Properties of the Sockeye Salmon Virus (Oregon Strain)* (33719)

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A viral disease of sockeye salmon, *Oncorhynchus nerka,* which occurred in hatcheries at Winthrop and Leavenworth, Washington, was described by Rucker *et al.* in 1953 (1). Watson *et al.* (2) reported further experimental evidence for the viral etiology of the disease in 1954. In 1958 an outbreak of disease with a high mortality rate appeared among young sockeye salmon at the Willamette River Salmon Hatchery in Oregon. An infectious agent was isolated from diseased fish during this outbreak by one of us (J.L.F.), which could transmit infection to juvenile or fingerling sockeye salmon in the form of bacteria-free filtrates of infected tissue. This agent has been found to have many of the characteristics of animal viruses and is presumed to be the same as or closely related to the virus reported by Rucker *et al.* (1).

Materials and Methods. Cell cultures. Sockeye salmon cells employed in most of the experiments were derived from embryos at the eyed egg stage, by a trypsinization method (3). They were maintained as monolayer cultures in Eagle's minimum essential medium (4) with 20% agamma calf serum (Hyland Laboratories). The complete medium also contained 100 units of penicillin, 100 μg of streptomycin, and 25 units of Mycostatin/ml. Cultures were transferred at intervals of 12–15 days, but during the course of this work had not yet become heteroploid. Other cells used in certain experiments included steelhead trout (*Salmo gairdneri*) cell line STE 137 derived from embryos (3, 5); HeLa cells (Microbiological Associates); and bovine kidney cells (Veterinary Diagnostic Laboratory, Oregon State University). All cell lines were cultivated in the medium described above.

Viruses. The sockeye salmon virus was maintained by inoculation of sockeye salmon cell cultures, harvesting the culture fluid after 7 days incubation at 18°, centrifuging, dispensing in 0.5-ml volumes in small Pyrex tubes, and storing at —60°. The stock virus used in these experiments contained 1.1×10^7 $TCID_{50}$/ml.

Other viruses used were maintained in a similar manner by inoculating cell cultures of the appropriate type and harvesting the infectious culture fluid. For the virus of infectious pancreatic necrosis, (obtained from Dr. Ken Wolf, Eastern Fish Disease Laboratory), cell line STE 137 was used. Bovine kidney cells were employed with infectious bovine rhinotracheitis virus, (obtained from the Veterinary Diagnostic Laboratory, Oregon State University). HeLa cell cultures were used with type 2 poliovirus. Lee influenza virus was prepared as infected allantoic fluid from chick embryos inoculated on day 10. Both of the latter agents were obtained from the stock virus collection of this laboratory.

Infectivity titrations. The end point dilution method was used for infectivity titrations of the sockeye salmon virus. Pyrex tubes, 10×75 mm were inoculated with 200,000 sockeye cells in a 0.5-ml volume of the complete medium and incubated for 2 days at 18°. A series of 10-fold dilutions of the virus preparation to be titrated was then prepared, using complete medium as the diluent. The original nutrient medium was then withdrawn from all the tube cultures. Each virus dilution was inoculated into a group of 5 tubes in a volume of 0.2 ml and all tubes then received 0.3 ml of complete medium. They were incubated at 18° for 7 days and were then examined microscopically for the cytopathic effect of the virus. The 50% end point was estimated by the method of Reed and Muench (6). The other viruses with the exception of influenza, were titrated by essentially the same method, except that the ap-

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propriate cell line was substituted for the sockeye salmon cells. Influenza virus was titrated by inoculating groups of 10-day-old chick embryos in the allantoic sac, and infection was detected by testing the allantoic fluid for hemagglutinating activity against chicken erythrocytes.

Measurement of cell associated virus. The relative proportions of virus in infected sockeye cell cultures which was free in the culture fluid and which was cell associated were determined by separating the fluids and cells from cultures 3 days after virus infection and measuring the concentration of virus in each by infectivity titration. Cells were removed from glass surfaces by means of a rubber policeman and separated from fluids by centrifugation. They were washed once in Earle's balanced saline and resuspended in the original volume of fresh complete medium, and then were disrupted either by ultrasonic vibration, by 3 cycles of freezing and thawing in a dry ice acetone bath, or by high speed homogenization in a Virtis tissue homogenizer. The latter method was the most effective in releasing virus from the cells without significant loss of infectivity, and was employed in the experiments reported.

Ether sensitivity of the virus. The ether sensitivity of the sockeye salmon virus was determined by the method described by Andrewes and Horstmann (7). To a fresh preparation of virus in tissue culture fluid 20% by volume of ethyl ether was added; the mixture was shaken and held at 4° for 23 hr, with vigorous shaking at intervals of several hours during this period. A control preparation of virus containing no ether was treated in a similar manner. The ether treated virus in a sterile beaker was held in a tissue culture cubicle supplied with filtered air at 16° to permit evaporation of the ether, which required about 2.5 hr. The active virus in both treated and control preparations was then measured by infectivity titration. In order to establish confidence in the method employed, Lee influenza virus, known to be ether sensitive (8), and the virus of infectious pancreatic necrosis of trout, which is ether resistant (9), were studied using the same procedure. The influenza virus was prepared as infectious allantoic fluid from 12-day-chick embryos and was found to contain $10^{7.5}$ chick embryo ID_{50}/0.2 ml. The infectious pancreatic necrosis virus, in the form of tissue culture fluid from infected cells of steelhead trout, contained $10^{6.3}$ $TCID_{50}$/0.2 ml.

Results. Initial studies with the sockeye salmon virus indicated it would replicate in monolayer cultures of sockeye cells, with the production of a distinct cytopathic effect. Cellular changes were first noted on the second day of infection, and were most marked by the sixth day, when many cells had become granular, round, and detached from the glass surface. This cell culture–virus system provided a method for titrating virus infectivity, and a convenient source of virus for the experiments reported herein.

An estimate of the particle size of the virus was made by filtration of infectious tissue culture fluids through Millipore membrane filters having average pore diameters of 0.45, 0.30, 0.22, and 0.10 μ. Infectivity of the filtrates was detected by inoculation of each one into a group of 10 Leighton tube cultures of sockeye salmon cells in a volume of 0.2 ml, and examination of each tube microscopically for the typical cytopathic effect of the virus after 7-days incubation at 18°. Filtrates from the 0.22-μ membranes and those of larger pore diameter were uniformly infectious, while those from the 0.10-μ membrane repeatedly failed to produce infection. Employing the ratio of particle diameter to pore diameter obtained by Elford (10) the virus particle size may be estimated as 110–165 mμ. This data is similar to that reported by Parisot *et al.* (11).

The influence of temperature upon the replication rate of the virus was studied by inoculating groups of Leighton tube cultures of sockeye salmon cells with 0.2 ml of tissue culture fluid containing $10^{3.5}$ $TCID_{50}$ of virus. Subgroups of the inoculated cultures were incubated at various temperatures in the range from 4 to 23°. The virus concentration in each subgroup was measured by infectivity titrations performed upon the pooled fluid from 3 replicate cultures after incu-

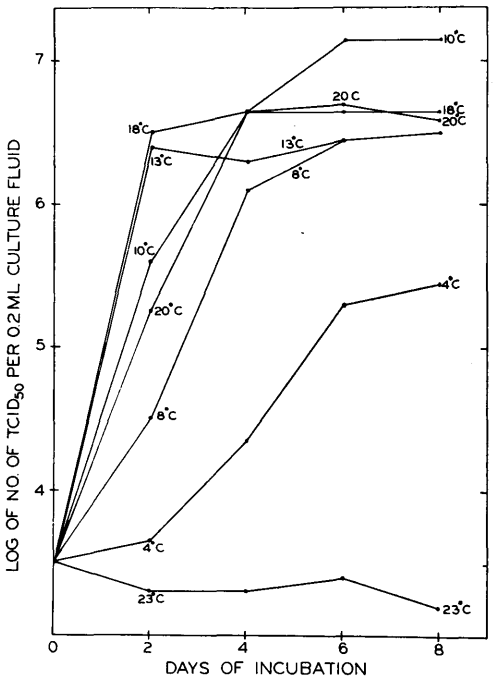

FIG. 1. Effect of temperature on replication of sockeye salmon virus; each point on the curves represents the mean value from 2 experiments.

bation periods varying from 1 to 8 days. The most significant results of these studies are shown in Fig. 1. Note that no infectious virus was formed at 23°, though this is within the optimum range for multiplication of the host cells. At 4° the rate of replication was relatively low, as was the virus titer found on the eighth day. The maximum rate of virus replication was observed in the range from 13 to 18°, as shown by the higher concentrations of infectious virus at these temperatures on the second day. Within this optimum temperature range the virus concentration in the culture fluid increased by about 3 log units.

The relative concentrations of virus released into the cell culture fluid on the third day of infection and of virus still closely associated with the cells was determined by infectivity titrations on the cell free fluid and on a homogenate of the washed cells. The virus recoverable from the washed cells was only a small fraction, not more than 5%, of that present in the culture fluid. From this data it was concluded that infectivity titrations could be performed on culture fluids alone without the introduction of a significant error due to the small amount of residual virus in the cells.

The sensitivity of viruses to ether is important in characterizing these agents, as it reflects the presence or absence of lipids essential for infectivity. The sockeye salmon virus, as well as a known ether sensitive virus and a known ether resistant one, were studied with respect to this property. The results of infectivity titrations on all 3 viruses before and after the ether treatment are shown in Table I. It is apparent that the virus of infectious pancreatic necrosis was completely resistant to ether, while the infectivity of the influenza virus was reduced by 3 log units. In the ether treated preparation of sockeye salmon virus no infectivity could be detected. This result offers convincing evidence for the presence of essential lipids in this virus.

The virus produced distinct plaques in monolayer cultures of sockeye salmon cells when the latter were inoculated with proper dilutions of the agent, overlayed with tissue culture medium containing 0.75% Noble agar, and incubated at 18°. The plaques first became discernible after 5-days incubation, but were larger and more distinct on the seventh

TABLE I. Ether Sensitivity of the Sockeye Salmon Virus Compared to that of Lee Influenza and IPN[a] Viruses.

Virus	Log of infectivity titer (ID_{50}/0.2 ml)
Lee influenza	
Before ether treatment	7.4[b]
After ether treatment	3.4
Infectious pancreatic necrosis	
Before ether treatment	6.3[c]
After ether treatment	6.3
Sockeye salmon disease	
Before ether treatment	4.7[c]
After ether treatment	0.0

[a] Infectious pancreatic necrosis virus of trout. This virus and the sockeye salmon agent were employed as tissue culture fluid. The influenza virus was chick embryo allantoic fluid.

[b] Chick embryo ID_{50}.

[c] Tissue culture ID_{50}.

TABLE II. Presumptive Identification of Nucleic Acid Type in the Sockeye Salmon Virus.

Virus[a]	Host cells	10^{-3} *M* 5-bromodeoxyuridine during virus replication period	Virus titer in culture fluid after 48 hr (log of $TCID_{50}$/0.2 ml)
Infectious bovine rhinotracheitis[b]	Bovine kidney	Absent	5.6
		Present	0.0
Poliomyelitis type 2 (MEF-1)[c]	HeLa	Absent	8.4
		Present	8.6
Sockeye salmon disease	Sockeye salmon	Absent	5.9
		Present	5.6

[a] Each virus was added to monolayer cell cultures in a concentration of about $10^{3.5}$ $TCID_{50}$/0.2 ml of complete medium. After 1 hr this medium was removed and fresh medium containing 10^{-3} *M* 5-bromodeoxyuridine was added to half the cultures; fresh medium alone was added to the other half; virus titrations were made 48 hr later.

[b] A known DNA virus.

[c] A known RNA virus.

day. A plaque counting virus assay method was investigated and found to be practical. It was compared with the end point dilution assay for measuring the infectivity of the same stock virus preparation on 3 separate occasions and appeared to be much more precise than the end point method. For studies where the maximum precision in measuring virus concentration is required the plaque assay would be the method of choice. It was not employed routinely in these experiments because of the large quantity of sockeye salmon cells required.

The antimetabolite 5-bromodeoxyuridine inhibits the replication of many DNA containing viruses, while lacking similar activity with those containing RNA (12). This selective effect thus provides a presumptive method for identifying the type of nucleic acid present in a viral agent. The replication of sockeye salmon virus in cultures of sockeye cells in the normal tissue culture medium and in this medium containing a 10^{-3} *M* concentration of 5-bromodeoxyuridine was measured over a 48-hr incubation period at 18°. Similar experiments were carried out with the virus of infectious bovine rhinotracheitis, a known DNA virus (13) in cultures of bovine kidney cells; and with type 2 polio virus, strain MEFI, known to contain RNA (14) in HeLa cell cultures. This concentration of the compound showed no evidence of toxicity for any of these cell systems during this time period, that could be detected microscopically. The results of experiments with all 3 viruses are shown in Table II. It is evident that the infectious bovine rhinotracheitis virus failed to replicate in the presence of the antimetabolite, while replication of the poliovirus was unaffected. Similarly, the sockeye salmon virus replicated as well in the presence of the compound as in its absence. Thus the data offer presumptive evidence that the nucleic acid of the latter virus is RNA.

Discussion. Of particular interest in these studies of the sockeye salmon virus is the observation that the optimum temperature range for virus replication was 13 to 18°, with no replication at 23°, while the optimum range for growth of the host cells was 18–23°C (3). Apparently some step in the biosynthesis and assembly of virus components, possibly mediated by an enzyme, is unable to proceed at 23°. Whatever the step may be, it appears to be separate and distinct from any metabolic processes of the host cells, which grow readily at this temperature. It would appear that this temperature effect offers a useful selective mechanism for the study of the various phases of new virus formation, such as adsorption, and synthesis of virus nucleic acid and protein, in a cell culture system.

It has also been reported that the virulence of a virus which was presumably the same agent, for fingerling sockeye salmon was influenced significantly by water temperature (2). It was most virulent at water temperatures from 10 to 15.5°. At 20° it was still infectious, but fatal infections were much less frequent. These observations suggest that the proper manipulation of water temperature might offer an effective method for controlling an outbreak of the disease in hatcheries, if facilities for temperature control were available.

The data presented indicate that the sockeye salmon virus resembles agents of the arbovirus or the myxovirus group. Final classification will require a study of the ultrastructure of the virus particle by electron microscopy, and confirmation of nucleic acid type by chemical means. These studies are now in progress.

Summary. Several important characteristics of the sockeye salmon virus have been determined. It replicates abundantly in cultures of sockeye salmon cells, producing characteristic cytopathic effects. Most of the new virus produced is released into the culture fluid. The particle size was estimated by membrane filtration to be in the range of 110–165 mμ. The maximum rate of virus replication was found in the temperature range from 13 to 18°, and no replication occurred at 23°, although host cells grew well at that temperature. Virus infectivity was destroyed by ether, indicating the presence of essential lipids. Resistance of the agent to inhibition by 5-bromodeoxyuridine offers presumptive evidence that its nucleic acid is RNA.

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