic acid production were due to a combination of factors involving the population density of the cells. During the course of the experiments, the fish cells increased in number by more than

five fold. This would mean that even with medium changes, the amount of glucose available per cell was decreasing. Also, the larger number of cells would be producing CO_2 at a more rapid rate which could cause some of the pyruvate or phosphoenol pyruvate to be converted to malate or oxalacetate as was suggested by Whitfield and Rixon (1961). A larger number of cells in the same volume of medium would also be less susceptible to metabolite leakage. In addition, cell density has been shown to influence a number of factors related to cell growth in vitro (Macieira-Coelho, 1967). It therefore seems most likely that the decrease in glucose utilization and lactic acid production found during the growth of fish cells in vitro is related to the concomitant increase in population density.

It appears that cells grown in vitro use glucose at a rate that far exceeds that required for cell growth and multiplication (Wilson, Jackson and Brues, 1942) (Graff et al., 1965) (Eagle et al., 1958). During the initial phases of growth, a high glycolytic rate occurs in all cell cultures regardless of the origin of the cells. Differences observed in the rates of utilization of glucose and production of lactic acid between salmonid fish and human cells may simply reflect the lower growth rate and incubation temperature of the fish cells. Christofalo and Kritchevsky (1965) suggested that metabolically, cells grown in culture resemble each other more than they resemble their respective tissues of origin.

On the other hand, the fact that the proportion of glucose utilized which appeared as lactic acid was lower in the fish cell lines than the human cell line deserves further consideration. This suggests a possible difference in the pathway by which the fish cells use glucose and could be elucidated by further experimentation.

CO_2 Requirements

The CO_2 requirements of the coho salmon cells (CSE 119) and the steelhead trout cells (STE 137) were studied. In addition, HeLa cells were used in order to make comparisons with a mammalian cell line. All the cells were grown in medium in which the bicarbonate was replaced with Tris buffer and the serum was dialyzed to remove any bicarbonate from that source. The fish cells were grown at 18° C and the HeLa cells at 35° C.

Both fish cell lines showed a requirement for carbon dioxide when grown in medium without bicarbonate in CO_2 free Conway microdiffusion dishes. This is in agreement with studies on a number of other cell types (Harris, 1954) (Geyer and Chang, 1958) (Swim and Parker, 1958). However, different levels of CO_2 depletion showed that differences exist in the CO_2 requirements of fish and mammalian cells. The CO_2 requirement of fish cells seemed considerably lower as it was demonstrated that the fish cells grew well in bicarbonate free medium with free gas exchange with the air

(approximately 0.03% CO_2). HeLa cells as well as other mammalian cells (Swim and Parker, 1958) fail to show significant growth under the same conditions.

Carbon dioxide fixation could play a role in one or more of a number of reactions in living cells in vitro. A number of these reactions have been summarized for heterotrophic CO_2 fixation (Lachica, 1968). Some of the reactions have been noted in microorganisms, some in animal cells and some in both cell types. These reactions include CO_2 condensations with pyruvate to give oxalacetate, with pyruvate to give malate, with phosphoenolpyruvate to give oxalacetate, with α-ketoglutarate to give isocitrate, with ribulose-5-phosphate to give 6-phosphogluconate, with ribulose-1, 5-diphosphate to give 2-phosphoglyceric acid, with 5-aminoimidazole ribonucleotide to give 5-amino-4-imidazole carboxylic acid ribonucleotide, with acetyl-CoA to give malonyl-CoA and with propinyl-CoA to give methylmalonyl-CoA. CO_2 has also been shown to be incorporated into proteins. However, Wiame and Bourgeois (1955) suggested that the synthesis of the tricarboxylic acid (TCA) cycle intermediates, malate and oxalacetate, by the $C_3 + CO_2$ condensation reaction, in combination with the role played by these intermediates in the synthesis of amino acids, represents the CO_2 fixation involved in protein synthesis.

Two basic approaches have been used in an attempt to show the

role of CO_2 in mammalian cell metabolism. These involve the use of labeled $C^{14}O_2$ and the use of various CO_2 substitutes.

Since oxalacetate was known to be formed in some cases by the fixation of CO_2, experiments have been reported in which CO_2 depleted cultures were supplemented with oxalacetate. The different levels of CO_2 depletion used in various mammalian cells gave rise to different results. Oxalacetate was found to be an adequate substitute for CO_2 in strain L mouse and HeLa cells (Gwatkin and Siminovitch, 1960), in Yoshida ascites tumor cells (Kieler, 1960), and in three neoplastic lines of human cells (Kelley, Adamson and Vail, 1960). However, using more stringent CO_2 depletion techniques, it was shown that oxalacetate alone did not completely substitute for CO_2 in conjunctival cells (Chang, Liepins and Margolish, 1961) or in strain L mouse or HeLa cells (Runyan and Geyer, 1967).

In the work reported here, CO_2 free microdiffusion dish cultures were used to study substitutions for CO_2 by oxalacetate. It was found that oxalacetate did not substitute for CO_2 in the growth of either the coho salmon or steelhead trout cells in vitro. Under the same conditions, partial substitution did occur in HeLa cells. A possible explanation of this effect might involve a CO_2 fixing reaction necessary for growth of both fish and mammalian cells in vitro and a separate reaction in which CO_2 is released from oxalacetate. The mechanism for release of CO_2 from oxalacetate may be available

to HeLa cells, thereby allowing the released CO_2 to substitute for atmospheric CO_2 in these cells.

Other materials have been used in attempts to substitute for CO_2. Among these, various precursors for purines and pyrimidines have been the most successful (Runyan and Geyer, 1967). Similar experiments could be run using the salmonid fish cells.

Chang, Liepins and Margolish (1961) found that most of the CO_2 from labeled bicarbonate incorporated into HeLa or conjunctival cells was recovered from the purines and pyrimidines. They found the activity was higher in the purines. McCoy, Maxwell and Kruse (1961) found CO_2 incorporated from labeled sodium carbonate in aspartic acid, glutamic acid, proline and in the purines and pyrimidines of mouse cells in vitro. Similar studies with salmonid fish cells may give some indications of the mechanisms of CO_2 fixation involved in these cells.

Another approach to this mechanism would involve assays for the various enzymes known to be involved in CO_2 fixation. An example would be aminoimidazole ribonucleotide carboxylase which catylizes the incorporation of carbon dioxide into the purine ring. This reaction was first demonstrated by Lukens and Buchanan (1957) in extracts of avian liver.

In summary, it appears that salmonid fish cells in vitro fix CO_2 in at least one reaction that is essential for growth of these cells.

Mammalian cells show a greater dependence on CO_2 than either fish cell line studied. This may be due to any of a number of CO_2 fixing reactions. The mechanism of CO_2 fixation in the salmonid fish cells could include reactions found in mammalian cells but the fish cells require much less CO_2. Further elucidation of the CO_2 fixing mechanism in fish cells could be achieved by several suggested studies. Finally, it is worth mentioning that growth of the salmonid fish cells in open cultures could make possible the use of petri dishes and plastic culture vessels for growing these cells and for virus studies without the need of a CO_2 incubator.

Virus Susceptibility

One of the original reasons for the development of these salmonid fish cell lines was as a tool for virus research. Work was initiated by Miss Linda Nims (1968) on the range of viral susceptibility of salmonid cell lines to both viruses from fish and from homoiothermic animals. Both Oregon sockeye salmon virus and Sacramento river chinook disease virus replicated well in the two salmonid cell lines studied. Western equine encephalitis virus replicated in the salmonid cells but Newcastle disease virus did not. To continue this work studies were conducted to determine if reoviruses and reovirus-like agents would replicate in either salmonid cell line studied.

The reoviruses were chosen for study for several reasons. First, because the viruses and antibodies to them are so widespread in nature, it was felt they might occur in fish. This hypothesis was strengthened by the report of Stanley et al. (1964) that hemagglutination inhibition antibody to Reovirus type 3 had been detected in trout sera. Secondly, a fish virus (IPN) and a plant virus (WTV) appeared to be morphologically indistinguishable from the reoviruses. Both of these viruses had been demonstrated to infect poikilothermic cells.

During preliminary work, a technique was developed for the detection of reovirus infected HeLa cells. This technique involved the demonstration of a viral inclusion body by acridine orange staining. The technique was adapted to the titration of Reovirus types 1 and 3 using HeLa cell cultures at 35° C.

Although preliminary staining experiments using cell line CSE 119 and STE 137 infected with Reoviruses types 1 and 3 at 26° C showed some green inclusions, repeated attempts to pass both viruses in either cell line at that temperature were unsuccessful. Similar reported attempts to infect leafhopper nymphs and leafhopper cell cultures with reoviruses failed to show any viral multiplication (Streissle, Rosen and Tokumitsu, 1968).

Since it was possible that the low incubation temperature of 26° C was preventing virus replication, an attempt was made to pass Reovirus type 3 in HeLa cells at 26° C. Definite viral inclusion

bodies were demonstrated in the HeLa cells at this temperature but no increase in virus titer was detected.

It appears that there are two possible explanations for these results. Either an essential viral or an essential cell reaction was inhibited at this temperature. Scholtissek and Rott (1969) reported that in chick fibroblasts infected with fowl plague virus at 25° C, all subunits of the virus were slowly synthesized to normal yields while infectious progeny was not produced. They attributed this effect to the low activity of the viral RNA polymerase at this temperature. Officer (1964) reported that although Venezuelan and Eastern equine encephalitis viruses would replicate in a fish cell line at 22° C, neither virus would replicate in a homoiothermic mouse cell line below 25° C. Since normal replication of both viruses occurred in the mouse cells at 37° C, it appears that some cellular reaction necessary for viral replication was inhibited below 25° C.

Further experimentation would have to be done to determine why the reoviruses would not replicate at 26° C. One approach might be the isolation of a cold reovirus mutant. Jabłoński (1968) isolated a "cold" mutant of ECHO 9 virus by repeated passage at 30° C. If such a mutant was isolated, an attempt could be made to pass it in the fish cells. Another approach would be to attempt to adapt the fish cells to growth at 30° C. Present attempts to grow the salmonid cells at this temperature have been unsuccessful, but a period of

gradual temperature increase over a number of transfers may be successful.

Infectious Pancreatic Necrosis (IPN) virus is the etiological agent of an acute, virulent and highly fatal disease in trout. IPN virus has been shown to have a morphology which is very similar to the reoviruses (Moss and Gravell, 1969). The virus replicated well in both cell line CSE 119 and STE 137 at 18° C. The virus was also successfully carried through five serial passages in both cell lines.

Since IPN virus has been shown to have many of the characteristics of the reovirus group, infected cells of both salmonid cell lines were stained with acridine orange. If the virus contained double stranded RNA and viral replication took place in the cytoplasm of infected cells, one would expect to see green inclusion bodies similar to those observed in reovirus infections. No such inclusions were observed in either salmonid cell line. It appears that final classification of the virus must await a determination as to whether the virus contains double stranded RNA.

Studies were made of the infectious process in both salmonid fish cell lines infected with IPN virus using phase contrast microscopy. The course of the infection was the same in both cell lines. An early webbing of the cytoplasm was noted at 18 hours post infection. A similar effect has been noted at a very low frequency in uninfected fish cells, but the great number of cells showing this

cytoplasmic effect was no doubt due to the viral infection. This was followed by a rounding up of the cells, shrinkage of the nuclei and a margination of nuclear chromatin. By 36 hours, the cell monolayer had been almost completely destroyed in both cell lines.

These results show that either cell line could be used for the isolation and study of IPN virus. The possibility also exists for the development of an attenuated strain of the virus for use for immunization against this highly virulent agent. This may be of particular significance since the virus, previously not known on the west coast of the United States, has recently been isolated in both California and Washington states.

Wound Tumor virus (WTV) is a plant virus that is known to infect some 43 species of plants causing the formation of stem and root tumors. The morphology of the virus is almost identical to that of the reoviruses and it contains double stranded RNA. The virus has been shown to replicate and has been passed serially in cell lines derived from the leafhopper.

Because of its similarity to the reoviruses and its ability to replicate in an insect cell line, attempts were made to infect the salmonid fish cell cultures with WTV at 23° C. Because the agent is not presently found in the northwestern United States, government restrictions prevented titration of the virus in insects. Instead passage material was stained with acridine orange and observed

for the presence of inclusion bodies as well as for possible cytopathic effects. No indication of virus replication was found in either fish cell line using this technique.

SUMMARY

Poikilothermic cell lines can be of great value both in studies of cell metabolism and in studies of viruses that can replicate in them. Salmonid fish embryos are ideal for the establishment of such cell cultures. The research presented in this thesis is concerned with the characterization of two cell lines, CSE 119 derived from embryonic tissues of coho salmon (Oncorhynchus kisutch) and STE 137 derived from embryonic tissues of steelhead trout (Salmo gairdneri). The work was divided into three sections dealing with growth and glycolysis, CO_2 requirements, and susceptibility to reovirus and reovirus-like agents.

Glycolysis was studied in terms of the amounts of glucose utilized and lactic acid produced during active growth of the salmonid cells. These results were compared to data from parallel experiments using a human embryonic cell line. The salmonid cells were grown at 23 ° C over a 10 day period for CSE 119 and an 18 day period for STE 137. The human embryonic cells were grown at 35 ° C over a seven day growth period. All cells were grown in Eagle's minimal essential medium supplemented with a 20% dialyzed newborn agamma calf serum. At intervals during the growth period, the levels of glucose and lactic acid were determined using Glucostat from Worthington Biochemical Corp. and the Barker and Summerson lactic

acid determination. This information, together with the values for the mean dry weight per cell for each cell type, was used to calculate the rates of glucose utilization and lactic acid production in μmoles per mg of dry weight per day. Values of 5.81 and 8.56 were calculated for glucose utilized and lactic acid produced, respectively, for the coho cell line, CSE 119. Values of 3.65 and 4.82 for glucose utilized and lactic acid produced were determined for the steelhead cell line, STE 137. Values of 8.18 and 14.13 for glucose utilized and lactic acid produced were determined for the hyman embryonic cells. The $Q^{O_2}_{CO_2}$ values (equivalent microliters CO_2 produced per mg dry weight of cells per hour) were calculated for each cell type during the first portion of each experimental growth period. Values of 7.97 and 4.50 obtained for CSE 119 and STE 137, respectively, were somewhat lower than the value of 13.19 calculated for the human cells.

In all three cell lines studied, the rates of glucose utilized and lactic acid produced per cell decreased over the growth periods observed. It was demonstrated that these decreasing rates in the salmonid cells were probably not due to changes in pH or in mean cell volume, but could most likely be related to an increase in population density. In addition, it was shown that the very gradual fall in pH observed during the growth of the salmonid cells is apparently due in part, at least, to the smaller amount of lactic acid produced

by these cells.

During the course of the studies with the salmonid cell lines, an investigation of the CO_2 requirements of these cells was undertaken. For these experiments, Tris buffer was used to replace the bicarbonate in the medium and 20% dialyzed serum was again used. Results from the salmonid cells grown at 18° C were compared to parallel experiments using HeLa cells grown at 35° C.

The coho salmon cells, CSE 119, and the steelhead trout cells STE 137, were grown under 2% atmospheric CO_2, open to the air (0.03% CO_2), and in sealed cultures containing KOH traps to remove CO_2 produced during growth. HeLa cells were grown under the same conditions except that 3% CO_2 was used due to the lower solubility of CO_2 at the higher temperature. In both cases growth was compared to growth in rubber stoppered cultures. Growth under 2% and 3% atmospheric CO_2 was comparable to growth in the stoppered cultures in all cases. The salmonid cells showed good growth in the cultures that were open to the air, but little or no growth occurred in the HeLa cells under the same conditions. Only STE 137 cells grew when the KOH traps were used.

To determine if CO_2 was actually required for growth of the salmonid cells, CO_2 free cultures were prepared using Conway microdiffusion dishes in which the cells were grown in the center well and a 10% solution of KOH was added to the outer well. The

cultures were sealed with high vacuum grease. Under these conditions, both salmonid cell lines demonstrated a growth requirement for CO_2 comparable to that shown by the HeLa cells. Attempts were made to use oxalacetate to substitute for CO_2 in these cultures. Oxalacetate partially substituted for CO_2 in the HeLa cell cultures, but little or no growth occurred in the salmonid cell cultures under the same conditions.

As part of a continuing effort to determine the viral susceptibility of salmonid cell lines, the coho cells (CSE 119) and the steelhead cells (STE 137) were tested for their susceptibility to Reovirus types 1 and 3, Infectious Pancreatic Necrosis (IPN) virus and Wound Tumor virus (WTV). Characteristic inclusions could be demonstrated in HeLa cells infected with both reoviruses at 35° C and stained with acridine orange. When salmonid cells infected with both reovirus types at 26° C were stained in a similar fashion, smaller inclusions of a similar nature were often observed. However, attempts to demonstrate the production of infectious virus in single and multiple passage experiments at this temperature with both salmonid cell lines failed. HeLa cells were then infected at 26° C and although the characteristic inclusions were formed, infectious virus was again not produced. The salmonid cells would not tolerate 30° C for even short periods of time.

IPN virus replicated well in both salmonid cell lines at 18° C.

Since IPN virus has been reported to have a similar morphology to the reoviruses, attempts were made to demonstrate reovirus-like inclusions in infected salmonid cells using acridine orange staining. No such inclusions were observed. Phase contrast studies of IPN infected salmonid cells showed an early webbing of the cytoplasm, followed by a rounding up of the cells, shrinkage of the nuclei and a heavy margination of nuclear chromatin.

Because of its similarity to the reoviruses and its ability to replicate in an insect cell line, attempts were made to infect the salmonid cells with WTV at 23 ° C. At selected passages, infected cells were stained with acridine orange and observed for the presence of inclusion bodies as well as for possible cytopathic effects. No indication of virus replication was found in either salmonid cell line.

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