The virion protein patterns of 72 isolates of infectious hematopoietic necrosis virus (IHNV) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of S-methionine labeled virus. This analysis led to the classification of these virus isolates into 4 or more types. Type 1 virus is characterized by a nucleocapsid protein with an approximate molecular weight of 40,500 daltons. The type 2 and 3 viruses have N proteins of 42,800 and 43,250 daltons respectively. Virus belonging to Type 2 was responsible for the recent epizootics of IHN among fish in the lower Columbia River. The California IHNV isolates were Type 3 with the exception of those isolated from fish at Coleman Hatchery on the Sacramento River. These Coleman Hatchery isolates belonged to a Type 4 virus group which are characterized by a larger G or glycoprotein of approximately 70,000 daltons. All other viruses examined have G proteins of 67,000 daltons. The Type 5 virus isolates were not sufficiently distinct to warrant classification into a separate type. These findings have been useful in determining that (1) a particular virus type is
characteristic for a geographic area and will infect many different salmonid species in that area and (2) the same type isolated from parental fish is responsible for the subsequent outbreak of the disease in progeny.

The specific radioactive labeling of virus proteins in the infected cell has been used to study the intracellular synthesis of viral proteins. The temporal synthesis of the viral polypeptides suggest that they were derived from the translation of independently transcribed monocistronic mRNAs.

Antibody to IHNV was measured by solid phase direct binding assays with Iodinated Protein A or with immunoperoxidase staining. The high binding antibody titer of rabbit anti-IHNV serum made possible the development of two immunological tests for IHNV. Both methods were specific, sensitive to less than 10 ng of virus protein and represented a new method of characterizing different strains of IHNV.
A Molecular Study of the Viral Proteins of Infectious Hematopoietic Necrosis Virus

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A MOLECULAR STUDY OF THE VIRAL PROTEINS OF INFECTIOUS
HEMATOPOIETIC NECROSIS VIRUS

I. LITERATURE REVIEW

Historical Perspective

In 1953, a viral disease among sockeye salmon (Oncorhynchus nerka) was reported in hatcheries at Winthrop and Leavenworth, Washington (Rucker et al., 1953). A viral etiology was suspected when the condition was transmitted by cell-free filtrates and the disease did not respond to antibiotic therapy (Watson, 1954). This particular isolate of the viral agent was not retained in storage.

An epizootic of a similar disease with high mortality occurred among juvenile sockeye salmon at the Willamette River Salmon Hatchery in Oregon (Wingfield et al., 1969) in 1958. The virus was isolated from the diseased fish and a characterization of its physical-chemical properties was made (Wingfield, 1968; McCain et al., 1974).

In 1958 an epizootic also occurred among Chinook salmon (Oncorhynchus tschawytscha) fry at a California hatchery. A few months later, another outbreak was reported at the Sacramento River Hatchery in California (Ross et al., 1960; Parisot, 1962). The disease was named "Sacramento River Chinook Disease" after the species and site of
isolation. Although the natural host of this agent was chinook salmon, both healthy chinook salmon and cutthroat trout (Salmo clarki) were susceptible to infection with cell-free filtrates from infected fish.

Two identical viruses from rainbow trout (Salmo gairdneri) and sockeye salmon in British Columbia, Canada, were isolated in 1967 (Amend et al., 1969). The name "infectious hematopoietic necrosis" was proposed to describe the outstanding necrosis of the hematopoietic tissue of the kidney and spleen in infected fish. Subsequently, Amend and Chambers (1970) proposed that all three viruses: British Columbia Virus, the Oregon sockeye salmon virus, and the virus of the Sacramento River chinook disease, be referred to as infectious hematopoietic necrosis virus (IHNV). This proposal was based on the fact that all three viruses possessed the same bullet-shaped structure with similar dimensions upon electron microscopic examination and all three produced the same type of cytopathic effect in cell culture.

Geographic Distribution:

Infectious hematopoietic necrosis virus was originally found to be endemic to the Pacific Northwest of the United States. However, it has since been found in Alaska, British Columbia, Japan, and most recently, Taiwan (Chen, Kou, Hedrick, and Fryer, in press). The disease was first reported in sockeye salmon in Washington State in 1953 (Rucker et al., 1953). Since then, the virus has been found to be widely distributed among six salmonid fish species reared in hatcheries

In Oregon, the first report of IHNV was from the Willamette River Salmon Hatchery in 1958 where it was first isolated and grown in tissue culture (Wingfield et al., 1969). In 1975, rainbow trout and kokanee salmon (landlocked \textit{O. nerka}) suffered mortalities from IHNV at the Wizard Falls Hatchery on the Metolius River. Later, kokanee salmon in Suttle Lake, which drains into the Metolius River, were also found to be virus carriers. The kokanee in Suttle Lake had been used as an egg source for the Wizard Falls Hatchery. During the same year, mortalities due to IHNV also occurred in juvenile steelhead trout (\textit{S. gairdneri}) at Round Butte Hatchery which is located below the Metolius and Deschutes rivers (Mulcahy et al., 1980). Since then, IHNV virus has been isolated periodically from both adult and juvenile steelhead trout at the Round Butte Hatchery. During the winter of 1975-76, adult chinook salmon in the Elk River Hatchery on the south coast of Oregon were found to be IHNV carriers. Later, mortalities occurred in the progeny. Another outbreak occurred in juvenile chinook salmon in 1978 at the same hatchery but not in juvenile steelhead (Mulcahy et al., 1980).

The first suspected viral agent responsible for annually recurring mortalities in juvenile Chinook salmon was reported in Northern California in 1960 by Ross et al., 1960. In 1962, a disease with mortality for Sacramento River juvenile Chinook salmon but not steelhead trout was reported (Parisot and Pelnar, 1962). This agent was partially characterized by Nims et al. (1970) and Wingfield and Chan
Recently, IHNV-caused mortality in juvenile steelhead was reported in the Sacramento River basin (Chen, 1984).

Grischkowsky and Amend (1976) found that IHNV caused mortalities in juvenile sockeye salmon in Alaska. Adult chinook salmon were found to be the carriers (Wertheimer and Winton, 1982).

In 1967, IHNV was first isolated from rainbow trout and sockeye salmon in British Columbia (Amend et al., 1969). In 1976, it was found widely distributed in Western Canada among populations of sockeye salmon fry (Williams and Amend, 1976).

Epizootics of IHN have also occurred in rainbow trout in Minnesota, Montana, South Dakota, and Idaho. The first IHNV isolation in West Virginia was made in 1973 from rainbow trout (Wolf et al., 1973). The disease is believed to have been introduced into these areas by the importation of contaminated eggs or diseased fish (Pilcher and Fryer, 1980).

Infectious hematopoietic necrosis virus outbreaks were first reported in land locked sockeye salmon in Hokkaido, Japan in 1972 (Kimura and Awakura, 1972). The virus has since spread throughout Japan where it is now found in rainbow trout and chum salmon (Oncorhynchus keta)(Sano et al., 1977). There has been a suggestion that the disease was introduced into Japan from the importation of contaminated eggs from Alaska in 1968 (Wolf, 1976). The virus has also been isolated from rainbow trout in Taiwan (Chen, Kon, Hendrick, and Fryer, in press).
Species Susceptibility and Resistance

The first experiments determining the susceptibility of different fish species to IHNV were conducted by Wingfield et al., (1968). They reported that the virus, isolated from juvenile sockeye salmon during the Willamette River Salmon Hatchery outbreak in 1958, was virulent for young kokanee salmon but not for juvenile Chinook salmon, coho salmon (Oncorhynchus kisutch), or rainbow trout. In fish cell lines, this virus isolate replicated well in sockeye and steelhead trout cells but grew in chinook salmon cells only after several passages (Nims et al., 1970).

In 1975, a series of IHNV outbreaks and isolations began to occur in rainbow trout, kokanee salmon, chinook salmon and steelhead trout in the Metolius and Deschutes river watershed in Central Oregon. Although IHNV was found in adults of chinook salmon and steelhead trout at Round Butte Hatchery, mortalities due to IHNV were found to occur only in juvenile steelhead trout (Mulcahy et al., 1980).

In Alaska, the mortality due to IHNV was reported only in juvenile sockeye salmon, but not in juvenile chinook salmon (Grischkowsky and Amend, 1976). Yet, virus has been detected in adult chinook and sockeye salmon in Alaska.
Both chinook salmon and steelhead trout are reared in hatcheries in the Sacramento and Feather rivers. However, mortality due to IHNV has been observed only in juvenile chinook salmon in California (Parisot and Pelnar, 1962; Wingfield and Chan, 1970). Recently, juvenile steelhead trout have died from IHNV in the Sacramento River basin (Chen, 1983).

No mortalities due to IHNV have been reported in coho salmon. Neither experimental injection with two different strains of IHNV (Watson et al., 1954; Parisot and Pelnar, 1962) nor waterborne exposure to IHNV (Wingfield et al., 1970; Wingfield and Chan, 1970) have produced mortality in coho salmon. Apparently, coho salmon are resistant to the pathogenic aspects of IHNV infection. Recently, Chen (1984) has reported that coho salmon may be susceptible to large doses of IHNV (Chen, 1984).

Signs of Infectious Hematopoietic Necrosis

The rapid onset of mortalities in alevins and young fish is usually the first sign of an epizootic of IHNV. Among individual fish, the presence of fecal casts trailing from the vent is one of the characteristic signs of the disease. Dark coloring, anemia, exophthalmia, distension of the abdomen with ascites, and petechial hemorrhages at the base of the fins are also found in infected fish.
In addition, the diseased fish may exhibit behavior such as lethargy or frenzy (Rucker et al., 1953; Ross et al., 1960; Amend et al., 1969).

Internally, anemia of the moribund fish can be seen by a pale liver, spleen, and kidney. Ascites with hemorrhages in the mesenteries is also observed. Histological extensive necrosis of the hematopoietic tissues in kidneys, spleen, and pancreas are the most characteristic histopathological signs (Amend et al., 1969; Yasutake et al., 1965).

Diagnostic Methods

Infectious Hematopoietic Necrosis Virus can be isolated from fish tissue. The tissue extract is prepared by homogenization and preincubation in antibiotic media before inoculation onto suitable fish cell cultures. Susceptible cell lines include fathead minnow cells (FHM), rainbow trout gonad cells (RTG-2), steelhead trout embryo cells (STE-137), Chinook salmon embryo cells (CHSE-214) and epithelioma papillosum cyprin (EPC) (Fendrick et al., 1982). Since these different cell lines exhibit differences in sensitivity to virus infection, the choice of proper cells for IHNV cultivation is critical.

The infected cells are incubated at 12 to 15 °C and examined for cytopathic effects daily. Infected cells will round up and form clusters during a typical IHNV infection. Margination of chromatin at the nuclear membrane is also observed. After positive IHNV infected cell cultures are obtained, the confirmed diagnosis requires
neutralization of the virus by specific anti-IHNV sera. This requirement may cause the diagnosis of an IHNV infection to take from 3 to 8 weeks for completion (Leong et al., 1983; American Fisheries Society, 1979; Fisheries and Environment, 1977).

Transmission

Transmission routes for IHNV have been examined intensively. One of the major routes of transmission is thought to be vertical. Virus cannot be detected in trout surviving an IHNV epizootic (Amend, 1975). However, at the time of sexual maturity, IHNV can be isolated again from survivors of epizootics in the semen, ovarian fluid, and internal organs (Watson et al., 1954; Wingfield and Chan, 1970; Amend, 1975; Mulcahy et al., 1983). Thus, it seems that the adult carriers become a reservoir for congenital transmission from adult to fry. Vertical transmission has been used to explain the outbreaks of IHN in isolated cases of the disease in West Virginia, Minnesota, and Nebraska where contaminated eggs or fish were transported into these states.

There is even more evidence for horizontal, water-borne transmission of IHNV. Fish have been infected with IHNV when placed in live boxes downstream from hatcheries carrying infected fish (Rucker et al., 1953; Wingfield and Chan, 1976). Recently water-borne transmission of IHNV has been documented among spawning sockeye salmon at the Cedar River Hatchery in Washington (Mulcahy et al., 1983). The virus was isolated from water taken from a side channel at Cedar River. The side
channel contained spawning sockeye salmon. Virus concentrations in this water ranged from 32.5 to 1,600 Plaque Forming Units (PFU)/ml. No virus was detected in water from the Cedar River itself. This low level of virus was sufficient to cause a localized gill infection in healthy fish held in the side channel. Experimental evidence for water-borne transmission of IHNV has been obtained in many laboratories (Watson et al., 1954; Wingfield et al., 1970; Amend and Nelson, 1977). Direct contact of healthy fish with moribund or dead fish also leads to the transmission of the disease (Watson et al., 1954).

Transmission of the disease by ingestion of unpasteurized viscera of adult salmon or salmon carcasses has been implicated as the cause of IHNV epizootics in the 1950's (Watson et al., 1954; Groberg et al., 1982). Several investigators have shown that the feeding of infected viscera to juvenile sockeye salmon or other susceptible species will result in the transmission of IHNV (Watson et al., 1954; Groberg et al., 1982).

Control of Infectious Hematopoietic Necrosis Disease

The methods currently available for controlling the spread of IHNV are avoidance procedures designed to eliminate the vertical generation-to-generation transmission and the horizontal individual-to-individual or water-borne transmission. Extermination of infected stocks of fish and the quarantine of infected populations are the major methods for the control of fish viral diseases today. These
measures limit the spread of infection and maintain those fish populations identified as being IHNV-free. Fishery managers are often reluctant to exterminate their fish stocks and thus, these available methods are not popular (Groberg and Fryer, 1983; Mulcahy, 1983).

McMichael (1974) developed an attenuated strain of IHNV by passing an isolate of Oregon Sockeye virus IHNV from rainbow trout repeatedly on a steelhead trout cell (STE-137) at 18°C. After 41 passages under these conditions, the virulence of the virus for kokanee salmon fry was greatly reduced. Tebbit (1976) tested the attenuated virus by water-borne exposure or intraperitoneal injection of sockeye salmon fry or kokanee juveniles. The attenuated IHNV strain was 103 times less virulent than the parental wild type IHNV strain in a water-born exposure tests. A high degree of protection with the vaccine was obtained 25 days after the vaccination. Ninety percent or more of the vaccinated fish were protected against fatal infection with the wild type virus by 48 hour water-borne exposure or intraperitoneal injection. The control, ninety percent or more of unvaccinated fish succumbed to IHN (Fryer et al., 1976). Amend and Smith (1974) also demonstrated that rainbow trout developed protective antibody upon intraperitoneal injection of a nonlethal dose of IHNV. Virus neutralizing antibody from immunized fish was shown to develop within 54 days after vaccination. Despite the success of the experimented attenuated vaccines in sockeye salmon, attenuated viruses have not been used to vaccinate fish for production in hatcheries. The risk of reversion to virulence and the dependence of isolating virus for
diagnostic purposes have limited the usefulness of attenuated vaccines.

Coleman National Fish Hatchery on the Sacramento River in California, has successfully controlled IHNV mortality among chinook salmon by holding infected salmon fry at elevated water temperatures (Amend, 1970). At water temperatures above 15 °C fish infected with IHNV do not die. It is possible that the growth of the virus at this temperature is inhibited and the fish immune system is capable of mounting a protective immune response before the onset of massive tissue necrosis. Recently, the Coleman Hatchery IHNV isolate was shown to be temperature sensitive for growth. Since, it is the only isolate that is temperature-sensitive, this control method is not useful at other hatcheries where different strains are present. The high cost of heating hatchery water is also an obvious disadvantage. The method does not eliminate the carrier state and survivors of an IHNV epizootic may transfer the virus if shipped to other areas (Mulcahy, 1983).

Serological and Biochemical Properties

An immunological comparison between the Oregon sockeye virus, the Sacramento River Chinook Salmon virus, and the isolate from diseased rainbow trout in British Columbia was conducted by McCain et al., (1971). The results from reciprocal cross plaque neutralization tests using rabbit specific serum against each virus indicated that Oregon sockeye virus was identical with the rainbow trout isolate. Sacramento
River chinook virus was closely related to these two isolates, but not identical.

The virus is ether-sensitive and will grow in the presence of 5-bromo-deoxyuridine. These properties suggest that IHNV is a lipid-enveloped RNA virus (Wingfield et al., 1969). The RNA character of the viral genome was confirmed by McCain et al. (1974) when the nucleic acid extracted from purified IHNV was tested by the orcinol and phenylamine reaction and also found to have the density of RNA in cesium sulfate. The viral nucleic acid is ribonuclease sensitive.

The nonequimolar ratio of adenylic and uridylic acid content of the viral RNA and its ribonuclease sensitivity suggested that the RNA was single-stranded (McCain et al., 1974; Hill et al., 1975). The bullet-shape morphology of the virus particle under electron microscopic examination, ether sensitivity, and single-stranded RNA character suggested that IHNV is a member of the family Rhabdoviridae.

Characteristics of the viral proteins of Rhabdoviridae.

There are two genera in the family Rhabdoviridae: Vesiculovirus (Vesicular Stomatitis virus group) and Lyssavirus (Rabies virus group). These genera are distinguished by distinct virion protein patterns which may be resolved in sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE). The Vesiculovirus protein pattern consists of one large polypeptide which is the polymerase L (200,000 daltons), two
forms of a glycoprotein \( G_1 \) and \( G_2 \) (67,000 and 65,000 daltons), a nucleoprotein \( N \) (50,000 daltons), a matrix protein \( M \) (29,000 daltons), and a very faint band of a nonstructural protein \( NS \) (45,000 daltons). The Lyssavirus protein pattern is found characteristically in rabies virus and is composed of the polymerase \( L \) (170,000 daltons), two forms of the glycoprotein, \( G_1 \) and \( G_2 \) (70,500 and 65,000 daltons), nucleoprotein \( N \) (58,500 daltons), and two matrix proteins, \( M_1 \) and \( M_2 \) (39,500 and 25,000 daltons) (Coslett et al., 1980).

The virion protein pattern for IHNV has been characterized for purified virus preparations by two groups of investigators. McAllister and Wagner (1975) estimated that the molecular weights of the five virion proteins were 157,000 daltons for the polymerase \( L \), 72,000 for the glycoprotein \( G \), 40,000 for the nucleocapsid phosphoprotein \( N \), 25,000 for the matrix phosphoprotein \( M_1 \), and 20,000 for the matrix protein \( M_2 \). The \( G \) was shown to be a glycoprotein by labeling with \(^3\text{H}\)-glucosamine, and the \( N \) and \( M_1 \) proteins were found to be phosphoproteins by the incorporation of \(^{32}\text{P}\)-orthophosphate. This report was partially confirmed by Hill et al. (1975). The molecular weights reported by these investigators were 150,000 daltons for \( L \), 90,000 for \( G \), 55,000 for \( N \), 40,000 for \( M_1 \), and 35,000 for \( M_2 \). The variation in the reported size of the virion proteins may have resulted from strain differences or variations in the gel electrophoresis system. Both groups agreed that IHNV protein pattern more closely resembled that of rabies virus than VSV.
Two dimensional peptide mapping of iodinated proteins of rabies virus suggested that L, N, M\textsubscript{1}, and M\textsubscript{2} were unique gene products. The typical peptide maps for G\textsubscript{1} and G\textsubscript{2} were identical and indicated that they were derived from the same gene product (Coslett et al., 1980). Dietzschold et al., (1979) also provided evidence that the G contained 40\% more carbohydrate than G\textsubscript{2}.

Therefore G\textsubscript{1} exhibited a slower electrophoretic mobility on SDS-PAGE and an apparently higher molecular weight. Differences between the two G proteins reflected the different degrees of glycosylation. The approximate number of protein molecules per virion has been determined (Coslett et al., 1980). Additional nonstructural virus-specific proteins were not observed (Coslett et al., 1980).

There are three major size classes of RNA in rabies infected cells: 42S virion RNA, 30S RNA and a heterogeneous class of RNA from 12S to 16S. The 30S and 12S to 16S RNA's were separated by acid-urea agarose gel electrophoresis into 5 bands. A wheat germ cell-free translation system was used to translate the RNA bands 3, 4, and 5 into polypeptides which co-migrated on SDS-PAGE with the rabies virion proteins N, M\textsubscript{1}, and M\textsubscript{2} (Pennica et al., 1980). Rabies 30S RNA directed the synthesis of a large polypeptide which co-migrated with the L protein from rabies virus. The P60 polypeptide was synthesized from RNA band 2 and it migrated faster than either G\textsubscript{1} or G\textsubscript{2}. The peptide patterns obtained after proteolysis of G\textsubscript{1}, G\textsubscript{2} and P60 showed that P60 and G\textsubscript{2} were identical. The patterns showed that G\textsubscript{1} was
similar to G₂ and P60, but contained 2 to 3 additional fragments (Coslett et al., 1980). Rabies virus proteins appeared to be derived from the translation of independently transcribed monocistronic messenger RNA molecules.

Two forms of the glycoprotein, G₁ and G₂, appear in vesicular stomatitis virus (VSV) infected cells (Knipe et al., 1977). Vesicular stomatitis virus mRNA species have been resolved into 4 bands by electrophoresis on formamide-polyacrylamide gels. These mRNA species were complementary to the genome RNA and contained tracts of poly(A). Vesicular stomatitis virus mRNA from 3 of 4 bands have been eluted from formamide-polyacrylamide gels and translated in a Krebs II ascites cell-free translation system. Band 2 mRNA (0.7 x 10⁶ daltons) directed the synthesis of the protein moiety of the glycoprotein (G) and band 3 (0.55 x 10⁶ daltons) directed the synthesis of the N protein. Band 4 mRNA (0.28 x 10⁶ daltons) coded for NS and M proteins (Knipe et al., 1975). These results were consistent with measurements of the viral genome size by complexity analysis and the coding capacities for the VSV mRNAs (Rose and Knipe, 1975; Both et al., 1975).

The order of genes on the VSV genome is 3'-N-NS-M-G-L-5' (Ball and White, 1976). It has been shown that transcription is sequential from the 3' to the 5' end of the genome (Ball and White, 1976). The gene order on the rabies virus genome is similar with 3'-N-M¹-M₂-L-5' (Flamand and Delagneau, 1978). The gene order of the IHNV genome has not been determined.
A virion transcriptase exists in all rhabdoviruses. The activity of this enzyme has been demonstrated in purified virions of VSV (Baltimore et al., 1970; Moyer and Banerjee, 1975), rabies virus (Kawai, 1977), IHNV (McAllister and Wagner, 1977) and viral hemorrhagic septicemia virus (VHSV) (McAllister and Wagner, 1977). The concentration of detergent required to release the virion nucleocapsid for transcription is higher in rabies than in VSV. The optimal temperature for in vitro transcription of fish viruses was 18°C for IHNV and 15°C for VHS, whereas it is 30°C for VSV and rabies (McAllister and Wagner, 1977).
REFERENCES


II. ANALYSIS OF THE QUANTITY AND SYNTHESIS OF THE VIRION PROTEINS OF INFECTIOUS HEMATOPOIETIC NECROSIS VIRUS
INTRODUCTION

Infectious hematopoietic necrosis (IHN) virus is a member of the Rhabdoviridae family and more closely resembles the rabies virus group than the vesicular stomatitis group (McAllister and Wagner, 1975; Lenoir and deKinkelin, 1975; Leong et al., 1981). Five major structural proteins have been identified in purified preparations of IHN virions. These proteins have been designated L for the polymerase, G for the surface glycoprotein, N for the nucleocapsid protein, M\textsubscript{1} and M\textsubscript{2} for the envelope proteins. The glycoprotein nature of the virion protein G has been confirmed by specific labeling of the G protein with \textsuperscript{3}H-glucosamine and two phosphoproteins have been identified, N and M\textsubscript{1} (McAllister and Wagner, 1975). Estimates for the molecular weights of these proteins have varied between 150,000 to 190,000 for L, 67,000 to 80,000 for G, 38,000 to 40,500 for N, 22,500 for M\textsubscript{1}, and 17,000 to 20,000 for M\textsubscript{2}. These variations in the reported size of the virion proteins might be accounted for by differences in the virus strain used in each study. Thus, the molecular weights of the virion proteins were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for eleven different strains of IHN virus. Striking differences in the migration of the N and G proteins were observed among the virus strains.

In this report, the synthesis of the structural proteins of the Round Butte strain of IHNV was examined. The time of appearance of each virion protein during the course of infection
was determined in Chinook salmon embryo cells. Unlike rabies virus, IHNV infection results in the inhibition of cellular protein synthesis. Virus protein synthesis was detected in vivo without resorting to hypertonic shock treatment of the infected cells. The time of appearance of each protein in vivo is unique and suggests that each structural protein is synthesized on a monocistronic mRNA species.
MATERIALS AND METHODS

Cells and Virus

Chinook salmon \textit{(Oncorhynchus tschawytscha)} embryo cells (CHSE-214) were obtained from J. L. Fryer, Dept. Microbiology, Oregon State University, Corvallis, Oregon. The epithelioma papillomatus cyprini (EPC) cells (Tomasec and Fijan, 1971) were obtained from D. Mulcahy, National Fisheries Research center, Seattle, Washington. Both cells were grown in minimum essential medium (MEM) in Earle's salts (Autopow MEM, Flow Laboratories) supplemented with new born calf serum (Grand Island Biologicals Company) to 10%, glutamine to 10 mM, penicillin to 500 units/ml, and streptomycin to 500 ug/ml.

Infectious hematopoietic necrosis virus was isolated from fish tissue taken during IHN epizootics at selected locations in Alaska, Washington, Oregon, and California. The virus was isolated and propagated on EPC cells. For growth and preparation of large quantities of unlabeled and radioactively labeled virus, CHSE-214 cells were used. No difference in the virion proteins was observed for virus grown in CHSE-214 or EPC cells. With the exception of the Round Butte strain, studies on all other strains of IHNV were made on virus that had been passed in tissue culture three to five times.

Virus Purification

The virus was propagated in CHSE-214 cells on 150 cm$^2$ plastic tissue culture flasks (Corning). The cell monlayers were infected at a multiplicity of infection (MOI) of 0.001 plaque forming units
(PFU)/cell and incubated at 16°C for 7 days or until the monolayers were destroyed. At this time the culture fluid was harvested and the cell debris removed by centrifugation at 4000 x g for 10 min at 4°C. All the following procedures were conducted at 0-4°C. The supernatant fluid was layered onto a 0.3 ml pad of 100% glycerol and centrifuged in a Beckman SW27 rotor, 90 min, 80,000 x g. The virus pellet was resuspended in 0.01 M Tris(hydroxymethyl)amino methane-HCl buffer, pH 8.3 containing 0.15 M NaCl and 0.01 M EDTA (STE) and 10 ml of this virus suspension was recentrifuged through a discontinuous gradient composed of 50% sucrose-STE, 7 ml; 35% sucrose-STE, 10 ml; and 20% sucrose STE, 10 ml. After centrifugation in the SW27 rotor for 90 min at 80,000 x g, the virus band was collected from the interphase between the 20% and 35% sucrose layers. The virus suspension was concentrated by centrifugation in a Beckman SW50.2 at 150,000 x g for 30 min. The resulting virus pellet was resuspended in STE and then centrifuged through a continuous gradient of 5% to 30% sucrose-STE in a SW41 rotor at 48,000 x g for 30 min. The virus band was collected and centrifuged in SW41 rotor for one hour at 150,000 x g. The virus pellet was resuspended in SDS-denaturing electrophoresis buffer (SDS-sample buffer).
SDS polyacrylamide Gel Electrophoresis

The separation of SDS-dissociated proteins by polyacrylamide gel electrophoresis was done on glass slides using a modified procedure of the "mini-gel" system described by Matsudaira and Burgess (1978). The 0.5 mm thick gels were composed of a slab of 10% polyacrylamide with a stacking gel of 4.75% polyacrylamide. Before electrophoresis protein samples were boiled for 1.5 min in SDS-sample buffer (0.0625 M Tris, pH 6.8, 10% glycerol (w/v), 5% 2-mercaptoethanol, and 2.3% SDS). The samples were electrophoresed under constant current conditions at 20 mA/gel for 45 min. The Laemmli gel buffer system was used in preparation of running and gel buffers (Laemmli, 1970).

Silver Stain for Protein in Gels

Following the electrophoretic separation, the gels were soaked in 30% methanol, 10% acetic acid, and 10% trichloroacetic acid (TCA) for one hour. The gels were stained with silver nitrate in a procedure modified from that described by Allen (1980). As little as 10 ng of protein is detectable by this procedure.

The standard protein molecular weight markers that were used to determine the molecular weight of the viral proteins were prepared as described in the Bio-Rad Laboratories catalog (February, 1981). The protein markers were phosphorylase B (94,000), bovine serum albumin (68,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (21,000) and lysozyme (14,000).
Isotopic Labeling of Virion Proteins

Confluent CHSE-214 cell monolayers in 35 mm petri dishes (Corning) were washed twice with MEM and were either mock-infected or infected with 5-10 TCID$_{50}$/cell in 0.1 ml of MEM supplemented with 2% dialyzed fetal bovine serum (Grand Island Biologicals Co.) (MEM-2d). After one hour adsorption at 16°C, 2 ml of MEM-2d was added to each dish and the cells were incubated at 16°C. At various times as indicated in the test, the tissue culture fluid was removed and 1 ml of methionine-free MEM-2d added to each dish. After 15 min, this media was removed and replaced with 0.5 ml of methionine-free MEM-2d containing 100 μCi of $^{35}$S-methionine (specific activity, >900 Ci/m mole). The dishes were incubated for 1 hr at 16°C. Then, label was removed and the cell monolayers were washed twice with 1.0 ml of phosphate buffered saline (PBS, 0.135 M NaCl, 0.008 M Na$_2$HPO$_4$, 0.0015 M KH$_2$PO$_4$, 0.0027 M KCl). The cells were lysed with 250 μl of SDS-lysis buffer (0.5 M urea, 2% Nonidet P-40, 5% 2-mercaptopethanol). The lysates were stored at -70°C in cryotubes (Nunc).

In the pulse-chase experiments, the IHN infected cells were labeled with 85 μCi $^{35}$S-methionine for 5 min at 16°C, then washed with PBS. The cells were then washed with PBS before the addition of PBS containing 10 mM methionine. At intervals as described in the text, sample cells were washed twice with PBS before the addition of a lysis buffer described above. The viral proteins were labeled with $^3$H-amino acids at 10 μCi/ml. The $^3$H-amino acids labeled viruses were harvested and purified as described before.
The virion phosphoproteins were labeled with $^{32}$P-orthophosphate at 50 μCi/ml or $^3$H-amino acids at 10 μCi/ml. The radioactively labeled virus was harvested and purified as above.

**Autoradiography**

The slab gels were dried on to cellophane in a Bio-Rad slab gel dryer. Kodak NS-5T X-ray film was exposed to the dried gels for 1-7 days at room temperature before development.

**Densitometer Scans of Protein Gels**

The average densities of protein bands stained with silver or Coomassie Blue R-250 were measured at 620 nm with a Cary 219 spectrophotometer equipped with a scanning densitometer. Although the relative density of silver stain versus protein concentration is linear for most proteins (Switzer, Merril, and Shifrin, 1979), the correlation for some proteins like albumin is not linear. Similar findings have been reported for the coomassie blue stain (Wilson, 1979). Thus, the contribution of each virion protein to the total virus protein was calculated from four separate determinations of the area under each peak detected in stained gels by the silver and coomassie blue procedure.

In addition, the contribution of each virion protein to the total virus protein was estimated with purified virus labeled with $^3$H-amino acids.
RESULTS

Structural Proteins of IHNV Virus

The structural polypeptides of the Round Butte strain of IHNV were separated by SDS-PAGE in a mini-slab gel system (Matsudaira and Burgess, 1978) and stained with silver nitrate in a procedure modified from Allen, 1980. The relative mobility of the marker proteins was inversely related to the logarithm of the respective molecular weights in this gel system (Figure II. 1). Five virion proteins were identified with molecular weights of 150,000 for L (polymerase), 67,000 for G (glycoprotein), 40,500 for N (nucleocapsid protein), 22,500 for M\(_1\) (matrix protein), and 17,000 for M\(_2\) (matrix protein) (Figure II. 2). In some virus preparations even after several additional centrifugations, there appeared two G proteins at 67,000 and 65,000. In addition, the relative contribution of each protein species to the total protein content of the virus was determined by scanning for optical density (630 nm) absorption peaks in the silver and Coomassie blue stained gels and by measuring the radioactivity for virus labeled with \(^3\)H-amino acids (Table II. 1). An approximation of the number of molecules of each protein per virion was made as described by Obijeski et al., 1976. The determination is based upon the assumptions that: (1) the estimates of the molecular weights of the viral proteins are correct; (2) the virions contain only one molecule of viral RNA; and (3) the gram molecular weight of the viral genome is 3.57 x 10^6 (Kurath and Leong, unpublished data).
To determine the ratio of virion RNA to protein, a suspension of purified IHNV labeled with $^3$H-uridine containing 0.48 mg of protein per ml, and a total of $8.36 \times 10^5$ cpm was used. After extraction, the purified RNA had a specific activity of $3.6 \times 10^4$ cpm per $\mu$g of RNA. It was calculated from these figures that there was 0.48 mg of protein to 0.023 mg of viral RNA in the original virus suspension corresponding to a protein to RNA weight ratio of 21:1. Similar ratios were obtained in three different experiments.

**Structural Proteins of Different strains of IHNV**

When the virion proteins of six other strains were compared in the same manner, major differences in N and G were seen (Figure II. 3). Striking differences in the apparent molecular weight of the N protein were immediately obvious. The Coleman Hatchery strain from California exhibited a G protein which was considerably larger than the G proteins of the other strains. Since all virus strains were grown in CHSE-214 cells for these studies, the differences in G and N were not due to differences in host cell glycosylation or phosphorylation.

Strain differences in the structural proteins of IHNV were also examined by specifically labeling the viral proteins in vivo with $^{35}$S-methionine. Approximately 24 h after infection at a multiplicity of infection of 5 to 10, the cells were exposed to $^{35}$S-methionine for 1 hour. The cells were lysed with SDS and the lysate applied directly to a polyacrylamide gel. After
electrophoresis, the gel was stained and dried before exposure to X-ray film for 24-48 h. A comparison of the silver stained gel and its accompanying autoradiogram for eleven different strains of IHNV is shown in Figure 4. It is clear that major differences in the apparent molecular weights of the N and G proteins were detectable in the autoradiogram. In fact the same strain differences that appeared in the silver stained gel for purified IHNV also appeared in the autoradiogram of the different infected cell lysates. Thus, direct $^{35}$S-methionine labeling and SDS-PAGE of infected cells is a simple method for making comparisons of the IHNV strains.

**Intracellular IHN Virion Protein Synthesis**

It is clear that the rate of synthesis of cellular proteins is reduced after IHNV infection (Figure II. 4). This observation suggested that it should be possible to determine the time of the intracellular appearance for each virion protein during the infectious cycle. At one hour intervals after infection, cultures of CHSE-214 cells were exposed to $^{35}$S-methionine for 1 h and then analyzed by SDS-PAGE. The synthesis of N, M$_1$ and M$_2$ is apparently initiated at 6-7 h after infection (Figure II. 5A). The G protein appears at approximately 9-10 h after infection. When earlier samples were taken and an excess of radioactively labeled material was analyzed by SDS-PAGE, N protein synthesis was observed as early as 2-3 h in the infection cycle (Figure II. 5B). Since cellular protein synthesis is not inhibited during early infection, it is difficult to distinguish viral proteins such a M$_2$ and L from host proteins in these early samples.
Two forms of the glycoprotein G is observed intracellularly (Figure II. 5A). These two forms, G₂ for the lower band (65,000) and G₁ for the upper band (67,000), appear to have a product precursor relationship. In an experiment designed to determine the kinetics of intracellular viral protein synthesis and accumulation, cells infected with IHNV (MOI approximately 20) for 24 h were exposed to $^{35}$S-methionine for 1, 5, 10, 30, and 60 min. At the indicated times, the cells were lysed and the lysate analyzed by SDS-PAGE. After one minute, both G₁ and G₂ appeared and after 5 and 10 min of label, G₂ was the dominant glycoprotein being synthesized in the cell (Figure II. 6A). However, at 30 min, the amount of radioactivity appeared equally distributed in both bands as labeled G₁ protein accumulated in the cell. By 60 min, the G₁ band was the predominantly labeled glycoprotein band.

When infected cells were exposed to $^{35}$S-methionine for 5 min followed with excess unlabeled methionine for 20, 40, 60, and 120 min, similar results were observed (Figure II. 6B). A 5 min exposure to the labeled precursor resulted in a predominant G₂ band. After a chase of 20 min with excess unlabeled methionine, the predominantly labeled band was G₁. These results do suggest that G₂ is synthesized immediately and then is further glycosylated to form G₁. Similar precursors for the glycolyated G protein have been reported for vesicular stomatitis virus (Knipe et al., 1977) and rabies virus (Coslett et al., 1980).
In both labeling experiments, comparatively higher rates of synthesis for the N and M₂ proteins were observed. The rates of synthesis for G and M₁ proteins were lower. A labeled band corresponding to the L protein did not appear until 20 min after a 5 min exposure to \(^{35}\)S-methionine (Figure II. 6B). However, continuous exposure to the labeled amino acid for more than 30 min did not produce a labeled L band on the autoradiogram (Figure II. 6A). In both experiments, each gel lane received 50,000 cpm of labeled material. This apparent paradox in the results may be explained if L protein is synthesized at a very low rate and constitutes a very small portion of the total virion protein synthesized in the cell. Thus, during continuous exposure to \(^{35}\)S-methionine, L protein synthesis occurs at such a low rate that its presence is undetectable. The appearance of L in the pulse-chase experiment indicates that L protein accumulates at a faster rate than the other virion proteins.
DISCUSSION

The virion proteins of IHNV have been identified and their intracellular synthesis has been examined. There are five virion proteins with molecular weights of 150,000 (L); 67,000 and 65,000 (G₁ and G₂); 40,500 (N); 22,500 (M₁); and 17,500 (M₂). The glycoprotein nature of the G proteins was verified by specific labeling with $^3$H-glucosamine (data not shown) and their location of the surface of the virion was determined by treatment with Triton X-100 (data not shown). The nucleocapsid protein, N, was found to be associated with the viral nucleic acid and specifically labeled with $^{32}$P-orthophosphate (data not shown). Thus, the work of McAllister and Wagner, 1975, has been confirmed.

An estimate of the number of molecules per virion for each protein was made for IHNV. The ratio of virus protein to RNA was determined to be 21:1, a low figure in comparison to VSV and rabies. These viruses have ratios of 92:1 and 72:1 respectively (Bishop and Roy, 1972; Coslett et al., 1980). The low protein to RNA ratio for IHNV is unusual and may reflect differences in the membrane structure of fish and mammalian cells (Moore et al., 1976). It is similar to that obtained for the bunyavirus, La Crosse virus, which has a ratio of 30:1 when grown in BHK/21 cells (Obijeski et al., 1976). This ratio is used for calculating the total molecular weight of the virion protein.

The relative contribution of each protein to the total molecular weight of the virion was estimated from densitometer
tracings of SDS-polyacrylamide gels of purified virus. For both silver and Coomassie Blue stained gels, the relative proportion of each virion protein was different from that obtained with $^3$H-amino acid labeled virus and that reported by McAllister and Wagner (1975) for $^{14}$C-amino acid labeled virus (Table II. 1). The differences probably reflect differences in methods for determining the proportion of each virion protein. For most proteins a nearly linear relationship exists between the relative density of a band and the micrograms of protein in the band (Switzer et al., 1979). However, variations in staining do exist between different proteins (Oakley et al., 1980). Thus, densitometer tracings of Coomassie Blue stained gels were also included in the study. Several investigators have used Coomassie Blue stained gels to estimate relative virion protein concentrations (Bishop and Roy, 1972, Coslett et al., 1980, and Obijeski et al., 1976). No major differences were found in the staining patterns produced by silver or Coomassie Blue. Any error in these calculations would be an overestimate of the number of G protein molecules since these stains may bind more extensively to glycoproteins (McKnight, 1977).

The dalton equivalents of protein in IHN virions were calculated by reference to the amount of viral RNA per total viral protein. The ratio obtained (21:1) is equivalent to 4.8% RNA per IHN virion, whereas the VS virion has an estimated 0.7 to 1.09% RNA and rabies is composed of 1.39% RNA. These differences are reflected in the estimated number of virion proteins per IHNV.
particle. The numbers of L, G, N, M₁, and M₂ molecules per virion for rabies is reported as 79 (L), 1723 (G₁ and G₂), 1975 (N), 402 (M₁), and 1156 (M₂) (Coslett et al., 1980). In contrast, IHN virions contain approximately a third as many L, a tenth as many G, and a half as many N molecules (Table II. 1). The remarkable difference is in the number of G molecules per virion. We and many others have found that it is difficult to produce antibody to IHN virus in warm-blooded animals. The poor immunogenicity of G or the poor neutralizing activity of the antisera may be a direct result of the low numbers of G molecules on the surface of the virion. In fact, purified G-protein is very immunogenic in rabbits and will induce the formation of rabbit anti-IHNV with a neutralizing titer of 1:10,000 (Engelking and Leong, personal communication).

The nucleocapsid protein, N, is phosphorylated and found in association with the viral RNA and other N proteins inside the virion. In addition, N protein interacts with L and possibly M, proteins in the virion. These interactions should impose strict demands on the N-protein and therefore, this sequence should be conserved among virus strains. That is the case for rabies and VSV. However, this report shows that the apparent size of the N protein varies among the IHNV strains. We are determining whether these variations in electrophoretic mobility result from differences in phosphorylation or size of the polypeptide chain.

Infection of salmon cells with IHNV results in an inhibition of cellular protein synthesis. In this characteristic, IHNV
differs from other members of the rabies virus group of the Rhabdoviridae. Cellular protein synthesis is not inhibited after infection with rabies virus (Coslett et al., 1980) and any study of rabies protein synthesis in the cell requires exposure to hypertonic shock to reduce the background of host protein synthesis. However, it has been possible to examine the synthesis of IHN viral proteins in the cell without resorting to this drastic treatment.

There are two species of virion glycoproteins, $G_1$ and $G_2$. Differences between these two glycoproteins may reflect incomplete glycosylation or some degradation of the carbohydrate moiety during virus purification. Since SDS-PAGE gels of fresh virus preparations do not exhibit the two glycoproteins, it is more likely that $G_2$ results from some degradation of $G_1$ in purified virions. Although peptide mapping data are not available for $G_1$ and $G_2$ it is probable that $G_1$ and $G_2$ have identical amino acid sequences. The pulse-chase experiment (Figure II. 6B) suggests that $G_2$ is synthesized and chased into the $G_1$ form which migrates slower. The slower form should contain more carbohydrate. Recent work by Coslett et al., 1980, has shown that in $G_1$ and $G_2$ of Rabies have identical peptide maps but $G_2$ protein contains less carbohydrate than $G_1$ (Dietscheld et al., 1979).

The first protein for rabies to appear in the course of infection is the N, or nucleocapsid protein, at 2-3 h after infection. At 6-7 h after infection, the membrane proteins, $M_1$ and
M₂, can be identified in the autoradiograms. The two forms of the
glycoprotein, G₁ and G₂, are found at 9-10 h after infection. It
was not possible to distinguish the virion L protein from other
host proteins in the gel until late in infection when cellular host
protein synthesis was completely inhibited. The L protein appears
to be synthesized at such a low rate that it constitutes a very
small portion of the total protein synthesis in the infected
cell. However, L protein accumulates faster than the other virion
proteins, indicating that L is not turned over as quickly. These
experiments show that IHNV proteins are synthesized
independently. Virus production begins at 12-14 h after infection
(Leong et al., 1981).

It appears that IHNV protein synthesis in salmon cells is
similar to that of rabies. Each virion protein seems to be derived
from the translation of independently transcribed monocistronic
mRNAs. In fact, five polyadenylated mRNA species are synthesized
in vitro. These mRNA species correspond appropriately in sizes to
the RNA species expected for each virion protein.
Table II.1. Estimated number and molecular weight of Infectious Hematopoietic Necrosis virus proteins.

<table>
<thead>
<tr>
<th>Protein Species</th>
<th>Mol. St. (x 10^3) (^a)</th>
<th>Percentage of Total Virus Proteins</th>
<th>Mol. Wt. of Protein per Virion (x 10^-6) (^d)</th>
<th>Number of Molecules per Virion (^e)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Incorporation (^b)</td>
<td>Stained (^c)</td>
<td>Incorpor.</td>
</tr>
<tr>
<td>L</td>
<td>150.0</td>
<td>8.6</td>
<td>6.1</td>
<td>6.5</td>
</tr>
<tr>
<td>G</td>
<td>67.0</td>
<td>14.9</td>
<td>26.1</td>
<td>11.1</td>
</tr>
<tr>
<td>N</td>
<td>40.5</td>
<td>41.4</td>
<td>30.4</td>
<td>31.0</td>
</tr>
<tr>
<td>M1</td>
<td>22.5</td>
<td>8.1</td>
<td>15.5</td>
<td>6.1</td>
</tr>
<tr>
<td>M2</td>
<td>17.0</td>
<td>27.0</td>
<td>19.9</td>
<td>20.3</td>
</tr>
</tbody>
</table>

\(^{a}\)Molecular weight estimates were determined as described in Materials and Methods.

\(^{b}\)Percentage of each protein determined by using virus labeled with \(^3\)H amino acids.

\(^{c}\)Percentage of each protein was calculated four separate determinations of the area under each peak of gels stained with Coomassie blue or silver and scanned at 620 nm. The average percentage of each protein for silver staining and for Coomassie Blue staining was again averaged for the final result.

\(^{d}\)Total virion protein was derived from a 21:1 ratio of virus protein to RNA and the estimate that the mol. wt. of the IHN viral genome is 3.57 x 10^6 (Kurath and Leong, unpublished observation).

\(^{e}\)Number of protein molecules per virion by dividing the daltons of protein per virion by their molecular wt.
Figure II. 1 A plot of the relative migration of Infectious Hematopoietic Necrosis structural proteins, L, G, N, M\textsubscript{1} and M\textsubscript{2}, and molecular weight marker proteins as a function of the logarithm of the molecular weight. The proteins were separated by electrophoresis in SDS-polyacrylamide gels as described in Materials and Methods. The marker proteins included phosphorylase B (94,000), bovine serum albumin (68,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (21,000), and lysozyme (14,000).
Figure II. 1.
Figure II.2. Densitometer tracings of SDS-polyacrylamide gels of purified Infectious Hematopoietic Necrosis virus stained with Coomassie blue, broken line, or silver nitrate, solid line.
Figure II. 2.
Figure II.3. **Electrophoretic profile of different strains of Infectious Hematopoietic Necrosis virus.** Purified Infectious Hematopoietic Necrosis virus was disrupted and electrophoresed in SDS-polyacrylamide gels as described in Materials and Methods. Lane 1 contains the molecular weight marker proteins. The other lanes are identified as follows: Lane 2 (Round Butte Hatchery), Lane 3 (Nan Scott Lake), Lane 4 (Elk River), Lane 5 (Coleman Hatchery), Lane 6 (Karluk River), Lane 7 (Suttle Lake), Lane 8 (Coleman Hatchery), Lane 9 (Round Butte Hatchery), Lane 10 (Karluk River), Lane 11 (Round Butte Hatchery), and Lane 12 (Cedar River).
Figure II. 3.
Figure II.4. A comparison of eleven different strains of Infectious Hematopoietic Necrosis virus was made by autoradiographic analysis of the intracellular proteins labeled by $^{35}$S-methionine. Infected cells were labeled as described in Materials and Methods. The lanes are marked KL (Karluk River, Alaska), TA (Tamgas Creek, Alaska), CD (Cedar River, Washington), LE (Lewis River, Washington), RB (Round Butte, Oregon), NS (Nan Scott Lake, Oregon), ER (Elk River, Oregon), SL (Suttle Lake, Oregon), CO (Coleman Hatchery, California), TR (Trinity River, California), and FE (Feather River, California).
Figure II.5. Gel electropherogram of Infectious Hematopoietic Necrosis virus proteins synthesize in CHSE-214 cells at different times after infection. Infected cells were exposed to S-methionine for one hour at the times indicated after infection. Each lane received 50,000 cpm. The film in Fig. 5B was exposed for a longer time so that early virus protein synthesis was detectable.
Figure II. 5.
Figure II.6. Gel electropherogram of viral proteins synthesized in CHSE-214 cells after pulse-labeling with $^{35}$S-methionine. In 6A, the infected cells were labeled for 1, 5, 10, 30, or 60 minutes before samples were taken for SDS-PAGE. In 6B, the infected cells were labeled for 5 minutes and sampled or the label was removed and fresh media containing excess cold methionine was added for 20, 40, 60, or 120 minutes before samples were taken for SDS-PAGE.
Figure II. 6.
REFERENCES


III. OCCURRENCE OF DIFFERENT TYPES OF INFECTIOUS HEMATOPOIETIC NECROSIS VIRUS IN FISH.
INTRODUCTION

Infectious hematopoietic necrosis virus (IHNV) causes acute and extensive necrosis in the hematopoietic tissues of the salmon and trout. It has caused extensive mortalities in hatchery fish populations since the 1950's in the States of Washington (Watson, Guenther and Rucker, 1954), Oregon (Yasutake, Parisot, and Klontz, 1965), and California (Ross, Pelnar, and Rucker, 1960). The disease is also enzootic among wild populations of sockeye salmon (Oncorhynchus nerka) in the Pacific Northwest and Alaska, British Columbia and in the Chinook salmon (Oncorhynchus tschawytscha) of the Sacramento River system in California (Amend et al. 1973, Grischkowsky and Amend, 1976; Yasutake, Parisot, and Klontz, 1965). Isolations of IHNV have also been made in Japan (Sano et al., 1977) and Taiwan (Chen, Kou, Hedrick, and Fryer, in press). Despite the prevalence of this disease in salmon and trout in the Northwest, there has been no comparative study of the different isolates of IHNV. Previously, a comparative study was not possible because serological differences between strains of virus were not readily detected. Only one study of strain differences in IHNV has been made. A serological comparison of three different isolates of IHNV were made in cross-neutralization studies. The Oregon sockeye salmon virus and the British Columbia strain of IHNV were found to be closely related. The Sacramento River Chinook salmon virus was less closely related to the other two isolates (McCain et al., 1971).
A sensitive method for distinguishing IHNV strains by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the virion polypeptides have been developed (Leong et al., 1981). Major strain differences were observed in the molecular weight of the nucleocapsid protein (N) and the envelope glycoprotein (G). This technique has made it possible to compare 72 different isolates of IHNV and group them into 4 or more types. An analysis of the geographic distribution of these IHNV isolates among selected 6 species of fish has indicated that a particular strain will be found among all salmonid species in one geographic area. The virus exhibits a geographic preference and not a species-specificity. Moreover, recent outbreaks of the disease in the Columbia River basin were all caused by the same virus strain indicating the spread of the virus by horizontal water-borne transmission.
MATERIALS AND METHODS

Cells and Viruses

Chinook salmon (*Oncorhynchus tshawytscha*) embryo cells (CHSE-214) were obtained from Dr. J. L. Fryer, Department of Microbiology, Oregon State University, Corvallis, Oregon. Epithelioma papillomatum cyprini (EPC) cells (Tomasec and Fijian, 1981) were obtained from B. J. Hill, Ministry of Agriculture, Fisheries and Food, Fish Diseases Laboratory, Weymouth, Dorset, U.K. Both CHSE-214 and EPC cell lines were grown in minimum essential medium (MEM) containing Eagle's salts (Autopow MEM, Flow Laboratories) supplemented with 10% newborn calf serum (Sterile Systems), 10 mM glutamine, 500 units/ml penicillin, and 500 μg/ml streptomycin (GIBCO).

Infectious hematopoietic necrosis virus was isolated from fish tissue collected during IHNV epizootics at several locations in Alaska, Washington, Oregon, Idaho, California, and Western Canada. The virus isolates were obtained from W. Groberg (Oregon Department of Fish and Wildlife), D. Mulcahy (National Fisheries Research Center, U.S. Fish and Wildlife Service, Washington), R. Grishkowsky (Alaska Department of Fish and Game), G. Tebbit (Wildlife Vaccines, Wheatridge, Colorado), G. Traxlen (Pacific Biological Station, Nanaimo, British Columbia, Canada), G. Traxler (Pacific Biological Station, Nanaimo, British Columbia, Canada) and N. Wood (Rangen Research Laboratories, Idaho). Each virus isolate was passed no more than three times in tissue culture before
analysis of the virus proteins by SDS-PAGE. When large quantities of virus were required, the virus was grown on CHSE-214 cells.

Virus Purifications

The virus was propagated in CHSE-214 cells grown in 150 cm² plastic tissue culture flasks (Corning). The cell monolayers were infected at a multiplicity of infection (M.O.I.) of 0.001 plaque forming units PFU/cell and incubated at 16°C for 7 days or until the monolayers showed complete cytopathic effect (CPE). At this time the culture fluid was harvested and the cell debris removed by centrifugation at 4,000 x g for 10 min at 4°C. All the following procedures were conducted at 0-4°C. The supernatant fluid was layered onto a 0.3 ml pad of 100% glycerol and centrifuged in a Beckman SW27 rotor for 90 min at 80,000 x g. The virus pellet was resuspended in 0.01 M Tris (hydroxymethyl) amino methane-HCl buffer, pH 8.3, containing 0.15 M NaCl and 0.01 M EDTA (STE). Ten ml of this virus suspension was centrifuged through a discontinuous gradient composed of 7 ml of 50% sucrose-STE, 10 ml of 35% sucrose-STE, and 10 ml of 20% sucrose-STE in an SW27 tube. After centrifugation for 90 min at 80,000 x g, the virus band was collected from the interphase between the 20% and 35% sucrose layers. The virus suspension was concentrated by centrifugation in a Beckman SW27 at 80,000 x g for 90 min. The resulting virus pellet was resuspended in STE and then centrifuged through a continuous gradient of 5% to 30% sucrose-STE in a SW41 rotor at 21,000 rpm for 30 min. The virus band was collected and then
centrifuged in a SW41 rotor for one hour at 35,000 rpm. The virus pellet was resuspended in SDS-denaturing electrophoresis buffer (2.3% SDS; 0.05 mM Tris, pH 6.8; 10% glycerol (w.v); 5% 2-mercaptoethanol), boiled for 2 min, and stored at -20°C.

Isotopic Labeling of Virion Proteins

Confluent CHSE-214 cell monolayers in 35 mm² petri dishes (Corning) were washed twice with MEM and were either mock-infected or infected with an MOI of 5-10 TCID₅₀/cell (tissue culture infection dose for 50% infectivity) of IHNV in 0.1 ml of MEM supplemented with 2% dialyzed fetal bovine serum (GIBCO) (MEM-2d). Virus was adsorbed for one hour at 16°C, diluted with 2 ml of MEM-2d and then the cells incubated at 16°C. When the infected cells exhibited 70% of CPE, the tissue culture media was removed and 1 ml of methionine-free MEM was added to each dish. After one hour, this medium was removed and replaced with 0.5 ml of methionine-free MEM containing 100 µCi of ³⁵S-methionine (specific activity, 900-1000 Ci/mmole, NEN). The dishes were incubated for one hour at 16°C, at which time the medium was removed and the cell monolayers washed twice with 1.0 ml of phosphate buffered saline (PBS; 0.135 M NaCl, 0.008 M Na₂HPO₄, 0.0015 M KH₂PO₄, 0.0027 M KCl). The cells were lysed with 100-200 µl of SDS-lysis buffer (9.5 M urea, 2% Nonidet P-40, 5% 2-mercaptoethanol) and the lysate stored at -70°C in cryotubes (NUNC). For electrophoresis, the lysates were mixed with SDS and glycerol to give final concentrations of 2.5% SDS, 10% glycerol, 1% non-P40, 5% 2-
mercaptoethanol, 4.75 M urea, and boiled for 2 min. Samples were then loaded on the gel immediately or stored at -20°C.

**Sodium Dodecyl Sulfate polyacrylamide Gel Electrophoresis**

The separation of SDS-dissociated proteins by polyacrylamide gel electrophoresis was done on glass slides using a modified procedure of the "mini-gel" system described by Matsudaira and Burgess (1978). The 0.5 mm or 0.8 mm thick gels were composed of a slab of 10% polyacrylamide with a stacking gel of 4.75% polyacrylamide. Samples were prepared as described above, and electrophoresed at 10 mA through the upper gel and then, 20 mA for the lower gel. The running and gel buffers used were identical to those described by Laemmli (1970).

**Silver Stain for Protein in Gels**

Following the electrophoretic separation, the gels were soaked in 30% methanol, 10% acetic acid, and 10% Trichloroacetic acid (TCA) for one hour. The gels were stained with silver nitrate (Allen, 1980). The standard protein molecular weight markers (Bio-Rad) that were used to determine the molecular weight of the viral proteins were prepared as described by the manufacturer. The protein markers included phosphorylase B (94,000), bovine serum albumin (68,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (21,000) and lysozyme (14,000).

**Autoradiography**

The slab gels were dried onto cellophane in a BioRad slab gel drier. Kodak NS-5T or X-Omat AR X-ray film was exposed to the dried gels for 1-4 days at room temperature.
RESULTS

**Typing of Infectious Hematopoietic Necrosis Virus Strains**

The analysis of the virion protein patterns of different IHNV isolates by SDS-PAGE has been extensively studied (Leong et al., 1981). Only virus-specific proteins are labeled with $^{35}$S-methionine when the radioactive label is added to infected cells. Host cell protein synthesis is inhibited by the virus infection. Therefore, the viral G (glycoprotein), N (nucleocapsid protein), $M_1$ and $M_2$ (matrix proteins) are easily detected on an autoradiogram of an SDS-PAGE analysis of infected cell lysates. The L (polymerase) protein is detectable with prolonged exposure of the X-ray film. Major strain differences are apparent in the electrophoretic mobility of the N protein and the G protein, Figure 1 and 4. An analysis of 72 different IHNV isolate (Table III. 1) has been used to place these isolates into five different types.

Isolates belonging to Type 1 have a faster migrating N protein with an approximate molecular weight of 40,500 daltons. The first isolation of this type was made at the Round Butte (RB) Hatchery in Oregon in 1975 (Mulcahy et al., 1980). Type 1 IHNV was also found at Dworshak (DW) National Fish Hatchery Entiat (EN) National Fish Hatchery and Rapid River (RR) Hatchery (Fig. III. 1 and III. 2). The same type 1 IHNV was also isolated from fish in the Deschutes River watershed at Round Butte Hatchery, Warm Springs (WS) National Fish Hatchery, Metolius River (ME), and Suttle Lake (SL). Moreover, virus taken from steelhead fry, yearlings, and adults at
Round Butte Hatchery all belong to Type 1. In Alaska and Canada, Type 1 IHNV has been found in fish at Tamgas Creek (TA), Lake Auke (LA), Lake Nerka (LN) and several lakes and rivers in British Columbia (Table III. 1).

Virus isolates characterized as Type 2 IHNV have N proteins with a molecular weight of approximately 42,800 daltons. The first reported isolation of Type 2 IHNV was made in Idaho in 1978 (Fig. III. 2). More recently, Type 2 IHNV appeared in the lower Columbia River basin for the first time in 1980 at the Lewis River Hatchery (LE) (WDF) in Washington (Fig. III. 1, Table III. 3). Since that time, the Type 2 virus has appeared at Skamania (SK), Mossyrock (MO), Minto Pond (MP), Gnat Creek (GN), Beaver Creek (BC1), and Little White Salmon (LS) hatcheries in the lower Columbia River basin (Fig. III. 1, Table III. 3). All of these virus isolates have a slower migrating N protein than the Round Butte and Dworshak Type 1 viruses. In addition, Type 2 strains have been isolated from the Pahsimeroi (PA), Niagra Spring (NI), Clear Spring (CS) hatcheries and at other fish hatcheries in the Hagerman Valley (HA-1, HA-2) in Idaho (Figs. III. 1, III. 2, III. 6).

The IHNV taken from fish at Nan Scott Lake (NS) and Elk River (EK) in Oregon; Trinity River (TR), Feather River Hatchery (FE), Sacramento River (SV) and Coleman Hatchery (CO1) in California are grouped together as Type 3 viruses (Figs. III. 3, III. 4, III. 7). The N protein of these strains migrate with an apparent molecular weight of 43,500 SDS-polyacrylamide gels (Fig. III. 3 and III. 4).
Viruses grouped under Type 4 were some of the isolates from Coleman Hatchery (CO2 and CO3) in California (Fig. III. 4). This virus type is characterized by a G protein with a molecular weight of 70,000, and is a temperature sensitive strain. Type 4 viruses do not replicate well at 18-22°C (Leong et al., 1981). The virus isolate, CO2, corresponds to CO mentioned in this earlier report (Leong et al., 1981).

Type 5 virus include those that have not been adequately analyzed by multiple cross comparison of the virion protein patterns. Included in this category are the IHN virus isolates taken from the Cedar River in Washington (CD and CD2). The N protein of CD2 migrates at an electrophoretic position midway between the N proteins of Type 1 and Type 2 (Fig. III. 1). In contrast, CD has an N protein which migrates even more slowly than that of Type 3 (Fig. III. 4). The CD and CD2 isolates are different from each other and are not distinct enough to be considered as a separate type. Karluk River isolates (KL and KL2) from Alaska also possess N proteins which migrate between the N proteins of Types 1 and 2 viruses. The Karluk River strain resembles CD2. However, a more detailed analysis of these two isolates must be made.

**Relationship between virus type and virulence to fish**

An analysis of the species of fish infected with each virus type is presented in Tables III. 2, III. 3, III. 4 and III. 5. Type 1 strains of IHNV have been found in steelhead trout, sockeye
salmon, kokanee salmon (landlocked sockeye salmon), pink salmon, and Chinook salmon (Table III. 2). Type 2 and Type 3 viruses have never been isolated from sockeye salmon (Tables III. 3 and III. 4). However, Type 2 IHNV strains have been found in steelhead, cutthroat, and rainbow trout, and Chinook salmon. Type 3 IHNV has been found mainly in chinook salmon with only one reported isolation from rainbow trout (Table III. 4, Fig. III. 7). It seems likely that Type 3 virus is the same virus responsible for "Sacramento River Chinook Disease." The Sacramento River virus causes were severe mortalities in chinook salmon but not in steelhead trout although these two species were cultured in the same hatcheries in Sacramento River and Feather River (Parisot and Pelnar, 1962; Wingfield and Chan, 1970). Type 4 and 5 of IHN virus were isolated from chinook and sockeye (Table III. 5).

**Differentiation of IHNV and IPNV by SDS-PAGE**

The utility of the detection method was further demonstrated by comparing the intracellular IHN-specific proteins with that produced in cells infected with infectious pancreatic necrosis virus (IPNV). The latter virus is also found in salmonid species of fish in the Northwest. Although IPNV produces a different CPE in tissue culture cells, there is always the possibility of mistaking one virus of the other. Confirmation of a diagnosis for either virus requires serum neutralization studies. However, the SDS-PAGE method does distinguish between these two viruses easily. It is clear that the $^{35}$S-methionine labeled virus proteins
in IPNV-infected cells are different from that synthesized in IHNV-infected cells (Fig. III. 5). Three different isolates of IPNV gave identical patterns.

The IHNV Associated with Recent Outbreaks in 1980-82 in the Lower Columbia River Are Similar to the Type of IHNV from the Hagerman Valley in Idaho

The similarity between the virus types isolated in the lower Columbia River and in Idaho suggests that a single source of infection and horizontal transmission may have been responsible for the sudden increase in the IHNV incidence in the Columbia River (Fig. III. 6).
DISCUSSION

The finding that IHNV may be typed by the virion protein patterns has made possible a study of the regional distribution of 72 different IHNV isolates in several fish species. An analysis of these results indicate that (1) there are at least 4 major types of IHNV, (2) a particular type of IHNV is characteristic for a specific area and not for a particular species of fish in that area, (3) the same type of virus isolated from spawning adults is responsible for the subsequent IHNV epizootic in the progeny fry, (4) the same type of virus, Type 2, was responsible for IHNV outbreaks among fish in the lower Columbia River, and (5) Type 2 virus is also found in fish in the Snake River, Idaho, which drains into the Columbia River.

In addition, some correlation between virus type and virus virulence was found for Types 1 and 3 IHNV virus. The first isolation of IHNV, the Oregon Sockeye virus, in Oregon was made at the Willamette Salmon Hatchery in juvenile sockeye salmon in 1958. In 1967, a virus referred to as IHNV was isolated from rainbow trout and sockeye salmon in hatcheries in Western Canada (Amend et al., 1969). Although these isolates are no longer available, the finding of only Type 1 virus in sockeye salmon in Canada today and the serological similarity of the Oregon Sockeye Virus and the Canadian IHNV isolates (McCain et al., 1971) suggest that Type 1 virus is the original sockeye salmon IHNV. This Type 1 virus is virulent in sockeye salmon and steelhead trout. Type 1 virus does not produce mortalities among Chinook salmon fry.
The IHNV virus isolated from Sacramento and Feather river Chinook salmon belong in the Type 3 group. This virus type causes high mortalities in chinook salmon in California but does not readily kill steelhead trout. Such differences in the virulence of different strains of IHNV for different species of fish has been reported (M. Chen, Ph.D. thesis, Oregon State University, Corvallis, 1984).

The recent epizootics (1980-82) of IHNV at hatcheries located in the lower third of the Columbia River (Fig. III. 6) were all due to Type 2 IHNV (Fig. III. 1). The earliest appearance of Type 2 IHNV among the isolates tested was found in steelhead adults at Niagra Springs Hatchery (NI) and Hagerman Valley (HA 1 and HA 2) in 1978 (Table III. 3 and Fig. III. 2). Later isolations of the virus were made in rainbow trout from Clear Springs (1982), and Hagerman Valley (1981, 1982, 1983) in Idaho. The similarity between the virus types isolated in the lower Columbia River and Idaho suggests that a single source of infection and horizontal transmission may have been responsible for the sudden increase in the IHNV incidence in the Columbia River (Groberg and Fryer, 1983).

The finding that IHNV strains can be typed by the virion protein patterns and that a particular virus type is endemic for a particular area is significant for several reasons. The most important reason is that once an area has been "typed" with a particular virus type it becomes possible to detect the introduction of a new strain into that area. For example, the N
protein of CD2 which was obtained in 1981 is different from the CD strain of the previous year (Fig. III. 4). There is also a difference between N proteins of DW and DW2 (Fig. III. 1, III. 4). Thus, it is possible that new strains of IHNV were introduced at Cedar River and at Dworshak hatcheries. If the appearance of a new strain of IHNV coincided with the introduction of fish or eggs, then the origin of the new strain might be traced. It should be possible to distinguish between an IHNV epizootic which originated from contaminated eggs or from infected fish in the watershed upstream from the hatchery.
### Table III. 1. Summary of Infectious Hematopoietic Necrosis Virus strains isolated from Northwest America.

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StS, Steelhead trout, summer  
StW, Steelhead trout, winter  
Ct, Cutthroat trout  
SS, Sockeye salmon  
CH, Chinook salmon  
ChF, Chinook salmon, fall  
ChS, Chinook salmon, summer  
K, Kokanee  
Rb, Rainbow trout  
Abbreviations as in Mulcahy et al., 1980.
Table III. 2. Different species of fish that Type 1 of Infectious Hematopoietic Necrosis virus was isolated from.

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<td>kokanee</td>
<td>OR</td>
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<td>1981</td>
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</tr>
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<td>steelhead</td>
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<td>RB3</td>
<td>steelhead</td>
<td>OR</td>
<td>adult</td>
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<td>RB4</td>
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<td>OR</td>
<td>yearling</td>
<td>1983</td>
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<td>SL</td>
<td>kokanee</td>
<td>OR</td>
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<td>1980</td>
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<td>adult</td>
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<tr>
<td>EN</td>
<td>steelhead</td>
<td>OR</td>
<td>-</td>
<td>1982</td>
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<td>pink salmon</td>
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<td>juvenile</td>
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<td>AK</td>
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<td>1981</td>
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<td>-</td>
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<td>1978</td>
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<td>1978</td>
</tr>
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<td>CW2</td>
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<td>1978</td>
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<td>-</td>
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<td>1974</td>
</tr>
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<td>-</td>
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<td>CAN</td>
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<td>1974</td>
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<td>1974</td>
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</tr>
<tr>
<td>WC2</td>
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<td>CAN</td>
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<td>1976</td>
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</table>

*aCode as described in Table 1.
*bOR = Oregon
*cAK = Alaska
*dID = Idaho
*eCAN = Canada
Table III. 3. Different species of fish that Type 2 IHN virus was isolated from.

<table>
<thead>
<tr>
<th>Code</th>
<th>Host Name</th>
<th>State</th>
<th>Stage of Development</th>
<th>Date of Isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC1</td>
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<td>WA</td>
<td>juvenile</td>
<td>1981</td>
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<td>BC2</td>
<td>cutthroat trout</td>
<td>WA</td>
<td>adults</td>
<td>1982</td>
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<tr>
<td>CL2</td>
<td>steelhead, winter</td>
<td>WA</td>
<td>adults</td>
<td>1982</td>
</tr>
<tr>
<td>CL3</td>
<td>steelhead, summer</td>
<td>WA</td>
<td>juvenile</td>
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<td>CL4</td>
<td>steelhead, summer</td>
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<td>adult</td>
<td>1981</td>
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<td>steelhead, winter</td>
<td>WA</td>
<td>adult</td>
<td>1981</td>
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<td>adult</td>
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<td>HA1</td>
<td>rainbow trout</td>
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<td>1978</td>
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<td>HA3</td>
<td>rainbow trout</td>
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<td>1983</td>
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<td>HA4</td>
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<td>juvenile</td>
<td>1983</td>
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<td>1979</td>
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<td>LE</td>
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<td>adult</td>
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<td>SK</td>
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<td>juvenile</td>
<td>1981</td>
</tr>
</tbody>
</table>

\(^a\)Code as described in Table 1
\(^b\)OR = Oregon
\(^c\)WA = Washington
\(^d\)ID = Idaho
\(^e\)AK = Alaska
Table III. 4. Different species of fish that Type 3 Infectious Hematopoietic Necrosis virus was isolated from.

<table>
<thead>
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<th>Code</th>
<th>Host Name</th>
<th>State</th>
<th>Stage of Development</th>
<th>Date of Isolation</th>
</tr>
</thead>
<tbody>
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<td>ER</td>
<td>Chinook</td>
<td>OR^b</td>
<td>juvenile</td>
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<td>ER2</td>
<td>Chinook</td>
<td>OR</td>
<td>juvenile^e</td>
<td>1977</td>
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<tr>
<td>FE</td>
<td>Chinook</td>
<td>CA^c</td>
<td>juvenile</td>
<td>1977</td>
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<tr>
<td>SV</td>
<td>Chinook</td>
<td>CA</td>
<td>adult</td>
<td>1979</td>
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<td>Chinook</td>
<td>CA</td>
<td>juvenile</td>
<td>1980</td>
</tr>
<tr>
<td>NS</td>
<td>rainbow trout</td>
<td>OR</td>
<td>juvenile</td>
<td>1971</td>
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<td>CO</td>
<td>Chinook</td>
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<td>1980</td>
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</table>

^aCode as described in Table 1
^bOR = Oregon
^cCA = California

Table III. 5. Different species of fish that Type 4 and 5 Infectious Hematopoietic Necrosis virus was isolated from.

<table>
<thead>
<tr>
<th>Code</th>
<th>Host Name</th>
<th>State</th>
<th>Stage of Development</th>
<th>Date of Isolation</th>
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</thead>
<tbody>
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<td>CA</td>
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<td>1979</td>
</tr>
<tr>
<td>CD^b</td>
<td>sockeye</td>
<td>WA^d</td>
<td>juvenile</td>
<td>1980</td>
</tr>
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<td>CD2^b</td>
<td>Chinook</td>
<td>WA</td>
<td>juvenile</td>
<td>1981</td>
</tr>
<tr>
<td>KL^b</td>
<td>sockeye</td>
<td>AK^e</td>
<td>juvenile</td>
<td>1979</td>
</tr>
<tr>
<td>KL2^b</td>
<td>Chinook</td>
<td>AK</td>
<td>juvenile</td>
<td>1978, 1979</td>
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</table>

^abelongs to Type 4
^bbelongs to Type 5
^cCA = California
^dWA = Washington
^eAK = Alaska
Figure III. 1. An autoradiogram of the Type 1 and 2 IHNV isolates in the Columbia River basin. Gel electropherogram of different isolates of IHNV infected CHSE-214 cells labeled by $^{35}$S-methionine as described in Materials and Methods. The lanes are marked RB1 (Round Butte Hatchery), DW (Dworshak), PA (Pahsimeroi Hatchery), NI (Niagra Springs), EN (Entiate), LE (Lewis River), SK (Skamania), MO (Mossyrock Hatchery), MP (Minto Pond), GN (Gnat Creek), BC1 (Beaver Creek), LS (Little White Salmon).
Figure III. 2. An autoradiogram of Type 1 IHNV and the IHNV isolated in Idaho (Type 2). Infected cells were labeled as described in Materials and Methods. The lanes are marked RB (Round Butte Hatchery), DW (Dworshak), PA (Pahsimeroi Hatchery), NI (Niagra Springs), CS (Clear Springs), HA1 (Hagerman River 1), HA2 (Hagerman River 2), RR (Rapid River), SK (Skamania), GN (Gnat Creek), RB1 (Round Butte Hatchery).
Figure III. 2.
Figure III. 3. A comparison of Infectious Hematopoietic Necrosis virion protein patterns for the California (Type 3) and Type 2 isolates. Infected cells were labeled as described in Materials and Methods. The lanes are marked BC2 (Beaver Creek 2), ER (Elk River), PA (Pahsimeroi Hatchery), GN (Gnat Creek), NS (Nan Scott Lake), SK (Skamania), TR (Trinity River), FE (Feather River).
Figure III. 3.
Figure III.4. A comparison of Infectious Hematopoietic Necrosis virion protein patterns for Type 3, 4 and 5 isolates. Infected cells were labeled as described in Materials and Methods. The lanes are marked CO (Coleman River), SV (Sacramento River), CO3 (Coleman River 3), TR (Trinity River), RB1 (Round Butte Hatchery), CD2 (Cedar River 2), CD (Cedar River), TR (Trinity River), SV (Sacramento River), CO3 (Coleman River 3), CO2 (Coleman River 2), ER (Elk River), ER2 (Elk River 2).
Figure III. 4.
Figure III.5. The differences in virion protein patterns between Infectious Pancreatic Necrosis virus (IPN) and Infectious Hematopoietic Necrosis Virus infected cells were labeled as described in Materials and Methods. The lanes are marked IPN 1 (Canada), IPN 2 (Canada), IPN 3 (Canada), WR (White River), CNTL (Control uninfected cells), RB 1 (Round Butte 1).
Figure III. 5
Infectious Hematopoietic Necrosis Virus was isolated from fish in the Columbia River basin.
Figure III.7. The locations where Infectious Hematopoietic Necrosis Virus was isolated from fish in Northern California.
Figure III.7.

NORTHERN CALIFORNIA SITES

○ TYPE III VIRUS

▲ TYPE IV VIRUS

0 24 48 MILES
REFERENCES


IV. A COMPARISON OF DETECTION METHODS FOR INFECTIOUS HEMATOPOIETIC NECROSIS VIRUS
INTRODUCTION

Infectious hematopoietic necrosis (IHN) disease has become an increasingly serious problem for hatchery raised salmon and trout in the United States. Prior to 1980 the IHN virus was isolated sporadically at only a few hatcheries in the Columbia River basin (CRb) (Mulcahy, Tebbit, Groberg, McMichael, Winton, Hedrick, Philippon-Fried, Pilcher, and Fryer, 1980). From 1980 to 1981, a dramatic increase of IHN disease was observed in fish at CRb hatcheries. Estimated losses of fish to this viral agent increased more than twenty-fold from 1980 to 1981 and forty-fold from 1980 to 1982 (Groberg and Fryer, 1983).

Quarantine and complete destruction of infected fish and eggs are currently used to control the spread of IHN disease (Groberg and Fryer, 1983). Since these measures are costly and taken with great reluctance, the decision to destroy the resource must be based on an accurate and rapid diagnostic method for IHN virus. Present diagnostic procedures for IHN rely primarily on classical methods of virus isolation in tissue culture and virus identification by serum neutralization. The confirmation of IHN infection may take as long as eight weeks (Mulcahy et al. 1980; Leong, J. C., Hsu, Y. L., Engelking, H. M., Fendrick, J. L., Durrin, L. K., Kurath, G. 1983). Since IHN disease can spread quickly and kill up to 90% of the fish within two to three weeks, a more rapid method for the diagnosis of IHN is desirable.
We have investigated several methods for detecting virus in tissue samples and report here a comparison of those methods utilizing serum neutralization of the IHNV virus, radioactive-labeling of the virus-specific proteins, and immunological detection of virus-specific proteins in protein blots. Less than 1 ng of virus protein was detected by the radiolabeling of virus-specific proteins.

The development of an immunological assay for IHNV was made possible with the finding that rabbit anti-IHN sera does bind efficiently to the virus despite its poor neutralizing activity. The binding of antibody to IHNV virus on protein blots was detected with either $^{125}$Iodine-labeled *Staphylococcus aureus* Protein A ($^{125}$I-Prot. A) or horse radish peroxidase-conjugated goat anti-rabbit gamma globulin sera (Per-anti-rab. IgG). Both reagents produced protein blots of equal intensity. The immunological detection of virus proteins offered advantages in cost, accuracy, speed and, in the case of the Per-anti-rab. IgG reagent, the stability and ease in handling of a nonradioactive compound. The immuno-protein blots also yielded information about the immunological relatedness of different IHNV isolates.
MATERIALS AND METHODS

Cell and Viruses

Chinook salmon (Oncorhynchus tshawytscha) embryo cells (CHSE-214) were obtained from Dr. J. L. Fryer, Department of Microbiology, Oregon State University, Corvallis, Oregon. CHSE-214 cells were grown in minimum essential media (MEM) in Earles' salts (Autopow MEM, Flow Laboratories) supplemented with newborn bovine serum to 10%, glutamine to 10 mM, penicillin to 500 units/ml, and streptomycin to 500 µg/ml.

Infectious hematopoietic necrosis virus was isolated from fish tissue samples taken during IHNV epizootics at several locations in Alaska, Washington, Oregon and California. The virus was isolated and propagated on Epithelioma papillosum cyprini (EPC) cells (Tomasec and Fijan, 1971). Virus required for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) studies was grown on CHSE-214 cells.

Virus Purification

Virus was grown in 150 cm² plastic tissue culture flasks (Corning). Cell monolayers were infected at a multiplicity of infection (MOI) of 0.001 plaque forming units (PFU)/cell and incubated at 16°C for 7 days. The culture fluid was harvested and the cell debris was removed by centrifugation at 4000 x g for 10 min at 4°C. All the following procedures were conducted at 0-4°C. The supernatant fluid was layered onto a 0.3 ml pad of 100% glycerol and centrifuged in a Beckman SW 27 rotor for 90 min at
80,000 x g. The virus pellet was resuspended in STE [0.15 M NaCl; 0.01 M Tris-HCl, pH 8.3; 0.01 M Ethylene-diamine-tetracetic acid (EDTA), pH 7.0] and 10 ml of the virus suspension was recentrifuged through a discontinuous gradient composed of 50% sucrose-STE, 7 ml; 35% sucrose-STE, 10 ml; and 20% sucrose-STE, 10 ml. After centrifugation in the SW 27 rotor for 90 min at 80,000 x g, the virus band was collected from the interphase between the 20% and 35% sucrose layers. The virus suspension was concentrated by centrifugation in a Beckman SW 50.1 rotor at 150,000 x g for 30 min. The resulting virus pellet was resuspended in STE and then centrifuged through a continuous gradient of 5% to 30% sucrose-STE in a Beckman SW41 rotor at 48,000 x g for 30 min. The resulting virus band was pelleted in SW41 rotor for 1 h at 150,000 x g. The pellet was resuspended in SDS-denaturing electrophoresis buffer.

**Immunization**

Purified IHNV was emulsified with (1.05 mg in 1.5 ml STE) an equal volume of complete Freund's adjuvant; 2.4 ml of the mixture were injected subcutaneously into a rabbit in 3 sites in the neck region and 0.3 ml of the mixture were injected into each hind foot pad. Immunization by this method was repeated at day 30. At 2, 3 and 4 weeks following the first immunization, the antibody titer was measured by examining the binding of $^{125}$I-protein A to serially diluted sera which was adsorbed to IHN virus bound to the bottom of wells in a microtiter plate (See Antibody Titration by Solid Phase Direct Binding of $^{125}$I-Protein A, Materials and Methods).
Infectious Hematopoietic Necrosis Virus Infected Cell Lysate

Confluent CHSE-214 cell monolayers in 35 mm petri dishes (Corning) were infected at an MOI of 5-10 TCID\textsubscript{50}/ml in 0.1 ml of MEM supplemented with 2% dialyzed fetal bovine serum (GIBCO) (MEM-2d). Virus was adsorbed for 1 h at 16°C, diluted with 2 ml of MEM-2d and the cells were incubated at 16°C. Following 30-40 h, the tissue culture fluid was removed and the monolayers were washed twice with 1.0 ml of phosphate buffered saline (PBS) (0.135 M NaCl, 0.008 M Na\textsubscript{2}HPO\textsubscript{4}, 0.0015 M KH\textsubscript{2}PO\textsubscript{4}, 0.0027 M KCl). The cells were lysed with 100 μl of lysing buffer (9.5 M urea, 2% Nonidet P-40, 5% 2-mercaptoethanol) and the lysates were stored at -70°C in screw-capped plastic vials.

Polyacrylamide gel electrophoresis

Cell lysates were prepared by mixing with a solution of 10% SDS and 100% glycerol to give final concentrations of 2.5% SDS, 10% glycerol, 1% Nonidet P-40, 2.5% 2-mercaptoethanol and 4.75 M urea. The SDS-treated samples were boiled for 2 min and then loaded on the gel immediately or stored at -20°C. The electrophoresis was performed as described by Laemmli (1970) using a 10% acrylamide slab gel (10 cm x 8.2 cm x 0.8 mm mini gel system) with a 4.75% acrylamide stacking gel (Matsudaira and Burgess, 1978). Gels were subjected to an electrophoretic current of 20 mA/gel until the bromophenol blue dye marker reached the bottom.
Contact Diffusion Blotting

Following electrophoresis, contact diffusion blotting was used to transfer the protein to nitrocellulose membranes (Bowen, Steinberg, Laemmli and Weintraub, 1980). The gels were soaked in immersion buffer (50 mM NaCl, 2 mM EDTA, 10 mM Tris-pH 7.4, 0.1 mM dithiothreitol, 4 M urea) and shaken at room temperature for 1 h. The gel was sandwiched between prewetted sheets of nitrocellulose in transfer buffer (50 mM NaCl, 2 mM EDTA, 10 mM Tris-pH 7.4, 0.1 mM dithiothreitol) and shaken for 24 h at room temperature. The transfer buffer was changed twice during the shaking. Following protein transfer, the sandwich was dismantled and the nitrocellulose membranes were immersed at a 45° angle in blocking solution (5% BSA-PBS) and shaken for 1 h at room temperature. The resulting protein blots were then developed with the $^{125}\text{I}$-Prot. A or Per-anti-rab. IgG.

$^{125}\text{I}$-Protein A Binding Assay

Following blocking, the nitrocellulose membranes were rinsed briefly in PBS, placed into a container of rabbit anti-IHN virus sera (1:100 dilution in 5% BSA-PBS) and gently shaken at 4°C overnight. The membranes were washed twice with PBS containing 0.05% Nonidet P-40 for 10 min, rinsed with PBS, and placed into a PBS solution containing $10^5$ counts per min of $^{125}\text{I}$-protein A from Staphylococcus aureus (New England Nuclear) and shaken at 4°C for 4 h. After the labeling, the membranes were rinsed as described above, and blotted dry on Whatmann 3 MM filter paper. The
membranes were then wrapped in polyethylene film (Handi-Wrap) and exposed to X-Omat AR X-ray film (Kodak) with an intensifying screen at -70°C for 12-14 h.

Peroxidase Conjugate Staining

The protein blots were developed in a solution containing rabbit anti-IHN virus serum which had been diluted 100-fold in a solution of Tris-buffered saline (TBS) (20 mM Tris-pH 7.5, 500 mM NaCl), containing 1% gelatin (BioRad) and gently shaken overnight at 4°C. The membranes were rinsed briefly with deionized distilled water and then washed twice with TTBS (20 mM Tris pH 7.5, pH 7.5, 500 mM NaCl, 0.05% Tween-20 for 10 minutes. The membranes were transferred to a solution containing Per-anti-rab. IgG (Bio-Rad) (1:2000 dilution in 1% gelatin-TBS) and incubated for 1 h with gentle agitation. Following the second antibody solution, the membrane was rinsed as described above. Next, the blots were immersed in freshly prepared horse radish peroxidase color development solution (60 mg 4-chloro-1-naphthol in 20 ml cold methanol plus 60 ul cold 30% H₂O₂ in TBS, Bio-Rad Immun-Blot Assay Kit), shaken for 30 min until the purple bands or dots appeared, and washed with deionized distilled water. The membranes were photographed immediately because some fading of the stain occurred in 1-2 weeks.

Antibody Titration by Solid Phase Direct Binding: ¹²⁵I-Protein A

A solid phase direct binding assay was used to measure the binding of anti-IHN virus antibody to IHN virus. Purified IHN
virus was diluted to 2-8 ug/ml protein in PBS and 50 ul portions were added to wells of polyvinyl microtiter plates (Costar). The virus protein was allowed to incubate in the well for 12-14 h in a humidified 37°C incubator. The virus protein-containing wells were then treated with 5% BSA (RIA grade, Sigma Co.) in PBS at 37°C in a CO2 incubator for 2 h. Antibody from a two-fold series dilution in 1% BSA in PBS was placed into each well. After incubation at 37°C for 45 min, the wells were washed extensively. Bound anti-IHN antibody was detected by adding $10^5$ cpm of $^{125}$I-protein A to each well and incubating the microtiter plate for an additional 45 min. The plate was washed free of unbound $^{125}$I-protein A and subjected to autoradiography.

Antibody Titration by Solid Phase Direct Binding: Peroxidase-conjugated Sera

The "dot blot" was prepared as described by Hawkes, Niday, and Gordon (1982). The nitrocellulose membrane was cut into the appropriate size (12.3 cm x 15.8 cm) for the Hybri-dot apparatus (Bethesda Research Laboratories). The membrane was prewetted with TBS and placed on filter paper for air drying for 5 min. Then, the membrane was put into the Hybri-dot apparatus for sample application. Five microliters of purified IHN virus (2-8 ug/ml protein) in PBS was applied to the surface of the membrane in the wells created by the Hybri-dot apparatus and air dried. Following binding of virus protein, the membrane was immersed in 3% gelatin-TBS for 1 h to block any remaining unbound sites. The membrane was
treated then with rabbit anti-IHN virus antibody serum and Per-
anti-rab. IgG. The membrane was developed as described above.
RESULTS

Antibody Titer Determined by Solid Phase Direct Binding or Dot Blot

Peroxidase Staining

It was previously found that rabbit antisera prepared against IHN virus had neutralization titers of 1:250 or less (Leong et al., 1983). However, our preliminary studies indicated that the neutralization test does not quantitate the anti-viral antibodies in a sensitive and reproducible manner. In our effort to find an alternative test we found that antisera with a 50% plaque neutralization titer of 1:250 exhibit a titer of greater than 1:32,000 by solid phase direct binding assay. In this particular assay, the antigen-antibody complex was detected by the binding of 125I-labeled protein A (Figure IV. B). Another lot of antisera with a 50% plaque neutralization titer of 1:32 was found to have a titer of 1:4,096 in a solid phase direct binding assay which was developed with Per-anti-rab. IgG (Figure IV. 1A). For both assays, the binding antibody titer was 128-fold higher than the 50% plaque neutralization titer (Table IV. 1).

Detection of Infectious Hematopoietic Necrosis Viral Proteins by
125I-protein A or Peroxidase-conjugated Antisera Binding

Two different methods of detecting viral proteins in contact diffusion blots were examined with rabbit antisera raised against purified IHNV (Figure IV. 2). A polyacrylamide gel containing lanes of increasing concentrations of IHNV protein was subjected to electrophoresis as described in Materials and Methods. Identical
protein diffusion blots of this single gel were prepared by creating a sandwich of the gel between two nitrocellulose membranes. Each membrane was treated with the same lot and dilution of rabbit anti-IHN virus sera. Then, one membrane was developed with $^{125}$I-labeled protein A and the other membrane was treated with Perox-anti-rab. IgG. The five structural proteins of IHN virus were identified and designated as L (polymerase), G (glycoprotein), N (nucleocapsid phosphoprotein), M$_1$ (matrix phosphoprotein), and M$_2$ (matrix protein) (McAllister and Wagner, 1975; Leong, J. C., Hsu, Y. L., Engelking, H. M., and Mulcahy, D., 1981). It is clear that the limits of detection for both methods are identical. All five viral proteins were detected at 2 ug of virus protein (Figure IV. 3A and IV. 3B). The major viral proteins, G, N, and M$_1$, were detected by both methods at a lower limit of 10 ng of total viral protein and N was detected at 2.5 ng of virus protein. The differences in detection of the viral proteins reflect the relative amount of each protein in the virion (Hsu and Leong, in preparation).

**Strain Typing by $^{125}$I-Protein A and Peroxidase Staining**

Poor neutralization titers of rabbit antisera to IHN virus have made the detection of strain variation in IHN virus difficult. Thus, a method of typing IHN virus strains was developed by comparing the virion protein patterns of different isolates of IHN virus by SDS-PAGE (Leong et al., 1981). Major strain differences were noted in N and G proteins. However, the
SDS-PAGE method required that the virus sample be taken when 70% of the cell monolayer exhibited virus-induced CPE. This requirement results from the fact that host cellular protein synthesis is not inhibited until late in the infectious process. Thus, the synthesis of cellular and viral proteins in early infection cannot be easily distinguished by $^{35}$S-methionine labeling.

The detection of viral proteins by specific immune sera eliminates the problem of background host protein synthesis. Intracellular virus-specific proteins were distributed in gels by SDS gel electrophoresis and then transferred to nitrocellulose membranes as described previously. The resulting protein blots were then bathed in a solution containing rabbit anti-IHN virus sera. The protein blots were subsequently developed with Per-anti-rab. IgG or $^{125}$I-Prot. A as described in Figure IV. 3. The viral proteins, G, N, and M₁, were clearly detected by this technique (Figure IV. 4A and IV. 4B). The background of host protein synthesis was virtually eliminated by preadsorbing the rabbit anti-IHN virus sera with uninfected CHSE-214 cells prior to use. The apparent differences in background shown for the peroxidase stained gel versus the $^{125}$I-Prot. A stained gel were the result of using two different lots of anti-IHNV sera. The sera used with the $^{125}$I-Prot. A stained gel was not adequately adsorbed with uninfected cells.

It is apparent that the protein blotting technique may be used to type different strains of IHNV (Figure IV. 4). We have
confirmed our previous studies with $^{35}$S-methionine labeling in the typing of the different strains of IHN virus (Leong et al., 1981).
DISCUSSION

For many virological studies, the transfer of viral proteins to nitrocellulose paper and the localization of these proteins with specific ligands is rapidly developing as an important research tool (Towbin, Stahelin and Gordon, 1979; Bittner, Kupferer and Morris, 1980; Bollum and Chang, 1981; Burnette, 1981; Symington, Green and Brackmann, 1981; Legocki and Verma, 1981; Ramirez, Bonilla, Morino and Leon, 1983). We report here the application of this technique in the detection of IHN virus.

Localization of virus-specific proteins in gels by reaction with immune sera and subsequent detection of the reacted antibody with $^{125}$I-Prot. A or Per-anti-rab. IgG yielded results of equal sensitivity (Table IV. 2). The sensitivity of the assay was determined by the anti-IHNV antibody titer and not by the developing reagent, i.e. $^{125}$-Prot. A or Per-anti-rab. IgG. Approximately 10 ng of total virus protein was detectable with immune sera. The minimum level of detection obtained by radioactive labeling of virus-specific proteins with $^{35}$S-methionine was much lower. Less than 1 ng of virus protein was detected with $^{35}$S-methionine (Table IV. 2). Although the radioactive labeling requires less time for completion of the assay (Figure IV. 2), the radiolabeling method is not useful until 70% CPE is observed in the monolayer of infected cells. The background of host protein synthesis is not reduced sufficiently until 70% CPE in the culture has been reached. This requirement is not necessary with assays employing specific immune sera.
Previously, the major detraction in the use of immune sera for the detection of IHN virus had been the poor neutralizing titer of rabbit anti-IHN virus sera. This problem has been eliminated by the fact that rabbit antisera does have a high antibody binding titer. Moreover, methods employing specific binding of anti-IHN virus antibody may be used to characterize the IHN virus strains (Figure IV. 4). Immune sera will detect virus-specific proteins in protein blots at early times in infection. As soon as 2-3 hours after infection, fish cells will begin to synthesize the viral N-protein. The G-protein will appear at approximately 9-10 h postinfection (Leong, Hsu and Engelking, 1983). Since strains are differentiated on the basis of the electrophoretic mobility of N and G-proteins in SDS-gels, it should be possible to make an early diagnosis and strain identification of IHN virus (Figure IV. 3).

A close examination of the protein blot developed with rabbit anti-IHN serum revealed that the G, N, and M\textsubscript{1} proteins were readily demonstrated. However, L and M\textsubscript{2} proteins were not as readily detected (Figures IV. 3 and IV. 4). The missing L-protein in these protein blots may be explained by the fact that large proteins are not transferred efficiently from gel to nitrocellulose membrane (Burnette, 1981). Also, the relative number of L-protein molecules per virion is very small. There are approximately 40 L-molecules per virion as compared to 560 N-molecules per virion (Leong, Hsu and Engelking, 1983).
Finding a suitable explanation for the missing $M_2$ protein in Figure IV. 3 is more difficult. There are approximately 900 $M_2$ molecules per IHN virion and thus, $M_2$ is the most numerous virion protein (Leong, Hsu and Engelking, 1983). We have shown that $M_2$ does transfer to nitrocellulose membranes efficiently by following the transfer with Coomassie blue staining. It seems that the antisera does not detect $M_2$ as efficiently as $G$, $N$, and $M_1$ proteins. In Figure IV. 3A and IV. 3B, $M_2$ is detected at a minimum virion protein concentration of 200 ng whereas $M_1$ and $G$ are readily detected at a virion protein concentration of 10 ng. There is a 20-fold difference in the apparent level of detection for the $M_2$ protein and the $G$, $N$, and $M_1$ proteins. If the relative contribution of each protein to the total virion protein concentration is taken into account, that difference is even greater. These results may indicate that the $M_2$ protein is less immunogenic and therefore it does not induce a strong binding antibody reaction. Another explanation may be that the small $M_2$ protein is sufficiently denatured in the protein blots, that it is no longer recognized efficiently by the rabbit antibody (Burnette, 1981).

A comparison of different IHN virus strains by detecting virion proteins with immune sera has several other uses. The technique allows us to compare the immunologic relatedness of the different IHN virion proteins. It is clear that all $N$ and $M_1$ proteins are closely related (Figure IV. 4). However, there are
differences in the staining of the G-protein. Antisera to the Round Butte strain of IHNV did not light up the G-protein for the IHNV strain isolated in pink salmon from Tamgas Creek, Alaska (Leong et al., 1981). This result suggests that this isolate may be antigenically different from the other IHNV isolates. However, a more complete study is required before these two isolates are classified as separate immunological groups.
Table IV. 1. A Comparison of Antibody Titer by Solid Phase Direct Binding and Virus Neutralization Assays.

<table>
<thead>
<tr>
<th>Antibody Lot</th>
<th>(A) 50% Plaque Neutralization</th>
<th>(B) Solid Phase Direct Binding</th>
<th>Relative Activity$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1:250 125I-Prot.A</td>
<td>1:32,768</td>
<td>131</td>
</tr>
<tr>
<td>2</td>
<td>1:32 Per-anti-rab.IgG</td>
<td>1:4,096</td>
<td>128</td>
</tr>
</tbody>
</table>

$^a$A comparative measure of each lot of sera for binding vs. plaque neutralization activity, (B)/(A).
Table IV. 2. A Comparison of Diagnostic Methods for IHNV.

<table>
<thead>
<tr>
<th>Method</th>
<th>Level of Detection</th>
<th>Time Required for Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus Neutralization</td>
<td>0.007 ng&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7-13 days</td>
</tr>
<tr>
<td>Radiolabeling</td>
<td>&lt;1.000 ng&lt;sup&gt;b&lt;/sup&gt;</td>
<td>48.0 hours</td>
</tr>
<tr>
<td>Per-anti-rab.IgG</td>
<td>10.000 ng&lt;sup&gt;c&lt;/sup&gt;</td>
<td>51.5 hours</td>
</tr>
<tr>
<td>^{125}I-Prot.A</td>
<td>10.000 ng&lt;sup&gt;c&lt;/sup&gt;</td>
<td>66.0 hours</td>
</tr>
</tbody>
</table>

<sup>a</sup>The virus protein present in a plaque neutralization assay was calculated from the estimated mass of the virion, 1.31 x 10<sup>-16</sup> grams, and the particle to infectivity ratio, 1,000:1 (Leong et al., 1983). Each plaque neutralization assay contained 50 infectious particles.

<sup>b</sup>The level of detection was determined by first developing the gel with a silver stain. The minimum protein concentration detected with a silver stain was 1 ng. However, ^{35}S-methionine labeling produced virus bands where no protein band was found in a silver stain of the same gel.

<sup>c</sup>The level of detection was determined in the experiment shown in Figure 3B. The amount, 10 ng, is the total virus protein applied to the gel. At least 3 virion proteins were detected at this protein concentration. This represents an approximation of the minimum level of detection of ^{125}I-Protein A binding.
Figure IV.1. Titration of Anti-Infectious Hematopoietic Necrosis Virus sera by Solid Phase Direct Binding Assay. Serial two-fold dilutions of rabbit anti-IHNV sera was added to wells containing purified IHN virus bound to the bottom of microtiter plates or to nitrocellulose membranes as described in Materials and Methods. The bound antibody was detected with Per-anti-rab. IgG in A or I-prot. A in B. The arrows indicate the last dilution of the antisera which produces a positive assay. In A, the last dilution is 1:4,096. In B, the last dilution is 1:32,768.
Figure IV. 1.
Figure IV.2. A comparison of the time required to complete each assay for Infectious Hematopoietic Necrosis Virus. A detailed account of each assay is described in the Materials and Methods.
Figure IV.2.

**TIME REQUIREMENTS FOR ASSAYS**

<table>
<thead>
<tr>
<th>RADIOACTIVE LABELING</th>
<th>IMMUNO-PEROXIDASE</th>
<th>125I-PROTEIN A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
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<tr>
<td>IHNV INFECTED CELLS</td>
<td></td>
<td></td>
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<tr>
<td>30 h incubation</td>
<td>9 h incubation</td>
<td></td>
</tr>
<tr>
<td>at 16°C</td>
<td>at 16°C</td>
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</tr>
<tr>
<td>35S-methionine(^a)</td>
<td>Cell Lysis</td>
<td></td>
</tr>
<tr>
<td>1 h, 16°C</td>
<td>with Lysing Buffer</td>
<td></td>
</tr>
<tr>
<td>Cell Lysis with</td>
<td>SDS-PAGE</td>
<td></td>
</tr>
<tr>
<td>Lysing Buffer</td>
<td>2 h</td>
<td></td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Immersion Buffer</td>
<td></td>
</tr>
<tr>
<td>2 h</td>
<td>1 h</td>
<td></td>
</tr>
<tr>
<td>Fix in Methanol-</td>
<td>Transfer to</td>
<td></td>
</tr>
<tr>
<td>Acetic Acid</td>
<td>Nitrocellulose</td>
<td></td>
</tr>
<tr>
<td>1 h</td>
<td>24 h</td>
<td></td>
</tr>
<tr>
<td>Wash in H(_2)O</td>
<td>Blocking Solution</td>
<td></td>
</tr>
<tr>
<td>1 h</td>
<td>1 h</td>
<td></td>
</tr>
<tr>
<td>X-Ray Film</td>
<td>Rabbit Anti-IHV</td>
<td></td>
</tr>
<tr>
<td>12 h</td>
<td>sera</td>
<td></td>
</tr>
<tr>
<td>Develop</td>
<td>125I-Protein A</td>
<td></td>
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<tr>
<td></td>
<td>Staining</td>
<td></td>
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<tr>
<td></td>
<td>1.5 h</td>
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<tr>
<td>Peroxidase Staining</td>
<td>Staining, 4 h</td>
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<tr>
<td>X-ray Film</td>
<td>12 h</td>
<td></td>
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<tr>
<td>12 h</td>
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<tr>
<td>48 HOURS</td>
<td>51.5 HOURS</td>
<td>66 HOURS</td>
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</tbody>
</table>

\(^a\)35S-methionine labeling as described by Leong et al., 1981.
Figure IV.3. A comparison of protein blots developed with $^{125}$I-Prot. A and with Par-anti-rab. IgG reagents. Different concentrations of purified IHN virus were subjected to SDS-PAGE. After electrophoresis, identical protein blots were prepared from the same gel as described in Materials and Methods. The virus protein concentration in each lane were as follows: a. 2 ug, b. 1 ug, c. 200 ng, d. 100 ng, e. 50 ng, f. 10 ng, g. 5 ng, h. 2.5 ng, i. 0.5 ng. A. Protein bands were developed with horseradish peroxidase-conjugated goat anti-rabbit gamma globulin sera. B. Protein $^{125}$I bands were developed with I-labeled Protein A.
Figure IV. 3.
Figure IV.4. A comparison of different strains of IHNV with protein blots developed with Per-anti-rab. IgG and with $^{125}$I-Prot. A. Lysates of cells infected with different strains of IHNV were subjected to SDS-PAGE as described. Protein blots were prepared from the gel and reacted with rabbit anti-IHNV sera. The immune complexes were localized with either Per-anti-rab. IgG (A) or $^{125}$I-Prot. A (B). Each lane contained a different IHNV strain as follows: (1) Feather River, (2) Warm Springs, (3) Trinity River, (4) Tamgas River, (5) Nan Scott Lake, (6) Suttle Lake, (7) Elk River, (8) Coleman Hatchery, (9) Round Butte Hatchery, (10) Cedar River, (11) Karluk River, and (12) Beaver Creek Hatchery.
<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
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<th>4</th>
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<th>9</th>
<th>10</th>
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<tbody>
<tr>
<td><strong>Peroxidase</strong></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>125I-Protein</td>
<td>A</td>
<td>G</td>
<td>N</td>
<td>M</td>
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Figure IV. 4.
REFERENCES


V. BIBLIOGRAPHY


