AN ABSTRACT OF THE THESIS OF

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Title: CHARACTERIZATION OF THE OREGON Sockeye
SALMON VIRUS

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Characterization of a virus requires that its physical, biochemical and biological properties be determined. In this study the Oregon sockeye salmon virus was characterized. It was isolated in the fall of 1958 from juvenile sockeye salmon, Oncorhynchus nerka, being reared at the Oregon Fish Commission's Willamette River Hatchery.

When inoculated into tissue cultures of sockeye embryonic cells the virus produced a definite sequence of cytopathic changes. First there was a thickening of the nuclear membrane accompanied by alteration of the nucleoli. This was followed by changes in the nuclear chromatin and in some cases fragmentation of the nuclei. Finally the cells rounded up and detached from the glass surface.

"In vivo" experiments carried out to determine the host range of this virus in salmonids indicated young kokanee salmon, Oncorhynchus nerka, were susceptible while juvenile chinook salmon,
Oncorhynchus tshawytscha, coho salmon, Oncorhynchus kisutch, and rainbow trout, Salmo gairdnerii were resistant.

Histological examination of kokanee salmon infected with the virus indicated the kidney was the only organ showing any appreciable change.

Primary cultures of chinook salmon, coho salmon and steelhead trout, Salmo gairdnerii gairdnerii embryonic cells did not become infected upon exposure to this virus. Cell cultures from these species after being maintained for several months in continuous culture were again exposed to the virus. Now the chinook salmon and steelhead trout cells were susceptible to the virus while the coho salmon cultures remained resistant. This was consistent with information of other workers who found that in some cases fish cells carried in cell cultures for long periods of time became susceptible to a wide variety of animal viruses.

When the Oregon sockeye salmon virus was inoculated into monolayer cultures of sockeye embryonic cells, easily discernible plaques were formed within seven days. The plaque titration method of quantitating this virus was found to be more precise than the infectivity titration used in this study. It was not used routinely for virus quantitation however because of the large number of cells required.

The data from plaque titrations and infectivity titrations made
it possible to determine the number of plaque forming units (pfu) per ID$_{50}$. Results indicated one ID$_{50}$ was equal to 0.55 pfu. This value was in good agreement with the hypothetical value of 0.69 pfu for one ID$_{50}$.

Attempts to measure active intracellular virus showed the amount to be negligible when compared to the concentration of extracellular virus. This suggested the virus was readily released from infected cells and might not be completely infectious until it passed through the cell membrane.

Exposure to ether at a concentration of twenty per cent by volume completely inactivated the virus. This was an indication that the virus particle contained lipid materials which were essential for infection. There was also presumptive evidence that this was an RNA virus since replication was not inhibited by 5-bromodeoxyuridine (an inhibitor of DNA viruses).

Experiments carried out at various temperatures showed the virus multiplied most rapidly between 13$^\circ$ C and 18$^\circ$ C. In this temperature range a maximum titer was reached within two days after inoculation. Temperatures outside the optimum range reduced the rate of replication and in some cases the final titer was at least one log lower than in the 13$^\circ$ to 18$^\circ$ range. At a temperature of 23$^\circ$ C no new virus was formed. This was rather surprising since the optimum temperature for cell growth has been
reported as 23°C.

Before the Oregon sockeye salmon virus could be studied with the electron microscope it was necessary to concentrate and partially purify the virus. Concentration was achieved by centrifugation at 20,000 rpm for one hour. The concentrated material was then placed on a sucrose density gradient which yielded a band of virus material after centrifuging at 23,000 rpm for one hour. This band material was observed with the electron microscope and particles which exhibited evidence of cubic symmetry were observed. Measurement of these particles indicated their size range was between 92 and 145 mµ in diameter. This value was in good agreement with the particle size of the virus as determined with Millipore filters (110 mµ to 165 mµ).

Based on the information obtained during this study, the Oregon sockeye salmon virus appears to be closely related to the arbovirus group. This however is only a tentative placement and more information will be needed before the virus can be definitely assigned to a particular group of viruses.
CHARACTERIZATION OF THE OREGON
SOCKEYE SALMON VIRUS

by

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Review of Literature</td>
<td>3</td>
</tr>
<tr>
<td>Experimental Materials and Methods</td>
<td>9</td>
</tr>
<tr>
<td>Chemicals</td>
<td>9</td>
</tr>
<tr>
<td>Processing Glassware</td>
<td>9</td>
</tr>
<tr>
<td>Filters</td>
<td>9</td>
</tr>
<tr>
<td>Sterilization of Glassware</td>
<td>10</td>
</tr>
<tr>
<td>Sterility Tests</td>
<td>10</td>
</tr>
<tr>
<td>Tissue Culture Medium</td>
<td>10</td>
</tr>
<tr>
<td>Ammonium Bicarbonate</td>
<td>11</td>
</tr>
<tr>
<td>Neutral Buffered Formalin</td>
<td>11</td>
</tr>
<tr>
<td>May-Grünwald-Giemsa-Stain</td>
<td>12</td>
</tr>
<tr>
<td>Phase Microscopy</td>
<td>13</td>
</tr>
<tr>
<td>Source of Tissue Used</td>
<td>13</td>
</tr>
<tr>
<td>Method of Preparing Primary Cultures</td>
<td>13</td>
</tr>
<tr>
<td>Maintenance of Stock Cultures</td>
<td>14</td>
</tr>
<tr>
<td>Counting of Cells</td>
<td>14</td>
</tr>
<tr>
<td>Procedure for &quot;In Vivo&quot; Experiments</td>
<td>14</td>
</tr>
<tr>
<td>Recovery of Virus from Experimental Fish</td>
<td>15</td>
</tr>
<tr>
<td>Histological Examination</td>
<td>16</td>
</tr>
<tr>
<td>Infectivity Titration of the Virus</td>
<td>16</td>
</tr>
<tr>
<td>Ether Sensitivity</td>
<td>17</td>
</tr>
<tr>
<td>Determination of Virus Size by Filtration</td>
<td>18</td>
</tr>
<tr>
<td>Plaque Titration</td>
<td>18</td>
</tr>
<tr>
<td>Determination of Intracellular Virus</td>
<td>19</td>
</tr>
<tr>
<td>Temperature Range</td>
<td>20</td>
</tr>
<tr>
<td>Presumptive Identification of Nucleic Acid Type</td>
<td>21</td>
</tr>
<tr>
<td>Virus Susceptibility of Cell Cultures of Other Species</td>
<td>23</td>
</tr>
<tr>
<td>Purification of the Virus</td>
<td>23</td>
</tr>
<tr>
<td>Shadow Casting Virus Material</td>
<td>26</td>
</tr>
<tr>
<td>Potassium Phosphotungstate</td>
<td>26</td>
</tr>
<tr>
<td>Negative Staining Procedure</td>
<td>27</td>
</tr>
<tr>
<td>Experimental Results</td>
<td>28</td>
</tr>
<tr>
<td>Cytopathogenic Effect of the Oregon Sockeye Virus in Cultured Sockeye Embryonic Cells</td>
<td>28</td>
</tr>
<tr>
<td>Cellular Changes Observed in Infected Cells Using May Grünwald-Giemsa Stain</td>
<td>28</td>
</tr>
<tr>
<td>Phase Microscopy of Infected Cells</td>
<td>29</td>
</tr>
<tr>
<td>Table</td>
<td>Title</td>
</tr>
<tr>
<td>-------</td>
<td>-----------------------------------------------------------------------</td>
</tr>
<tr>
<td>1</td>
<td>Species specificity of the Oregon sockeye salmon virus &quot;in vivo&quot;</td>
</tr>
<tr>
<td>2</td>
<td>Susceptibility of cell cultures from different species of fish to the Oregon sockeye salmon virus</td>
</tr>
<tr>
<td>3</td>
<td>Estimate of the particle size of the Oregon sockeye salmon virus by filtration</td>
</tr>
<tr>
<td>4</td>
<td>Comparison of the plaque method to the infectivity method of titration</td>
</tr>
<tr>
<td>5</td>
<td>Number of plaque forming units required to produce infection in a monolayer of cells</td>
</tr>
<tr>
<td>6</td>
<td>Comparison of methods used in determination of the amount of intracellular virus present in sockeye embryonic cells after three days of infection</td>
</tr>
<tr>
<td>7</td>
<td>Validity of the testing procedure for ether sensitivity; comparison of sockeye virus with Lee influenza and IPN viruses</td>
</tr>
<tr>
<td>8</td>
<td>Presumptive identification of type of nucleic acid in virus as determined by use of 5-bromodeoxyuridine</td>
</tr>
<tr>
<td>9</td>
<td>The effects of temperature on the &quot;in vitro&quot; replication of the Oregon sockeye salmon virus</td>
</tr>
<tr>
<td>10</td>
<td>Virus titers resulting from concentration and purification of virus samples</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>1</td>
<td>Sockeye embryonic cell cultures in Rose chambers for observation with the phase contrast microscope. The culture shown here had been incubated for 24 hours and served as control for cells which had been exposed to virus for 24 hours. These cells are characterized by moderately dark nucleoli within the clear nuclear area. The cytoplasm of the cells shows many various sized granules (X533)</td>
</tr>
<tr>
<td>2</td>
<td>Sockeye embryonic cells exposed to the Oregon sockeye salmon virus for 24 hours. These cells were cultured and observed in the same manner as the cells in Figure 1. The changes noted in the infected cells were: nucleoli of greater density and a thickening of the nuclear membrane (X533)</td>
</tr>
<tr>
<td>3</td>
<td>Forty-eight hour control culture of sockeye embryonic cells. These cells again show the structures as they were described in the 24-hour control cultures (X533)</td>
</tr>
<tr>
<td>4</td>
<td>Sockeye embryonic cells 48 hours after inoculation with the virus. The dense nucleoli and thicker nuclear membranes are even more obvious in Figure 2. Also noticeable at this point is the tendency of the cells to round up (X533)</td>
</tr>
<tr>
<td>5</td>
<td>Sockeye embryonic cells which had been incubated for 96 hours. These cells served as controls for cells which had been exposed to the virus for 96 hours (X533)</td>
</tr>
<tr>
<td>6</td>
<td>A Rose chamber culture of sockeye embryonic cells which have been exposed to the virus for 96 hours. By this time many of the cells have rounded up and large clear areas appear in the cell sheet where infected cells detach from the glass (X533)</td>
</tr>
</tbody>
</table>
Rate of replication of the Oregon sockeye salmon virus in sockeye embryonic cells at 18° C.

Sockeye embryonic cell cultures after seven days exposure to the Oregon sockeye salmon virus. The bottle on the left has the highest concentration of virus and many plaques are discernible in the cell sheet. The center bottle shows only a few plaques in the monolayer and the bottle on the right no plaques. The virus dilution between each bottle is ten fold.

Effect of temperature on the rate of replication of the Oregon sockeye salmon virus.

Effect of temperature on the rate of replication of the Oregon sockeye salmon virus.

Shadow cast pellet material with polystyrene latex spheres (264 mµ. in diameter) added as reference particles. Most of the particles in the pellet had a diameter between 91 and 145 mµ. and appear to have cubic symmetry (see arrows) (X76, 000).

Pellet material negatively stained with potassium phosphotungstate. In this case reference particles were not added as with shadowed material (Figure 10) since they cause the stain to accumulate in large droplets. In the lower part of the picture is a particle which appears to have cubic symmetry (X152, 000).

A shadow cast particle from the band obtained by density gradient centrifugation. The larger spherical particle is a polystyrene latex sphere (266 mµ. in diameter) which serves as a reference particle. The smaller particle shows some signs of cubic symmetry and has a diameter of approximately 118 mµ. (X152, 000). 

Figure 7 Rate of replication of the Oregon sockeye salmon virus in sockeye embryonic cells at 18° C. ........ 44

Figure 8 Sockeye embryonic cell cultures after seven days exposure to the Oregon sockeye salmon virus. The bottle on the left has the highest concentration of virus and many plaques are discernible in the cell sheet. The center bottle shows only a few plaques in the monolayer and the bottle on the right no plaques. The virus dilution between each bottle is ten fold ......................... 47

Figure 9 Effect of temperature on the rate of replication of the Oregon sockeye salmon virus.............. 57

Figure 10 Effect of temperature on the rate of replication of the Oregon sockeye salmon virus.............. 58

Figure 11 Shadow cast pellet material with polystyrene latex spheres (264 mµ. in diameter) added as reference particles. Most of the particles in the pellet had a diameter between 91 and 145 mµ. and appear to have cubic symmetry (see arrows) (X76, 000) .... 63

Figure 12 Pellet material negatively stained with potassium phosphotungstate. In this case reference particles were not added as with shadowed material (Figure 10) since they cause the stain to accumulate in large droplets. In the lower part of the picture is a particle which appears to have cubic symmetry (X152, 000) ......................... 66

Figure 13 A shadow cast particle from the band obtained by density gradient centrifugation. The larger spherical particle is a polystyrene latex sphere (266 mµ. in diameter) which serves as a reference particle. The smaller particle shows some signs of cubic symmetry and has a diameter of approximately 118 mµ. (X152, 000) ......................... 68
Another particle found in material from the band after density gradient centrifugation. This particle has a diameter of approximately 86 μm, and as in Figure 12 there is some suggestion that the particle may possess cubic symmetry (X152, 000).
CHARACTERIZATION OF THE OREGON SOCKEYE SALMON VIRUS

INTRODUCTION

In recent years the addition of many newly discovered animal viruses to those already known has created a great deal of interest in trying to classify them into specific taxonomic groups (1, p. 1-401; 29, p. 1-58). A virus is assigned to a particular group on the basis of its physical, biochemical and biological properties. Each group is made up of agents having similar properties.

Determination of the biochemical, physical and biological properties of a virus is termed virus characterization. In the present study the virus characterized was the Oregon sockeye salmon virus. This virus was isolated in the fall of 1958 by J. L. Fryer from infected juvenile sockeye salmon, Oncorhynchus nerka, at the Oregon Fish Commission's Willamette River Hatchery. From these diseased fish a bacteria-free filtrate was prepared which would produce the disease in healthy juvenile sockeye salmon. This was presumptive evidence that the infectious agent was of a viral nature.

Further in vivo experiments indicated the etiological agent of the disease was specific for young sockeye salmon. These
experiments also strengthened the evidence for the viral nature of the agent.

Several difficulties were encountered in carrying out the in vivo experiments. These difficulties prompted efforts to develop cell culture methods for the cultivation of fish tissues before attempting further study of the virus. After several years of experimentation an excellent cell culture method for fish was developed by Fryer, Yusha and Pilcher (11, p. 566-586). This method for cultivating cells from fish tissues has been utilized in the present study of the Oregon sockeye salmon virus.
LITERATURE REVIEW

The sockeye salmon virus disease was first recognized in young sockeye salmon, *Oncorhynchus nerka*, at the Winthrop, Washington, hatchery in 1950. It was not known at this time however, that the etiological agent of the disease was a virus. During the next three years the disease appeared at other hatcheries in Washington with mortalities in some cases reaching 90 percent of the young sockeye salmon population.

The first report on the sockeye salmon disease was made by Rucker and co-workers (25, p. 35-46). Their study covered outbreaks of the disease in juvenile sockeye salmon and kokanee salmon, *Oncorhynchus nerka*, at the Winthrop and Leavenworth hatcheries during 1950 and 1951. They found that bacteria-free filtrates prepared from diseased fish and inoculated intraperitoneally into healthy juvenile sockeye salmon would transmit the disease. This indicated a probable viral etiology for the disease. Further evidence supporting this view was the ineffectiveness of drugs and chemicals normally used in treating bacterial fish diseases. The disease could also be transmitted to healthy juvenile sockeye salmon by placing infected fish among them; however infected sockeye salmon did not transmit the disease to fingerlings of chinook salmon, *Oncorhynchus tshawytscha*, coho salmon,
Oncorhynchus kisutch or cutthroat trout, *Salmo clarki clarki.*

Diseased fish were described as being lethargic but displaying sporadic hyperactivity. They were darker in color than normal fish, with distended abdomens and displayed hemorrhagic areas at the base of the fins. Spinal deformities developed in some fish that survived the disease. Internally, the liver and spleen were pale and the stomach was found to be filled with a milky-colored fluid (25, p. 35-46).

In 1954, Watson, Guenther and Rucker reported further experimental evidence which proved the etiological agent of the sockeye salmon disease to be a virus (28, p. 1-36). Investigations on the source of the virus pointed to infected fish products which were incorporated into the diet of juvenile sockeye salmon. No evidence was obtained to indicate transmission of the virus from adult to fingerlings through the egg. The virus was reported to be most virulent at water temperatures from 50° to 60° F. At temperatures above and below these two points the virus was still infective but mortalities were much reduced. It was found to be infective for sockeye salmon between one and 14 months old. Adult sockeye salmon returning to spawn were found to be carriers of the virus even though showing no symptoms. The virus was found to be completely inactivated by heating for 15 minutes at 60° C. (28, p. 1-36).
In 1959 a report was made by the same investigators which summarized the results of their studies on the sockeye salmon virus disease (13, p. 1-6). They proved conclusively that the source of the virus had been adult salmon viscera incorporated into the diet of young sockeye salmon. Pasteurization of such viscera inactivated the virus and made it safe for feeding to the young salmon. After instituting the process of pasteurization of fish products in fish diets the sockeye salmon virus was no longer a problem for fish culturists in Washington. However, one outbreak of the disease was diagnosed in young sockeye salmon which were not receiving fish products in their diet. The source of the virus in this case was thought to be adult salmon which were in the water supply being used by the hatchery (13, p. 1-6).

The histopathologic changes which occurred in infected sockeye salmon have been described by Wood and Yasutake (30, p. 85-90). Their observations were made on infected fish from epizootics which occurred in Washington hatcheries. Microscopic lesions were observed in liver, spleen, brain, eyes, muscle, air bladder, intestines and blood. A characteristic of the disease was ceroid deposits in both the spleen and in areas of focal necrosis in the liver. Focal necrosis was also found in the muscle wall of the intestine and air bladder. They reported no inclusion bodies, rickettsia, or bacteria in any of their sections and found no changes
in the spinal cord, gills, heart, kidney, gall-bladder, testes or ovaries.

The cellular hematology of healthy and virus-infected sockeye salmon have been compared as a possible means for rapid diagnosis of this disease. For this purpose specimens of the peripheral blood of normal and infected sockeye salmon were examined with a phase contrast microscope. Two changes were observed in the blood of infected fish. Before mortalities occurred there was degeneration of leukocytes and thrombocytes as evidenced by the presence of highly refractile bodies in the cytoplasm of these cells. During the height of the epizootic, after mortalities had begun, extra cellular granules from basophils appeared and the leukocytes and thrombocytes were completely degenerated. Hematocrit values well below average were recorded for infected fish (27, p. 27-39).

The sockeye salmon virus was first isolated in Oregon in the fall of 1958 by J. L. Fryer. Heavy mortalities were occurring in young sockeye salmon at the Willamette River hatchery. From these infected fish a filterable agent was isolated which would transmit the disease to healthy fingerlings of sockeye salmon. By further in vivo experiments the viral nature of the agent was confirmed and the virus was shown to be specific for young sockeye salmon (10, p. 2). This virus appeared to be similar to the
one previously described in Washington (25, p. 35-46).

Further information on the Oregon sockeye virus has been provided by Parisot, Yasutake and Klontz. This information was obtained by injecting the virus into one-inch rainbow trout, Salmo gairdneri, which they reported to be susceptible to the virus. Their results indicated the Oregon sockeye salmon virus was inactivated by heating at 60° C. for 30 minutes; however, 10 percent ether, alcohol or chloroform had no effect on it. It passed a millipore filter with 300 mµ pore diameter but not a filter with 100 mµ pore diameter, and tolerated a pH range of 4 through 10. (23, p. 502-519).

Yasutake, Parisot and Klontz have reported on the histopathological changes observed in sockeye salmon infected with the Oregon sockeye salmon virus. They observed such changes in the kidney hematopoietic tissue, spleen, pancreas and granule cells in the intestine tissue (31, p. 520-530).

Klontz, Yasutake and Parisot have investigated the host response of one-inch rainbow trout to the Oregon sockeye salmon virus. The first response, noted approximately two days after injection, was an increase in the number of macrophages in the connective tissue at the injection site. "This was followed by a marked increase in lymphocytes and neutrophils in the same area and in the peripheral vascular system." Four to five days after
injection, an increase was noted in the lymphocytopoietic activity in the anterior kidney and the number of pyroneophilic cells in the anterior kidney and spleen increased (16, p. 531-542).
MATERIALS AND METHODS

Chemicals

Reagent grade chemicals were used in the preparation of all solutions. In all cases the solvent was deionized or double distilled water.

Glassware

The containers used for starting and maintaining stock cultures of cells were 32-ounce prescription bottles. For plaque titration of the virus the cells were grown in two-ounce prescription bottles. Tissue cultures used for infectivity titration of the virus were grown in Kimax test tubes (12 mm x 75 mm). The other types of glassware used in the present study have already been described by Fryer (10, p. 15).

Processing Glassware

The procedure used in washing glassware was the same as that previously described by Fryer (10, p. 16).

Filters

Seitz and Millipore filters were used in sterilization of certain solutions and in some experiments. Preparation of these filters
followed the method described by Fryer (10, p. 17).

**Sterilization of Glassware**

Most glassware was sterilized by heating in an oven for at least 12 hours at 110° C. Items which could not be sterilized in the above manner were autoclaved at 121° C. for at least 15 minutes.

**Sterility Tests**

The complete medium used for cell cultures was tested for sterility before being placed on the cells. The tissue culture fluid on cells when experiments were terminated was also checked for sterility. These sterility checks were carried out to make sure that results were not being influenced by contaminating microorganisms. These tests were carried out in sterile thioglycollate broth according to the procedure of Fryer (10, p. 18).

**Tissue Culture Medium**

Eagle's minimum essential medium containing 20 percent agamma calf serum (Hyland Laboratories), penicillin, streptomycin and mycostatin was the nutrient fluid used for cell cultures. This medium was prepared and stored as described by Fryer
(10, p. 24).

Ammonium Bicarbonate

A solution of ammonium bicarbonate has been found to facilitate the resuspension of virus pellets which will not resuspend readily in distilled water.

Ammonium bicarbonate solution was required with the Oregon sockeye salmon virus, and was prepared in the following manner. First, a 1.2 molar solution of ammonium carbonate solution was prepared. Then carbon dioxide was bubbled through the solution until the pH was below 7.8. Once the pH was below 7.8 the solution was converted to ammonium bicarbonate. This stock solution of ammonium bicarbonate was diluted 1:10 in distilled water for resuspension of virus pellets.

Neutral Buffered Formalin

37-40% Formaldehyde 100 ml

\[ \text{NaH}_{2}\text{PO}_4 \cdot \text{H}_2\text{O} \quad 4.0 \text{ gm.} \]

\[ \text{Na}_2\text{HPO}_4 \text{ (anhydrous)} \quad 6.50 \text{ gm.} \]

Dilute to one liter with distilled water. This solution had a pH of seven.
May-Grünwald-Giemsa-Stain

Sockeye salmon cell cultures were grown on coverslips in Leighton tubes; then the coverslips were removed, stained and mounted on slides. The sequence of cytopathic events which occurred in sockeye embryonic cells from the time of infection with the Oregon sockeye salmon virus, to detachment of the cells from the coverslip, were observed and photographed.

Stock May-Grünwald stain was prepared by dissolving 2.5 grams of the dye in 1000 ml of absolute methanol and allowing the solution to age at least one month. Stock Giemsa stain made by adding 1.0 gram of the dye to 66 ml of glycerol and holding at 55-60°C for 1.5 to 2 hours, followed by the addition of 66 ml of absolute methanol. The procedure for staining was as follows (20, p. 182):

1. Wash the coverslip with cells 15-20 minutes in BSS.
2. Fix for 5 minutes in absolute methanol.
3. Stain for 10 minutes in stock May-Grünwald stain.
4. Stain for 20 minutes in dilute Giemsa stain (1:10 dilution of stock in deionized water).
5. Wash quickly in two rinses of acetone. Do not let the coverslip dry.
6. Rinse 3 times in acetone-xylol (2:1), 3 times in
acetone-xylol (1:2) and once in fresh xylol.

7. Mount on slide in mounting medium.

Phase Microscopy

Sockeye embryonic cells were cultivated in Rose chambers and then infected with the Oregon sockeye salmon virus. By use of the phase microscope it was possible to observe and photograph the sequence of cytopathic events caused by the virus as they occurred in living cells.

Source of Tissue Used

In the majority of experiments the cell cultures used were prepared from embryos taken from eyed eggs of sockeye salmon. This stage in development occurs approximately 16 days after fertilization when the water temperature is 50°C. Tissue cultures used in a few cases were from established cell lines of fish, human and bovine origin.

Method of Preparing Primary Cultures

The primary cultures were prepared from embryos taken from eyed sockeye salmon eggs. The method followed in preparing these cultures was that of Fryer, Yusha & Pilcher (11, p. 566-586).
Maintenance of Stock Cultures

Since the eyed salmon eggs are only available for a short time during the year, enough primary cultures had to be started and then expanded throughout the year to supply the cells needed for experimental purposes. The techniques employed for maintenance of cell cultures were those described by Fryer, Yusha and Pilcher (11, p. 566-586).

Counting of Cells

In setting up experiments it was necessary to know the approximate number of cells per ml of culture fluid. The number of cell nuclei was determined by staining a cell suspension with crystal violet stain (0.1 percent crystal violet and 0.1 molar citric acid in deionized water) and then performing a hemocytometer count. The procedure followed in carrying out the hemocytometer count was that reported by Fryer (10, p. 33).

Procedure for "In Vivo" Experiments

Species of fish used in "in vivo" experiments were kokanee salmon, chinook salmon, coho salmon and rainbow trout. These fish ranged in size from one to four inches.

The different species of fish were obtained from various hatcheries and transported to the laboratory. Here they were held for 24 hours prior to experimentation, in a constant temperature room.
(16°C). This holding period gave the fish a chance to recover from the rigors of transportation and handling.

For the experiment fish were placed in one gallon glass jars (10 fish to a jar) in the constant temperature room at 16°C. The experiment was initiated by adding four ml of virus suspension to the water containing the fish. During the course of an experiment, the fish were fed and the water changed at 48-hour intervals. Activity of the virus was determined by the difference in mortalities between test lots of fish exposed to the virus and those receiving no virus.

**Recovery of Virus from Experimental Fish**

Fish which died during an experiment were put in 8 ml of Eagle's minimum essential medium and homogenized with the Virtis homogenizer for one minute at 23,000 rpm. The homogenate was then centrifuged at 3,000 rpm for 20 minutes. After centrifugation the supernate was collected and assayed for possible virus activity. This assay was carried out in two different ways. In the first method one ml of the supernate was assayed by diluting it in nine ml tissue culture medium and then placing the medium on cultures of sockeye embryonic cells. The tissue culture medium in this case contained double the concentration of antibiotics normally used. The second assay method consisted of filtering a portion of the supernatant fluid through a Millipore filter (pore size 0.45μ) and then diluting the filtrate 1:4 in tissue culture medium. The diluted material was placed in cultures of sockeye embryonic cells. In both
methods the cells in the presence of supernatant fluids were observed daily for cytopathic changes that would indicate presence of the virus.

**Histological Examination**

Some of the kokanee salmon from "in vivo" experiments were preserved in buffered formalin. Later they were embedded in blocks of paraffin and sectioned serially. The serial sections were stained with hematoxylin and erosin and observed microscopically. By comparison of normal sections to virus infected ones the histological changes caused by the virus were determined.

**Infectivity Titration of the Virus**

A series of ten-fold dilutions were prepared using a portion of the virus suspension. The diluent was complete tissue culture medium with 20 percent agamma calf serum added. After the dilution series (10^-1 through 10^-7) was prepared each dilution was inoculated into five tubes (10mm x 75mm) of sockeye embryonic cells (200,000 cells per tube) in a volume of 0.2 ml. After adding the virus 0.3 ml of complete medium containing 20 percent agamma calf serum was added to each tube. The cells were then incubated at 18° C. for seven days. After incubation the cells were checked microscopically to determine the highest dilution showing virus activity. Then by use of the Reed and Muench method (24, p. 493-497) the log of the number of tissue culture ID\(_{50s}\) in 0.2 ml of original virus suspension was
determined. When five tubes were used at each dilution and the Reed Muench method was employed the least significant difference between two titrations was assumed to be $0.62$ of a log (17, p. 487-495).

Ether Sensitivity

The exposure of different viruses to ether inactivates some of them. Those viruses which were inactivated are considered to have lipid material which is essential to their infectivity. This presence or lack of essential lipid material is one of the main characteristics used in virus classification.

The ether sensitivity of the Oregon sockeye salmon virus was determined by the method described by Andrews and Horstmann (2, p. 290-297). Pure diethyl ether was added to a buffered aqueous virus suspension, in tissue culture fluid at a concentration of 20 percent by volume. The mixture was then refrigerated along with a control tube of virus containing no ether for 23 hours at $4^\circ$ C. The tubes were given a vigorous shaking periodically during refrigeration. After refrigeration the ether treated sample was placed in an open beaker and both samples held in a sterile transfer room at $16^\circ$ C. Approximately two hours were needed for complete evaporation of the ether from the open beaker. Then both samples were titrated for infectivity to determine the effect of the ether.
Determination of Virus Size by Filtration

The approximate diameter of a virus can be calculated by determination of the filter pore sizes which will allow the virus to pass and those which will retain it.

The approximate size of the Oregon sockeye salmon virus was determined by filtering a virus suspension of known concentration through Millipore filters having average pore diameters of 0.8μ, 0.45μ, 0.3μ, 0.22μ and 0.14μ. The filtrate was then tested for virus activity to see which pore sizes allowed passage of the virus.

Plaque Titration

Five ml of a suspension of sockeye embryonic cells at a concentration of approximately 600,000 cells per ml were planted in two-ounce prescription bottles. After two days incubation at 18° C. a plaquing experiment could be started.

A series of 10-fold dilutions of a virus suspension were prepared in tissue culture medium and after removing the medium from the bottles of cells, 0.5 ml of each virus dilution was added to each of three bottles. The virus was distributed over the cell layer by tilting and allowed to absorb to the cells for one hour at 20° C. Every 15 minutes during the hour the bottles were gently rocked back and forth to get an even dispersal of the virus over the cell
sheet. After one hour of incubation the bottles were placed at 4°C.

for 15 minutes. Then three ml of complete tissue culture medium
containing 0.75 percent Noble agar, which was held at 45°C., was
added to each bottle and allowed to spread over the monolayer. The
bottles were then incubated overnight at 18°C. The next day they
were inverted to prevent moisture, which condensed in the bottles,
from running down between the agar overlay and the cell sheet.

After seven days of incubation at 18°C. the agar overlay was re-
moved by adding two ml of Earle's balanced salt solution to each
bottle and then pouring off the diluted agar. Each cell sheet was
then washed with two ml of balanced salt solution. Finally, the cell
sheets were stained for five minutes with Wright's stain and plaques
were counted at each dilution.

Determination of Intracellular Virus

With some viruses infective particles are found inside the host
cell while other viruses do not become infective until they are out-
side the host cell. In the case of the Oregon sockeye salmon virus
the following method was used to determine if a significant amount
of infective intracellular virus was present.

To determine the intracellular virus the host cells must in
some manner be broken up without inactivating the virus. In the
present study disruption by freezing and thawing and by sonic
energy was first tried. However, it was found that treating suspensions of infected sockeye embryonic cells in the Virtis homogenizer for one minute at 23,000 rpm disrupted the host cells but did not inactivate the virus while the other two methods caused some loss in infectivity. To determine if infective virus was present inside infected cells the following procedure was used. First the virus titer was determined on tissue culture fluid removed from sockeye embryonic cells three days after virus infection. Then the total virus titer in the tissue culture fluid and inside the infected cells was determined by disrupting the cells from another group of cultures incubated at the same time. This released any intracellular virus present into the tissue culture fluid. The total virus in the homogenate was titrated. The final step was to separate the infected cells, from a third group of cultures, from the tissue culture fluid by centrifugation. The cells were then washed in Earle's balanced salt solution. After washing they were re-suspended in the original volume of 12 ml fresh tissue culture medium and disrupted in the Virtis homogenizer. Then the virus titer, which represented intracellular virus, was determined.

Temperature Range

The range of temperature over which cold blooded animal viruses will replicate is of special interest, since the body
temperature of the host can be varied over a wide range without ill
effects. It is possible that the host might be able to survive at a
temperature which would not allow replication of the virus.

The temperature range for the Oregon sockeye salmon virus
was determined in the following manner. Sockeye embryonic cells
were planted in Leighton tubes at a concentration of 450,000 cells
per ml. These tubes were then incubated at 18°C. for two days.
The tissue culture fluid was then removed and 0.5 ml of new tissue
culture medium containing the virus at a concentration of 10^{3.5}
ID\textsubscript{50} per 0.2 ml was added to each tube of the cells. The cells were
then placed in an incubator at the temperature to be tested in that
particular experiment. Virus titrations were made on the tissue
culture fluid at the time of virus inoculation and at 2, 4, 6 and 8 days
after exposure of the cells to the virus. The replication rate of the
virus was tested at 4°C., 8°C., 10.5°C., 13°C., 18°C., 20°C.,
and 23°C.

Presumptive Identification of Nucleic Acid Type

All viruses discovered to date have been found to contain either
deoxyribonucleic acid or ribonucleic acid. No virus has been found
to have both types of nucleic acid. This has made nucleic acid type
one of the basic characteristics of virus classification. To deter-
mine the nucleic acid type of the Oregon sockeye salmon virus the
compound 5-bromodeoxyuridine was used. It has been found that replication of viruses which contain DNA is inhibited by 5-bromodeoxyuridine in concentrations that are not toxic to the host cells. RNA containing viruses are not affected by this compound. Experiments were carried out in the following manner. Cells were planted in Leighton tubes at a concentration of 450,000 cells per ml. Two days later the tissue culture medium was removed and new medium containing $10^{3.5} \text{ID}_{50}$s of virus per 0.2 ml was added. The virus was allowed to absorb to the cells for one hour. Then the medium was drawn off and tissue culture medium containing $10^{-3}$ molar 5-bromodeoxyuridine was added to half the tubes. Normal tissue culture medium was added to the remainder of the tubes. Virus titrations were carried out after incubation at $18^\circ C$. for 48 hours to see if the compound inhibited virus multiplication. Experiments were first carried out with known RNA and DNA viruses to see if the compound would give the expected results. The virus of infectious bovine rhinotracheitis grown in bovine kidney cells was the known DNA virus. Polio virus grown in He La cells was the known RNA virus. The Oregon sockeye salmon virus was tested using the same procedure that had been used on the viruses of known nucleic acid type.
Virus Susceptibility of Cell Cultures of Other Species

Tissue cultures started from embryonic tissues of chinook salmon, coho salmon and steelhead trout were exposed to the Oregon sockeye salmon virus to determine the specificity of the virus in vitro. Experiments were carried out by planting 600,000 cells per ml, of the species to be tested, in Leighton tubes. Sockeye embryonic cells were planted at the same concentration to serve as a control. Two days after planting the cells the tissue culture medium was removed and a virus suspension containing $10^{4.0} \text{ID}_{50}$s per 0.2 ml was added to the cells being tested and to the control. The virus titer of the tissue culture fluid was determined at the time of inoculation and at three and seven days intervals after exposure to the virus. The cells were also observed microscopically for any signs of cytopathic effect.

Purification of the Virus

The observation of a virus with the electron microscope requires that the purest possible virus preparation be used. Extraneous particles in the virus suspension make it difficult to recognize the actual virus.

The most satisfactory method found for purification of the Oregon sockeye salmon virus included a single cycle of
differential centrifugation followed by density gradient centrifugation in a sucrose density gradient. The virus to be purified was grown in cultures of sockeye embryonic cells. Thirty-five ml of tissue culture fluid containing 600,000 cells per ml was placed in a 32-ounce prescription bottle and allowed to incubate at 18° C. for two days; then the tissue culture medium was removed and new medium containing $10^{3.5} \text{ID}_{50}$ per 0.2 ml virus was added to the cells. Five days after inoculation the virus containing medium was removed from the cells. The total volume of virus containing fluid used was approximately 100 ml. The virus containing medium was first centrifuged at 3,000 rpm for 20 minutes in an International model UV centrifuge. The supernate from the first centrifugation was then transferred to polycarbonate tubes and centrifuged at 24,000 rpm in the Spinco ultracentrifuge for one hour to sediment the virus. The virus pellets in each tube were resuspended in 0.5 ml of 0.1 molar NH$_4$HCO$_3$. The total volume of virus suspension from the pellets was 2.5 ml. Two ml of this virus suspension was layered on top of a 5% to 40% sucrose density gradient. The sucrose density gradient had previously been formed in a 30 ml lusteroid cellulose tube using the Buchler density gradient former (Buchler Instruments). After layering the virus suspension on top of the sucrose gradient the tube was placed in the number 25 Beckman swinging bucket rotor and
centrifuged at 23,000 rpm for two hours. After centrifugation the gradient tube was carefully removed and any visible bands were collected by using the Beckman fractionating system (Beckman Instruments). A tube was placed in the area of the band and a positive pressure was applied to the contents of the gradient tube. The pressure forced the material in the band area out through the tube where it was easily collected. The fractions collected were then titrated to determine which one contained the virus. After identifying this band, it was isolated in subsequent experiments and diluted together with a small amount of the fluid adjacent to the band to approximately 30 ml with ammonium bicarbonate solution. The virus was then centrifuged again at 24,000 rpm for one hour. The supernate was removed and each virus pellet was re-suspended in 1.5 ml of ammonium bicarbonate. A final low speed centrifugation of 3000 rpm for 20 minutes was used to remove clumps of virus. The supernate from this centrifugation was used in electron microscope studies.

The polystyrene latex spheres used in the present study had a particle diameter of 0.264µ with a standard deviation of 0.006µ. The stock solution contained 3.5% solids. For use as reference particles a portion of the stock solution was first diluted in distilled water to contain 1% solids. Then the solution containing 1% solids was diluted 1:10 with virus suspension. This gave a
desirable distribution of polystyrene particles when viewed with the electron microscope.

**Shadow Casting Virus Material**

The grids used were of 200-mesh copper and were coated with formvar according to the procedure of Drummond (7, p. 56-73). The following procedure was used when the virus material was to be shadow cast. Nine-tenths ml of purified virus suspension was mixed with 0.1 ml of the dilute suspension of polystyrene particles (diameter 264μ) and placed on the grids with a fine-tipped dropper. After the drop had been on the grid for 30 seconds most of it was drawn off. The grids were placed under 15 pounds vacuum in a vacuum desicator jar for 24 hours allowing them to dry. After drying the grids were shadow cast with a paladium platinum alloy.

**Potassium Phosphotungstate**

A 2% solution of phosphotungstic acid adjusted to a near neutral pH with one normal potassium hydroxide has been reported to act as a negative stain when mixed with virus particles which are then observed with the electron microscope (3, p. 103-110).

The negative stain used to study the Oregon sockeye salmon virus was potassium phosphotungstate. This was prepared by
adjusting a 2% solution of phosphotungstic acid to pH 7.0 with one normal potassium hydroxide.

**Negative Staining Procedure**

With the negative stain, virus particles were imbedded in an electron dense material. It has been found that this technique allows the shape and sub-structure of a virus to be seen more clearly than with positive staining procedures.

To observe the Oregon sockeye salmon virus by negative staining, purified virus material was mixed 1:1 with the 2% potassium phosphotungstate at pH 7.0. This mixture was then placed in a nebulizer and sprayed onto formvar-coated electron microscope grids. The grids were then dried under vacuum in a vacuum desiccator jar. After drying the grids were observed with the electron microscope. The microscope used was an RCA built in 1950 and was not a high resolution instrument, but was the only one available. Upon finding droplets of electron dense materials containing visible particles photographs were taken. These photographs were studied to determine the shape and sub-structure of the virus.
EXPERIMENTAL RESULTS

Cytopathogenic effect of the Oregon sockeye salmon virus (O.S. V.) in cultured sockeye embryonic cells

(1) Cellular changes observed in infected cells using May Grunwald-Giemsa stain.

Sockeye embryonic cells which had been grown in tissue culture for four months were planted, in Leighton tubes containing coverslips, at a concentration of 800,000 cells per ml of complete medium. Forty-eight hours later the medium was removed and new complete medium containing \(2 \times 10^{5.5}\) ID\(_{50s}\) of virus per 0.2 ml was added to half of the tubes. The other half of the tubes received only complete medium and served as the control. All cultures were incubated at 18°C. Starting 24 hours after addition of the virus two coverslips containing infected cells and two normal cells were stained at 24 hour intervals for the next seven days.

The first change noted after initiation of virus infection was disappearance of the nucleoli in most of the cells after 24 to 48 hours incubation. Another early change observed was a thickening of the nuclear membrane. After 48 hours of exposure to the virus some cells began to show additional nuclear changes. The chromatin became dense and fibrous, and in some cases
fragmented, giving the appearance of a multinucleated cell.

After three days of virus infection all cell division appeared to have ceased and some cells were beginning to round up and detach from the glass.

Most of the cytopathological changes associated with infection were in the nucleus of the cell. However, vacuolization was observed in the cytoplasm of some cells which had been exposed to the virus.

(2) Phase microscopy of infected cells.

The changes observed in infected living cells with the phase microscope were nearly the same as those seen in stained cells except that in living cells the nucleoli did not disappear after 24 to 48 hours.

For these observations four Rose chambers were inoculated with sockeye embryonic cells at a concentration of 800,000 cells per ml of complete medium. Forty-eight hours after inoculation the medium was removed and complete medium containing $10^{4.8} \text{ID}_{50s}$ of virus per 0.2 ml was added to two of the Rose chambers. The other two chambers received only complete medium and served as the control cultures.

Photographs were taken of cell cultures at 24, 48 and 96 hours after exposure to the virus. Photographs of uninfected cells were
taken at the same intervals. The uninfected cells show the nucleus, nucleoli, and various cytoplasmic granules as they appear normally in fish cell cultures (Figures 1, 3, and 5).

Sockeye embryonic cells which has been exposed to the virus for 24 hours showed a thickening of the nuclear membrane and the nucleoli appeared more dense by dark phase microscopy than normal cell nucleoli (Figure 2). Cultures 48 hours after virus inoculation, when compared to control cells, showed again the thickened nuclear membrane and the dense nucleoli and were beginning to round up (Figure 4). After 96 hours of exposure to the virus most of the cells had rounded up and many had become detached from the glass growing surface of the chamber. Within the rounded up cells can be seen the characteristics described previously (Figure 6).

Species specificity of virus infection

(1) Virus specificity "in vivo"

The "in vivo" experiments were carried out in a refrigerated sterile transfer room at a temperature of 16\degree C. Fingerlings of each species of fish, in groups of 10, were placed in one-gallon jars. Each jar contained two liters of water. Four ml of tissue culture medium containing $10^{5.67}$ ID$_{50}$s of virus per 0.2 ml were added to each jar containing one species of fish. For each
Figure 1. Sockeye embryonic cells cultured in Rose chambers for observation with the phase contrast microscope. The culture shown here had been incubated for 24 hours and served as control for cells which had been exposed to the virus for 24 hours. These cells are characterized by the moderately dark nucleoli within the clear nuclear area. The cytoplasm of the cells shows many various size granules. (X533)

Figure 2. Sockeye embryonic cells exposed to the Oregon sockeye salmon virus for 24 hours. These cells were cultured and observed in the same manner as the cells in Figure 1. The changes noted in the infected cells were: nucleoli of greater density and a thickening of the nuclear membrane. (X533)
Figure 3. Forty-eight hour control culture of sockeye embryonic cells. These cells again show the structures as they were described in the 24-hour control cultures. (X533)

Figure 4. Sockeye embryonic cells 48 hours after inoculation with the virus. The dense nucleoli and thicker nuclear membranes are even more obvious than in Figure 2. Also noticeable at this point is the tendency of the cells to roundup. (X533)
Figure 5. Sockeye embryonic cells which had been incubated for 96 hours. These cells served as controls for cells which had been exposed to the virus for 96 hours. (X533)

Figure 6. A Rose chamber culture of sockeye embryonic cells which have been exposed to the virus for 96 hours. By this time many of the cells have rounded up and large clear areas appear in the cell sheet where infected cells have detached from the glass. (X533)
fish species, a second jar was prepared, with no added virus to serve as a control. An additional control was run with kokanee salmon in which the virus inoculum was heated at 60°C for 30 minutes before adding it to the water.

After exposure of fingerling kokanee salmon, chinook salmon, coho salmon and rainbow trout to the virus, the kokanee salmon was the only species which experienced significant mortalities (Table 1). The cause of death was confirmed by infection of sockeye cell cultures and production of typical cytopathogenic effects with material from kokanee salmon which had died during the experiment. "In vivo" specificity was checked a second time and the results substantiated those of the first experiment. (Table 1)

(2) Susceptibility of fish cell cultures from different species to the Oregon sockeye salmon virus

A series of experiments were conducted to determine if established cell lines started from embryonic tissues of different species of salmonids were susceptible to the virus. The cell lines used were chinook salmon (started January 1963; 32 transfers), coho salmon (started February 1963; 41 transfers), and steelhead trout (started June 1963; 34 transfers). The cell cultures were grown in Leighton tubes inoculated with 600,000 cells per ml of complete medium. Each of the cell lines was
Table 1. Species specificity of the Oregon sockeye salmon virus "in vivo".

Virus concentration of initial inoculum was $10^{5.67} \text{ID}_{50}^\text{s}$ per 0.2 ml. Four ml was added to each jar.

<table>
<thead>
<tr>
<th>Time of exposure to the virus (days)</th>
<th>Experiment I</th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Heated control</td>
<td>Active virus</td>
<td>Control</td>
<td>Virus</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>Virus</td>
<td>Control</td>
<td>Virus</td>
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<tr>
<td>1</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>1</td>
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</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Mortalities</strong></td>
<td>1/10</td>
<td>2/10</td>
<td>8/10</td>
<td>0/10</td>
</tr>
</tbody>
</table>

**Experiment II**

|                                    |  |  |  |
|                                    |  |  |  |
| 1                                  | 3 |
| 2                                  | 2 |
| 3                                  | 3 |
| 4                                  | 2 |
| 5                                  | 3 |
| 6                                  | 1 |
| 7                                  |  |  |
| 8                                  |  |  |
| 9                                  |  |  |
| 10                                 |  |  |
| **Mortalities**                    | 0/10 | 0/10 | 9/10 |

1 The virus material heated at 60°C for 30 minutes before exposing the fish to it.

2 The control fish were exposed to four ml of complete tissue culture medium which was added to 2 liters of water.
tested in parallel with sockeye embryonic cells (started 1964; 18 transfers) known to be susceptible to the virus. All experiments were carried out at 18° C. Forty-eight hours after the cell cultures had been planted the medium was removed and approximately $10^{3.96}$ ID$_{50}$s of virus in 1.0 ml of complete medium was added to each tube. At three days and seven days after adding the virus, medium from three tubes of the cell line being tested was combined and titrated on cultures of sockeye embryonic cells by the infectivity titration method. This titer was compared to the original virus concentration and the titer of tissue culture fluid from sockeye cells inoculated at the same time.

The virus replicated and produced a definite cytopathic effect in cultures of chinook salmon and steelhead trout as shown in Table 2. There was no evidence of virus infection in cultures of coho salmon. These three cell lines had all been carried in tissue culture for at least two years and are known to have become heteroploid. When the virus was inoculated into primary cultures of chinook salmon cells no evidence of infection was observed (Table 2). The primary chinook salmon cells had been grown in tissue culture for approximately two months before virus exposure. Apparently the established line of chinook salmon cells had undergone a change in susceptibility to the virus. Possibly this could have occurred with the steelhead cells also.
Table 2. Susceptibility of cell cultures from different species of fish to the Oregon sockeye salmon virus

Virus titer expressed in log of the $ID_{50s}/0.2\, \text{ml}$ of tissue culture medium

<table>
<thead>
<tr>
<th>Cell species</th>
<th>Number of transfers $^2/$</th>
<th>Number of days after virus inoculation of the cell culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sockeye salmon (October 1964 $^1/$)</td>
<td>18</td>
<td>4.17 6.63 6.63</td>
</tr>
<tr>
<td>Chinook salmon (January 1963 $^1/$)</td>
<td>32</td>
<td>4.17 6.83 6.63</td>
</tr>
<tr>
<td>Steelhead trout (June 1963 $^1/$)</td>
<td>34</td>
<td>3.83 6.50 6.50</td>
</tr>
<tr>
<td>Coho salmon (February 1963 $^1/$)</td>
<td>41</td>
<td>4.17 4.51 3.50</td>
</tr>
<tr>
<td>Primary chinook salmon (February 1966 $^1/$)</td>
<td>2</td>
<td>3.68 2.83 2.83</td>
</tr>
</tbody>
</table>

$^1/$ Date each culture was started from embryos removed from eyed eggs.

$^2/$ The number of times the cells have been transferred in tissue culture.
Histopathology of virus infected kokanee salmon

Serial cross sections of kokanee salmon, which had died during the "in vivo" experiments, were stained with hematoxylin and eosin. The only pathology noted was in the kidney where most of the parenchymal cells had been destroyed.

Approximate virus particle size as determined by filtration

Tissue culture medium from sockeye embryonic cells which had been exposed to the virus for seven days was centrifuged at 3000 rpm for 20 minutes. After this the supernatant was passed through Millipore filters with pore sizes of 0.45µ, 0.22µ, and 0.1µ. A group of 10 Leighton tubes of sockeye embryonic cells was inoculated from each of the four filtrates. The inoculum volume was 0.2 ml/tube. These cultures were observed for evidence of virus activity. It was found that the O.S.V. would pass through a Millipore filter of 0.22µ pore size but not one of 0.1µ pore size (Table 3). By use of a table, prepared by Elford (9, p. 284-386) giving the ratio of the size of particle which will pass through a certain pore diameter to the pore size of the filter, the approximate particle size of the virus was calculated to be between 110 mµ and 165 mµ. This experiment was repeated and the results were the same as those of the first experiment.
Table 3. Estimate of particle size of the Oregon sockeye salmon virus by filtration

<table>
<thead>
<tr>
<th>Average pore diameter 1/ of Millipore filter</th>
<th>Number of tubes 2/ showing infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.45µ</td>
<td>10/10</td>
</tr>
<tr>
<td>0.30µ</td>
<td>10/10</td>
</tr>
<tr>
<td>0.22µ</td>
<td>10/10</td>
</tr>
<tr>
<td>0.10µ</td>
<td>0/10</td>
</tr>
</tbody>
</table>

1/ Manufacturer's values.
2/ Tubes were cultures of sockeye embryonic cells. Each tube contained 0.8 ml of Eagle's MEM with 20% newborn agamma calf serum. The virus filtrate was introduced in a volume of 0.2 ml per tube. The tubes were incubated at 18°C and observed for a period of 10 days.
Replication rate of the virus at 18°C.

Sockeye embryonic cells were planted in Leighton tubes at a concentration of 800,000 cells per ml. After 48 hours incubation at 18°C. 0.2 ml of tissue culture medium containing \(10^{3.5}\) ID\(_{50}\)s of virus was added to several of the tubes. The volume was brought to one ml in each tube by addition of fresh complete medium. Then the cultures were incubated at 18°C. Each day, for six days after exposure to the virus, fluid from three infected tubes was combined and the virus concentration determined by infectivity titration.

On the basis of two experiments at this temperature the first new virus appeared in the tissue culture fluid 24 to 48 hours after inoculation of sockeye embryonic cell cultures (Figure 7). The maximum titer in the tissue culture fluid was reached 72 hours after exposure of the cells to the virus. After the virus reached maximum titer at 72 hours it remained at this level until the experiment was terminated six days after inoculation of the cells.

Plaque titration

When monolayer cultures of sockeye embryonic cells were exposed to proper dilutions of the virus, plaques were discernible
Figure 7. Rate of replication of the Oregon sockeye salmon virus in sockeye embryonic cells at 18°C. (The data for these curves was taken from infectivity titrations using four tubes per dilution.)
after five days of incubation at 18° C. If the cultures were incubated for seven days the plaques were larger and more distinct. (Figure 8) The seven-day incubation period allowed a more accurate plaque count.

Plaque titration and infectivity titration experiments were carried out using the same virus dilution series for both titrations. This type of experiment was repeated twice on the same virus stock in order to determine which titration method gave the most consistent results. Titration by the plaque method was found to be much more consistent than infectivity titration (Table 4). Calculations were also made to determine how many active virus particules (pfu) were required to produce cytopathic changes in a cell monolayer used for infectivity titration. Using the data in Table 5 the mean of the ID₅₀s from three experiments was 247,000 ID₅₀s per 0.2 ml of the original virus suspension. The mean number of plaque forming units (pfu) in 0.2 ml of a 1:81,000 dilution was 1.68.

\[
\frac{247,000}{81,000} = 3.05 \text{ ID}_{50}\s
\]

\[
\text{one ID}_{50} = \frac{1.68}{3.50} = 0.55 \text{ pfu}
\]

Theoretical calculations have found one ID₅₀ to be equal to 0.69 pfu (Cooper, 6, p. 319-374).
Figure 8. Sockeye embryonic cell cultures after seven days exposure to the Oregon sockeye salmon virus. The bottle on the left has the highest concentration of virus and many plaques are discernible in the cell sheet. The center bottle shows only a few plaques in the monolayer and the bottle on the right no plaques. The virus dilution between each bottle is ten fold.
Table 4. Comparison of the plaque method to the infectivity method of titration

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Method</th>
<th>Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Plaque formation</td>
<td>147,600 pfu/0.2 ml</td>
</tr>
<tr>
<td></td>
<td>Infectivity titration</td>
<td>201,000 ID_{50s} 0.2 ml</td>
</tr>
<tr>
<td>II</td>
<td>Plaque formation</td>
<td>151,000 pfu/0.2 ml</td>
</tr>
<tr>
<td></td>
<td>Infectivity titration</td>
<td>122,000 ID_{50s} 0.2 ml</td>
</tr>
<tr>
<td>III</td>
<td>Plaque formation</td>
<td>147,600 pfu/0.2 ml</td>
</tr>
<tr>
<td></td>
<td>Infectivity titration</td>
<td>607,000 ID_{50s} 0.2 ml</td>
</tr>
</tbody>
</table>

1/ In each experiment the same virus suspension was titrated by both methods.
2/ Plaque forming units.
3/ Number of infective doses determined by the method of Reed and Muench (24, p. 493-497).
Table 5. Number of plaque forming units required to produce infection in a monolayer of cells

| Virus dilution | Infectivity titration, 1/number of tubes infected | Log ID$_{50}$s per 0.2 ml | Plaque titration Average number of plaques per 0.2 ml 2, 3/
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1: 81,000</td>
<td>5/5</td>
<td>5.305</td>
<td>1.600</td>
</tr>
<tr>
<td>1:243,000</td>
<td>2/5</td>
<td></td>
<td>0.800</td>
</tr>
<tr>
<td>1:729,000</td>
<td>0/5</td>
<td></td>
<td>0.000</td>
</tr>
</tbody>
</table>

Experiment I

| 1: 81,000      | 5/5                                           |                          | 2.240                       |
| 1:243,000      | 5/5                                           |                          | 1.000                       |
| 1:729,000      | 2/5                                           | 5.783                    | Not known                   |

Experiment II

| 1: 81,000      | 4/5                                           |                          | 1.200                       |
| 2:243,000      | 0/5                                           | 5.087                    | 0.142                       |
| 1:729,000      | 0/5                                           |                          | 0.000                       |

1/ Volume of virus inoculated in each tube was 0.2 ml.

2/ Average number of plaques on three bottle cultures at each dilution.

3/ Volume of virus inoculated in each bottle was 0.5 ml.
Intracellular virus

Disruption of the cells for intracellular virus determination was done by sonic oscillation, alternate freezing (-40°C) and thawing (20°C) for three cycles and treatment with the Virtis homogenizer for 30 seconds at approximately 23,000 rpm. Virtis homogenizer treatment was the only method which gave complete cell disruption without inactivating significant amounts of the virus. The amount of infective intracellular virus was found to be so small that when added to the amount found in the tissue culture fluid it could not be detected by the infectivity titration method (Table 6). Results indicate this virus is one of those that is readily released from infected cells into the culture fluid.

Ether sensitivity of the virus

Diethyl ether at a concentration of 20 percent by volume was found to completely inactivate the O.S. V. after 23 hours exposure at 4°C. The same testing procedure was used on influenza virus (Lee strain) a known ether sensitive virus (14, p. 564-575) and infectious pancreatic necrosis virus (IPN) a known ether resistant virus (19, p. 320-327). The influenza virus sample was allantoic fluid from 12-day old chick embryos, which had been infected for 40 hours and had a titer of \(10^{7.5}\) ID\(_{50}\) per 0.2 ml. The IPN
Table 6. Comparison of methods used in determination of the amount of intracellular virus present in sockeye embryonic cells after three days of infection

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Cell disruption</th>
<th>Virus titer (Log of the ID$_{50}$ per 0.2 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sonic oscillation</td>
<td></td>
</tr>
<tr>
<td>Tissue culture fluid alone, without sonic treatment</td>
<td>5.33</td>
<td></td>
</tr>
<tr>
<td>Tissue culture fluid and disrupted cells</td>
<td>4.66</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Alternate freezing and thawing 1/</strong></td>
<td></td>
</tr>
<tr>
<td>Tissue culture fluid alone, unfrozen</td>
<td>6.50</td>
<td></td>
</tr>
<tr>
<td>Tissue culture fluid and disrupted cells</td>
<td>5.50</td>
<td></td>
</tr>
<tr>
<td>Washed cells 2/</td>
<td>4.66</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Virtis homogenizer</td>
<td></td>
</tr>
<tr>
<td>Tissue culture fluid alone</td>
<td>6.17</td>
<td></td>
</tr>
<tr>
<td>Tissue culture fluid and disrupted cells</td>
<td>6.27</td>
<td></td>
</tr>
<tr>
<td>Washed cells 2/</td>
<td>4.83</td>
<td></td>
</tr>
</tbody>
</table>

1/ This treatment consisted of three cycles of alternate freezing and thawing. Freezing of the sample was done by plunging the tube into a bath of acetone and dry ice. The temperature of this solution was -40°C. The sample was thawed by placing it in a water bath at 20°C.

2/ These cells were removed from the tissue culture medium by centrifugation and resuspended in an equal volume of Earle's BSS. Finally, they were resuspended in an equal volume of fresh complete medium and disrupted by the means indicated above.
virus sample was tissue culture fluid from infected steelhead embryonic cells and had a titer of $10^{6.33}$ ID$_{50}$s per 0.2 ml. The results agreed with previously reported information concerning the two viruses (Table 7).

**Type of nucleic acid**

A $10^{-3}$ molar concentration of 5-bromodeoxyuridine has been reported to inhibit replication of many DNA viruses without being toxic to host cells (8, p. 509-520; 26, p. 1-12). This concentration of the compound was found to be nontoxic to sockeye embryonic cells.

Cells used in nucleic acid experiments were planted in Leighton tubes at a concentration of 450,000 cells per ml.

Two days after planting the medium was removed and new medium containing virus was added to the cells. The virus was allowed to absorb to the cells for one hour. Then the unabsorbed virus was removed and complete medium containing $10^{-3}$ molar 5-bromodeoxyuridine was added to half the cell cultures. The remaining tubes received only complete medium. Two days after exposure to the virus and addition of 5-bromodeoxyuridine, the culture fluid was removed and the virus titer of control tubes was compared to tubes containing the metabolic inhibitor.

The nucleic acid type in the O.S.V. was tentatively identified
Table 7. Validity of testing procedure for ether sensitivity; comparison of sockeye virus with Lee influenza and IPN viruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>Titer Log of ID&lt;sub&gt;50&lt;/sub&gt; per 0.2 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lee influenza virus (ether sensitive)&lt;sup&gt;1/&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Before ether treatment</td>
<td>7.376</td>
</tr>
<tr>
<td>After ether treatment</td>
<td>3.376</td>
</tr>
<tr>
<td>IPN virus (ether resistant)&lt;sup&gt;2/&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Before ether treatment</td>
<td>6.333</td>
</tr>
<tr>
<td>After ether treatment</td>
<td>6.333</td>
</tr>
<tr>
<td>Sockeye virus</td>
<td></td>
</tr>
<tr>
<td>Before ether treatment</td>
<td>4.666</td>
</tr>
<tr>
<td>After ether treatment</td>
<td>0.000</td>
</tr>
</tbody>
</table>

1/ Titration of influenza virus was carried out in 10-day old embryonated chicken eggs. The number of eggs infected at each dilution was determined by testing for haemagglutination of chicken red blood cells exposed to chorio allantoic fluid from inoculated eggs.

2/ The IPN virus was assayed by infectivity titration using five tubes of steelhead embryonic cells at each dilution.

as RNA. The method used for making this determination was also used on a known DNA virus (bovine rhinotracheitis virus) and a known RNA virus (MEF 1 type 2 polio virus).

The results recorded in Table 8 show that a concentration of 10<sup>-3</sup> molar 5-bromodeoxyuridine completely inhibited replication of the known DNA virus. The replication of the known RNA virus was not inhibited by exposure to the metabolic inhibitor. Since
Table 8. Presumptive identification of type of nucleic acid in virus as determined by use of 5-bromodeoxyuridine

The host cells were exposed to the compound\(^1\) and the virus simultaneously for 48 hours

<table>
<thead>
<tr>
<th>Virus</th>
<th>Host cells</th>
<th>10(^{-3}) molar 5-bromodeoxyuridine(^2)</th>
<th>Virus titer Log of the ID(_{50})s per 0.2 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infectious bovine(^3)/</td>
<td>Bovine kidney</td>
<td>Absent</td>
<td>5.625</td>
</tr>
<tr>
<td>Rhinotracheitis virus (known DNA)</td>
<td></td>
<td>Present</td>
<td>0.000</td>
</tr>
<tr>
<td>Polio virus(^3)/</td>
<td>He La</td>
<td>Absent</td>
<td>8.375</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Present</td>
<td>8.550</td>
</tr>
<tr>
<td>Sockeye virus(^4)/</td>
<td>Sockeye embryonic</td>
<td>Absent</td>
<td>5.883</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Present</td>
<td>5.625</td>
</tr>
</tbody>
</table>

\(^1\) Concentration was 10\(^{-3}\) M.
\(^2\) The compound was dissolved in Eagles MEM with 20% newborn agamma calf serum added. This medium was placed on cells after removal of old tissue culture fluid.
\(^3\) Experiments with these viruses were carried out at 37\(^\circ\) C.
\(^4\) Experiments with sockeye salmon virus were performed at 18\(^\circ\) C.
replication of the sockeye salmon virus did not appear to be affected by 5-bromodeoxyuridine it was assumed this virus had the same type of nucleic acid as the known RNA virus which responded in a similar manner (Table 8).

**Influence of Temperature on Virus Replication "In Vitro"**

Groups of sockeye embryonic cell cultures were inoculated with 0.2 ml of tissue culture fluid containing $10^{3.5}$ ID$_{50}$s of virus. The cultures were incubated at 4°, 8°, 10.5°, 13°, 18°, 20°, and 23° C. The virus yield in the pooled culture fluid from three tubes was measured at 2, 4, 6, and 8 days after infection of the cultures. The virus was found to multiply at all temperatures except 23° C. (Table 9). At 4° C, the rate of replication was reduced and the maximum titer at eight days was approximately one log lower than at 8°, 10.5°, and 13° C. At 8° and 10.5° the virus titer was near maximal on the fourth day, while at 13° and 18° the maximum was reached in two days. (Figures 9 and 10) At 20° C, the replication rate of the virus decreased so that the maximum titer was not achieved until the fourth day of incubation. At 23° C, there was no evidence of viral replication and no cytopathic changes were observed in the cells.

Results have indicated the optimal temperature range for virus replication is between 13° C and 18° C. These results are
Table 9. The effects of temperature on the "in vitro" replication of the Oregon sockeye salmon virus grown in sockeye embryonic cells

<table>
<thead>
<tr>
<th>Days of exposure to the virus</th>
<th>Average log of the $\text{ID}_{50}$s per 0.2 ml of tissue culture medium from two experiment at each temperature 1, 2/</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$4^\circ C.$</td>
</tr>
<tr>
<td>0</td>
<td>3.50</td>
</tr>
<tr>
<td>2</td>
<td>3.65</td>
</tr>
<tr>
<td>4</td>
<td>4.35</td>
</tr>
<tr>
<td>6</td>
<td>5.30</td>
</tr>
<tr>
<td>8</td>
<td>5.45</td>
</tr>
</tbody>
</table>

1/ For each virus dilution five tubes of sockeye embryonic cells were each inoculated with 0.2 ml of the diluted virus. This procedure was used in each of the temperature experiments.

2/ The medium used in all experiments was Eagles MEM with 20% newborn agamma calf serum added. This medium was also used as the diluent in all virus titrations.
Figure 9. Effect of temperature on the rate of replication of the Oregon sockeye salmon virus
Figure 10. Effect of temperature on the rate of replication of the Oregon sockeye salmon virus.
particularly interesting in view of the fact that the optimal temperature range for growth of the host cells has been reported to be between 18° C. and 23° C. (11, p. 566-586). The host cells did not multiply at 13° C. although this same temperature was found to be optimum for virus multiplication.

Purification of the virus

Tissue culture fluid from infected cells having a titer of approximately $10^6$ ID$_{50}$s per 0.2 ml was centrifuged at 24,000 rpm for one hour in the Spinco Mod. L centrifuge in order to sediment the virus. The pellet material after suspension in a small volume of 0.12 molar ammonium bicarbonate solution had a titer of approximately $10^7.3$ ID$_{50}$s per 0.2 ml. Two ml of this solution was layered on the surface of a 5% to 40% sucrose density gradient solution which had a volume of 32 ml. The density gradient solution was centrifuged at 23,000 rpm for one hour. After centrifugation a grayish white band appeared approximately 4.45 cm. from the top of the tube. The approximate density at the level of the band was 1.1270. The virus concentration in this band was two to three logs higher than in samples taken from other areas of the gradient (Table 10). The material from the band had a titer very close to that of the pellet material. Material from the band area was diluted with 30 ml of 0.12 molar ammonium bicarbonate solution and the
Table 10. Virus titers resulting from concentration and purification of virus samples

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Pellet 1/</th>
<th>Band 2/</th>
<th>Sample from fluid above band in sucrose gradient</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.83</td>
<td>6.32</td>
<td>4.50</td>
</tr>
<tr>
<td>2</td>
<td>7.83</td>
<td>7.32</td>
<td>4.15</td>
</tr>
<tr>
<td>3</td>
<td>7.15</td>
<td>6.83</td>
<td>4.63</td>
</tr>
</tbody>
</table>

1/ The pellet was virus material centrifuged out of 100 ml of tissue culture fluid. The pellet was resuspended in 2.4 ml of 0.12 molar ammonium bicarbonate and titrated by the infectivity titration method.

2/ The band resulted from density gradient centrifugation of the pellet material in a sucrose density gradient for one hour at 23,000 rpm. The volume of the band fraction was approximately 1.5 ml.
virus was sedimented by centrifugation at 24,000 rpm for one hour, to remove the sucrose from the band sample.

Electron microscopy of the O. S. V.

A suspension of virus pellet material (titer $10^7.3\text{ID}_{50}$ per 0.2 ml) in 0.12 M ammonium bicarbonate containing $4 \times 10^{11}$ polystyrene particles per ml was placed on electron microscope grids with a fine-tipped dropper. The grids were then placed under vacuum in a vacuum desiccator jar overnight for drying of the suspension and removal of volatile salts. After drying the grids were shadow cast with a platinum palladium alloy.

When photomicrographs were made of representative areas, the predominant particles in the pellet had a diameter between 91 μ and 145 μ. Some of these particles exhibited evidence of cubic symmetry as shown by their shape and that of their shadows in photomicrographs (Figure 11).

Material from the virus pellet was also examined after negative staining with potassium phosphotungstate. The virus suspension was mixed 1:1 with 2% potassium phosphotungstate, placed in a nebulizer and sprayed onto formvar-coated grids. After drying the grids under vacuum in a desiccator jar they were observed with the electron microscope. With this stain particles were again photographed which appeared to have cubic symmetry based
Figure 11  Shadow cast pellet material with polystyrene latex spheres (264 m\(\mu\) in diameter) added as reference particles. Most of the particles in the pellet had a diameter between 91 and 145 m\(\mu\) and appear to have cubic symmetry (see arrows). (X76, 000).
their hexagonal profile (Figure 12).

Material recovered from the density gradient band did not show as great a concentration of particles as was seen in the pellet. However, particles were found of approximately the same size and shape as those seen in the pellet material (Figures 13 and 14). It seems probable that the particles described represent the virus, but further work will be required to determine this with certainty.
Figure 12. Pellet material negatively stained with potassium phosphotungstate. In this case reference particles were not added as with shadowed material (Figure 10) since they cause the stain to accumulate in large droplets. In the lower part of the picture is a particle which appears to have cubic symmetry. (X152,000)
Figure 13. A shadow cast particle from the band obtained by density gradient centrifugation. The larger spherical particle is a polystyrene latex sphere (264 μm in diameter) which serves as a reference. The smaller particle shows some signs of cubic symmetry and has a diameter of approximately 118 μm. (X152,000)
Figure 14. Another particle found in material from the band after density gradient centrifugation. This particle has a diameter of approximately 86 mμ, and as in Figure 12 there is some suggestion that the particle may possess cubic symmetry. (X152,000)
DISCUSSION

The large number of animal viruses which have been isolated in recent years have created interest in devising a systematic classification for them. Classification schemes based on physical, biochemical and biological properties of viruses have resulted in the recognition of several distinct classes of viruses (1, p. 1-401; 29, p. 1-58). Each class is made up of viruses which have several characteristics common to that particular group.

The purpose of experiments in this study was to determine some of the physical, chemical and biological properties of the O. S. V.; then, using known characteristics of the virus, tentatively to assign it to the group of viruses to which it seemed to be most closely related.

In cultures of sockeye embryonic cells most of the changes attributed to the virus were associated with the nucleus. The nuclear membrane thickened and the chromatin material became coarse and granular. The nucleoli were certainly affected since they disappeared in stained virus infected cells and became more dense in infected cells viewed with the phase contrast microscope. The apparent disappearance of the nucleoli in stained cells suggests that they have been changed biochemically, losing the staining reaction of RNA-protein and acquiring that of the rest of the nucleus.
which contains DNA-protein.

The increase in density of the nucleoli in living infected cells proves they do not really disappear; at present there is no explanation for the increased density. In some cases the nuclei were divided into several segments giving the appearance of a multinucleated cell.

Vacuolization was the only change noted in the cytoplasm of the cell. There was no evidence of inclusion bodies in either the nucleus or the cytoplasm.

Generally within 7 to 10 days after infection of sockeye embryonic cell cultures all of the cells had rounded up and many had detached from the glass. The cytopathic sequence produced by this virus in cell culture was not particularly useful in assigning it to a certain group of viruses. But, its failure to cause inclusion bodies tentatively eliminated it from groups of viruses such as the Herpes, Adeno, Reo and Pox viruses which produce inclusion bodies in infected cells.

When various species of young salmonid fishes were exposed to the virus, the kokanee salmon (land locked variety of sockeye salmon) was the only susceptible species. Fingerling chinook salmon, coho salmon and rainbow trout were found to be resistant. These results agree with earlier work on the Washington sockeye salmon virus which was infectious only to young sockeye salmon.
and kokanee and not to young coho and chinook salmon or fingerling rainbow trout (28, p. 1-36).

There has been a report by Parisot (23, p. 502-519) that the Oregon sockeye salmon virus will infect one-inch rainbow trout when injected intraperitoneally. However, he found the rainbow trout were only susceptible when they were approximately six weeks old. He found young chinook salmon and cutthroat trout to be resistant to the virus.

From our experimental results and the reports of others it appears this virus is quite specific, infecting only its natural host when a natural route of exposure is used. When the virus was injected intraperitoneally its host range was extended to fingerling rainbow trout.

Kokanee salmon which died from virus infection during "in vivo" experiments were sectioned and stained to determine pathological changes resulting from infection. The principal change was associated with the kidney where there was marked degeneration of the parenchymal cells. All other organs and tissues appeared normal. Other investigators have also found the kidney to be the organ most affected by the O. S. V.; with the spleen, pancreas and liver involved in some extensive cases (30, p. 85-90). In the present study the pathological examination was preliminary in nature and involvement of other organs such as the spleen,
pancreas and liver could have been overlooked if changes were not extensive.

The host range of this virus on embryonic fish cell lines was less specific than with the intact animals. Embryonic cell lines of chinook salmon and steelhead trout, which had been cultured for approximately three years were susceptible to the virus. Salmon (coho) cell cultures of the same age were still resistant to virus infection. These cell lines were heteroploid in chromosome number (18, 1-50). Embryonic chinook cells which had been cultured for two months and still had the diploid number of chromosomes were resistant to this virus. From these results it appears that cells cultured over a long period of time, during which the chromosome number becomes heteroploid, may in some cases lose their original resistance to a virus. The mechanism of this loss is somewhat of a mystery since primary cell cultures are often more susceptible to virus infection than cells cultured for long periods of time.

This breakdown of resistance in fish cells cultured for long periods of time may be a character unique to fish cell cultures. The fathead minnow cell line started in 1962 and subcultured 58 times has been found to be susceptible to viruses from fish, amphibians and even mammals (12, p. 555-565). A cell line established from grunt fin cells, a marine fish, has shown a
marked susceptibility to IPN virus which "in vivo" infects only the
brook and rainbow trout, both fresh water fish (20, p. 328-342).
An orphan virus isolated from grunt fish cell cultures will infect
and multiply in an established goldfish cell line but not in primary
cultures of goldfish cells (4, p. 343-361). Further evidence for
the increased host range of fish cell lines is the susceptibility of
a cell line derived from rainbow trout gonad tissue to Venezuelan
equine encephalomyelitis virus and Eastern equine encephalomye-
litis virus (22, p. 190-194).

On the basis of this evidence it is not too surprising that the
O. S. V. will infect chinook salmon and steelhead trout cell lines
although it will not infect these species "in vivo."

The multiplication of the Oregon sockeye salmon virus at 18°C.
in sockeye salmon embryonic cell cultures was found to give a
growth curve characteristic of viruses in cell culture. The first
new virus appeared in the medium 24 to 48 hours after infection.
The eclipse phase for this virus (24 to 48 hours) is longer than the
latent period for animal viruses in general. More specifically the
O. S. V. has a much longer eclipse phase than that of the IPN virus
of trout. New IPN virus was found in the medium of cell cultures
seven to eight hours after initial infection (19, p. 320-327). The
O. S. V. reached maximum titer in the culture fluid after three
days and remained at this level until the experiment was terminated
at seven days.

The preceding information on multiplication of this virus was concerned with extracellular or free virus. The amount of intra-cellular virus was also determined by disrupting the cells with the virtis homogenizer and measuring the amount of infective virus released. There was not a significant increase in virus titer after disruption of the cells. This would suggest that particles may not be infective until they pass through the cell membrane into the surrounding medium. This virus appears to be one of those which is readily released from the cells.

Distinct plaques were first observed in cell monolayer cultures of sockeye embryonic cells five days after exposure of the cells to the virus. Seven days after infection of cell cultures the size of the plaques had increased and they were easily discernible when the monolayers were stained. Since this virus will produce plaques a comparison was made between the plaque assay method and infectivity titration method of quantitating virus. The same virus dilutions were used for both methods. The plaque assay method gave more consistent results than the infectivity titration method. From these experiments it was also possible to calculate approximately how many infectious virus particles were necessary to equal one ID$_{50}$ (0.55 pfu = one ID$_{50}$).
A theoretical value for the number of plaque forming units per ID$_{50}$ has been reported by Cooper (6, p. 319-374). The value was derived on the supposition that, if there is no error in dilution, at the 50 percent end-point distribution will be Poissonian. By chance half of the hosts will receive no particles while a calculable portion of those infected will receive more than one particle (average = 1.38 particles). Thus, the overall average including tubes not infected will be 0.69 particles per tube. If both systems have equal sensitivity 0.69 pfu = one ID$_{50}$ (Cooper 6, p. 319-374). This value is in good agreement with the 0.55 pfu per ID$_{50}$ reported in the present study. The small difference in the two values could be attributed to dilution error in experiments carried out for this report.

In most cases the plaque assay method would have been used to quantitate virus suspensions since it was found to give more consistent results than the infectivity titration method. However, the plaque method required eight times more cells to carry out a titration than the infectivity titration method. Since the amount of time which could be devoted to expanding cell cultures was restricted, the decision was made to use the infectivity titration method as a measure of virus concentration even though it was not as accurate as the plaque assay method.

The reduction in titer of a virus after exposure to ether
indicates it has lipid materials which are essential to initiate an infection. This is one of the major characteristics used in virus classification. Using the method of Andrewes and Horstmann (2, p. 290-297), the Oregon sockeye salmon virus was completely inactivated by exposure to 20% diethyl ether, indicating the presence of essential lipids. However, Parisot (23, p. 502-519) reported this virus was not affected by exposure to 10% ether at 5°C for 24 hours. In an effort to clarify this apparent conflict of results the procedure used to determine the ether sensitivity of this virus was applied to a known ether sensitive and ether resistant virus. The results were consistent with previously recorded information concerning the known viruses. The O.S.V. was then tested once more and the results indicated as before that the virus contained essential lipids.

Several possible explanations exist for the discrepancy between the findings of Parisot and those of the present study. The concentration of ether used in this study was 20% as opposed to 10% used by Parisot (23, p. 502-519). The 20% by volume concentration of ether was chosen since this was the concentration most commonly used in determining the ether sensitivity of different viruses. This concentration of ether has also been recommended by Wilner (29, p. 1-58) as the method for determining the ether sensitivity of viruses.
The method of quantitating the virus after exposure to ether was also different in each study. Parisot injected the virus intraperitoneally into one-inch rainbow trout for quantitation. In the present study cultures of sockeye embryonic cells were used to quantitate the amount of virus before and after exposure to ether.

The concentration of virus exposed to ether in the two studies was also different. Parisot exposed $10^{10} \text{ID}_{50}$ of virus to ether. In the present investigation $10^3 \text{ID}_{50}$ of virus was exposed to ether. After carefully assessing the results and methods used, we must conclude that the O. S. V. is sensitive to ether and therefore possesses lipids essential to infection.

The type of nucleic acid which a virus contains is another of the characteristics which is important in attempting to classify it. To date all viruses discovered have either deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) but never both types of nucleic acid (5, p. 302-305).

The O. S. V. was exposed to 5-bromodeoxyuridine, a compound reported to inhibit the replication of DNA viruses (8, p. 509-520; 26, p. 1-12). A known DNA virus and RNA virus were also tested, using the same procedure on all three agents. The two known viruses responded as expected, the replication of the DNA virus being inhibited but not that of the RNA virus. The
O. S. V. cultivated in the presence of 5-bromodeoxyuridine produced an infectivity titer as high as that of control cultures. This indicated the virus probably contained the nucleic acid RNA.

The replication rate of the virus was determined in sockeye embryonic cells for temperatures between 4°C and 23°C. The virus replicated slowly at 4°C with maximum titer being reached after six days. The maximum titer at 4°C was approximately one log lower than at higher temperatures. At 8°C and 10.5°C, the rate of multiplication increased and maximum titer was reached after four days. At 13°C and 18°C, the virus replicated most rapidly reaching a maximum two days after infection of cells. At 20°C, the rate of replication began to decrease, indicating the optimum temperature was between 13°C and 18°C. At 23°C, the virus did not multiply at all and no cytopathic effect was observed in cells exposed to the virus at this temperature.

Although the virus multiplied most rapidly between 13°C and 18°C, this is not the optimum temperature for growth of the host cells. It has been reported that salmonid cells multiply most rapidly at 23°C (11, 566-586). This temperature was found to completely inhibit virus replication. A possible explanation for this could be a difference in the optimum temperature for enzymes responsible for virus production and cell
multiplication.

Another phase of this study was development of a method for purification of the O. S. V. Purification of the virus was necessary before any experiments could be carried out with the electron microscope. Centrifugation at 20,000 rpm for one hour was sufficient to sediment the virus from tissue culture fluid. After removal of the supernate the virus pellet was resuspended in a small volume of 0.12 molar ammonium bicarbonate solution. This solution has been used as the resuspending medium for several viruses which were to be photographed with the electron microscope. Ammonium bicarbonate is a volatile salt which can be removed by vacuum without any residue contaminating the virus preparation. This solution also causes clumps of virus material to break up more readily during resuspension. After the virus pellet had been resuspended the titer was approximately 10 times greater than in the original tissue culture medium. Some of this resuspended material was placed on grids for observation with the electron microscope. The rest of the virus suspension was layered on top of a sucrose density gradient which ranged from 5% to 40% sucrose. The gradient tube contained 32 ml of sucrose solution. After the virus was layered on top of the gradient the tube was placed in a Beckman swinging bucket rotor and centrifuged at 22,000 rpm for one hour. The brake on the centrifuge was disengaged to allow the
rotor to decelerate very slowly. Stopping the rotor too rapidly would have disrupted the bands in the gradient tube.

After centrifugation one band was visible in the gradient tube. It was approximately 4.45 cm from the top of the tube and had a virus titer two or three logs higher than samples from other levels in the gradient. The titer of the band area was nearly the same as that of the pellet material.

The band material was collected using the Beckman fractionating system which places a positive pressure on the gradient tube and forces the band material out through a small needle lowered into the band area. Then it was diluted with 30 ml of 0.12 molar ammonium bicarbonate solution and centrifuged again to separate the virus from sucrose collected with the band material. The virus pellet was then resuspended in a small volume of ammonium bicarbonate solution. This suspension was used to prepare grids for electron microscope study.

In electron photomicrographs of virus containing material sedimented from tissue culture fluid the predominant particles were between 91 mµ and 145 mµ. These particles appeared to be polyhedral in shape. From filtration experiments run with the O.S.V. its size was calculated to be between 110 and 165 mµ. The virus would pass a 220 mµ millipore filter but not a 100 mµ filter. The particle size arrived at by filtration experiments agrees quite
well with the size of the predominant particles seen with the
electron microscope using reference particles of known size.
The slightly smaller size arrived at by use of the electron
microscope could be caused by drying of the virus particles
after they were placed on the grids.

Many different animal and plant viruses have been found to
have a polyhedral shape (15 p. 101-170). The polyhedral
appearance of particles in the 92 mµ to 145 mµ size range is
presumptive evidence that these particles are the Oregon sockeye
salmon virus.

The material collected from the band after density gradient
centrifugation also exhibited particles of the same shape and in
the same size range as material from the pellet. However, the
number of particles on grids from the band was less than seen
on grids from the pellet. This was probably due to loss of
material in manipulations carried out after the initial sedimenta-
tion of the virus.

Information concerning the shape and size of the virus particle
is only presumptive evidence. Further work is needed before it
can be proved that the particles described are actually the Oregon
sockeye salmon virus.

The O. S. V. seems to be most closely related to the arbo-
virus group based on the characteristics determined in this study.
This group shows the following characteristics: has cubic symmetry and ribonucleic acid; forms no inclusion bodies in infected cells; is sensitive to ether; may be in the size range of 40 to 122 nm. These characters are all found at least presumptively in the sockeye salmon virus.

The arboviruses are so named because in nature they infect the salivary glands and bodies of arthropods. They cause no damage to the arthropod but are transmitted to the definitive host by means of arthropod bites.

The possibility of an arthropod vector for the Oregon sockeye salmon virus is good since many arthropods are present in the same environment as the fish. However, no effort has yet been made to determine if this virus can be transmitted by arthropods so this point must remain at least for the present an unknown factor.
SUMMARY AND CONCLUSIONS

1. The Oregon sockeye salmon virus will infect sockeye embryonic cells causing a well defined sequence of cytopathic events. The cytopathic changes include thickening of the nuclear membrane, alteration of the nucleoli and occasionally division of the nucleus into several segments.

2. "In vivo" experiments have indicated this virus will infect young kokanee (sockeye) salmon, but not chinook salmon, coho salmon or rainbow trout.

3. Histopathological examination of virus infected kokanee salmon revealed destruction of the parenchymal cells in the kidney as the only change noted in preliminary observations.

4. Cell cultures of chinook salmon and steelhead trout which had been maintained for several months were susceptible to the virus. Primary cell cultures from these species were virus resistant. Coho salmon cells were resistant to the virus at all stages.

5. Free virus in the nutrient fluid of infected cultures increased 2.5 to 3.5 logs 24 to 48 hours after initial infection. The
maximum titer was reached 48 to 72 hours after infection and remained at this level until the experiment was terminated seven days after exposure of the cell culture.

6. This virus produced easily discernible plaques in stained cell cultures of sockeye embryonic cells. The number of plaques was directly related to the virus concentration as determined by infectivity titration. Although plaque titration is more accurate than the infectivity titration it was not used in this study because of the large number of cells required.

7. The amount of intracellular virus in infected cell cultures was negligible compared to the free virus. This appears to be a virus which is readily released from cells. The small amount of intracellular virus also suggests this virus may not be a complete infectious unit until it passes through the cell membrane.

8. The virus was completely inactivated after exposure to a buffered aqueous mixture containing approximately 20 percent ether by volume. This indicates the presence of essential lipids associated with the particle.

9. Failure of 5-bromodeoxyuridine (an inhibitor of DNA viruses) to inhibit the replication of the Oregon sockeye salmon virus
indicates this may be an RNA virus.

10. Temperature studies in tissue culture have shown that the virus multiplies most rapidly at 13° C. and 18° C. reaching a maximum titer within two days after infection. At 8° C. and 20° C. maximum titer was not reached until four days after initial infection. At 4° C. the rate of replication was further reduced and the maximum titer was one log lower than at temperatures between 8° and 20° C. The critical upper temperature for virus replication was between 20° C. and 23° C. At 23° C. no new virus was produced even though this is considered to be the optimum temperature for growth of the host cells.

11. This virus was sedimented by centrifugation at 20,000 rpm (45,000 xg) for one hour. For further purification the sedimented virus was placed on a sucrose density gradient and centrifuged at 22,000 rpm for one hour. After centrifugation a band appeared 4.45 cm from the top of the tube which contained a high concentration of virus. This purified virus material was used to prepare grids for electron microscope study.

12. Using various sizes of Millipore filters the size of this virus was calculated to be between 110 and 165µ. Electron micrographs of purified virus material, with indicator particles of
known size added, indicated the predominant particle was between 92 and 145 μ in diameter.

13. After observing electron micrographs of this virus it seems probable that the particles have cubic symmetry. The resolution of the micrographs was not good enough to attempt a count of the capsomeres.

14. Based on characteristics determined in this study the Oregon sockeye salmon virus appears to be most closely related to the arborvirus group. This is only a tentative placement as further work is needed before this virus can be definitely assigned to a particular virus group.


