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Title: ISOLATION AND CHARACTERIZATION OF A NEW REOVIRUS FROM CHUM SALMON (ONCORHYNCHUS KETA)

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This report describes the isolation of a new virus from adult chum salmon (Oncorhynchus keta) returning to the Tokushibetsu Hatchery, Hokkaido, Japan. The agent was isolated in the CHSE-214 cell line derived from chinook salmon (Oncorhynchus tshawytscha). The virus replicated in selected fish cell lines incubated between 10 and 20°C and produced a unique cytopathic effect characterized by the formation of circular plaque-like areas of cytoplasmic fusion. The nuclei of the cells were not affected.

Physical and chemical studies have shown the agent, tentatively named chum salmon virus, to be a member of the family Reoviridae and distinct from any known virus of fish. Electron microscopy of negatively stained particles revealed a naked icosahedral virion 75 nm in diameter with a clearly defined double capsid. Treatment with α-chymotrypsin removed the outer capsid yielding 50-55 nm subviral

particles with enhanced infectivity. Viral infectivity was not reduced by treatment with ether or chloroform. The virus was resistant to freeze-thaw and pH 3, but unstable at 56°C. It did not hemagglutinate human type O erythrocytes. No cross-neutralization was observed with antiserum against reovirus types 1, 2 or 3 or against infectious pancreatic necrosis virus.

Viral replication was not inhibited by 5-fluoro-2'-deoxyuridine and infected cells stained with acridine orange showed typical reovirus-like cytoplasmic inclusions. Polyacrylamide gel electrophoresis of the viral genome revealed 10 segments of double-stranded RNA ranging in weight from 2.6×10^6 to 0.32×10^6 daltons with a total genome weight of 15.3×10^6 daltons. Analysis of virion polypeptides on SDS-polyacrylamide gels indicated five major virion proteins with molecular weights of 145,000, 130,000, 75,000, 43,000 and 33,000 daltons were present in the intact virion. Two minor polypeptides of 80,000 and 30,000 daltons were also present. The density of the intact virion in CsCl was 1.33 g/cc.

The virus had limited pathogenicity for salmonid fish. Chum and chinook salmon infected with the virus exhibited a focal necrotizing hepatitis. No mortality could be attributed to these lesions which were active sites of viral synthesis. Rainbow trout (Salmo gairdneri) and kokanee salmon (Oncorhynchus nerka) showed no pathological changes in any organ system examined. The virus replicated in all four species tested.

Isolation and Characterization of a New Reovirus From Chum Salmon (*Oncorhynchus keta*)

by

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ISOLATION AND CHARACTERIZATION OF A NEW REOVIRUS FROM CHUM SALMON (ONCORHYNCHUS KETA)

INTRODUCTION

In October of 1978, an examination was conducted on a stock of adult chum salmon (Oncorhynchus keta) returning to the Tokushibetsu Hatchery, Hokkaido, Japan. Samples from the fish were collected and tested for the absence of viruses in order that eggs from these fish could be imported into the United States for use in private aquaculture. During the course of the examination, a new virus was isolated which was unlike any known virus of fish. The importation of eggs from northern Japan was stopped while research was conducted to determine the nature of the virus.

The purpose of this study was three-fold: 1. to characterize the new virus and determine its taxonomic position with respect to other animal viruses, particularly those of fish, 2. to test the pathogenicity of the virus for several commercially important species of salmonid fish, and 3. to examine the geographic range of the virus in northern Japan and establish the presence of the virus in other hatcheries and stocks of fish.

To accomplish these goals, it was first necessary to determine the appropriate methods for the propagation and assay of the virus. Basic studies to evaluate culture media, cell lines, incubation temperatures, assay systems and purification techniques were conducted. Once these experiments were complete, a more detailed biochemical

and biophysical analysis was undertaken. These studies tested the resistance of the virus to physical and chemical treatments, examined the nucleic acid and protein composition of the virion, and looked for evidence of antigenic relatedness to other fish and animal viruses. The pathogenicity of the virus for certain salmonid fish was determined by injection of fish with the virus and looking for viral replication and evidence of pathology. Finally, an extensive effort was made to reisolate the virus from fish and shellfish at various locations in northeastern Hokkaido.

LITERATURE REVIEW

During the last twenty-five years, the development of fish cell culture techniques has allowed the isolation and characterization of many viruses from poikilothermic animals. A recent review by Wolf and Mann (1980), lists 61 cell lines and 32 viruses of fish. In the year since publication, two new viruses of fish have been described (Kimura et al., 1981; Winton et al., 1981). Two extensive reviews of the viral diseases of fish have been published recently (McAllister, 1979; Pilcher and Fryer, 1980).

The fish viruses which have been isolated or described represent several of the established groups of animal virus. Herpesviruses, iridoviruses, rhabdoviruses, orthomyxoviruses and several presumptive retroviruses have been identified. The remaining viruses of fish are unclassified because they are newly isolated, lack complete characterization, or do not fit into any of the established groups of animal viruses. Three of these ungrouped viruses have properties similar to reoviruses and are termed "reovirus-like" or reolike.

Reolike Viruses of Fish

The reolike viruses isolated from fish include infectious pancreatic necrosis virus (IPNV), the golden shiner (Notemigonus crysoleucas) virus (GSV) and a virus isolated from European eels (Anguilla anguilla) (EVE). Only one of these viruses (IPNV) has been extensively characterized.

Infectious pancreatic necrosis virus was first isolated from brook trout (Salvelinus fontinalis) by Wolf et al. (1960). Since the initial report, it has been found to be widely distributed. The virion is an unenveloped icosahedral particle but lacks the characteristic double capsid of the reoviruses (Kelly and Loh, 1972). There are some discrepancies concerning the size of the IPNV virion. The reported size of the particle ranges between 55-74 nm. These reports include those by Dobos et al. (1979) of 59.2 nm and Chang et al. (1978) of 71 nm. The capsid structure is composed of 92 capsomeres (Cohen and Scherrer, 1972). The IPNV virion has a density of 1.33 g/cc in CsCl as determined by Chang et al. (1978). Dobos (1977) reports four polypeptides are present in the purified virions. The genome of IPNV is composed of two pieces of double stranded RNA (Dobos, 1976) with a bouyant density of 1.60 g/ml in $CsSO_4$ (Macdonald and Yamamoto, 1976). Because the IPNV virion lacks a double capsid, has a smaller size and possesses only 2 genome segments and four virion polypeptides, Dobos et al. (1979) suggest that IPNV does not belong in any of the present genera of the family Reoviridae. They proposed the creation of a new group, the birna viruses to include various viruses with similar properties isolated from bivalve molluscs (Tellina tenuis) (Underwood et al., 1977), fruitflies (Drosophila melangaster) (Teninges et al., 1979), and chickens (Gallus gallus) (Nick et al., 1976).

Another reolike virus of fish has been isolated from European eels in Japan (Sano, 1976). This virus (EVE) has also been isolated from Japanese eels (Anguilla japonica) and is related antigenically to the

French 21 strain of IPNV. The virus, like IPNV, is resistant to heat (60°C for 30 min) and lipid solvents. Recent work has shown that the genome and polypeptide structure of EVE is similar, but not identical, to IPNV (R. P. Hedrick, Oregon State University, Dept. of Microbiology, personal communication).

Plumb et al. (1979) report the isolation of another reolike virus from diseased golden shiners. This virus is 70 nm in diameter with an icosahedral capsid structure. The virus is resistant to ether and heat (50°C for 30 min) and is stable at pH 3 and 10. Acridine orange stain revealed yellowish-green cytoplasmic inclusions and the lack of inhibition by 5-iodo-2 deoxyuridine and resistance to RNAse suggests this virus possesses a double stranded RNA genome. Schwedler and Plumb (1980) found no cross neutralization with polyvalent antiserum against IPNV.

Reolike Viruses of Shellfish

A reolike virus has been isolated from American oysters (Crassostrea virginica) and partially characterized by Meyers (1979). Electron micrographs revealed a naked icosahedral virion with a mean diameter of 79 nm and a double capsid layer. The virus replicated in fish cell lines incubated at 15 and 23°C. The virus is stable at pH 3 and resistant to chloroform. It did not hemagglutinate human type O erythrocytes and viral replication was not inhibited by 5-iodo-2-deoxyuridine. Infectivity was not neutralized by antiserum against IPNV or several known reoviruses.

Characteristics of the Family Reoviridae

Because the virus characterized in this study is a reovirus, a description of the family and the genera currently recognized is provided. Only those features of the family important in establishing the taxonomic position of a new virus are discussed. The reovirus family is one of the larger groups of animal viruses and has been the subject of several extensive reviews (Rosen, 1968; Wood, 1973; Joklik, 1974; Ramig and Fields, 1978; Gorman, 1979; and Verwoerd et al., 1979).

The name reovirus is an acronym for respiratory enteric orphan viruses which was proposed by Sabin (1959) for a group of viruses previously classified as ECHO type 10 (Enteric Cytopathic Human Orphan). Other viruses from arthropods (arboviruses) which have similar features were included in the family along with a number of plant viruses. Verwoerd (1970) proposed the name diplornavirus (diplo-rna-virus) for this group. The International Committee for Taxonomy of Viruses has determined that the family Reoviridae should include those viruses which (1) have a quasi-spherical morphology with elements of icosahedral symmetry and a diameter of 60-80 nm and (2) have several (usually 10-12) segments of double-stranded RNA with a molecular weight of 0.2 to 3.0 X 10^6 daltons. The combined molecular weight of the RNA is approximately 15 X 10^6 daltons contained in a single capsid (Fenner, 1976). Four genera were originally established: (1) Orthoreovirus (commonly referred to as reovirus), (2) Orbivirus, (3) an insect group (now referred to as Cypovirus) and (4) a plant virus group (Phytoreovirus). Recently, two new genera have been created. The genus

Rotavirus includes several mammalian viruses and the genus Fijivirus which was created to include some of the plant viruses (Joklik et al., 1980).

Viruses of the Genus Orthoreovirus

The viruses in the genus Orthoreovirus are the best characterized viruses of the Reoviridae and include viruses isolated from a wide range of vertebrates. The mammalian isolates group into 3 serotypes and the avian isolates into 5 serotypes (Joklik, 1974). These viruses grow in a wide range of cell lines incubated between 33 and 37°C. The morphology of the virion reveals two clearly visible capsid layers. Luftig et al. (1972) determined the diameter of the outer capsid was 76 nm and the inner capsid was 52 nm. The ultrastructure of the capsid has been controversial but 20 peripheral capsomeres can be seen in negatively stained preparations.

The outer capsid shell of orthoreoviruses is relatively stable to purification steps and centrifugation in CsCl but it can be selectively removed from the virion by heat or chymotrypsin (Shatkin and Sipe, 1968; Joklik, 1972). Spendlove et al. (1970) found that chymotrypsin treatment enhanced the infectivity of reovirus suspensions. After removal of the outer capsid layer, the nucleic acid in the core surrounded by the inner capsid is referred to as a sub-viral particle (Borsa et al., 1973a). Borsa et al., (1973b) found that monovalent cations were essential for enhancement of infectivity following chymotrypsin treatment.

The bouyant density in CsCl reported for the orthoreoviruses ranges from 1.32 to 1.41 g/cc (Ramig and Fields, 1978). Smith et al. (1969) found the intact particle had a density of 1.36 g/cc and the subviral particle (cores) had a density of 1.44 g/cc. Virions lacking nucleic acid have a density of 1.30 g/cc (Joklik, 1974).

The genome of the reoviruses, like all members of the family, contains double-stranded RNA. Gomatos et al. (1962) observed green-staining cytoplasmic inclusions which are indicative of double-stranded nucleic acids. Gomatos and Tamm (1963) showed that the genome was double-stranded RNA. The bouyant density of the RNA is 1.61 g/ml (Shatkin, 1965). Polyacrylamide gels have been used by Shatkin et al. (1968) to separate the genome into 10 segments of RNA which are present in equimolar amounts. The segments had a total molecular weight of approximately 15 X 10^6 daltons and were distributed into three size ranges (3 large, 3 medium, 4 small pieces).

The polypeptides of the virion were studied by Loh and Shatkin (1968) and Smith et al. (1969). Seven virion proteins were found which ranged in weight from 155,000 to 34,000 daltons and were distributed within 3 size ranges. The proteins of the top component were identical with those of the intact virion. Cores were found to contain only 4 polypeptides indicating 3 proteins made up the outer capsid. Cross and Fields (1977) have found eight proteins in the virion (3 outer capsid, 5 core).

The viruses of the genus Orthoreovirus are resistant to pH 3, high CsCl concentrations and heat (Joklik, 1974). These features are important taxonomic determinants. The mammalian reoviruses hemagglutinate cells of all human blood groups (Ramig and Fields, 1978).

Viruses of the Genus Orbivirus

The genus Orbivirus was first proposed by Borden et al. (1971) to include a group of arthropod borne viruses which are significantly distinct from other arboviruses in that they are stable to lipid solvents and possessed a double-stranded RNA genome. A large number of orbiviruses have been isolated from insects, but most remain poorly characterized. A few orbiviruses are known to cause disease in mammals and the best studied orbivirus is the virus which cause bluetongue disease in sheep. Serological relationships within the orbivirus group are extremely complex and based upon both complement fixation and neutralization (Gorman, 1979).

The morphology and some biophysical characteristics of the orbivirus virion are distinct from those of the orthoreoviruses. Orbiviruses possess only a diffuse outer capsid rather than a clearly structured outer layer. The diameter of the nucleocapsid, or inner layer, is 63 nm and the intact virion is 69 nm (Martin and Zweerink, 1972). Orbiviruses can also be separated from the reoviruses by their sensitivity to pH 3 (Borden, 1971). Bluetongue virus particles have a density of 1.38 to 1.36 g/cm^2 (Wood, 1973), however the virions are

reported to lose the outer capsid layer on exposure to high CsCl concentrations during centrifugation (Verwoerd et al., 1979).

Verwoerd et al. (1970) separated the RNA of bluetongue virus into 10 segments on polyacrylamide gels. Since then, several orbivirus genomes have been studied. To date, all have been found to contain 10 segments, typically distributed into three size classes (Verwoerd et al., 1979). The molecular weights of the segments range from 0.22 X 10^6 to 3.0 X 10^6 daltons with a total genome weight between 11 and 12.5 X 10^6 daltons (Gorman, 1979). Martin and Zweerink (1972) determined that the capsid of bluetongue virus is composed of seven polypeptides ranging in weight from 155,000 to 32,000 daltons. There were four major and three minor capsid polypeptides. Verwoerd et al. (1972) report that two polypeptides are associated with the outer coat. Most other orbiviruses characterized also have seven virion proteins (Gorman, 1979).

Viruses of the Genus Cypovirus

The genus Cypovirus of the family Reoviridae is composed of numerous serologically distinct viruses which are restricted to insects. The best studied is the virus causing cytoplasmic polyhedrosis of silkworms (CPV). The virion of CPV has a diameter of 60-65 nm but lacks the clear double capsid structure of the reoviruses (Joklik, 1974). Lewandowski and Traynor (1972) have characterized the morphology and polypeptide composition of the virion. Although twenty peripheral capsomeres could be seen, there was no evidence of an internal capsid and several (10-12) large spikes were observed on the surface of the

virion. The bouyant density of the particles was 1.435 g/cm^3 in CsCl and five virion polypeptides ranging in size from 151,000 to 33 daltons were detected.

Lewandowski and Leppla (1972), have reported 10 segments of double-stranded RNA ranging in size from 2.70 to 0.37 X 10^6 daltons with a combined weight of 14.6 X 10^6 daltons. However, unlike the reoviruses, the distribution of the molecular weights was bimodal.

Viruses of the Genus Phytoreovirus

The viruses of the genus Phytoreovirus replicate in many species of plants and in several species of insects (leafhoppers). The virion of wound tumor virus (WTV) possess a double capsid layer. Wood (1973) indicates the diameter of the complete particle is 70 nm while the core is 44 nm similar to the Orthoreovirus genus. Lewandowski and Traynor (1972) found that, unlike the orthoreoviruses, WTV was unstable in CsCl and upon isopycnic centrifugation was converted to cores with a density of 1.44 g/ml. They also found that the virions were quite stable in chymotrypsin. The virions are also reportedly unstable to treatment with halogenated hydrocarbons such as Freon 113 or pH 3 to which reoviruses are stable (Joklik, 1974).

Most reports indicate that the genome of WTV is composed of 12 pieces of double-stranded RNA which range in weight from 2.8 to 0.3 X 10^6 daltons. Wood (1973) reports 15 pieces and Lewandowski and Leppla (1972) report 13 segments. Further characterization will be necessary to resolve the exact number of segments. The polypeptides of the

virion have been characterized by Lewandowski and Traynor (1972) who described four major proteins ranging from 156,000 to 44,000 m.w.

Viruses of the Genus Fijivirus

The genus Fijivirus is a partially characterized collection of plant and insect viruses. Milne et al. (1973) report the intact virions are 63-70 nm in diameter with a double capsid. Treatment with chloroform or freezing removed the outer capsid yielding subviral particles of 53 nm in diameter. The virus was not infectious after chloroform treatment. The genome of the viruses in this group is composed of 10 segments of double stranded RNA with a total molecular weight of 18-19 X 10^6 daltons (Luisoni et al., 1979). Four serologically unrelated subgroups are described by Boccardo et al. (1979).

Viruses of the Genus Rotavirus

The rotaviruses are a newly formed genus of mammalian viruses many of which are associated with gastroenteritis and diarrhea. The intact virions have a double capsid structure and resemble reovirus particles except they possess a sharply defined outer edge (McNulty, 1978). The virion ultrastructure has been studied by Altenburg et al. (1980) who describe the sharp boundary layer as a "pseudoenvelope" because the virus is not inactivated by lipid solvents. The intact virions are 70 nm in diameter and the single-shelled cores measured approximately 60 nm in diameter. The stability of rotaviruses to various physical and chemical treatments has been determined by Estes et al. (1979). They found the virions were infectious after being subjected to freeze-thaw, sonication, ether, chloroform, genetron and treatment with pH 3.

The intact virions of rotaviruses have a density in CsCl of approximately 1.37 g/ml (Kalica and Theodore, 1979) and particles with a single capsid have a density of 1.39 g/ml. The virion contains 11 segments of double-stranded RNA ranging in weight from 2.2×10^6 to 0.2×10^6 daltons. The total genome weight was 11×10^6 daltons (Newman et al., 1975). The polypeptides of rotaviruses have been studied by Kalica and Theodore (1979) who determined the intact virion contains eight capsid proteins, three of which make up the outer capsid and five are located in the inner capsid.

MATERIALS AND METHODS

Cell Lines

The cell lines used for the isolation and propagation of virus for this study are listed in Table 1. Monolayer cultures of CHSE-214 and RTG-2 cells were used during the virological examination of samples of fish and shellfish from Japan. The chum salmon virus was isolated and routinely propagated in the CHSE-214 cell line. Virus replication and efficiency of plating studies were conducted with the CHSE-214, RTG-2, STE-137, CHH-1, KO-6, BF-2, BB, FHM, WC-1, EPC and LBF-2 cell lines. Stocks of other salmonid viruses were prepared in the CHSE-214 cell line. Mammalian reoviruses were propagated in BGM cells. Wolf and Quimby (1976) describe the methods for the routine cultivation of fish cell lines.

Glassware and Media

Methods for the processing of glassware are described by Wolf and Quimby (1976). The cell lines were maintained in 32 oz glass prescription bottles (West Co.) sealed with a white latex stopper, or in 75 cm^2 plastic tissue culture flasks (Corning Glass Works). Plaque assays were performed in 25 cm^2 plastic flasks (Corning Glass Works). The viral examination of fish and the 50% tissue culture infectious dose assay ($TCID_{50}$) used 96 well microplates (Flow Laboratories Inc.). Large quantities of virus were grown using 150 cm^2 plastic tissue culture flasks (Corning Glass Works).

Table 1. Cell lines used in this study for the isolation, propagation or identification of the viruses studied.

Cell Line	Abbreviation	Species of Origin	Reference
Chinook salmon embryo	CHSE-214	*Oncorhynchus tshawytscha*	Nims, Fryer and Pilcher, 1970
Rainbow trout gonad	RTG-2	*Salmo gairdneri*	Wolf and Quimby, 1962
Steelhead trout embryo	STE-137	*Salmo gairdneri*	Fryer, 1965
Chum salmon heart	CHH-1	*Oncorhynchus keta*	Unpublished data[1]
Kokanee salmon ovary	KO-6	*Oncorhynchus nerka*	Unpublished data[1]
Bluegill fry	BF-2	*Lepomis macrochirus*	Wolf and Quimby, 1966
Brown bullhead	BB	*Ictalurus nebulosis*	Wolf and Quimby, 1969
Fathead minnow	FHM	*Pimephales promelas*	Gravel and Malsburger, 1965
Walleye sarcoma	WC-1	*Stizostedion vitreum*	Kelly and Miller, 1978
Epithelioma papillosum cyprini	EPC	*Cyprinus carpio*	Tomasec and Fijan, 1971
Largemouth bass	LBF-2	*Micropterus salmoides*	Wolf and Quimby, 1966
Buffalo green monkey	BGM	*Cercopithecus aethiops*	Barron, Olshevsky and Cohen, 1970

1. Cell line initiated for this study.

Cells were grown in Eagle's minimum essential medium (MEM) with Earle's salts (Auto-Pow, Flow Laboratories Inc.). The medium was supplemented with fetal bovine serum (Flow Laboratories Inc.) at a concentration of 10% (MEM-10) for cell growth and 5% (MEM-5) for virus replication and viral examinations of fish. Salmonid cell lines were grown at 18°C and warmwater fish cell lines at 22°C. The BGM cells were incubated at 35°C.

Viruses Used in this Study

In addition to the virus isolated and described in this report, tentatively named chum salmon virus (CSV), several other viruses were used as controls in certain experiments. Infectious hematopoietic necrosis virus (IHNV) is a rhabdovirus of salmonid fish. A 1978 isolate of this virus from adult spawning kokanee salmon (Oncorhynchus nerka) in the Metolius River in central Oregon was used (Hedrick, 1980). Infectious pancreatic necrosis virus (IPNV), a reo-like virus of fish, was isolated from an asymptomatic infection of cutthroat trout (Salmo clarki, McMichael et al., 1975). An isolate of Herpesvirus salmonis (Wolf et al., 1978) was the gift of Dr. Dan Mulcahy. These salmonid viruses were propagated in the CHSE-214 cell line at 18°C for IHNV and IPNV and at 10°C for H. salmonis.

Three serotypes of mammalian reoviruses were received from Dr. Jo-Ann Leong. Reovirus type 1 (Lang), reovirus type 2 (Jones) and reovirus type 3 (Abney) were propagated in the BGM cell line at 35°C.

Preparation of Virus Stocks

After the discovery of CSV, a stock of the virus was prepared from one of the wells of the original CHSE-214 microplate which had a single plaque. The virus from this well was inoculated into a 75 cm^2 flask of CHSE-214 cells and incubated at 18°C until cytopathic effect (CPE) involved the entire cell monolayer. The culture fluid from this second passage was frozen at -65°C in 1.0 ml aliquots and used as a master stock. All experiments with CSV were conducted with third to sixth passage virus. Stocks of all viruses were prepared by inoculating 25 or 75 cm^2 flasks of appropriate cells and removing the culture fluid when the CPE was extensive. The culture fluid was clarified by low speed centrifugation (5000 g) for 20 min to remove cell debris and 1.0 ml aliquots were frozen at -65°C.

Virus Purification and Concentration

Monolayers of CHSE-214 cells in 150 cm^2 flasks were inoculated with chum salmon virus at a low multiplicity of infection (MOI) of approximately 0.01 plaque-forming units (PFU) per cell. The cells were incubated with 15 ml MEM-5 for 6 to 8 days at 18°C until CPE had destroyed the entire cell monolayer. Reovirus was prepared in BGM cells (MOI of 0.01 PFU/cell) incubated with 15 ml MEM-5 for 7-10 days at 35°C. After incubation when CPE was complete, the flasks were frozen at -20°C. The flasks were thawed and the cells dislodged by shaking or hitting the thawing flasks. The cells and culture fluid were pooled and centrifuged at 80,000 g for 90 min in an SW 28 rotor (Beckman Instruments).

The resulting cell and virus pellet was homogenized in SSC buffer (0.10 M sodium chloride, 0.015 M sodium citrate, pH 7.4) with 20 strokes of a Ten Broek homogenizer. The homogenate was placed in a screw-capped tube and an equal volume of trifluorotrichloroethane (Genetron 113, Allied Chemical Co.) added. This preparation was mixed for 5 min with a vortex mixer and centrifuged at 1500 g for 10 min in a clinical centrifuge to separate the Genetron from the aqueous phase. A second extraction with Genetron was made and the aqueous phase layered onto a 15-50% w/w continuous sucrose gradient in a polyallomer tube. This gradient was centrifuged at 80,000 g for 1 hr in an SW 28 rotor.

After centrifugation, two opalescent bands could be seen (complete and incomplete virions) in the middle of the sucrose gradient and were removed separately with a probe and peristaltic pump (Auto Densi-flow IIC, Buchler Instruments). The virus from the sucrose gradient was diluted with SSC buffer and pelleted at 115,000 g for 1 hr in an SW 50.1 rotor (Beckman Instruments). The viral pellet was resuspended in 0.2 ml SSC and applied to the top of a step CsCl gradient composed of 2 ml 40% w/w, 1.5 ml 30% w/w and 1.0 ml 20% w/w CsCl in SSC buffer. This gradient was centrifuged at 115,000 g for 20 hr in an SW 50.1 rotor. After centrifugation, the opalescent band in the center of the gradient was removed with a probe and peristaltic pump, resuspended in SSC buffer and pelleted at 115,000 g for 1 hr in an SW 50.1 rotor. This material was used for characterization of the virus. For protein analysis, the viral bands were subjected to additional purification cycles through sucrose and CsCl gradients.

Labeling of Viral RNA

Chum salmon virus RNA was labeled by infecting monolayer cultures of CHSE-214 cells in 150 cm^2 flasks with virus at an MOI of 0.1 PFU/cell. The cultures were incubated with 15 ml MEM-5 at 18°C for 24-36 hr. Then, the MEM-5 was decanted and 15 ml of MEM-5 containing 20 µCi/ml of ^{3}H-uridine (ICN Biochemicals Inc.) was added to each flask. These flasks were incubated at 18°C until CPE had involved the entire monolayer. The virus in the cells and culture fluid was pelleted by centrifugation at 80,000 g for 90 min in an SW 28 rotor. The pellet was homogenized, extracted with Genetron and subjected to purification and concentration through sucrose and CsCl gradients.

Examination of Fish for Viruses

The initial examination of adult chum salmon (Oncorhynchus keta) at the Tokushibetsu Hatchery in northern Hokkaido occurred on October 23, 1978 as part of a certification examination (Table 2). In that year, 150 semen samples, 150 ovarian fluid samples and 60 kidney and spleen samples were collected from sexually mature animals at the hatchery. The samples were prepared in five-fish pools according to the methods of The American Fisheries Society: Fish Health Section (1975). Semen and ovarian fluid samples were diluted 1:5 in MEM containing penicillin (1000 U/ml, Grand Island Biological Company), streptomycin (1000 µg/ml, Grand Island Biological Company), mycostatin (500 U/ml, Grand Island Biological Company), and gentamycin

Table 2. Locations and dates that fish and shellfish were collected for viral examination in Japan.

Date	Hatchery	Species	Number	Sample
Oct. 23, 1978	Tokushibetsu	Oncorhynchus keta	150 adult	Semen
			150 adult	Ovarian fluid
			60 adult	Kidney-spleen
Mar. 13, 1979	Tokushibetsu	Oncorhynchus keta	150 fry	Whole fry
		Oncorhynchus masou	150 fry	Whole fry
		Oncorhynchus gorbuscha	150 fry	Whole fry
Oct. 19, 1979	Tokushibetsu	Oncorhynchus keta	90 adult	Ovarian fluid
Nov. 30, 1979	Tokushibetsu	Oncorhynchus keta	40 adult	Ovarian fluid
Oct. 29, 1980	Tokushibetsu	Oncorhynchus keta	60 adult	Liver
			60 adult	Kidney-spleen
			60 adult	Ovarian fluid
		Oncorhynchus gorbuscha	28 adult	Ovarian fluid
			28 adult	Kidney-spleen
Oct. 29, 1980	Horonai	Oncorhynchus keta	29 adult	Kidney-spleen
			29 adult	Ovarian fluid
Oct. 30, 1980	Abashiri	Oncorhynchus keta	60 adult	Kidney-spleen
			60 adult	Ovarian fluid
Oct. 30, 1980	Abashiri port[1]	Sebastes schlegeli	7 adult	Kidney-spleen
		Sebastes trivittatus	3 adult	Kidney-spleen
		Platichthys stellatus	6 adult	Kidney-spleen
		Limanda herzensteini	6 adult	Kidney-spleen
		Limanda punctatissima	6 adult	Kidney-spleen
		Oncorhynchus masou	5 adult	Kidney-spleen

Table 2. Continued.

Date	Hatchery	Species	Number	Sample
		Scomber japonicus	4 adult	Kidney-spleen
		Eleginus gracilis	3 adult	Kidney-spleen
		Navodon tessellatus	6 adult	Kidney-spleen
		Ainocottus ensiger	4 adult	Kidney-spleen
		Raja pulchra	5 adult	Kidney-spleen
		Anahichas orientalis	2 adult	Kidney-spleen
Oct. 30, 1980	Tokoro area[2]	Patinopectin yessoensis	60 animals	Digestive organs
Oct. 31, 1980	Shari	Oncorhynchus keta	60 adult	Kidney-spleen
			60 adult	Ovarian fluid

1. At this location marine fish were obtained from commercial fishermen at the port.

2. At this location scallops were obtained from commercial growers.

(250 µg/ml, Sigma Chemical Company). Kidney and spleen pools were homogenized with a mortar and pestle in 20 volumes of Hanks' balanced salt solution (BSS, Flow Laboratories Inc.). The homogenate was diluted 1:5 in the antibiotic mixture. All tissues and fluids were maintained at approximately 4°C during their preparation and transportation. The samples were sent by air to the United States and driven to the laboratory at Newport, Oregon where they were placed on monolayer cultures of CHSE-214 and RTG-2 cells and incubated at 10 and 18°C.

After the discovery of a virus from fish collected at the Tokushibetsu Hatchery, extensive efforts were made to reisolate the virus. On March 13, 1979, scientists from the Laboratory of Microbiology, Hokkaido University at Hakodate sampled fry from the Tokushibetsu Hatchery. One hundred fifty chum salmon fry, 150 pink salmon (Oncorhynchus gorbuscha) fry and 150 masou salmon (Oncorhynchus masou) fry were collected in five-fish pools and homogenized whole in 9 volumes of BSS for 30 sec. The homogenate was diluted 1:10 with antibiotic mixture, placed on monolayers of CHSE-214 cells in screw-capped tubes and incubated at 18°C for 14 days.

In the fall of 1979, adult chum salmon at the Tokushibetsu Hatchery were again sampled. Ovarian fluid was collected from 90 females (30 three-fish pools) on October 19, 1979 and from 40 females (20 two-fish pools) on November 30, 1979. The ovarian fluid was removed from the fish with an automatic pipette and filtered through

a 0.45 µm membrane filter (Millipore). The samples were placed on cultures of CHSE-214 cells and incubated for 14 days at 18°C.

In October 1980, an effort was made to again isolate the virus from various fish along the northeast coast of Hokkaido. Scientists from the Laboratory of Microbiology, Hokkaido University, Hakodate, Japan and the Oregon State University laboratories at Corvallis and Newport, Oregon went to the Sea of Okhotsk hatcheries (Fig. 1). At the Tokushibetsu Hatchery 60 kidney-spleen samples, 60 liver samples and 60 ovarian fluid samples were collected from adult chum salmon and 28 kidney-spleen samples and 28 ovarian fluid samples were taken from adult pink salmon. At the Horonai Hatchery, 29 kidney-spleen and 29 ovarian fluid samples were collected from chum salmon. At both the Abashiri and Shari Hatcheries, 60 kidney-spleen and 60 ovarian fluid samples were taken from chum salmon. All the samples were collected in five-fish pools. Twelve species of marine fish were obtained from local fisherman at the Abashiri port. Kidney and spleen tissues from these 57 fish were sampled and pooled by species. Near the Tokoro area 60 cultured scallops (Patinopectin yessoensis) were obtained and part of the digestive tract was sampled in five-tissue pools.

The kidney-spleen, liver and tissue samples from all locations were homogenized for 30 sec in 9 volumes of BSS and diluted 1:4 in antibiotic mixture. Ovarian fluid samples were filtered through a 0.45 µm filter (Millipore). All samples were transported on ice to the Laboratory of Microbiology, Hokkaido University at Hakodate where

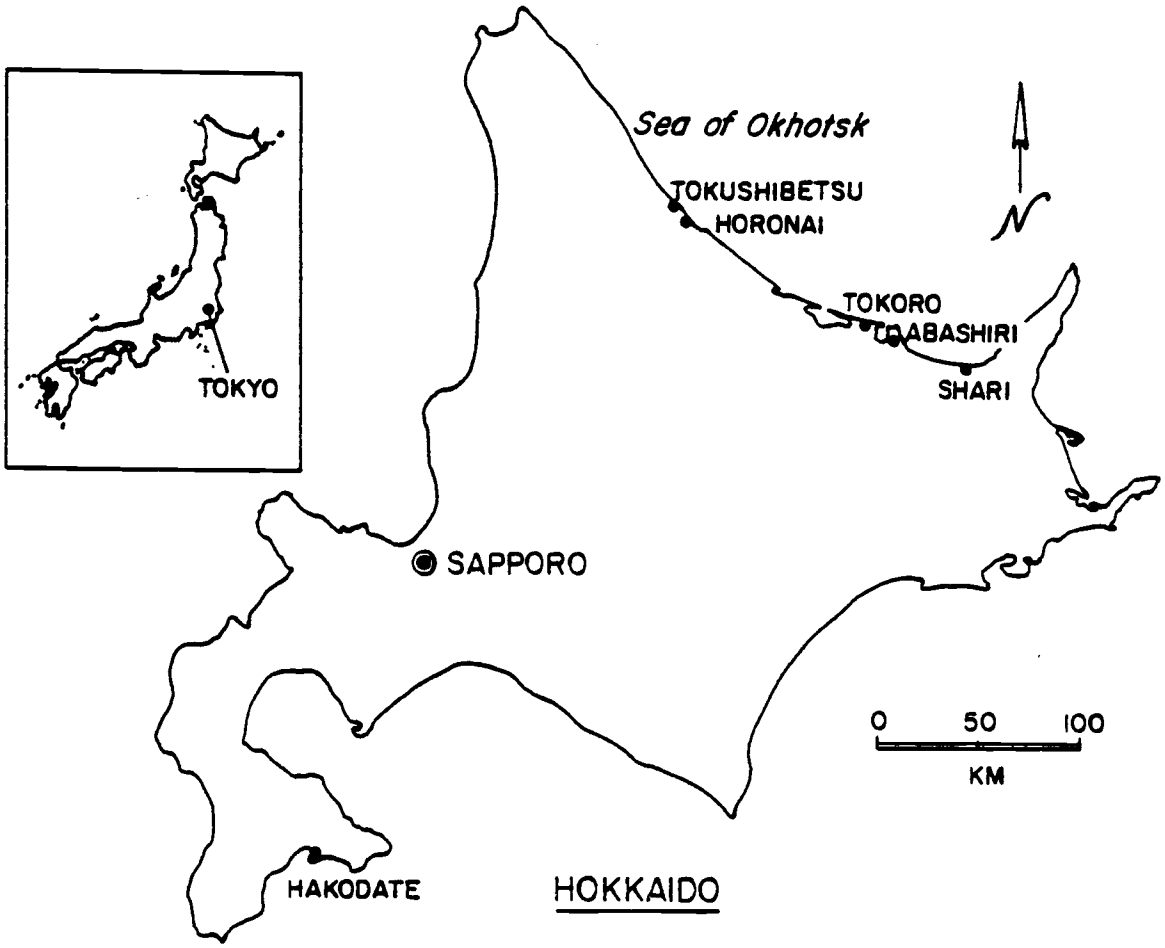


Figure 1. Map of Hokkaido, Japan showing the locations where fish and shellfish were examined for virus during 1978-1980. Salmon were sampled at the Tokushibetsu, Horonai, Shari and Abashiri Hatcheries. Shellfish from Tokoro and marine fish at Abashiri were also collected.

duplicate tubes of CHSE-214 and RTG-2 cells were inoculated with each sample and incubated at 18°C for 14 days.

Staining and Microscopy

Staining of Infected Cells

Monolayer cultures of CHSE-214 cells were grown on 1.5 cm coverslips in MEM-10 until confluent and inoculated with 10 PFU of chum salmon virus in 1.0 ml of MEM-5. Controls receiving only MEM-5 and infected cultures were incubated at 18°C for 72 hr. The coverslips were removed for fixation and staining with crystal violet, acridine orange and May-Grunwald-Giemsa stains.

The control and infected cultures for crystal violet stain were fixed in 37% formaldehyde for 5 min then rinsed in distilled water. The coverslips were stained with 1% crystal violet for 20 min, rinsed with distilled water and air dried. The cells were examined with a light microscope and photographed with Kodak High Speed Ektachrome film.

The acridine orange stain was performed by the method of Rovozzo and Burke (1973). Control and infected cultures were fixed in Carnoy's fixative, hydrated in a series of graded alcohols, and placed in McIlvaine's buffer (pH 3.8). The coverslips were stained in acridine orange (0.01% in McIlvaines's buffer, pH 3.8) for 5 min, rinsed in buffer and examined with a fluorescence microscope. The cells were photographed with Kodak High Speed Ektachrome film.

Cultures for the May-Grunwald-Giemsa stain were fixed in absolute methanol for 5 min, stained for 10 min in May-Grunwald stain, stained for 20 min with Giemsa (1:15) and rinsed with distilled water. The cells were examined with a light microscope and photographed with Kodak High Speed Ektachrome film.

Electron Microscopy

Negative stains of the chum salmon virus were prepared by removing the supernate from infected CHSE-214 cell cultures after CPE was complete. The culture fluid was centrifuged at 2000 g for 20 min to remove cell debris and the virions pelleted by centrifugation at 115,000 g for 1 hr. Virus pellets were resuspended in distilled water and stained for 1 min with 2% phosphotungstic acid (PTA) on Formvar coated grids. Virus from CsCl gradients was negatively stained by removing the virus bands from the centrifuge tube by side puncture. The virus was diluted with distilled water or SSC buffer and centrifuged at 115,000 g for 1 hr. The pellet was resuspended in distilled water and examined.

The diameter of the virion was determined using bovine catalase as an internal standard according to the method of Wrigley (1968). Bovine catalase crystals (Ladd Research Industries) were added with PTA to a drop of virus suspension on Formvar coated grids, dried and examined in a Phillips 300 electron microscope. Kodak electron image plates were used for photography of the preparations.

Thin sections of infected CHSE-214 cells were prepared from a pellet of cells fixed in 4% gluteraldehyde in Sorensen's phosphate buffer (0.2 M, pH 7.4). The cells were stained with 1% v/v osmium tetraoxide in PBS (pH 7.4) and embedded in a mixture of Epon-Araldite. Silver sections were cut on a Porter-Blum MT-1 ultramicrotome with a diamond knife and placed on 300 mesh grids. A drop of lead citrate was added and the grids examined using a Phillips 300 electron microscope. The preparations were photographed on Kodak electron image plates.

Propagation and Titration of Virus

Comparison of Two Growth Media

Medium 199 (Flow Laboratories Inc.) supplemented with 5% fetal bovine serum (199-5) was compared with MEM-5 for ability to support the replication of CSV. Monolayer cultures of CHSE-214 cells in 25 cm^2 flasks were inoculated with 0.2 ml of a virus stock. The virus was allowed to adsorb for 30 min and 5 ml of either MEM-5 or 199-5 was added to each flask. After incubation at 18°C for 4 days, the virus in the culture fluid was titered by plaque assay.

Comparison of Plaque Assay Overlays

Various overlay media were compared to find the method giving the highest plaque assay titer. The agarose overlay medium was prepared by adding equal volumes of double strength (2X) MEM-5 heated to 45°C and 1.5% ionagar (Seakem Inc.) autoclaved and cooled to 45°C. The

methyl cellulose overlay was prepared by mixing equal volumes of 2X MEM-5 and sterile 3% methyl cellulose (4000 cps, J.T. Baker Chemical Co.). Since CSV formed discrete plaques without any overlay, a control overlay of MEM-5 only was also included.

Serial ten-fold dilutions of stock virus were prepared in MEM and 0.2 ml of each dilution was added to six 25 cm^2 flasks of CHSE-214 cells. The virus was allowed to adsorb for 30 min and 5 ml of each overlay added to each duplicate flask. After incubation at 18°C for 4 days, 2 ml 37% formaldehyde was added to the flasks and allowed to stand 30 min. The overlay was decanted from each flask and the cells stained with 1% crystal violet for 15 min. The flasks were rinsed with water, air dried and the plaques counted.

Determination of Adsorbtion Time

The time required for virus adsorbtion was measured by performing plaque assays after incubation of the inoculum for 30, 60 and 120 min. The growth medium was removed from monolayers of CHSE-214 cells in 25 cm^2 flasks. Serial ten-fold dilutions of stock virus were made in MEM and 0.2 ml of each dilution was added to duplicate cultures for each incubation period. After 30, 60 and 120 min incubation at 18°C, the unadsorbed virus was washed from the monolayers of a set of cultures with 3 rinses of BSS. Five ml of MEM-5-methyl cellulose was added to each flask and the cultures incubated at 18°C for 4 days. The cells were fixed for 30 min by addition of 2 ml 37% formaldehyde and stained with 1% crystal violet for 15 min. The plaques from

each incubation period were counted. The 120 min adsorbtion was assigned a value of 100% and the percentage of virus adsorbed at 30 and 60 min was calculated.

Comparison of Plaque Assay and $TCID_{50}$ Titers

A viral suspension containing 1000 PFU/ml of CSV was thawed and a series of ten-fold dilutions was made in MEM-5. A 96 well microplate containing monolayers of CHSE-214 cells was used for the $TCID_{50}$ assay and 0.1 ml of each dilution was placed into each of 10 wells. The plate was sealed with a mylar film and incubated at 15°C. The number of wells at each dilution showing CPE was read at days 4, 7, 14, 21 and 28. The $TCID_{50}$ titer was calculated by the method of Reed and Muench (1938).

The plaque assay was performed by inoculating 0.2 ml of each dilution onto three 25 cm^2 flasks of CHSE-214 cells. The flasks were rocked every 15 min during a 90 min adsorbtion. Five ml of MEM-5-methyl cellulose was added to each flask and the cultures incubated at 15°C for 4 days. The flasks were fixed with 37% formaldehyde for 30 min and stained with 1% crystal violet for 15 min and the plaques counted on flasks having 20-200 plaques.

Comparison of Cell-Associated and Released Virus

Two 25 cm^2 flasks of CHSE-214 cells were inoculated with 0.2 ml of stock virus and incubated at 18°C for 4 days. The culture fluid was removed and held at 18°C while the monolayers were washed three times with BSS. The cells were scraped from the flask with a rubber

policeman and placed in a 2 ml Ten Broek homogenizer. The cells were disrupted with 15 strokes of the plunger. Serial ten-fold dilutions were made in MEM-5 of the cell homogenate and the culture fluid. The titer of each was determined by plaque assay.

Virus Replication at Selected Temperatures

The optimal temperature for replication of CSV was determined in EPC cells. This cell line had been shown to support the growth of CSV and was chosen because it was capable of growth at higher temperatures than the salmonid cell lines. Monolayer cultures of EPC cells in 75 cm^2 flasks were infected with 500 PFU of virus and 20 ml of MEM-5 was added to each flask. Duplicate flasks were incubated at 5, 10, 15, 20, 25 and 30°C. At 48 hr intervals for 14 days, 1.0 ml of the culture fluid was removed from each flask and the titer determined by $TCID_{50}$ assay.

Virus Replication in Selected Cell Lines

The following cell lines were tested for the ability to support replication of chum salmon virus: CHH-1, CHSE-214, STE-137, KO-6, LBF-2, BF-2, WC-1, EPC, FHM, RTG-2 and BB. Cells were grown in 25 cm^2 flasks in MEM-10 at 18°C until confluent. The growth medium was removed and 5 ml of MEM-5 containing approximately 200 PFU of virus was added to each of two duplicate flasks of each cell line. After 14 days incubation at 18°C, 0.1 ml of the culture fluid was removed from each flask and added to a second set of cultures. After an

additional 14 days incubation at 18°C, the virus produced by each cell line was titered by $TCID_{50}$ assay on CHSE-214 cells.

Efficiency of Plating in Selected Cell Lines

The efficiency of plating (EOP) of chum salmon virus was determined for CHH-1, CHSE-214, STE-137, KO-6, LBF-2, BF-2, WC-1, EPC, FHM, RTG-2 and BB cells. Each of three 25 cm^2 flasks of each cell line was inoculated with 0.2 ml MEM containing approximately 200 PFU of virus. The cells were incubated for 90 min to allow the virus to adsorb. Five ml of MEM-5-methyl cellulose was added to each flask and the cultures incubated at 18°C for 4 days. The cells were fixed with 37% formaldehyde for 30 min and stained with 1% crystal violet for 15 min. The plaques on each flask were counted and averaged.

Serologic Techniques

Preparation of Virus Antiserum

Chum salmon virus was purified and concentrated through alternate sucrose and CsCl gradients. The lower virus band composed of complete virions was removed from the gradient by side puncture and resuspended in TNE (0.01 M Tris-HCl, 0.1 M NaCl, 0.001 M EDTA, pH 7.5) buffer to a volume of 0.5 ml. An equal amount (0.5 ml) of Freund's complete adjuvant (Difco Laboratories) was added and the mixture homogenized with a Virtis 23 homogenizer (microblade) for 1 min at 23,000 rpm until the emulsion was complete. An adult New Zealand white rabbit was

injected with 0.25 ml of the emulsion into each hind foot pad and 0.5 ml was injected intrascapularly in the back. Bleedings were taken at days 14, 21 and 28. At each bleeding, 50 ml of blood was collected in a sterile plastic centrifuge tube and the blood was allowed to clot for 1 hr at room temperature before centrifugation at 12,000 g for 10 min. The serum was harvested, filtered through a 0.45 µm membrane filter (Millipore) and stored in 1 ml aliquots at -65°C until use.

The neutralization index (NI) of the antiserum was determined by comparing the $TCID_{50}$ titer of virus dilutions incubated with and without antiserum (constant serum varying virus). Two sets of serial ten-fold dilutions were made in MEM-5 from a stock of chum salmon virus. One set of dilutions was incubated with equal volumes of antiserum (day 21 bleeding 1:10 in MEM). The second set was incubated with equal volumes of MEM. The two sets of dilutions were incubated for 90 min at 18°C. After incubation, 0.1 ml of each dilution was placed into each of 4 wells of a microplate of CHSE-214 cells. The number of wells showing CPE was recorded at 4, 6, 9 and 12 days incubation at 18°C. The neutralization index was calculated as the logarithm of the difference in the titer of the two sets of dilutions.

Cross Neutralization Test

Cross neutralization tests were performed by the microtiter method of Kuchler (1977). Stocks of the following viruses and antisera to each of them were used: chum salmon virus, infectious pancreatic necrosis virus, infectious hematopoietic necrosis virus, reovirus types

1, 2 and 3. The tests were performed by preparing virus stocks in MEM at a titer of 100 $TCID_{50}$/0.1 ml. Each antiserum was heat-inactivated for 30 min at 56°C and diluted 1:10 in MEM for use. Serial two-fold dilutions of serum were made in test tubes containing 0.5 ml MEM and each serum dilution was reacted with an equal volume (0.5 ml) of the stock virus suspension at room temperature for 1 hr. Each virus-serum combination was assayed for infectivity by inoculating 0.2 ml of each serum dilution into each of 4 wells of a microplate of CHSE-214 cells (for CSV, IPNV and IHNV) incubated at 18°C or BGM cells (for Reo 1, 2 and 3) incubated at 35°C. Incubation was continued until obvious CPE had developed in the controls (virus suspension with normal rabbit serum). The neutralization titer is expressed as the highest dilution of the antiserum preventing CPE.

Hemagglutination Test

Hemagglutination with human O erythrocytes was determined using the microtiter method of Kuchler (1977). Human O erythrocytes were collected in a tube containing 1.0 ml 5% sodium citrate per 5 ml whole blood. The cells were pelleted by centrifugation at 200 g for 10 min, washed 5 times with 0.85% saline and resuspended to a concentration of 0.5% in PBS (pH 7.4). A 50 µl microdiluter (Cooke Engineering) was used to make serial two-fold dilutions of CSV and reovirus types 1, 2 and 3 in PBS. Fifty microliters of cell suspension was added to each well and the microplate incubated at room temperature for 2 hr until an agglutination pattern had formed. The titer was determined as the final virus dilution showing complete hemagglutination.

Fluorescent Antibody Staining

Monolayer cultures of CHSE-214 cells were grown on 1.5 cm coverslips in MEM-10 until the cells were confluent. The cultures were infected with 10 PFU of virus in 1.0 ml MEM-5. Controls received only MEM. The cells were incubated for 72 hr at 18°C, fixed in 100% acetone for 5 min and incubated at room temperature for 30 min with chum salmon virus antiserum (1:10 in PBS, pH 7.4). After incubation, the coverslips were rinsed 3X in BSS and incubated for 30 min at room temperature with goat-antirabbit serum conjugated with fluoroscein isothiocyanate (Baltimore Biological Lab). The coverslips were rinsed 3X with PBS and examined with a fluorescence microscope.

Biochemical and Biophysical Methods

Freeze-Thaw Stability

The stability of the virus to repeated freezing and thawing was tested by titering a virus suspension before and during three freeze-thaw cycles. A virus stock was titered by plaque assay and 2.0 ml of the suspension was frozen at -20°C in a screw-capped tube. Three times at weekly intervals, the tube was thawed rapidly and an aliquot of the suspension was removed for plaque assay. The tube was refrozen and the process repeated.

Stability at 37 and 56°C

Stability of the chum salmon virus to 37 and 56°C was tested by incubating suspensions of the virus at these temperatures and removing aliquots for plaque assay at selected intervals. A stock of the virus was titered at the beginning of the incubation period. Portions of the virus suspension were placed in screw-capped tubes and incubated at 18, 37 and 56°C.

Aliquots of the suspensions were removed for plaque assay from the 18 and 37°C tubes after 6 and 24 hr incubation and from the 56°C tube at 0.5, 1, 2, 4, and 6 hr incubation. After 6 hr at 56°C, a 1.0 ml aliquot was removed from the tube and inoculated into a 25 cm^2 flask of CHSE-214 cells. This flask was incubated at 18°C and observed for CPE caused by any residual infectivity.

Resistance to Lipid Solvents

The sensitivity of chum salmon virus to treatment with lipid solvents was tested by two methods. Infectious hematopoietic necrosis virus, an enveloped virus, and infectious pancreatic necrosis virus, which lacks an envelope, were included as positive and negative controls.

Ether sensitivity was tested by the method of Andrewes and Horstmann (1949). Two ml of a virus stock was placed into each of two screw-capped tubes; 0.5 ml anhydrous ethyl ether (Mallinckrodt Inc.) was added to one tube and 0.5 ml sterile 0.85% saline to the control. The tubes were shaken frequently and incubated at 4°C for 24 hr. The contents of the tubes were poured into sterile petrie dishes and the

ether evaporated. The virus in the remaining aqueous suspension and in the control was titered by plaque assay.

Chloroform sensitivity was determined by the method of Feldman and Wang (1961). One ml of chloroform (J.T. Baker Chemical Co.) was added to 2 ml of virus suspension at room temperature. A control tube received 1 ml of sterile 0.85% saline. The mixtures were shaken for 10 min and both tubes centrifuged at 600 g for 5 min to separate the chloroform from the aqueous phase in the treated sample. The infectious virus in the aqueous phase of the treated sample and in the saline control was titered by plaque assay.

Effect of Chymotrypsin

The ability of α-chymotrypsin to remove the outer capsid layer of the virion was tested by the method of Joklik (1972). Virus from the upper and lower bands of a CsCl gradient was suspended in SSC buffer and diluted 1:10 with SSC containing α-chymotrypsin (Sigma Chemical Co., 3X crystalized, 100 μg/ml in SSC). Treated virions and untreated controls (SSC without α-chymotrypsin) were incubated at 37°C for 60 min and a sample removed for titration of infectious virus by $TCID_{50}$ assay. The remainder of the incubation mixture was pelleted by centrifugation at 115,000 g for 60 min in an SW 50.1 rotor. The pellet was resuspended in two drops of distilled water, negatively stained with 3% PTA on Formvar coated grids and examined with an electron microscope.

Stability to pH 3

Stability to pH 3 was tested by the method of Ketler et al. (1962). A virus suspension was diluted 1:10 in MEM adjusted to pH 3 by addition of 0.1 N HCl. Controls were diluted 1:10 in MEM at pH 7. After 30 min incubation at 18°C, the infectious virus was titered by $TCID_{50}$ assay. Infectious hematopoietic necrosis virus, unstable at pH 3, and infectious pancreatic necrosis virus, stable at pH 3, were included as positive and negative controls.

In a second experiment, the purified upper and lower virion bands from a CsCl gradient were each suspended 1:10 in SSC at pH 3 and at pH 7. These suspensions were incubated at 25°C for 60 min and a sample removed for titration of infectious virus by $TCID_{50}$ assay. The remainder of the suspension was centrifuged at 115,000 g for 45 min in an SW 50.1 rotor. The pellets were resuspended in distilled water and negatively stained with 3% PTA for electron microscopy.

Replication of the Virus in the Presence of 5-fluoro-2'-deoxyuridine

The resistance of chum salmon virus to the halogenated pyrimidine 5-fluoro-2'-deoxyuridine (FUDR), was determined by the method of Rovozzo and Burke (1973). The growth medium was removed from 25 cm^2 flasks of CHSE-214 cells. Treated flasks received 5 ml of 10^{-4} M FUDR in BSS. Controls received 5 ml of BSS. Treated and untreated cultures were infected with 0.1 ml of CSV stock and incubated for 16 hr at 18°C. All cultures were washed 3X with BSS and the medium replaced with 5 ml of MEM-5 in all flasks. The flasks were incubated at 18°C until CPE was extensive in the untreated cell monolayers. The infectious virus

produced in each culture was titered by plaque assay. Herpesvirus salmonis, a DNA virus, and infectious pancreatic necrosis virus, an RNA virus, were included in the experiment as positive and negative controls.

Density of Virions in CsCl

Virus was labeled with ^{3}H uridine and purified through alternate sucrose and CsCl gradients. The lower viral band was removed from the gradient with a probe and peristaltic pump, diluted in SSC and pelleted by centrifugation at 115,000 g for 1 hr in an SW 50.1 rotor. The viral pellet was resuspended in 0.5 ml SSC buffer and layered on top of a step gradient of 2 ml 40% w/w, 1.5 ml 30% w/w and 1 ml 20% w/w CsCl. The gradient was centrifuged for 24 hr at 115,000 g in an SW 50.1 rotor. Six drop fractions were collected with the aid of an automatic gradient collector (Buchler Instruments) from the top of the gradient. The fractions were assayed for infectivity by making a series of ten-fold dilutions in MEM containing antibiotics and performing a $TCID_{50}$ assay. The fractions were assayed for radioactivity by removing 20 µl of each fraction and placing it directly in 10 ml of scintillation cocktail (Aquasolve II, New England Nuclear Corp.). The scintillation vials were counted in a Beckman LS 8000 using a single label program. The density of the fractions was determined by removing a drop of selected fractions and reading the refractive index with an Abbe refractometer (Bausch and Lomb). The readings at 25°C were corrected for the SSC buffer by subtraction of the refractive index of

SSC (0.0022) and the density of the fractions calculated from the International Critical Tables.

Density of Viral RNA

Virus was labeled with ^{3}H-uridine, purified and concentrated by centrifugation in sucrose and CsCl gradients. The virus was pelleted by centrifugation at 115,000 g for 1 hr and resuspended in SSC buffer. Pronase (Calbiochem-Behring Corp.) was made as a stock solution of 5 mg/ml in SSC buffer and preincubated at 37°C for 30 min. The Pronase solution was added to virus in SSC to a final concentration of 500 µg/ml and SDS was added as a 10% stock solution in SSC to a final concentration of 1%. This mixture was incubated at 43°C for 1.5 hr. After incubation, phenol, saturated with SSC buffer, was added in equal volumes and the resulting mixture shaken for 5 min. The mixture was centrifuged at 1500 g in a clinical centrifuge for 10 min to separate the phenol from the aqueous phase. The aqueous phase was removed and extracted twice more with phenol. After the third extraction, the RNA in the aqueous phase was precipitated by the addition of three volumes of cold 95% ethanol and overnight incubation at -20°C. The precipitate was collected by centrifugation at 1500 g in a clinical centrifuge and the pellet resuspended in 0.1 ml SSC.

The RNA was layered onto a 2 step gradient composed of 1 ml of $CsSO_4$ at 1.75 g/ml and 1 ml $CsSO_4$ at 1.40 g/ml. The remainder of the tube was filled with mineral oil and the gradient centrifuged for 48 h at 115,000 g in an SW 50.1 rotor. Four drop fractions were collected by probe and peristaltic pump from the top of the gradient. The

fractions were assayed for radioactivity by removing 20 µl from each fraction and placing it in 10 ml of scintillation cocktail (Aquasolv II, New England Nuclear Corp.) and counting the samples in a Beckman LS 8000 with single label program. The density of the fractions was determined by removing a drop from selected fractions and reading the refractive index with an Abbe refractometer. The readings at 25°C were corrected for the SSC buffer and the density of the fractions calculated from the International Critical Tables.

Protein Analysis in Polyacrylamide Gels

The virus was purified through sucrose and CsCl gradients. Two sucrose and two CsCl gradients were used. The viral bands were extracted with a probe and peristaltic pump, pelleted and resuspended in SSC buffer. Virus from the upper and lower bands and high and low molecular weight markers (Biorad Laboratories) were run simultaneously on SDS gels prepared according to the Laemmli system (Laemmli, 1970).

A 12% separating gel was prepared by combining 10.1 ml distilled water, 7.5 ml 1.5 M Tris-HCl, pH 8.8, 0.3 ml 10% w/v SDS, 12 ml 30% acrylamide (acrylamide:BIS, 30:0.8), 0.1 ml fresh 10% (w/v) ammonium per sulfate, and 0.0075 ml TEMED. The reagents were added except for the TEMED and the mixture deaerated under vacuum for 15 min. Polymerization was initiated by addition of the TEMED and the mixture poured into the electrophoresis unit. The gels (1.5 mm thick) were allowed to polymerize overnight. A 3% stacking gel was poured over the separating gel. This gel was prepared by combining 6.3 ml distilled water, 2.5 ml 0.5 M Tris-HCl, pH 6.8, 0.1 ml 10% w/v SDS, 1.0 ml

30% acrylamide (acrylamide:BIS, 30:0.8), 0.1 ml fresh 10% (w/v) ammonium persulfate, and 0.005 ml TEMED. Well forming combs were placed in the polymerizing gel which was allowed to stand for 1 hr before use.

The samples were diluted 1:5 in sample buffer (4.7 ml distilled water; 1.0 ml 0.5M Tris-HCl, pH 6.8; 1.0 ml glycerol; 1.0 ml 10% w/v SDS; 0.1 ml 2-mercaptoethanol; 0.2 ml 0.05% w/v bromphenol blue) and heated in a boiling water bath for 2-5 min. A micropipette was used to load 10-100 µl of sample into each well of the stacking gel. The electrode buffer was made by addition of 6.0 g Tris base, 28.8 g glycine, and 10 ml 10% w/v SDS to a flask and distilled water added to a volume of 1000 ml. Electrophoresis was conducted at 4 ma/gel for 16-20 hr until the bromphenol blue tracking dye had migrated to within 1 cm of the bottom of the gel.

The gels were removed from the unit and placed in a solution of 227 ml distilled water, 227 ml absolute methanol, 46 ml glacial acetic acid and 1.25 g Coomassie blue for fixation and staining for 4-10 hr. The gels were destained by successive changes of a solution made of 875 ml distilled water, 50 ml methanol, and 75 ml glacial acetic acid until the background was clear. The gels were dried onto filter paper in a gel drier (Biorad Laboratories). A semi-log standard curve was constructed of the relative mobilities of the known molecular weight markers. The molecular weights of the virion proteins were calculated from this curve by the methods described in Cooper (1977) and Kuchler (1977).

RNA Analysis in Polyacrylamide Gels

Virus was purified by sucrose and CsCl gradients and the lower viral band was extracted with a probe and peristaltic pump. The virus was pelleted and resuspended in SSC buffer. Equal volumes of virus in SSC and buffer-saturated, redistilled phenol were mixed with a vortex mixer for 5 min. The mixture was centrifuged at 1000 g for 10 min to separate the phenol and the aqueous phase was removed. This process was repeated twice. After the third phenol extraction, the aqueous phase was removed and the RNA precipitated by addition of 2 volumes of cold 95% ethanol followed by overnight incubation at -20°C.

The RNA was collected by centrifugation at 1500 g for 20 min and resuspended in buffer (0.004 M Tris, 0.002 M sodium acetate, 0.0002 M EDTA pH 7.8). Both tube and slab gels were poured for electrophoresis of RNA. The 5% gels were prepared by combining 6 ml 30% acrylamide (acrylamide:BIS, 30:0.8), 12 ml of 3X buffer (the 3X buffer is: 0.12 M Tris, 0.06 M sodium acetate and 0.006 M EDTA, pH 7.8), 17.7 ml distilled water, 0.15 ml fresh 10% w/v ammonium persulfate, and 0.015 ml TEMED. The tube or slab gels were allowed to polymerize overnight.

The tube gels were used without a stacking gel, but the slab gels had a 3% gel containing sample wells placed over them. This stacking gel was made by combining 1.0 ml 30% acrylamide (acrylamide: BIS, 30:0.8), 3.3 ml of the 3X buffer, 5.6 ml distilled water, 0.1 ml fresh 10% w/v ammonium persulfate and 0.005 ml TEMED. This solution was layered over the separating gel. After the stacking gel had polymerized for 1 hr, the RNA solution was diluted 1:1 with glycerol and 10-50 µl

of RNA solution placed into each well. Reovirus type 3 RNA was used as a known molecular weight marker and electrophoresis was performed in 1X buffer for 24 hr at 15 ma/gel for slabs and 16 hr at 15 ma/gel for the tube gels. The gels were removed and fixed for 15 min in 1 M acetic acid and stained for 1 hr in 0.2% methylene blue in 0.4 M sodium acetate, pH 5. After staining, the gels were destained by rinsing in tap water until clear. A standard curve was made on semilog paper of the relative mobilities of the reovirus RNA versus molecular weight. Using this graph, the molecular weights of the chum salmon virus RNA segments were determined by the method of Shatkin et al. (1968).

In Vivo Studies

Pathogenicity for Chum, Chinook and Kokanee Salmon and Rainbow Trout

The pathogenicity of the virus for fish was tested in chum salmon, chinook salmon (Oncorhynchus tshawytscha), kokanee salmon and rainbow trout fry. Fish weighing 1-2 g were injected intraperitoneally (i.p.) with 10^4 $TCID_{50}$ of virus in 0.02 ml MEM. Two groups of twenty fish each were injected with virus and two control groups receiving 0.02 ml MEM were held in 10 l static water aquaria at 12°C for 42 days and observed daily for mortality. Fry dying during the experiment were frozen at -20°C until they were titered for virus by $TCID_{50}$ assay.

Virus Replication in Chum, Chinook and Kokanee Salmon and Rainbow Trout

The amount of virus produced by infected chum, chinook and kokanee salmon and rainbow trout fry was tested by injecting 1-2 g fish i.p. with 0.02 ml of MEM containing $10^4 TCID_{50}$ of virus. At 48 hr intervals for 14 days and then at day 21, 28, and 42, three fish were removed, pooled and frozen at -20°C. At termination of the experiment, the fish were homogenized in BSS (1:10 w/v) using a Virtis homogenizer. The homogenate was centrifuged at 2000 g for 20 min, the supernate filtered through a 0.45 µm filter (Millipore) and the virus titered by $TCID_{50}$ assay.

Pathology in Virus Infected Chum, Chinook and Kokanee Salmon and Rainbow Trout

Histological changes in infected chum, chinook, and kokanee salmon and rainbow trout fry were determined after injecting 1-2 g fry with 10^4 $TCID_{50}$ of virus i.p. in 0.02 ml MEM. At days 8, 14, 21, 28, and 42, two fish were removed and placed in Bouin's fixative. The fish were embedded in parafin and serial 7 µm sections were cut. The mounted sections of internal organs were stained with hematoxylin and eosin and examined by means of light microscopy.

Acridine Orange, May-Grunwald-Giemsa and Fluorescent Antibody Stains of Infected Chum Salmon

Serial 7 µm frozen cross sections were cut on a cryostat (International Equipment Company) through the visceral portion of infected chum salmon fry. The sections were picked up on a warm slide and each of three serial sections was stained with a different technique.

Acridine orange stained sections were fixed in Carnoy's fixative, rehydrated in a series of graded alcohols, rinsed in McIlvaine's buffer (pH 3.8) and stained with 0.01% acridine orange in McIlvaines buffer. These sections were examined with a fluorescence microscope.

Sections for May-Grunwald-Giemsa were fixed in absolute methanol for 5 min, stained for 10 min in May-Grunwald stain, stained for 20 min with Giemsa (1:15) and rinsed in distilled water. The sections were examined with a light microscope.

For fluorescent antibody stain, the sections were fixed in 100% acetone for 5 min and incubated for 30 min with anti-CSV (1:10 in MEM). After incubation, the sections were rinsed with PBS and reacted for 30 min with goat-antirabbit conjugated with FITC. The slides were rinsed in PBS and examined with a fluorescence microscope. Photographs of all sections were taken with Kodak High Speed Ektachrome film.

RESULTS

Isolation of Chum Salmon Virus

Kidney, spleen, ovarian fluid and semen samples collected from chum salmon at the Tokushibetsu Hatchery, Japan were flown to the United States for virological examination. The samples were placed on monolayer cultures of CHSE-214 and RTG-2 cells and incubated at 10 and 18°C. After six days incubation at 18°C, one of the twelve kidney and spleen pools on the CHSE-214 microplate began to show several areas of CPE. These areas consisted of a clear central zone surrounded by what appeared to be cell fusion. No changes were observed in the ovarian fluid or semen samples nor in any of the control cultures. No CPE was observed in any of the samples placed on RTG-2 cells. The plaque-like areas on CHSE-214 cells continued to expand during the next two days, eventually involving the entire cell monolayer. When fluid from these cultures was diluted 1:100 in MEM-5 and inoculated onto fresh CHSE-214 cells at 18°C, an identical CPE was observed. A second 1:100 dilution and subculture was performed with the same results. A third subculture was made after filtering the culture fluid through a 0.22 μm membrane filter and the characteristic plaques were again observed.

Examination of Fish from Japanese Hatcheries

Following the isolation of the virus from the samples of adult chum salmon at the Tokushibetsu Hatchery in October 1978, repeated attempts were made to reisolate the virus from fry and adult salmon, scallops and various marine fish along the northeastern coast of Hokkaido. Except for the single five-fish kidney and spleen sample from which CSV was originally isolated, the virus was not observed again. A total of 1591 samples were examined in the course of this study.

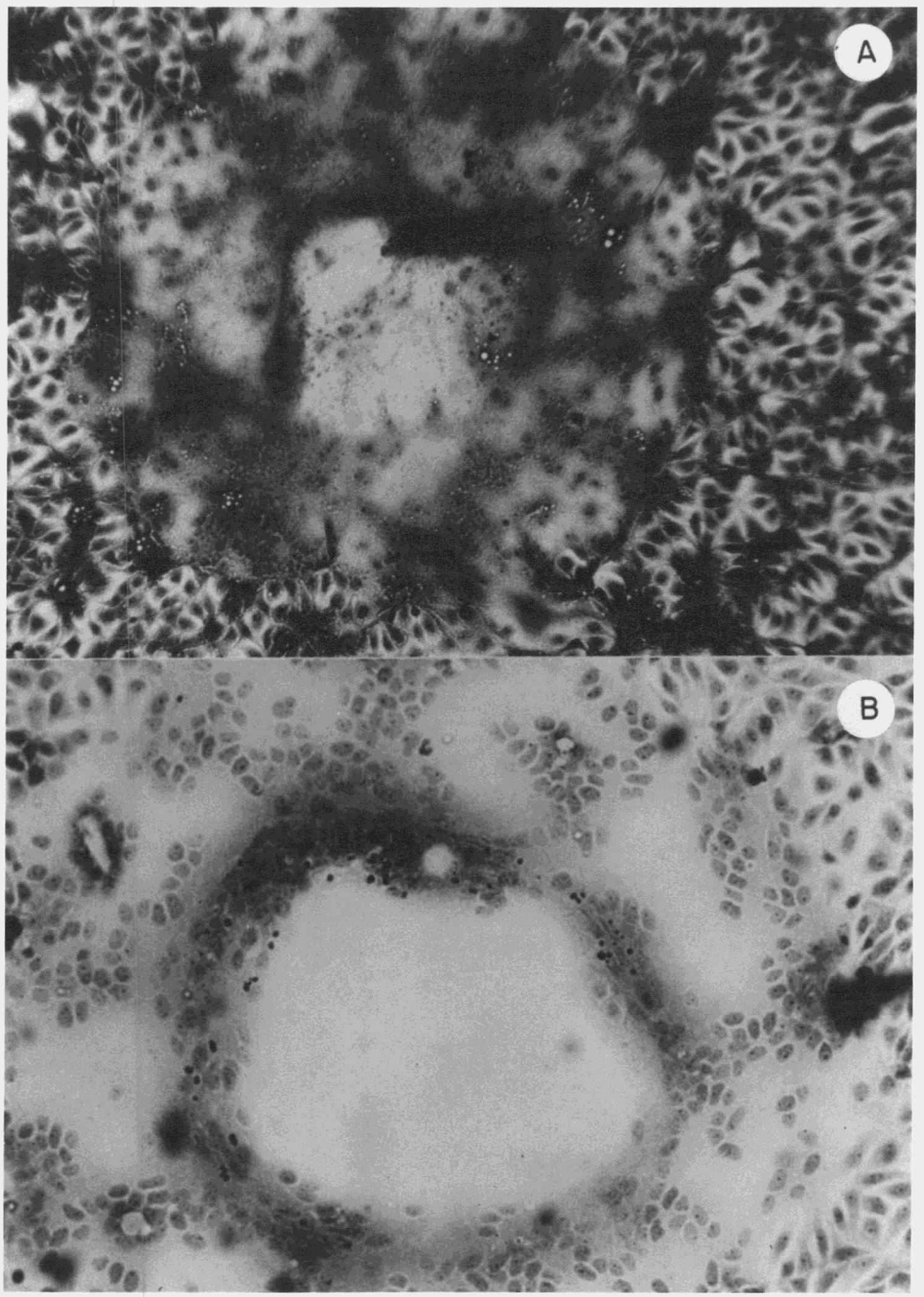
Staining and Microscopy

Staining of Infected Cells

The crystal violet stain was routinely used in the plaque assay. This staining procedure resulted in plaques with a red color which made them easily discernable against the purple-staining uninfected cells. Some features of plaque morphology could be seen in infected CHSE-214 cells on coverslips incubated for 72 hr at 18°C and stained with crystal violet. Intact nuclei were present at the margin of the plaque and the clear central area was surrounded by cytoplasmic fusion. The plaque had a characteristic donut shape (Fig. 2).

When infected CHSE-214 cells, incubated for 72 hr at 18°C, were stained with May-Grunwald-Giemsa, the plaque morphology could be seen to involve the destruction of the cell membranes and cytoplasmic fusion was apparent (Fig. 2). The nuclei were concentrated at the edge of the plaques and stained normally. Small dark staining bodies which are probably the virus inclusions seen with acridine orange stain

Figure 2. Photomicrographs of CHSE-214 cells infected with chum salmon virus for 72 hr at 18°C and stained with (A) 1% crystal violet, (B) May-Grunwald-Giemsa.


A
B

were observed at the periphery of the plaque. The cells surrounding the plaques and the control cultures were normal.

When monolayers of CHSE-214 cells on coverslips were stained with acridine orange, the uninfected cultures were normal with yellow-green nuclei and red staining cytoplasm (Fig. 3). Infected cultures demonstrated plaques with fused cytoplasm containing green inclusion bodies. The nuclei of the infected cells appeared normal, but there was nearly complete destruction of the cell membranes in the area surrounding these plaques and the nuclei were pushed together at the periphery of the central area. The green cytoplasmic inclusion bodies seen with CSV are identical to those observed with reoviruses, and are presumptive evidence that the virus possesses double-stranded nucleic acid (Ramig and Fields, 1978).

Morphology of the Virion

When observed by electron microscopy, negatively stained virions revealed particles with icosahedral symmetry and a double capsid structure (Fig. 4). The particles were 75 nm in diameter and both complete (containing nucleic acid) and coreless (incomplete) particles were seen. In profile, twenty peripheral capsomeres were visible around the virion. No envelope was detected. These morphological features are characteristic of the viruses of the family Reoviridae (Joklik, 1974).

Figure 3. Photomicrographs of (A) normal CHSE-214 cells and (B) CHSE-214 cells infected with chum salmon virus for 72 hr at 18°C stained with acridine orange. Arrows indicate inclusions. (250X)

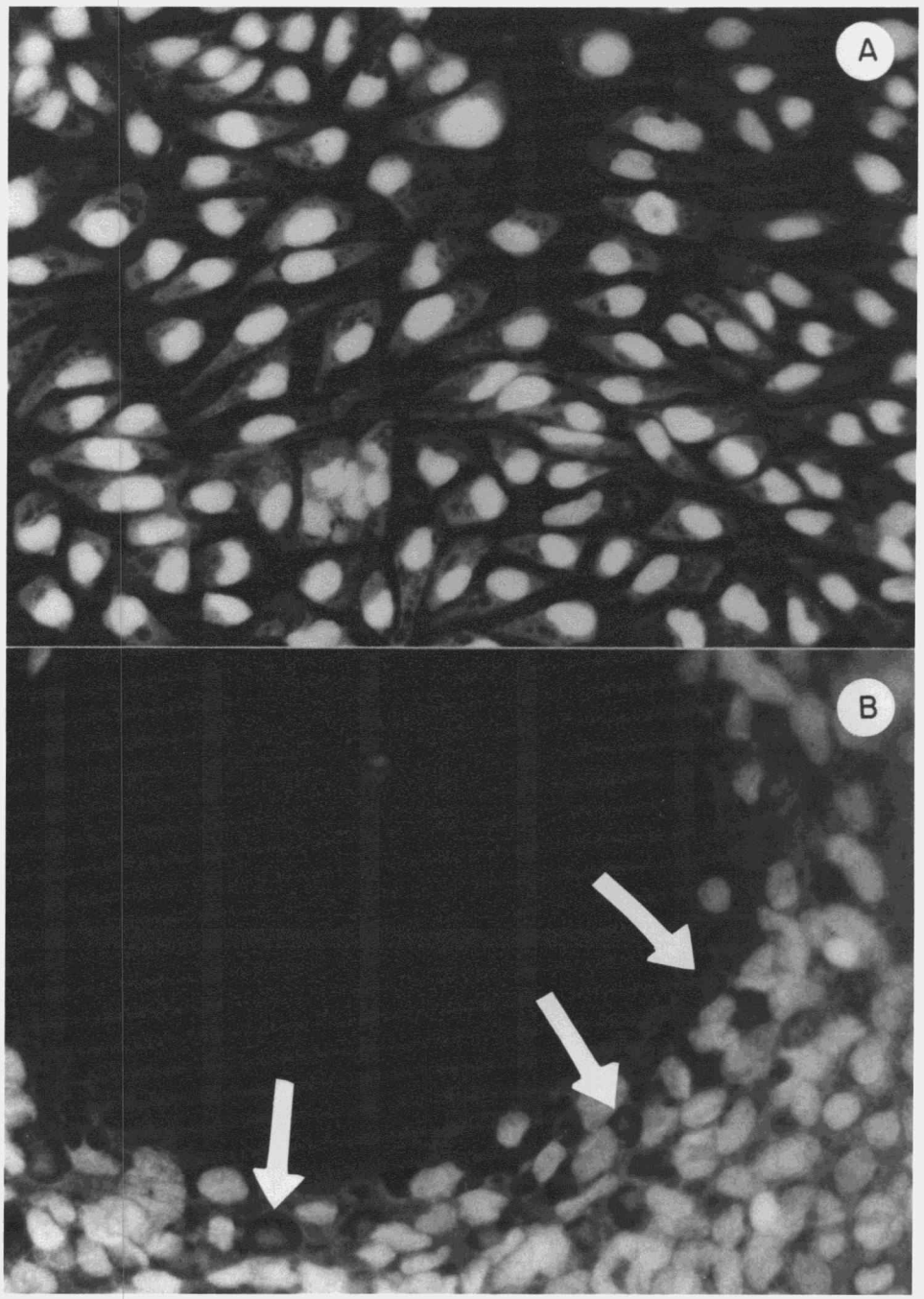
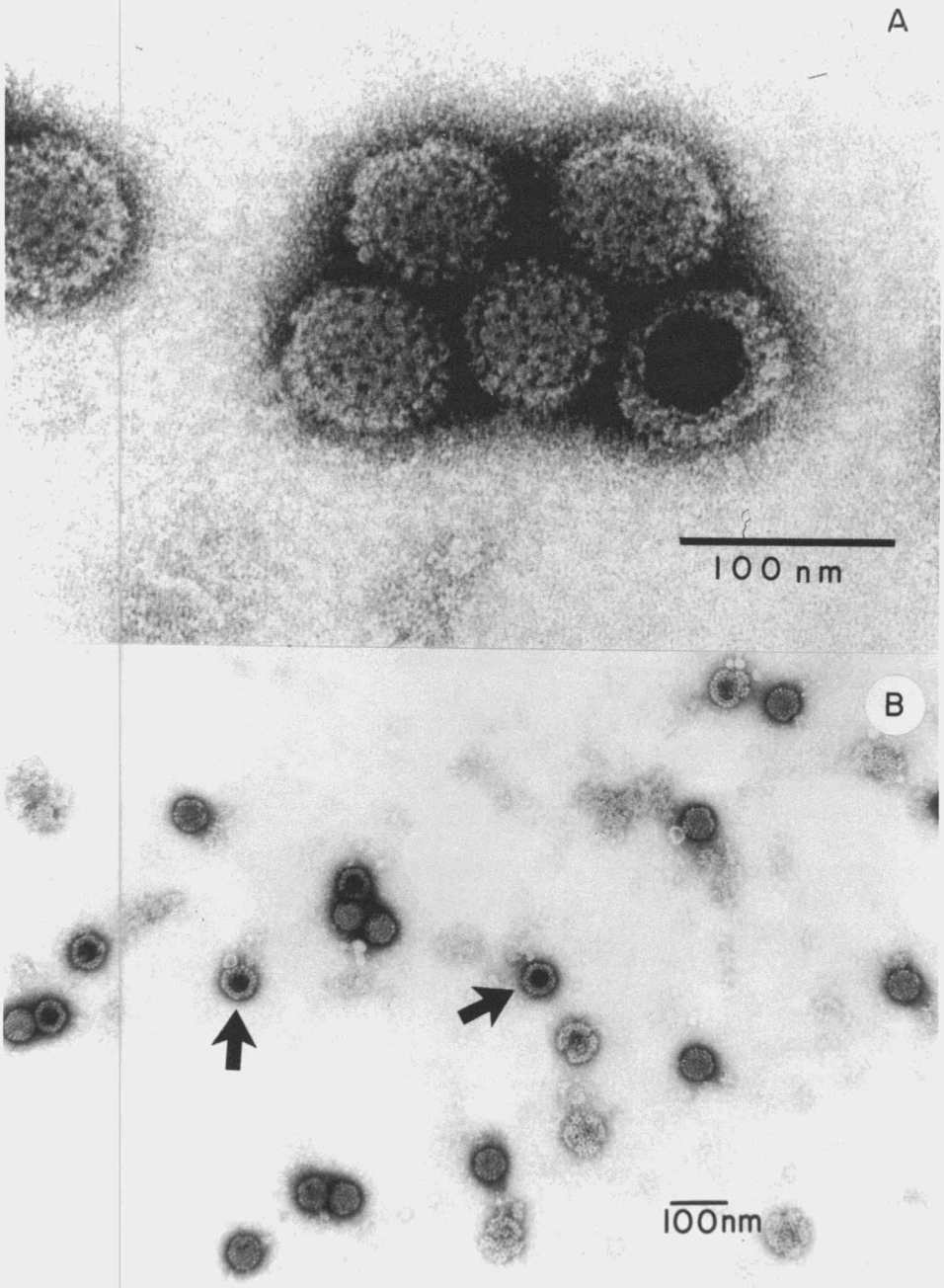

A
B

Figure 4. Electron micrographs of chum salmon virus negatively stained with 2% phosphotungstic acid. The mean diameter of the virion is 75 nm and a double capsid is present. Twenty peripheral capsomeres can be seen (A) and (B) complete and incomplete virions (arrows) are present in the preparations.


A
100 nm
B
100nm

Electron Microscopy of Infected Cells

Thin sections of infected CHSE-214 cells revealed numerous viral particles in the cytoplasm of infected cells (Fig. 5). The nuclei of the cells did not appear to be involved in viral replication. No evidence of enveloped or budding virions was detected. In some areas of the cytoplasm, large aggregates of virus were present, while in other cells large numbers of virions were detected in what appeared to be vacuoles.

Propagation and Titration of Virus

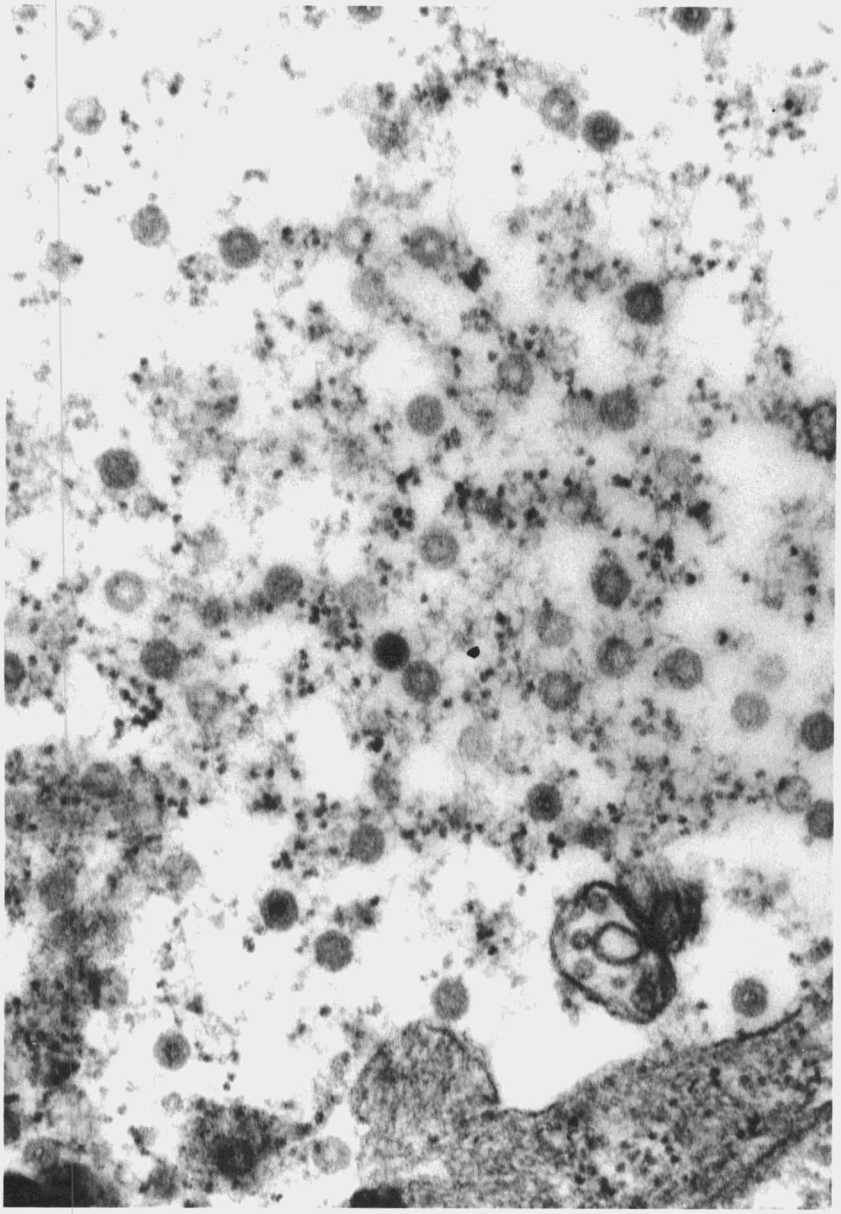
Comparison of Two Growth Media

After the initial isolation of the virus, a series of experiments was conducted to determine the optimum conditions for viral replication and assay. One of the first of these experiments compared the amount of virus produced by infected CHSE-214 cells incubated with the minimal essential medium, MEM-5, and the more complete medium, 199. No significant difference in virus titer was observed between the cells grown in MEM-5 and medium 199-5. In fact, the CSV titer in the culture fluid of the MEM-5 flask was slightly higher (7.6×10^4 PFU/ml) than that of the 199-5 flask (4.1×10^4 PFU/ml). Because MEM-5 seemed to support the replication of CSV adequately, it was used for all studies.

Comparison of Plaque Assay Overlays

The same suspension of virus was titered with a warm agarose overlay and a methyl cellulose overlay. Also, MEM-5 without any solid

Figure 5. Electron micrographs of thin sections through CHSE-214 cells infected with chum salmon virus. Virions are present in the cytoplasm of infected cells. (78,750X)



or semi-solid overlay was included as a control because the characteristic CPE of the virus suggested that discrete plaques would form and could be counted if the assay was not incubated for more than four days. The results of the comparison of the overlay media indicated that the MEM-5-methyl cellulose mixture was superior. When agarose overlays were used, the resulting titers were less than 10^2 PFU/ml perhaps because the warm agarose (45°C) inactivated some virus or damaged some of the cells. The cell sheet under this overlay appeared in poor condition when compared with the controls. The titers obtained in the assay with only MEM-5 were somewhat lower (5.2×10^3 PFU/ml) than when the methyl cellulose overlay was used (2.2×10^4 PFU/ml). This experiment indicated that the methyl cellulose overlay would produce the most reliable titers for the plaque assay.

Determination of Adsorption Time

In addition to the type of overlay, a second variable in determining viral titers by plaque assay involves the length of time required for the virus to adsorb to the cell sheet. A suspension of virus was titered by plaque assay after adsorption times of 30, 60 and 120 min. After 120 min adsorption, the plaque assay titer of the suspension was 2.5×10^4 PFU/ml. After 60 min adsorption, the titer was 2.1×10^4 PFU/ml or 81% of the 2 hr time. An adsorption time of 30 min gave a titer of only 1.8×10^4 PFU/ml or 71% of the 2 hr time. This information suggested that adsorption times in excess of 60 min should be used.

Comparison of Plaque Assay and $TCID_{50}$ Titer

Two methods are commonly employed to determine virus titers, the plaque assay and the 50% tissue culture infectious dose ($TCID_{50}$). These were compared to determine if one was superior. A second objective of the experiment was to establish a correlation between the two methods that would allow comparisons between experiments in which different assays were used. The plaque assay titer of the virus stock was 1.0×10^3 PFU/ml. The $TCID_{50}$ titer of the same stock rose during incubation. When read at day 4, the titer was 2.0×10^2 $TCID_{50}$/ml; at day 7, 2.5×10^2 $TCID_{50}$/ml and by day 14, 1.3×10^3/ml, or approximately the same as the plaque assay titer. If incubation was continued at 18°C, the titer continued to rise and at 21 days was 2.5×10^3 $TCID_{50}$/ml and after 28 days was 1.0×10^4 $TCID_{50}$/ml. This slow rise in $TCID_{50}$ titer may be related to the enhanced infectivity seen after pH 3 treatment as the cell cultures become acidic during incubation. A 14 day incubation period was chosen for the $TCID_{50}$ assay because it correlated with the plaque assay.

Comparison of Cell-Associated and Released Virus

The proportion of virus produced by infected cells which is liberated into the culture fluid may vary considerably depending on the type of virus. The rhabdoviruses and other enveloped viruses may release nearly all the virions, while many non-enveloped viruses tend to remain cell-associated until cell destruction is extensive. Only about 20% of the infectious CSV was liberated into the culture fluid

of infected CHSE-214 cells after 4 days incubation at 18°C. The titer of the virus released in the culture fluid was 7.6 X 10^4 PFU/ml, while the titer of cell-associated virus was 3.5 X 10^5 PFU/ml. The cytoplasmic inclusions seen with acridine orange stain also suggest that large aggregates of virus can be expected to be found associated with the cells until CPE is nearly complete. Because a significant amount of CSV was cell-associated, the cells from infected cultures were included in the procedures used to harvest the virus.

Virus Replication at Selected Temperatures

Replication of CSV at selected temperatures was examined for two purposes: (1) to determine the optimum temperature for virus replication in order to obtain the high yields of virus required for biophysical tests, and (2) to show that CSV was a virus of poikilotherms and was not capable of replication at mammalian temperatures. The EPC cell line was chosen for these experiments because it had been shown to support the replication of CSV and could tolerate higher incubation temperatures (30-35°C) than the salmonid cell lines. The optimal temperature for viral replication was determined to be 15-20°C (Fig. 6). The extent of CPE in the cultures correlated with the virus titer produced at each temperature. At 10°C, virus replication was slower and the CPE less extensive than at 15 or 20°C. At 25°C a slight initial CPE was noted, but this disappeared after 2 days and the cell monolayer assumed an appearance similar to the control.

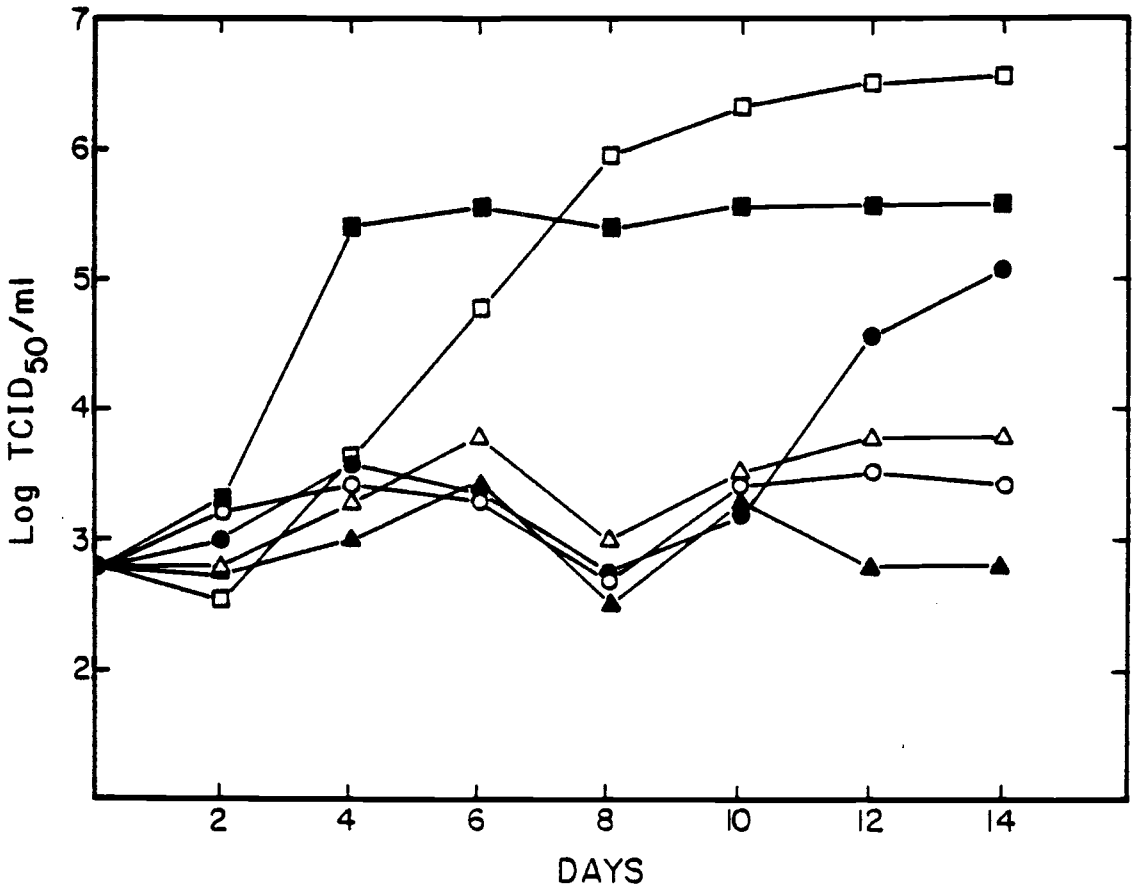


Figure 6. Concentration of infectious virus in the culture fluid of EPC cells infected with chum salmon virus and incubated at (0) 5°C, (●) 10°C, (□) 15°C, (■) 20°C, (Δ) 25°C, (▲) 30°C.

Virus Replication in Selected Cell Lines

The fish cell lines infected with CSV showed large differences in the ability to support virus replication. This information provides some indication of the potential host range, and was important in selecting a cell line to be used in the propagation of large quantities of virus. With the exception of the RTG-2 cell line, the best virus replication occurred in cells derived from salmonid fish (Table 3). The CHSE-214 and CHH-1 cell lines produced the highest titers. The RTG-2, FHM and BB cell lines showed no cytopathic effect and yielded very low titers of new virus. The other cell lines tested produced varying degrees of CPE and yielded moderate virus titers. This suggests that CSV may be capable of replicating in a variety of fish, especially salmonids. A similar property is seen with the mammalian reoviruses which are known to replicate in a wide range of cell lines and animals (Joklik et al., 1980).

Efficiency of Plating in Selected Cell Lines

The susceptibility to virus infection is an important criterion in selecting the appropriate cells to be used for virus assay. It also provides additional information on the potential host range of the virus. The sensitivity of selected fish cell lines to CSV was measured by efficiency of plating (EOP). The results, presented in Table 4, show the average number of plaques formed on each cell line after infection with 0.2 ml of a chum salmon virus suspension. The LBF-2 and EPC cells

Table 3. Titers of infectious virus released into the culture fluid from selected fish cell lines infected with 200 PFU of chum salmon virus and incubated 14 days at 18°C.

Cell Line	Abbreviation	Titer $TCID_{50}$/ml
Chum salmon	CHH-1	2.5×10^6
Chinook salmon	CHSE-214	1.6×10^6
Steelhead trout	STE-137	6.3×10^5
Kokanee salmon	KO-6	4.0×10^5
Largemouth bass	LBF-2	1.6×10^5
Bluegill	BF-2	6.3×10^4
Walleye	WC-1	6.3×10^4
Carp	EPC	3.9×10^4
Fathead minnow	FHM	5.0×10^2
Rainbow trout	RTG-2	5.0×10^2
Brown bullhead	BB	4.0×10^2

Table 4. Comparison of the plaquing efficiency of chum salmon virus on selected fish cell lines incubated at 18°C under a MEM-5-methyl cellulose overlay.

Cell Line	Abbreviation	Average Number of Plaques
Largemouth bass	LBF-2	604
Carp	EPC	393
Chinook salmon	CHSE-214	317
Steelhead trout	STE-137	231
Kokanee salmon	KO-6	224
Chum salmon	CHH-1	147
Walleye	WC-1	138
Bluegill	BF-2	42
Brown bullhead	BB	0
Fathead minnow	FHM	0
Rainbow trout	RTG-2	0

had the highest efficiency of plating while the CHSE-214, STE-137, CHH-1 and KO-6 cells were intermediate in susceptibility. The RTG-2, FHM and BB cell lines showed no evidence of infection. Although the CHSE-214 cell line had a lower efficiency of plating, the plaques were larger and more easily visible perhaps because the cell line produces greater amounts of virus. The CHSE-214 line was used for all virus assays as it represented a good compromise between EOP and accuracy.

Serologic Techniques

Neutralization Index of Virus Antiserum

Rabbit antiserum which was prepared against CSV was effective in neutralizing infectivity by this agent. After four days incubation on CHSE-214 cells at 18°C, the titer of virus treated with antiserum was 1.0×10^2 $TCID_{50}$/ml while the control titer was 1.6×10^6 $TCID_{50}$/ml. This reduction of over four $logs_{10}$ of infectivity gave a neutralization index (N.I.) of 4.2. After six days incubation, additional wells in the antiserum treated cultures began to exhibit CPE, however, the N.I. of 2.8 showed that the antiserum was still effective. Neutralization indicies at day 9 and 12 were both 2.0 indicating a reduction of two $logs_{10}$ or 99% of the infectious virus. This progressive increase in CPE is not unexpected as the neutralization of viral infectivity is a kinetic process and is reversible. The efficiency of neutralization depends on time, temperature and the nature of the virus, as well as the titer of the antiserum (Joklik et al., 1980).

Cross-Neutralization Tests

Cross neutralization tests were performed with antiserum against infectious pancreatic necrosis virus and the three serotypes of the mammalian reoviruses to test for the presence of common antigens. Chum salmon virus was incubated with anti-CSV serum and with antisera to each of the other viruses. The three reovirus serotypes and IPNV were each incubated with homologous sera and with antiserum against CSV. The titers are expressed as the highest dilution of the antiserum which prevented CPE in the cells.

No neutralization of chum salmon virus infectivity was observed by antisera against any of the other viruses tested (Table 5). Conversely, no cross-neutralization was seen by antiserum to CSV when tested against other viruses. These results indicate that no common antigens which can be detected by neutralization assay are present between CSV and any of the three mammalian reovirus serotypes. Likewise, no antigenic relationship was observed between IPNV and CSV.

Hemagglutination Test

The capacity to hemagglutinate human blood cells is an important taxonomic feature of the mammalian reoviruses. Chum salmon virus was tested for its ability to hemagglutinate erythrocytes by incubating a suspension of virus with human type O red blood cells. The virus did not show any hemagglutination. The three reovirus serotypes gave hemagglutination titers of 1:64 for type 1, 1:16 for type 2, and 1:32 for type 3. The lack of hemagglutination of human erythrocytes

Table 5. Cross-neutralization titers of chum salmon virus, infectious pancreatic necrosis virus and reovirus types 1, 2 and 3 incubated at 25°C for 1 hr with homologous or heterologous antiserum.

Virus	Antiserum Against	Titer[1]
Chum salmon virus	CSV	>1:20480
	Reo 1	<1:20
	Reo 2	<1:20
	Reo 3	<1:20
	IPNV	<1:20
Reovirus type 1	Reo 1	1:2560
	CSV	<1:20
Reovirus type 2	Reo 2	1:1280
	CSV	<1:20
Reovirus type 3	Reo 3	1:1280
	CSV	<1:20
Infectious pancreatic necrosis virus	IPNV	>1:20480
	CSV	<1:20

1. Titer is expressed as the highest dilution of the antiserum which prevented cytopathic effect in the cells used for the assay.

by CSV is a major point of difference between CSV and the orthoreoviruses.

Fluorescent Antibody Staining of Infected Cells

Infected and control CHSE-214 cells were incubated for 72 hr at 18°C and stained by the fluorescent antibody technique. The infected cultures exhibited specific fluorescence in the area immediately surrounding the plaques. Background fluorescence of the uninfected cells around these areas and in the controls was low. The specific fluorescence was uniform around the edge of the plaques. While the technique demonstrated that the antiserum was useful in detecting viral antigen, no concentrations of bright fluorescence which might indicate specific areas of active virus synthesis could be identified.

Biochemical and Biophysical Properties of Chum Salmon Virus

Resistance to Freeze-Thaw

Chum salmon virus was remarkably stable to repeated cycles of freezing and thawing. The titer of the original suspension was 2.7 X 10^5 PFU/ml. After three freeze-thaw cycles, the titer was 1.6 X 10^5 PFU/ml. This property of the virus made it possible to store aliquots of a stock virus suspension of known titer at -65°C.

Stability at 37 and 56°C

The stability of chum salmon virus at 37 and 56°C was measured by incubating a virus suspension at these temperatures and removing aliquots for titration at selected intervals. These titers were compared with a control suspension incubated at 18°C. The virus was stable during incubation at 18 and 37°C for 24 hr but unstable at 56°C (Fig. 7). At 56°C, the original suspension containing 2.7×10^5 PFU/ml declined in titer to 6.8×10^3 PFU/ml in one hr and no infectious virus remained after six hours. (A loss of infectivity of 1 $\log_{10}$ or greater in one hour is indicative of heat lability (Rovozzo and Burke, 1973).) No infectious virus was recovered when 1 ml of the suspension that had been incubated for 6 hr at 56°C was inoculated onto fresh monolayers of CHSE-214 cells, indicating a permanent inactivation had occurred.

Resistance to Lipid Solvents

The loss of infectivity after treatment with lipid solvents is evidence that a virus possesses a lipid containing envelope around the virion (Joklik et al., 1980). Chum salmon virus, infectious pancreatic necrosis virus (which lacks an envelope) and infectious hematopoietic necrosis virus (an enveloped virus) were tested for infectivity following treatment with chloroform or ether. Chum salmon virus was resistant to both chloroform and ether treatment (Table 6). The NaCl treated control and the ether or chloroform treated samples had similar titers. The IPNV control showed no loss of infectivity while the enveloped virus, IHNV, was completely inactivated by exposure to either

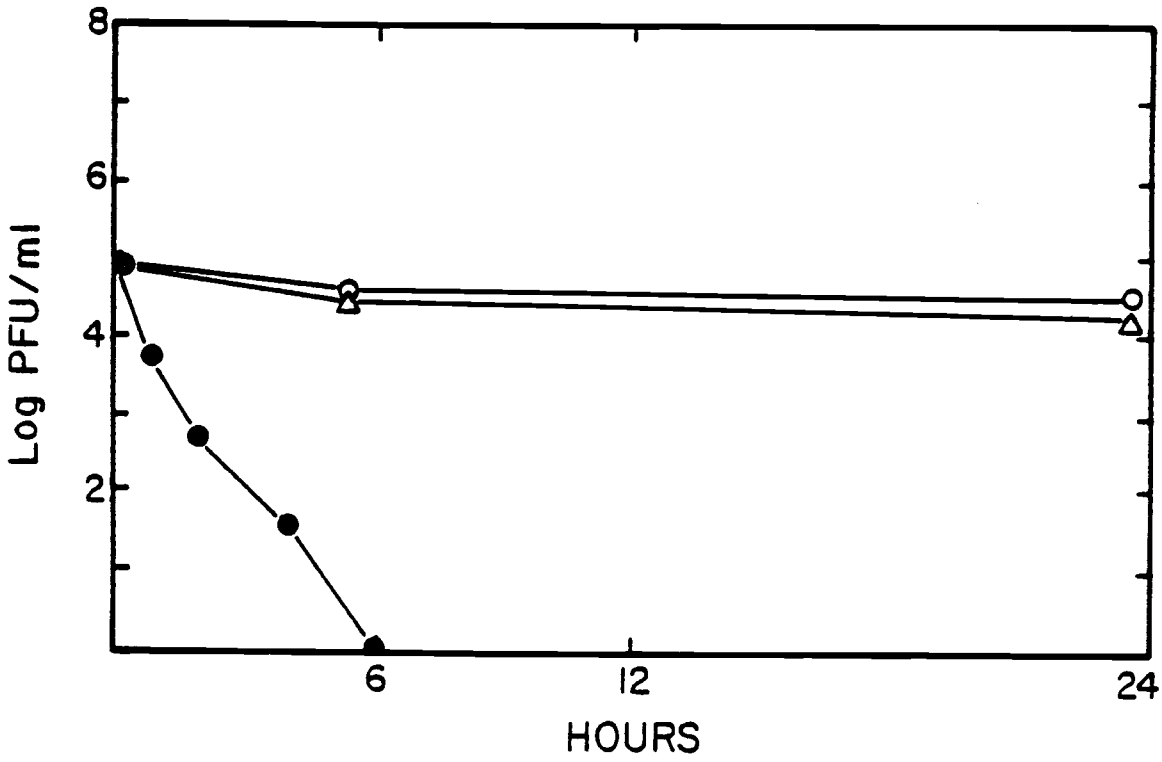


Figure 7. Concentrations of infectious virus in suspensions of chum salmon virus incubated at (0) 18°C, (Δ) 37°C and (●) 56°C.

Table 6. Titers of chum salmon virus, infectious pancreatic necrosis virus and infectious hematopoietic necrosis virus following treatment with lipid solvents.

Virus	Treatment[1]	Titer $TCID_{50}$/ml	Treatment[2]	Titer $TCID_{50}$/ml
Chum salmon virus	NaCl	1.5×10^5	NaCl	5.9×10^5
	Ether	2.6×10^5	Chloroform	1.7×10^6
Infectious pancreatic necrosis virus	NaCl	1.8×10^9	NaCl	1.3×10^9
	Ether	3.0×10^9	Chloroform	1.5×10^9
Infectious hematopoietic necrosis virus	NaCl	4.0×10^6	NaCl	8.1×10^6
	Ether	0	Chloroform	0

1. Treatment consisted of incubating a virus suspension with 0.85% NaCl or ether for 24 hr at 4°C.

2. Treatment consisted of incubating a virus suspension with 0.85% NaCl or chloroform for 10 min.

chemical. All viruses of the reovirus family are resistant to lipid solvents since they do not contain an essential lipid.

Effect of Chymotrypsin

To determine whether treatment with proteolytic enzymes would remove the outer capsid layer and result in enhanced infectivity, the chum salmon virus was treated with chymotrypsin and the preparations assayed for infectivity and examined by electron microscopy. A control suspension which was treated with buffer had a titer of 5.0×10^7 $TCID_{50}$/ml. The chymotrypsin treated suspension had a titer of 6.3×10^8 $TCID_{50}$/ml. Electron micrographs of each preparation revealed normal virions with a double capsid layer in the buffer treated controls. Numerous subviral particles 50-55 nm in diameter and much capsid debris could be seen in the chymotrypsin treated suspension (Fig. 8). The action of chymotrypsin on chum salmon virus demonstrated clearly the double capsid layer of the virion. It also served as an important taxonomic feature indicating a close relationship between chum salmon virus and the other viruses of the genus Orthoreovirus.

Resistance to pH 3

Incubation of chum salmon virus in MEM adjusted to pH 3 for 30 min did not reduce viral infectivity (Table 7). The titer after pH 3 treatment was, in fact, higher than the controls incubated at pH 7. The IPNV control was resistant to pH 3 and IHNV was inactivated.

Figure 8. Electron micrographs of purified chum salmon virus incubated for 1 hr at 37°C in (A) SSC buffer (B) SSC buffer containing 100 µg/ml α-chymotrypsin. Treated preparations show the removal of the outer capsid and the creation of 50-55 nm sub-viral particles. (129,200X)

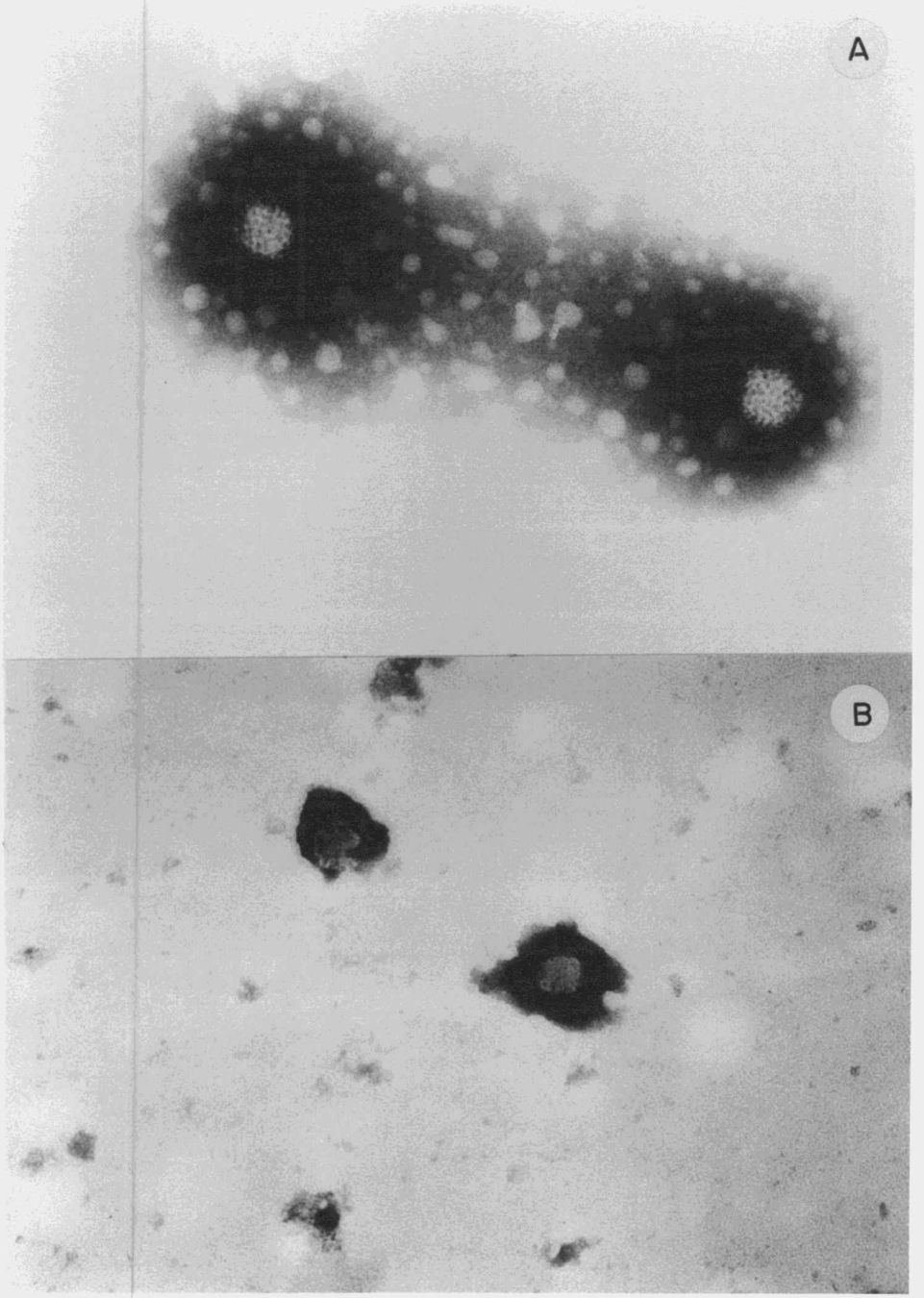

A
B

Table 7. Titers of chum salmon virus, infectious pancreatic necrosis virus and infectious hematopoietic necrosis virus following incubation at pH 3 for 30 min at 18°C.

Virus	Treatment[1]	Titer $TCID_{50}$/ml
Chum salmon virus	pH 7	3.2×10^5
	pH 3	5.0×10^6
Infectious pancreatic necrosis virus	pH 7	3.2×10^7
	pH 3	3.2×10^7
Infectious hematopoietic necrosis virus	pH 7	2.5×10^4
	pH 3	0

1. Virus was suspended in MEM adjusted to pH 3 or pH 7.

Electron micrographs of the virus preparations exposed to pH 3 revealed many 50-55 nm subviral particles and much capsid debris (Fig. 9). Electron micrographs of the controls incubated at pH 7 appeared normal and demonstrated that the removal of the outer capsid had not occurred during the preparation of the samples. The increase in titer of CSV after pH 3 treatment is probably due to the enhanced infectivity associated with the removal of the outer capsid.

Viral Replication in the Presence of 5-fluoro-2'-deoxyuridine

Viruses with an RNA genome are usually resistant to the action of 5-fluoro-2'-deoxyuridine (FUDR). Cultures of CHSE-214 cells were treated with either FUDR or BSS and the amount of chum salmon virus, infectious pancreatic necrosis virus and Herpesvirus salmonis produced by FUDR-treated or control cultures was compared. The deoxyuridine analog, FUDR, was not effective in blocking replication of CSV (Table 8). Control and treated cultures had similar titers and exhibited identical cytopathic effect during incubation. Replication of the DNA virus H. salmonis was inhibited, while IPNV, an RNA virus, was not affected by FUDR treatment. The lack of inhibition by halogenated pyrimidines is evidence that chum salmon virus possesses an RNA genome.

Density of the Virions in CsCl

Isopycnic centrifugation in cesium chloride gradients was used to determine the bouyant density of CSV. The density of the complete virion was 1.33 g/cc (Fig. 10). Refractive index measurements showed a

Figure 9. Electron micrographs of purified chum salmon virus incubated for 1 hr at 25°C in (A) SSC buffer at pH 7 (B) SSC buffer at pH 3. Subviral particles 50-55 nm in diameter and capsid debris are present in the pH 3 treated sample. (129,200X)

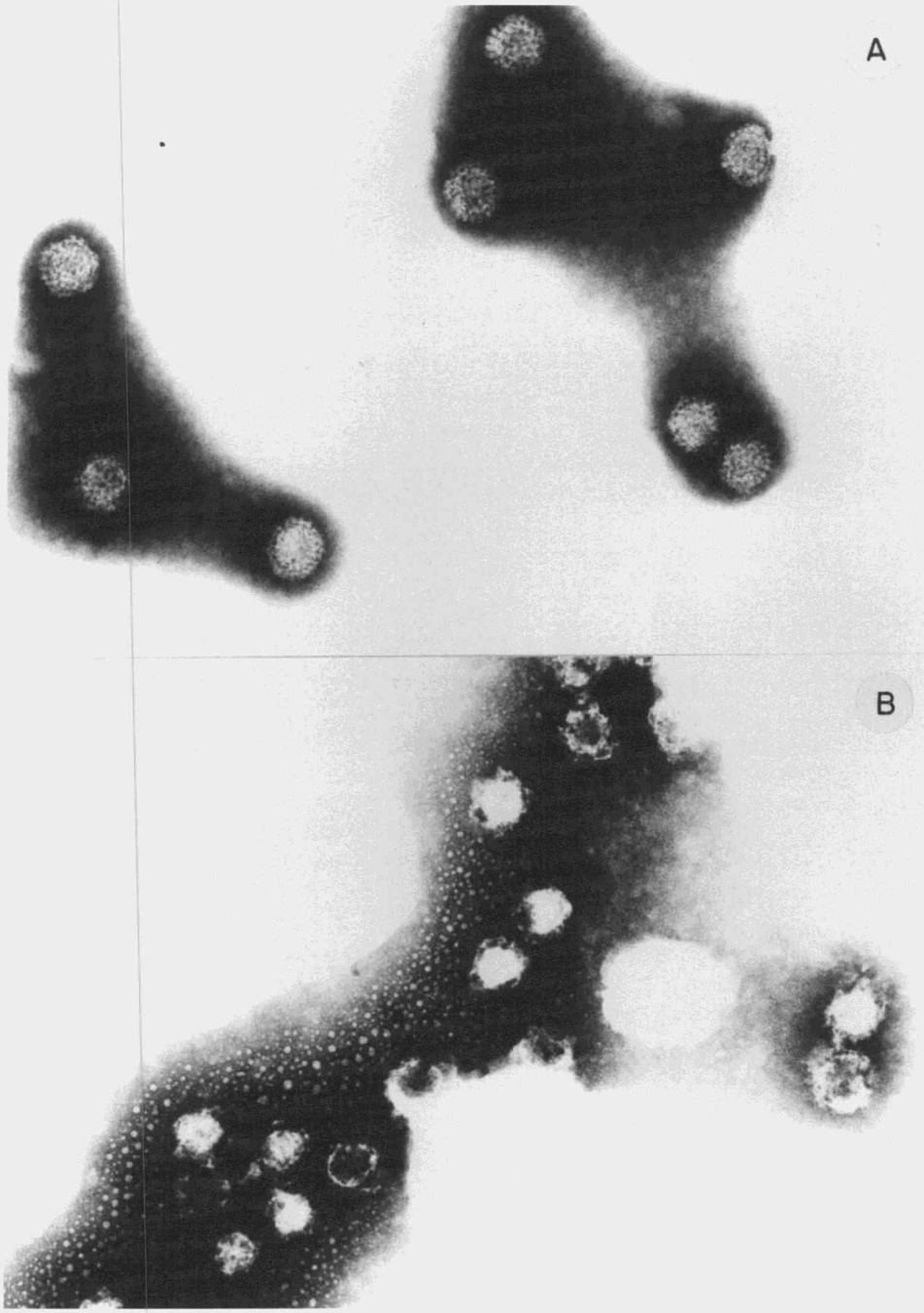

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Table 8. Titers of infectious virus released into the culture fluid from CHSE-214 cells infected with chum salmon virus, infectious pancreatic necrosis virus and Herpesvirus salmonis following treatment of the cells with 5-fluoro-2'-deoxyuridine.

Virus	Treatment[1]	Titer $TCID_{50}/ml$[2]
Chum salmon virus	BSS	1.0×10^{7}
	FUDR	6.3×10^{6}
Herpesvirus salmonis	BSS	1.0×10^{5}
	FUDR	1.0×10^{2}
Infectious pancreatic necrosis virus	BSS	3.2×10^{7}
	FUDR	2.0×10^{7}

1. Cells were incubated with BSS or BSS containing 10^{-4} M FUDR for 16 hr at 18°C before infection. Cells were infected with 0.1 ml stock virus and incubated at 18°C for CSV and IPNV, and 10°C for H. salmonis.

2. Titer of virus produced when CPE was extensive in BSS controls.

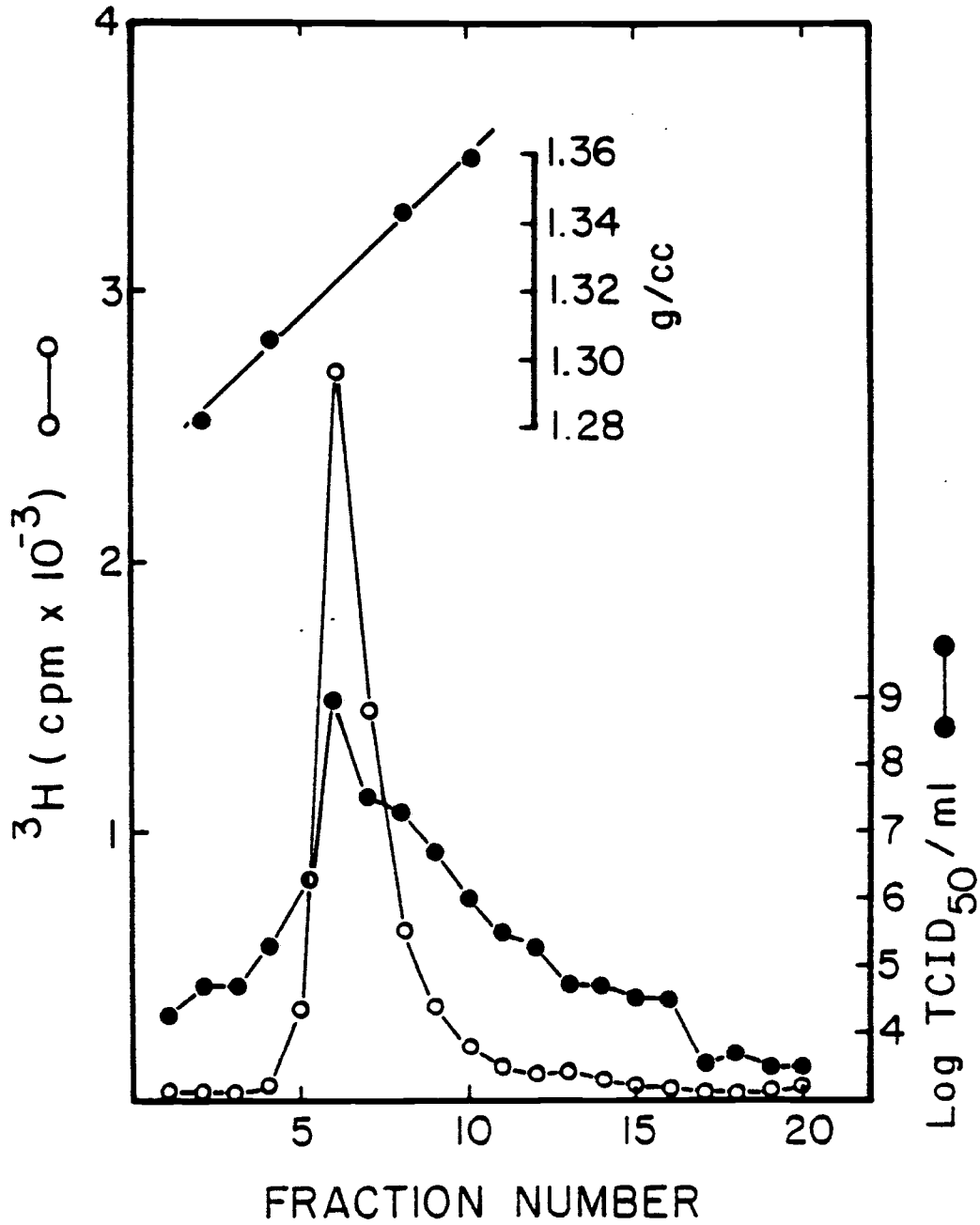


Figure 10. Isopycnic centrifugation in cesium chloride of chum salmon virus. The viral RNA was labeled with ^{3}H-uridine (0) and infectivity (●) determined by $TCID_{50}$ assay of each fraction. The density of selected fractions was determined by refractometer.

linear isopycnic gradient had formed. Repeated experiments gave the same density. In these tests, infectivity co-purified with radioactivity indicating the viral RNA had been labeled effectively and that the particles retained infectivity throughout the procedure. The density determined for CSV is within the reported range of the reovirus family (Cross and Fields, 1977).

Density of the Viral RNA

Isopycnic centrifugation in cesium sulfate gradients was used to determine the bouyant density of chum salmon virus RNA. The density of the viral RNA was determined to be 1.55 g/cc (Fig. 11). Repeated experiments gave the same results for centrifugation times between 24 and 60 hr. Density measurements of the gradient fractions indicated a linear isopycnic gradient had formed. This density is low compared to the reported density of the double-stranded reovirus RNA which is 1.60 to 1.61 g/cc. The reason for this discrepancy is unknown. Calibration checks of the refractometer used to determine the density of the fractions indicated no error was present.

Protein Analysis in Polyacrylamide Gels

Electrophoresis of chum salmon virus proteins on SDS-polyacrylamide gels revealed 5 major virion proteins (Fig. 12). At least two other protein bands were seen in many experiments. The extensive purification steps used (3 sucrose and 3 CsCl gradients for some preparations) suggest that the additional bands are probably of viral origin and may represent minor capsid proteins.

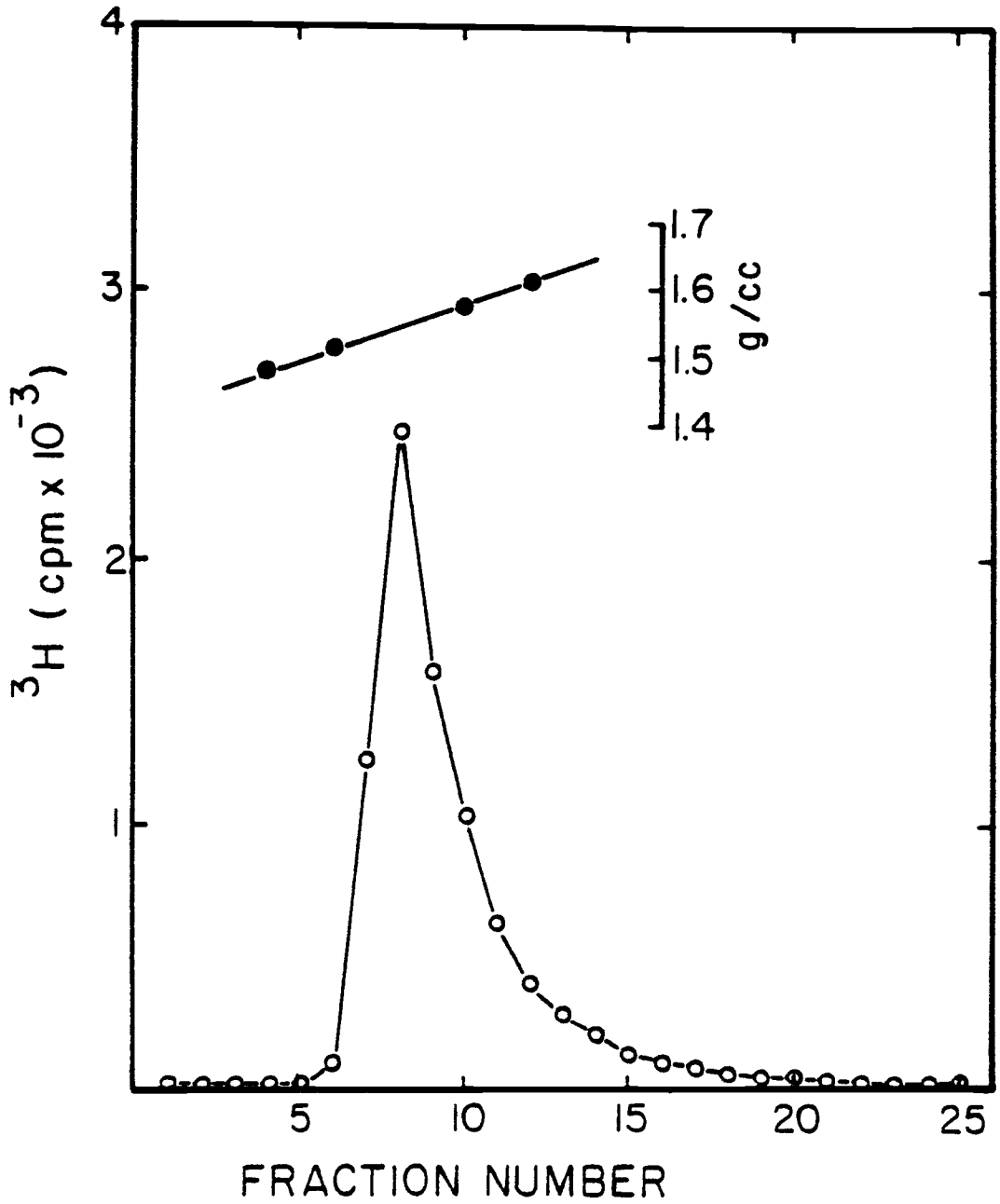
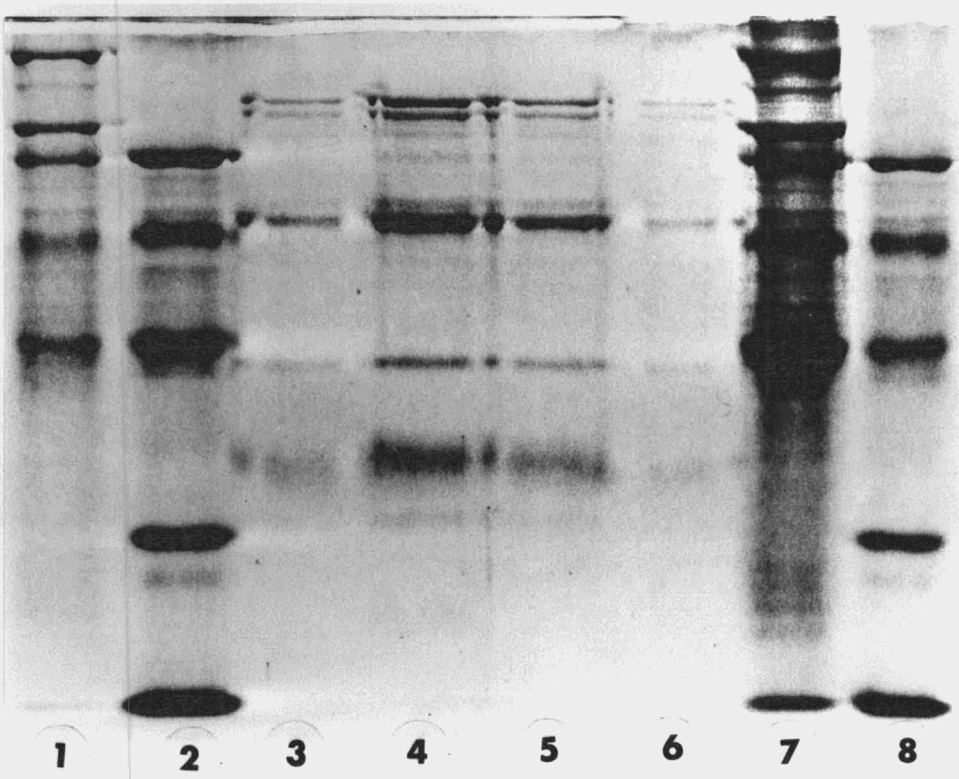


Figure 11. Isopycnic centrifugation in cesium sulfate of purified chum salmon virus RNA. The viral RNA was labeled with ^{3}H-uridine (O) and the density (●) of selected fractions determined by refractometer.

Figure 12. Photograph of a 12% polyacrylamide gel stained with Coomassie Blue after electrophoresis for 16 hr at 5 ma. The samples from left to right are 1. High molecular weight markers (top to bottom: myosin, 200,000; β-galactosidase, 116,250; phosphorylase B, 92,500; bovine serum albumin, 66,200; ovalbumin, 45,000 m.w.). 2. low molecular weight markers (top to bottom: phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; lysozyme, 14,400 m.w.). 3. and 4. Complete virions from lower band of CsCl gradient showing 5 major polypeptides of 145,000; 130,000; 75,000; 43,000 and 33,000 m.w. and 2 minor bands of 80,000 and 30,000 m.w. 5. and 6. Incomplete virions (upper band) showing same polypeptides as complete virions. 7. High molecular weight markers. 8. Low molecular weight markers.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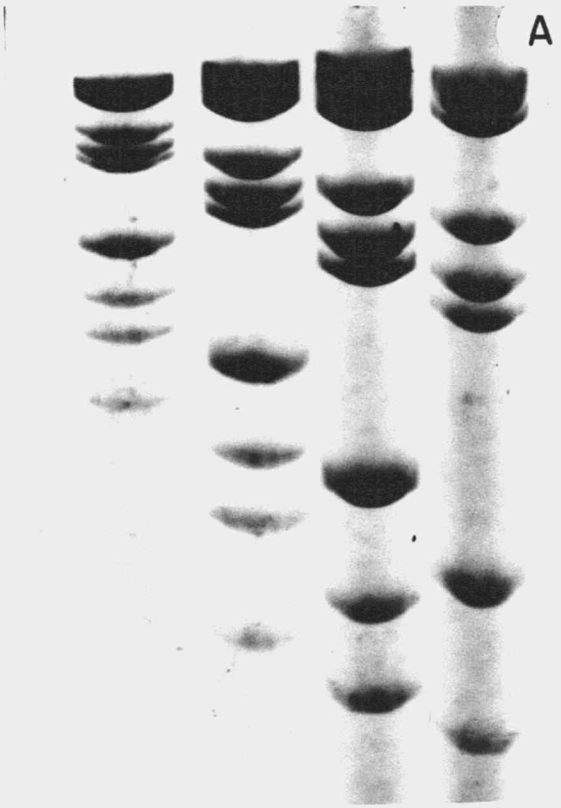
Most commonly, seven polypeptides, thought to be viral proteins, could be resolved. The molecular weight markers incorporated gave a linear plot of relative mobility versus molecular weight. The molecular weights of the five major virion proteins were: 145,000, 130,000, 75,000, 43,000 and 33,000 daltons. The two minor proteins had molecular weights of 80,000 and 30,000 daltons.

RNA Analysis in Polyacrylamide Gels

The viral genome of CSV was composed of 10 segments of RNA. Nine of these segments were routinely visible after electrophoresis in tube or slab gels (Fig. 13). The highest molecular weight segment at the top of the gel was more intensely stained than the rest of the RNA bands. Extensive electrophoresis in a 5% tube gel until nearly all the RNA segments had gone through the gel, resolved this high molecular weight band into two bands of RNA with only slightly differing molecular weights. The pattern resulting from the RNA analysis indicated the 10 segments fell into three size classes. A similar pattern is seen with the mammalian reoviruses (Joklik, 1974). The total genome weight was approximately 15.5 X 10^6 daltons, and the segments ranged in weight from 2.7 X 10^6 to 0.32 X 10^6 daltons (Table 9). All 10 segments of the reovirus type 3 RNA were visible and gave a linear plot of relative mobility versus molecular weight.

Figure 13. Photographs of polyacrylamide gels stained with methylene blue after electrophoresis. A. Tube gels (5%) after electrophoresis at 15 ma/gel for various times. Nine segments of chum salmon virus RNA are visible in some of the gels. B. Slab gels (5%) after electrophoresis at 15 ma/gel for 24 hr. Left to Right: Reovirus 3 RNA segments (10 segments in three size classes ranging from 2.5 X 10^6 to 0.61 X 10^6 daltons) chum salmon virus RNA segments (10 segments in three size classes ranging from 2.6 X 10^6 to 0.32 X 10^6 daltons).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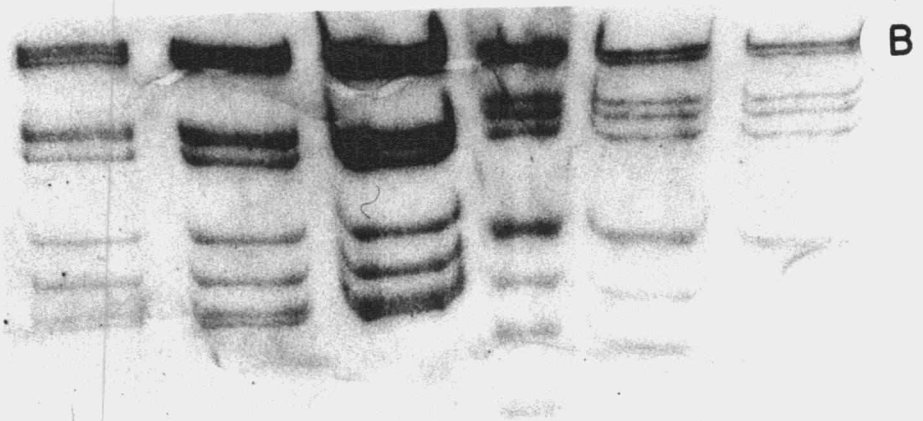
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Table 9. Comparison of the molecular weights of the RNA segments comprising the genome of chum salmon virus and reovirus type 3 determined by electrophoresis in polyacrylamide gels.

	Molecular Weight X 10^6 Daltons	
Segment[1]	Chum Salmon Virus	Reovirus Type 3
L 1	2.6	2.5
L 2	2.6	2.4
L 3	2.4	2.3
M 1	1.9	1.6
M 2	1.8	1.6
M 3	1.7	1.4
S 1	0.87	0.92
S 2	0.62	0.76
S 3	0.50	0.64
S 4	0.32	0.61
Total Weight	15.3 X 10^6	14.7 X 10^6

1. Segments are classed large (L), medium (M) and small (S).

In Vivo Studies

Pathogenicity for Chum, Chinook and Kokanee Salmon and Rainbow Trout

The pathogenicity of chum salmon virus for commercially important salmonids was tested. No substantial mortality occurred in the chum, chinook and kokanee salmon or in rainbow trout fry injected with chum salmon virus and held for 42 days at 12°C. No gross external signs of infection were seen nor was gross internal pathology observed among the few fry dying during the course of the experiment. The $TCID_{50}$ titers of those mortalities in the virus-injected group were not elevated compared to the titers of virus-injected fish which did not die.

Viral Replication in Chum, Chinook and Kokanee Salmon and Rainbow Trout

The virus titers of infected fish were determined at intervals to test for the production of new virus. Chum salmon virus replicates in all four species tested (Fig. 14). The virus titers increased gradually in the chinook salmon and rainbow trout fry. Virus replication in the chum and kokanee salmon fry increased 1.5 $\log_{10}$ in the second week of infection. Maximum titers in chum salmon ($10^{6.3}$ $TCID_{50}$/ml) occurred 21 days after injection and were somewhat above the titers of the other species. At the end of the 42 day experiment the virus titers in all four species were nearly the same (approximately $10^{-5.0}$ $TCID_{50}$/ml).

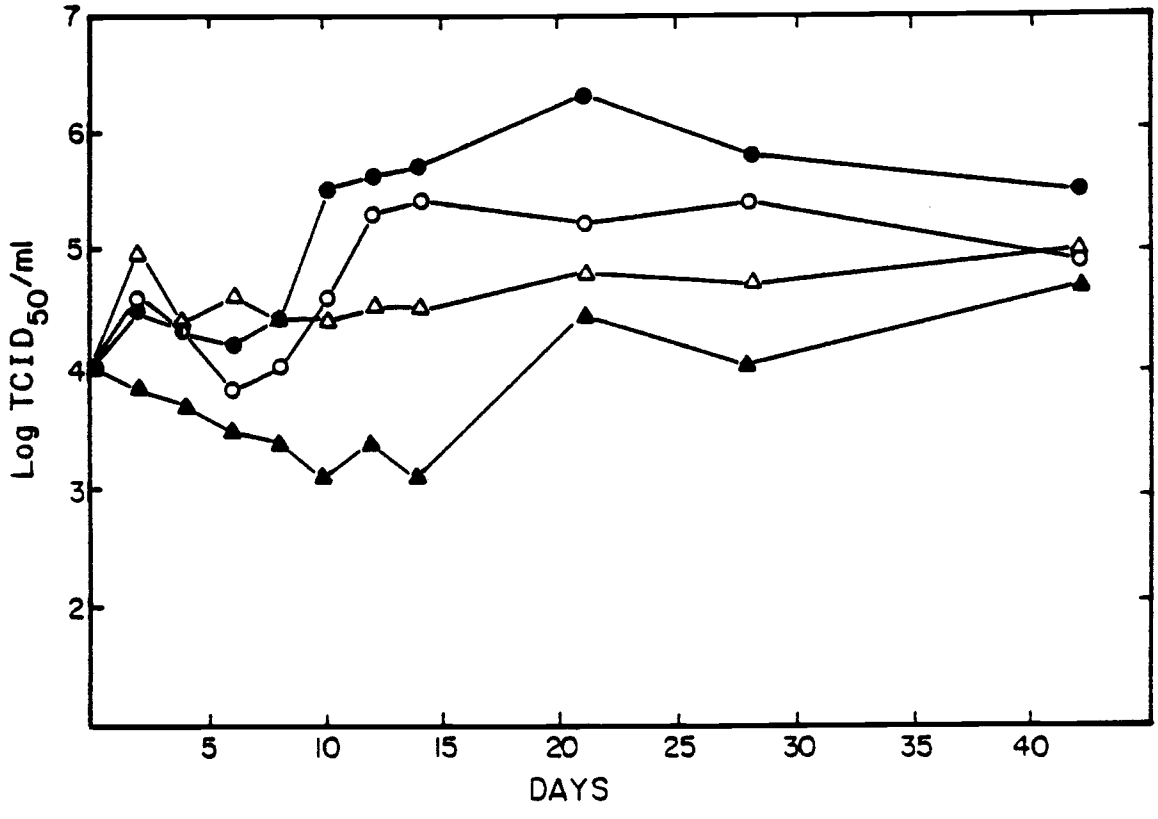

Figure 14. Titer of infectious chum salmon virus in homogenates of whole (0) kokanee, (●) chum, (Δ) chinook salmon fry and (▲) rainbow trout fry. Fry weighing 1.0-2.0 g were injected i.p. with 10^4 $TCID_{50}$ of purified virus in 0.02 ml MEM and held for 42 days at 12°C in 10 liter aquaria.

Histology of Virus Infected Chum, Chinook and Kokanee Salmon and Rainbow Trout

Histological examination provided evidence of pathology caused by CSV in chum and chinook salmon fry. Areas of focal necrosis were observed in the liver sections from infected fish. In the chum salmon fry (Fig. 15), these lesions began as small necrotic foci at day 8 post infection. After 14 days, the lesions were well developed with some large syncitial cells and a coagulation necrosis of the hepatocytes. The lesions began to heal by day 21. Normal cellular architecture began to return, however after 42 days, when the experiment was terminated, the hepatic architecture showed some remaining disorganization. A similar histological picture was seen in the chinook salmon fry (Fig. 16); however, the extent and severity of the lesions were less. No pathology was observed in the liver of infected kokanee salmon or rainbow trout. All other internal organs of the four species tested were unremarkable. The pathology observed in experimentally infected chum and chinook fry is best described as a focal necrotizing hepatitis.

Acridine Orange, May-Grunwald-Giemsa, and Fluorescent Antibody Stains of Infected Chum Salmon

A cryostat was used to cut serial, frozen, transverse sections through the visceral portion of a chum salmon fry which had been infected for 14 days. The serial sections were placed on separate slides and stained by acridine orange, May-Grunwald-Giemsa and acridine orange stain. Acridine orange stains of the first of the sections revealed sites of active nucleic acid synthesis in the liver.

Figure 15. Photomicrographs of hematoxylin and eosin stained sections through the liver of 1.0-2.0 g chum salmon fry held at 12°C in 10 liter aquaria. Control fry (A) were injected i.p. with 0.02 ml of MEM. Infected fish received 0.02 ml of MEM containing 10^4 $TCID_{50}$ of chum salmon virus and were examined at (B) 8 days, (C) 14 days, (D) 21 days, (E) 28 days, and (F) 42 days. Focal necrosis of the liver is most obvious in the 14 and 21 day sections.

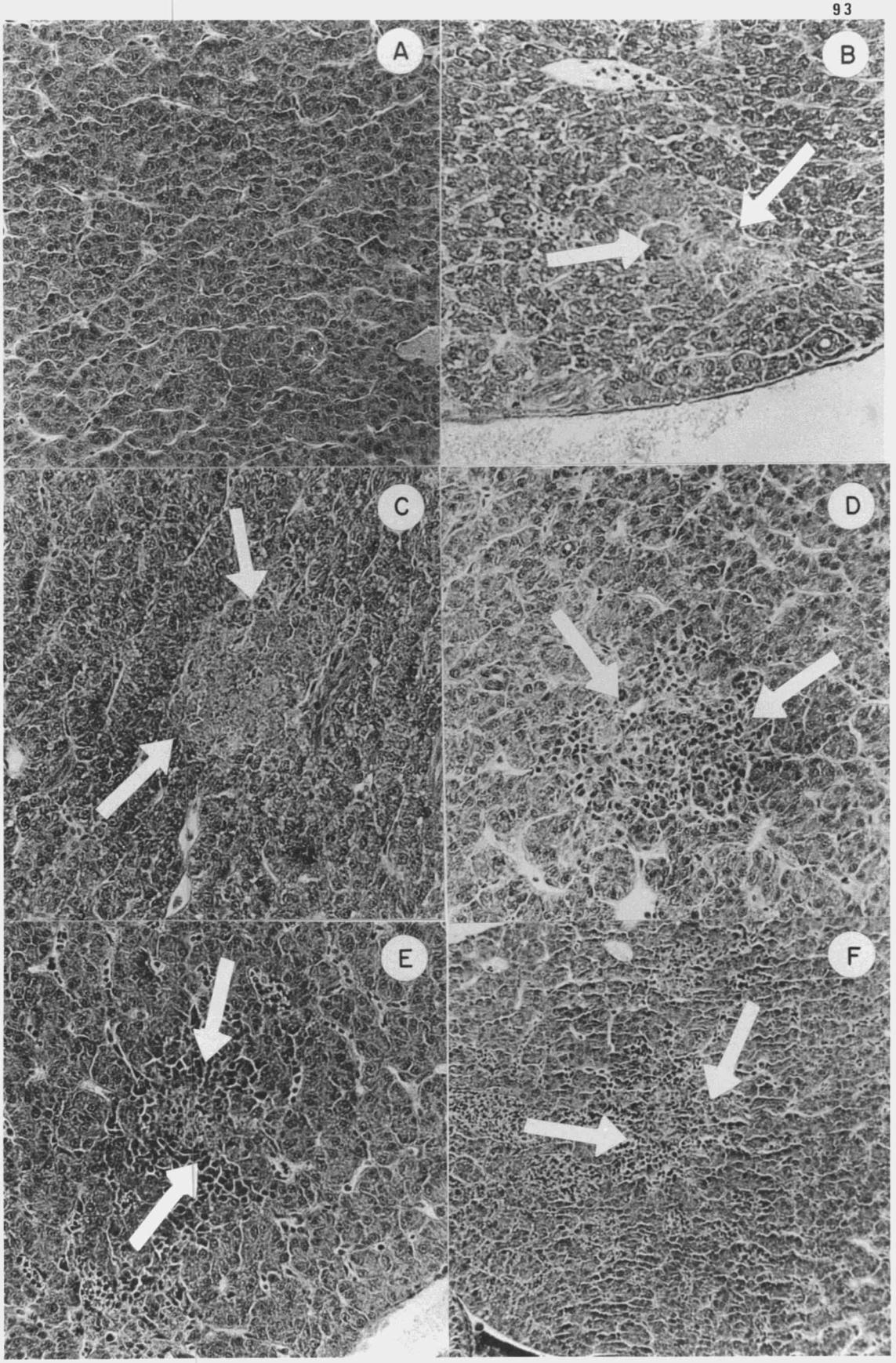
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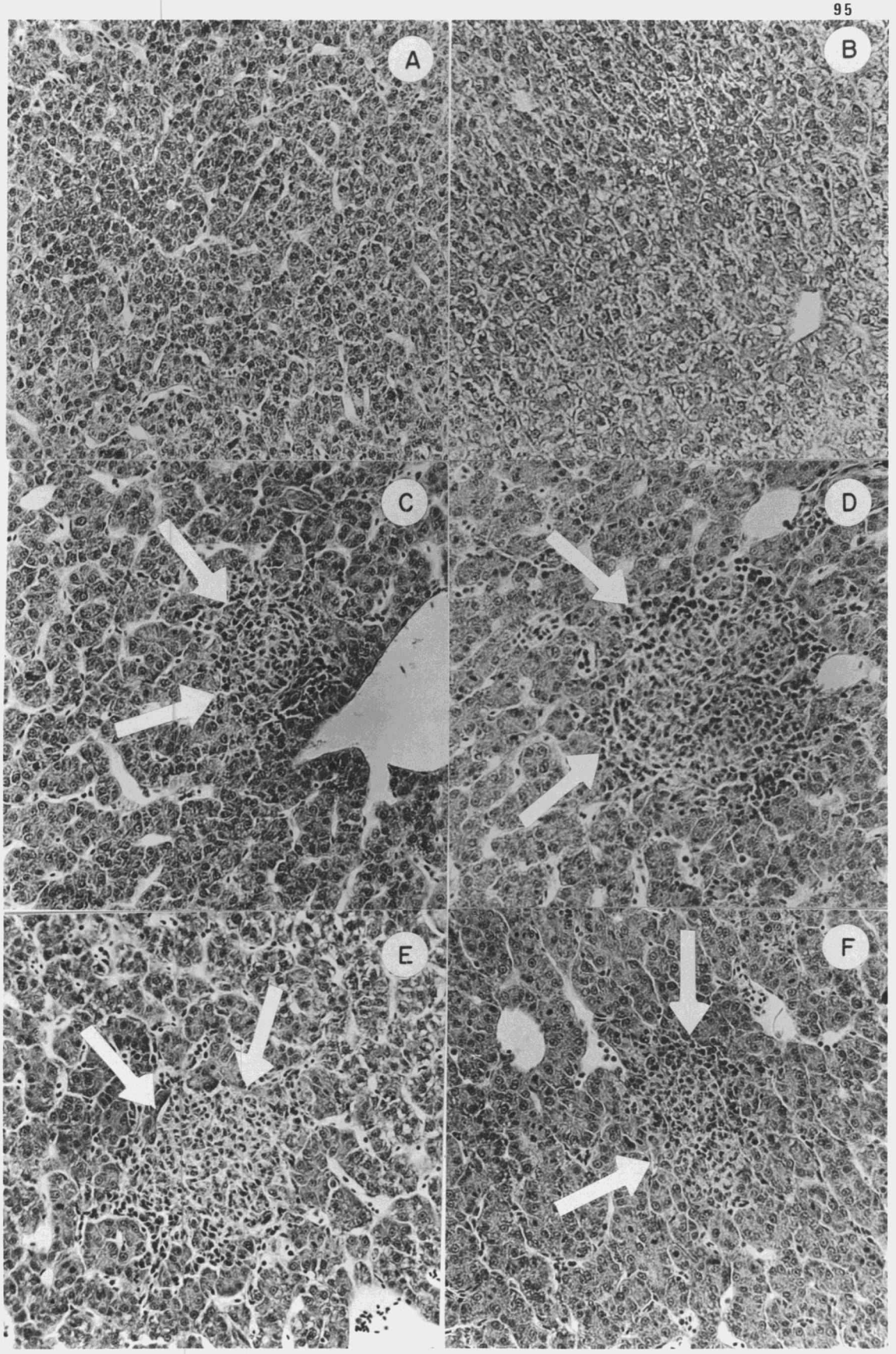
Figure 16. Photomicrographs of hematoxylin and eosin stained sections through the liver of 1.0-2.0 g chinook salmon fry held at 12°C in 10 liter aquaria. The control fry (A) were injected i.p. with 0.02 ml MEM. Infected fish received 0.02 ml MEM containing 10^4 $TCID_{50}$ of chum salmon virus and were examined at (B) 8 days, (C) 14 days, (D) 21 days, (E) 28 days and (F) 42 days.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These sites exhibited intense green fluorescence (Fig. 17). May-Grumwald-Giemsa stains of the adjacent section showed these areas were foci of necrosis. A third serial section also containing the lesion stained by the fluorescent antibody technique, indicated that these foci were sites of viral synthesis. High levels of specific fluorescence were observed. No other internal organs, the areas of normal liver surrounding these lesions nor the control fish showed any of these features.

Characteristics of Chum Salmon Virus

A listing of the known biological, biophysical, and chemical features of chum salmon virus is given in Table 10. This list will serve as a reference point for a discussion of the taxonomic position of this virus and as a summary of important characteristics.

Figure 17. Photomicrographs of stained serial frozen sections through the liver of a 1.0 g chum salmon fry injected i.p. with 10^4 $TCID_{50}$ of chum salmon virus and held for 14 days at 12°C in a 10 liter aquarium. The serial sections show the same lesion stained with (A) acridine orange (B) May-Grunwald-Giemsa and (C) fluorescent antibody methods.

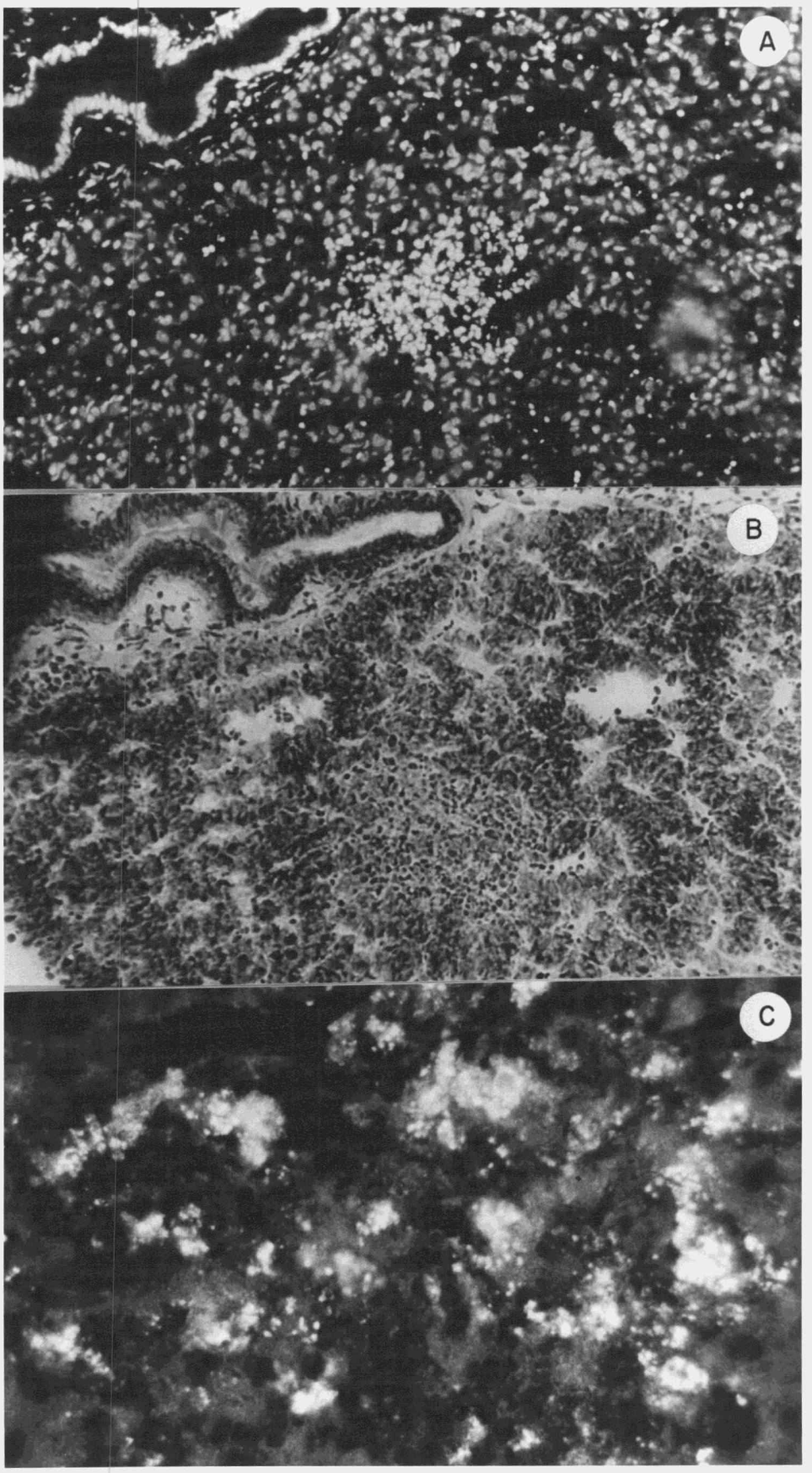
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Table 10. Summary of the known characteristics of chum salmon virus.

Isolated from:	Adult chum salmon (Oncorhynchus keta) October 23, 1978, Tokushibetsu Hatchery, Hokkaido, Japan
Good replication in:	CHSE-214, CHH-1, STE-137, KO-6
Poor replication in:	RTG-2, FHM, BB
Optimum temperature range:	15-20°C
Type of cytopathic effect:	Cell fusion, destruction of membranes, nuclei remain intact, forms plaques
Acridine orange stain:	Green cytoplasmic inclusions
Morphology of the virion:	Naked, quasi-spherical, non-enveloped icosahedral symmetry, double capsid, twenty peripheral capsomeres in profile
Diameter of virion:	Complete particle 73-78 nm Core 50-55 nm
Stability at 56°C:	Unstable
37°C:	Stable
Viral replication at 30°C:	Negative
25°C:	Negative
20°C:	Rapid
15°C:	Rapid
10°C:	Slow in 14 days
5°C:	Negative
Resistance to chloroform and ether:	Resistant - no loss of infectivity lacks essential lipids
Stability to CsCl:	Stable
Freeze-thaw:	Stable
Effect of pH 3 and chymotrypsin:	Outer capsid removed, enhanced infectivity
Hemagglutination:	Negative with human O

Table 10. Continued.

Genome:	10 Segments double-stranded RNA range 0.3-2.7 X 10^6 m.w. total 15.3 X 10^6 m.w.
Protein structure:	5 major capsid proteins 145,000, 130,000, 75,000, 43,000, 33,000 m.w. Two minor proteins 80,000 and 30,000 m.w.
Pathogenicity:	Focal necrosis in liver of chum and chinook salmon. Mortality limited in number. No pathogenicity in rainbow trout or kokanee salmon
Name of disease:	Focal necrotizing hepatitis

DISCUSSION

This report describes the isolation and characterization of a new reovirus from chum salmon. During the course of the study, it was of interest to determine specific properties of the virus which would lead to appropriate taxonomic placement. It was also desirable to test the pathogenicity of the isolate for commercially important salmonid fish and to define the geographic distribution of the virus.

After the isolation of the virus and confirmation by electron microscopy, the first experiments were designed to determine how to cultivate and enumerate the virus. These studies indicated that the virus could be propagated and titered by the methods commonly used for other fish viruses.

When the growth and assay conditions for CSV were established, experiments to characterize the virus were begun. The replication studies at selected temperatures or in selected cell lines indicated that CSV did not replicate at temperatures of 25°C or higher and that the virus was well adapted for growth in fish cells. This information provided strong evidence that the isolate was a virus of cold-blooded animals, probably fish, and not an accidental contaminant from human or other warm-blooded animal sources. The preference of CSV for salmonid cell lines seemed to suggest that the agent was a salmonid fish virus.

The determination that CSV was a reovirus was based on morphological and biochemical characteristics. Electron micrographs provided the initial evidence that CSV might be a reovirus. These showed 75 nm spherical particles which were morphologically similar to other reoviruses. Studies to establish the type of nucleic acid included acridine orange stains and inhibition by FUDR. These experiments indicated that the CSV genome was composed of double stranded RNA. Subsequently, the separation of the RNA into multiple genome segments in polyacrylamide gels provided additional evidence that CSV belonged to the reovirus group.

There are several biochemical and biophysical characteristics which are used to classify the Reoviridae into six genera. Among these characteristics are the effect of pH 3 and chymotrypsin, the number of segments of the RNA genome, the number of capsid proteins, and stability in CsCl and Freon 113. Using these criteria, the chum salmon virus would be included in the genus, Orthoreovirus.

However, members of the genus Orthoreovirus are normally isolated from mammals and birds and have two features which CSV does not possess. These viruses are stable at 56°C and hemagglutinate erythrocytes. Although CSV lacks these characteristics, it might be expected that an agent isolated from fish would not have the ability to withstand high temperatures nor to react with cells of a phylogenetically distant animal.

There are two possible taxonomic positions of CSV within the Reoviridae: 1) as a type species of a new, and seventh, genus of the family Reoviridae or; 2) as the only member of the genus Orthoreovirus with a cold-blooded host, perhaps as a separate sub-genus. Viral taxonomists have been slow to adopt the binomial nomenclature of the other biological sciences because the system implies a degree of genetic or evolutionary relatedness which is difficult to prove for many viruses. No viruses of the family Reoviridae are currently assigned species names, but, in the future, the name Orthoreovirus keta may be appropriate for CSV as it reflects the isolation from chum salmon (Oncorhynchus keta).

In addition to establishing the taxonomic position of this previously undescribed virus, the pathogenicity of CSV for salmonid fish was examined. This study was designed to assess the potential dangers that this virus might pose for these commercially important animals. Four species of fish, chum, chinook, and kokanee salmon and rainbow trout were examined for their susceptibility to CSV infection. The agent replicated in all four species tested; however, no mortality which could be ascribed to the virus occurred. The liver tissue of infected chum and chinook salmon fry developed areas of focal necrosis which were associated with viral replication. Although these lesions were not severe, this pathology could debilitate infected fish. Infection with CSV could decrease the animal's ability to resist physical or chemical stress or cause an increased susceptibility to other

potential infectious agents. Thus, although CSV has not been shown to be a highly virulent fish pathogen, the virus could be indirectly involved in mortality.

The histopathology associated with CSV infection is unlike that observed with other fish viruses. Chum salmon virus has a limited pathogenicity for the species tested with the liver as a target organ. The basis of this organotropy is unknown. Most virulent fish viruses demonstrate a widespread histopathology involving many internal organs. This pathology is often progressive, especially in fry, beginning in the most susceptible cells and eventually resulting in a generalized viremia, hemorrhage and necrosis in several internal organs. A few viruses of fish are confined to specific cells or areas of the body. Viral erythrocytic necrosis virus is observed only in blood cells while some of the neoplastic lesions caused by presumed retroviruses are found in specific locations.

The possibility that CSV could have deleterious effects on some fish populations stimulated examination concerning the geographic range of this virus in stocks of salmon around the Pacific rim. Fish from the northeastern coast of Hokkaido were of special interest. Virological examination of salmon along the Pacific coasts of the United States and Canada has been performed for many years without isolation of this virus. It was thought that the virus might have been undetected in Japan because, until recently, many Japanese microbiologists performed virus examinations of fish using only the RTG-2 cell line which does not support the replication of CSV.

Much effort went toward the reisolation of CSV from fish in Japan. The initial isolation occurred in the fall of 1978 from the stocks of adult chum salmon at Tokushibetsu Hatchery. The progeny of these fish and two other species at the hatchery, were sampled in the Spring of 1979 to determine if the virus had been transmitted to the fry. No virus was isolated from any of the three species of fry. That fall, adult fish were again sampled without success.

At the conclusion of the 1980 sampling program, it was apparent that the virus was not widespread among salmon stocks in northeastern Hokkaido. The cooperative sampling program that year included marine fish and shellfish in order to examine other possible host species. The shellfish were of interest because they serve as biological filters for concentrating viruses and because of a report by Meyers (1979) describing the isolation of a virus from oysters with properties similar to CSV.

The low temperature required for the replication of CSV (15-20°C) indicates that the natural host range of the virus is confined to cold blooded animals. Although, at this time it is impossible to determine the host range or potential reservoirs of CSV, the replication in salmonid fish and fish cell lines suggests that this agent is a "fish virus". Reoviruses, however, are noted for the ability to infect a wide range of cell lines and animal hosts. The natural reservoir of CSV is probably composed of several species of marine fish.

One of the most important areas of research regarding CSV, will be the examination of other species of marine and freshwater fish and shellfish for the presence of the virus to establish its possible origin. The single isolation of the virus from all the samples collected, suggests that the virus is not common among salmon stocks on Hokkaido. The susceptibility of marine fish and shellfish to CSV infection should also be studied in an effort to learn more about the potential host range.

In addition to the examination of other species of fish and shellfish for the presence of CSV or susceptibility to infection, there are some areas of future research which are of interest. A detailed comparison of CSV and the reolike viruses of fish and shellfish should be performed. While some of these viruses have been extensively characterized (IPNV), little is known about others. Two viruses, the golden shiner virus (Plumb et al., 1979) and the virus isolated from oysters by Meyers (1979) have some properties similar to CSV. Further work on the serology and molecular biology of these viruses and their relationship to CSV would be important in establishing if these isolates are related.

Another area of future research involves additional characterization of the virus. Tests for hemagglutination of selected fish erythrocytes by CSV would demonstrate if a hemagglutinin is present in the virion. The proteins of the inner capsid and the complete particle should be compared on polyacrylamide gels. This would allow identification of inner and outer capsid proteins.

An additional conclusion from this study which should be drawn in the importance of certification examinations of fish. After CSV was isolated from the adult salmon samples, eggs from these fish could not be imported. This process prevented the introduction of a new and potentially damaging virus. The primary control measure for viral diseases of fish is avoidance of the agent. Certification examination are designed to prevent the spread of these viral diseases among animal populations.

SUMMARY AND CONCLUSIONS

1. A previously undescribed virus, tentatively termed chum salmon virus (CSV), has been isolated from chum salmon in northern Japan. Based on morphology and biochemical and biophysical tests the virus is a member of the family Reoviridae. Of the genera contained in this family, CSV most resembles the viruses in the genus Orthoreovirus.

2. The virus replicated in salmonid cell lines incubated between 15 and 20°C producing CPE characterized by destruction of cell membranes, cytoplasmic fusion and the formation of plaques with intact nuclei. Acridine orange stains revealed green-staining cytoplasmic inclusions.

3. The virion had a quasi-spherical shape with icosahedral symmetry, a double capsid, and twenty peripheral capsomeres visible in profile in negatively stained preparations. The intact virion was 75 nm in diameter. The removal of the outer capsid resulted in a subviral particle 50-55 nm in diameter.

4. The virus was resistant to freeze-thaw, ether, chloroform, 37°C, pH 3 and chymotrypsin. Treatment with chymotrypsin and pH 3 removed the outer capsid creating subviral particles with enhanced infectivity.

5. The genome of the virus was composed of 10 segments of double-stranded RNA with molecular weights of 2.7 to 0.3 X 10^6 daltons. The total genome weight was 15.3 X 10^6 daltons.

6. The capsid of CSV was composed of 5 major virion polypeptides of m.w. 145,000, 130,000, 75,000, 43,000 and 33,000. Two minor proteins of m.w. 80,000 and 30,000 were thought to be present.

7. The virus caused limited mortality for chum and chinook salmon. Chum and chinook salmon developed a focal necrotizing hepatitis. Rainbow trout and kokanee salmon showed no evidence of pathology. The virus replicated in all four species tested.

8. The origin of the virus in the chum salmon examined is not known. The virus does not seem to be common among salmon populations in Japan.

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