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RAPID DIAGNOSIS OF IHN VIRUS INFECTION IN SALMON AND STEELHEAD TROUT

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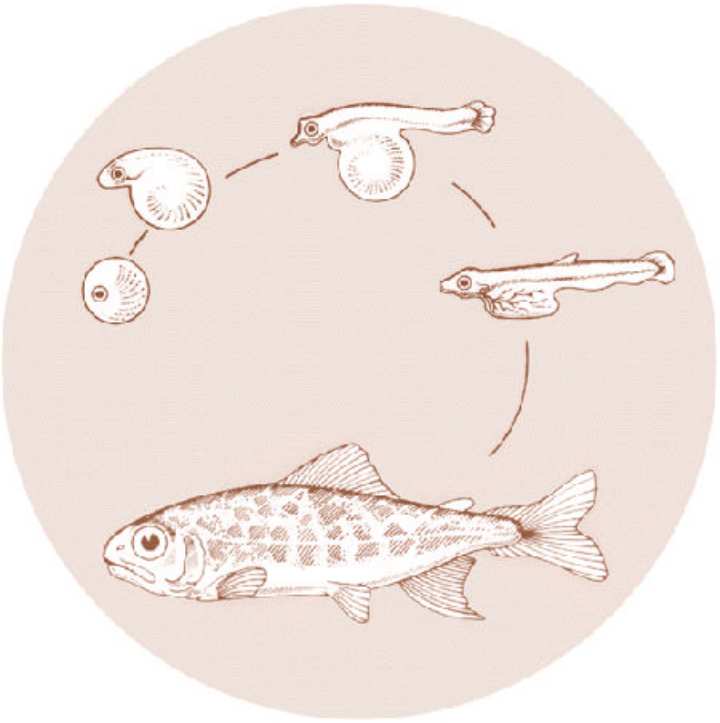

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Rapid Diagnosis of IHN Virus Infection in Salmon and Steelhead Trout

Final Report

by

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PREFACE

The main objective for this study was the development of a rapid diagnostic method for IHN virus in fish tissue samples. The rationale for developing new techniques for diagnosing IHNV infection was that present methods were time consuming and dependent on virus neutralization by specific antisera, a reagent that was not readily available or reliable. Fish pathologists required a rapid detection method which was sensitive enough to detect virus strain differences so that they could provide data for effective management decisions in controlling the spread of IHNV.

Bonneville Power Administration's (BPA) role in efforts in fish diseases and more generically the protection, mitigation, and enhancement of Columbia River salmon and steelhead populations, is mandated by Congress through the Pacific Northwest Electric Power Planning and Conservation Act (Regional Act), Pub. L. 96-501. Section 4 (h) of the Regional Act directs the Northwest Power Planning Council to develop a Fish and Wildlife Program. BPA's Administrator is authorized in Section 4(h)(10))(A) to "use funds and the authorities available to the extent affected by the development and operation of any hydroelectric project of the Columbia River and its tributaries." The fund is to be used to implement measures that are consistent with the Council's Fish and Wildlife Program.

The research detailed in this final report is consistent with these objectives. This final report has been prepared as part of BPA's policy to encourage the preservation and dissemination of research results by publication in scientific journals.

ABSTRACT

Three new methods for identifying IHNV in fish tissue samples have been developed. These methods are based on the initial growth of the virus in cells in culture and the subsequent analysis of the viral proteins by gel electrophoresis. These methods permit positive identification of IHNV infection as well as strain typing quickly.

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 ^{35}S-methionine labeled virus. This analysis led to the classification of these virus isolates into 5 types according to the apparent molecular weights of the virion N and G proteins. Type 1 virus is characterized by a nucleocapsid protein with an approximate molecular weight of 40,500 daltons. Types 2 and 3 viruses have N proteins of 42,800 and 43,250 respectively. Virus belonging to the Type 2 classification was responsible for the recent outbreaks of IHN disease among fish in the Lower Columbia River. The Type 3 virus isolates were obtained from California. In addition to Type 3 virus, California has a different IHN virus type at Coleman hatchery on the Sacramento River. This Type 4 virus is characterized by a slower migrating G protein with an apparent molecular weight of 70,000 daltons. All other virus isolates have G proteins of molecular weight of 67,000 daltons. The Type 5 IHN virus category contains isolates not sufficiently distinct to warrant

their classification as a separate type. These findings have been useful in determining (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 the progeny fish.

The specific radioactive labeling of virus proteins in the infected cell has also been used to study the intracellular synthesis of viral proteins. Using techniques originally developed for diagnosing an IHNV infection, we were able to characterize the sequence of events leading to the synthesis of viral proteins. The temporal synthesis of the viral polypeptides suggest that they were derived from the translation of independently transcribed monocistronic mRNAs.

Polyvalent antisera to purified IHNV was used to develop two additional methods for detecting IHNV infection in cells. Antibody to IHNV was measured by solid phase direct binding assays with radioiodinated Protein A or with immunoperoxidase staining. The high binding antibody titer of rabbit anti-IHNV sera made possible the development of two immunological tests for IHNV. Both immunological methods were highly specific, sensitive to less than 10 ng of virus protein and represented new methods of characterizing different strains of IHNV.

Table of Contents

List of Illustrations

III. Synthesis of the Structural Proteins of Infectious Hematopoietic Necrosis Virus

Figures:

Table:

Background

I. 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). The viral etiology of the disease was confirmed by its transmission with cell-free filtrates and the resistance of the disease to antibiotic therapy (Watson, 1954). This particular isolate of the viral agent was not retained in storage.

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

In 1958 an epizootic also occurred among chinook salmon (O. 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 (S. 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.

II. 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, personal communication). 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 many salmonid fish species in hatcheries of the Columbia River basin in Washington, Oregon and Idaho (Amend and Wood, 1972; Groberg and Fryer, 1983).

In Oregon, the First report of IHNV was from the Willamette River Salmon Hatchery in 1958 (Wingfield et al., 1969). In 1975, rainbow trout and kokanee salmon (landlocked 0. nerka) suffered high 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 (S. gairdneri) at Round Butte Hatchery which is located below the Metolius and Deschutes Rivers (Mulcahy et al., 1980). Since then, IHN 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, significant 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 high mortality for Sacramento River chinook salmon but not steelhead trout juveniles was reported (Parisot and Pelnar, 1962). This agent was isolated and characterized by Nims et al. (1970) and

Wingfield and Chan (1970). Recently, IHNV-caused mortality in juvenile steelhead was reported in the Sacramento River basin (Chen, 1983).

Grischkowsky and Amend (1976) found that IHNV caused high 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 sockeye salmon fry in Western Canada (Williams and Amend, 1976).

Epizootics of IHNV have occurred also 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 diseases of 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 dog salmon (O. 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, Hedrick, and Fryer, personal communication).

TTI. 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 (O. 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 and 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. More recently, Chen has reported that coho salmon may be susceptible to large doses of IHNV (Chen, 1983).

IV. Symptoms of IHNV

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).

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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. Necrosis of the hematopoietic tissues in kidney, spleen, and pancreas are the most characteristic histopathological signs (Amend et al., 1969; Yasutake et al., 1965).

V. Diagnostic Methods

In outbreaks of IHNV, infected fish exhibit behavioral changes such as lethargy or hyperactivity. When these fish are examined and internal signs of disease are present, IHN 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 cyprini (EPC) (Fendrick et al., 1982). Since these different cell lines do exhibit differences in their sensitivity to virus infection, the choice of a proper cell line for IHNV 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).

VI. Transmission

Transmission routes for IHNV have been examined intensively. One of the major modes of IHNV transmission is thought to be vertical transmission. 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 reservoirs for congenital transmission from adult to fry. Vertical transmission has been used to explain the outbreaks of IHNV in isolated cases of the disease in West Virginia, Minnesota, and Nebraska where contaminated eggs or fish were transported into these states.

There is also much 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; Winfield 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. Several investigators have shown that the feeding of infected viscera to juvenile sockeye salmon will result in the transmissison of IHNV (Watson et al., 1954; Groberg et al., 1982).

VII. Control of IHNV 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 the Oregon Sockeye virus isolate of IHNV from rainbow trout repeatedly on 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-borne 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. In the control, ninety percent or more of unvaccinated fish succumbed to IHN disease (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 also shown to develop within 54 days after vaccination. Despite the success of the attenuated vaccines in sockeye salmon, attenuated

viruses have not been used to vaccinate fish at the 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. 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 disease if shipped to other areas (Mulcahy, 1983).

VIII. 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, but not identical.

The virus is ether-sensitive and will grow in the presence of 5-bromo-deoxyuridine. These properties suggest that IHN virus is a lipid-enveloped RNA virus (Wingfield et al., 1969). The RNA character of the viral genome was determined by McCain et al. (1974). The nucleic acid extracted from purified IHN virus was tested by the orcinol and phenylamine reaction, found to have the density of RNA in cesium sulfate, and found to be 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 of the virus suggest that IHN virus should be classified as a member of the family Rhabdoviridae.

IX. Characteristics of the viral proteins of Rhabdoviridae

There are two genera in the family Rhabdoviridae: Vesculovirus (Vesicular Stomatitis virus group) and Lyssavirus (Rabies virus group). These genera are distinguished by distinct virion protein patterns which may be resolved in sodidum dodecyl sulfate

polyacrylamide gels (SDS-PAGE). The Vesiculovirus protein pattern consists of one large polypeptide which is the polymerase L (2,000 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 the NS protein (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 Ml, and 20,000 for the matrix protein M_2. The G was shown to be a glycoprotein by labeling with ^{3}H-glucosamine, and the N and Ml proteins were found to he phosphoproteins by the incorporation of ^{32}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, 93,000 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 viral strain differences or differences 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_1, and M_2 were unique gene products. The maps for G_1 and G_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_1 and G_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_1 contained 40% more carbohydrate than G_2.

Therefore G_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 observd (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_1, and M_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_1 or G_2. The peptide patterns obtained after proteolysis of G_1, G_2 and P60 showed that P60 and G_2 were identical. The patterns showed that G_1 was similar to G_2 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, G_1 and G_2, appear in vesicular stomatitis virus (VSV) infectd cells (Knipe et al., 1977). VSV 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). VSV mRNA from 3 of 4 bands have been eluted from formamide-polyacrylamide gels and translated in the Krebs II ascites cell-free translation system. Band 2 mRNA (0.7×10^6 daltons) directed the synthesis of the protein moiety of the glycoprotein (G) and 3 (0.55×10^6 daltons) directed the synthesis of the N protein. Rand 4 mRNA (0.28×10^6 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., 197).

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-M_1-M_2-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), and IHNV (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).

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II. A Comparison of Detection Methods for Infectious Hematopoietic Necrosis Virus

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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 et al., 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 et al., 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 IHN 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 IHN 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. IgC) (Figure 1C). 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.

Figure 1C. Schematic Diagram of Immunoblot Assay

MATERIALS AND METHODS

Cell and Viruses

Chinook salmon (Oncorhynchus tschawytscha) 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 ug/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 cm^2 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 buffer (0.15 M NaCl; 0.01 M Tris-HCl, pH 8.3; 9.01 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 $TCID_{50}$/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_2HPO_4, 0.0015 M KH_2PO_4, 0.0027 M KC1). 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) in Tris-glycine buffer. Gels were subjected to an electrophoretic current of 20 mA/gel until the bromephenol 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}I-Prot. A or Per-anti-rab. IgG.

^{125}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}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 l00-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 fresh1.y prepared horse radish peroxidase color development solution (60 mg 4-chloro-1-naphthol in 20 ml cold methanol plus 60 ul cold 30% H_20_2 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 l-2 weeks.

Antibody Titration by Solid Phase Direct Binding: ^{125}I-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 CO_2 incubator for 2 h. Antibody from a two--fold series dilution in 1% BSA in PES 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}L-protein A to each well and incubating the microtiter plate for an additional 45 min. The plate was washed free of unbond ^{125}T-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 Rlot 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 alternativeetest 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 ^{125}I-labeled protein A (Figure I LR). 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 I 1A). For both assays, the binding antibody titer was 128-fold higher than the 50% plaque neutralization titer (Table 1).

Detection of infectious Hematopoietic Necrosis Viral Proteins by ^{125}I-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 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 Per-anti-rab. IgG. The five structural proteins of IHN virus were identified and designated as L (polymerase), G (glycoprotein), N (nucleocapsid phosphoprotein), Ml (matrix phosphoprotein), and M_2 (matrix protein) (McAllister and Wagner, 1975; Leong et al., 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 3A and 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 syntheses is not inhibited until late in the infectious process. Thus, the syntheses or 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 3. The viral proteins, G, N, and M_1, were clearly detected by this technique (Figure 4A and 4B). The background of host proteinsyntheses 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 4). We have confirmed our previous studies with ^{35}S–methioninelabeling 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 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 2). Although the radioactive labeling requires less time for completion of the assay (Figure 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 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 3).

A close examination of the protein blot developed with rabbit anti-IHN serum revealed that the G, N, and Ml proteins were readily demonstrated. However, L and M_2 proteins were not as readily detected (Figures 3 and 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 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 3A and 3B, M_2 is detected at a minimum virion protein concentration of 200 ng whereas M_1 and C arereadily 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 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.

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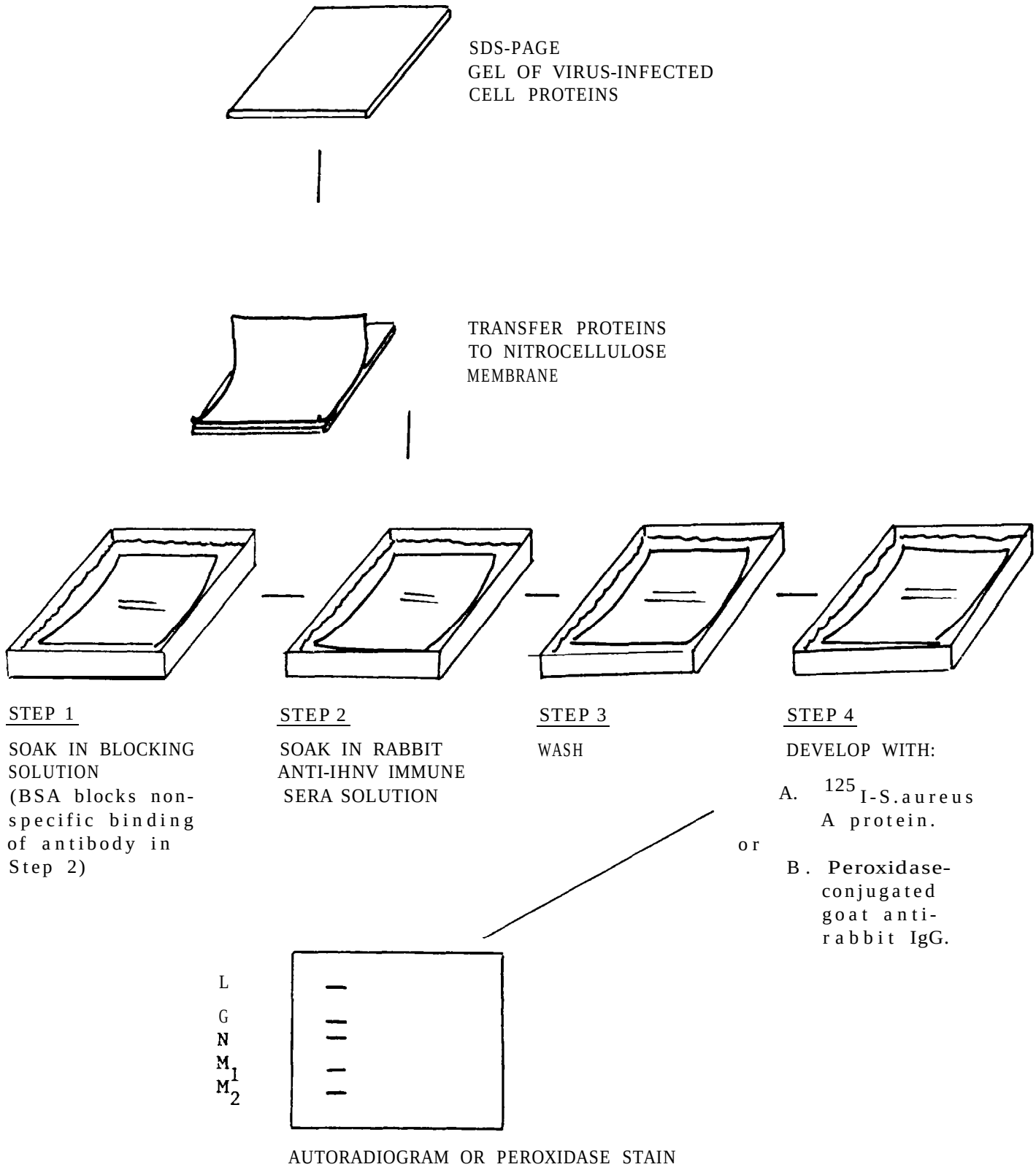


Figure 1A. Schematic Diagram of Immunoblot Assay

LEGENDS

Figure 1. Titration of Anti-IHN virus sera by Solid Phase Direct Binding Assay. Serial two-fold dilutions of rabbit anti-IHN virus 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 ^{125}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.

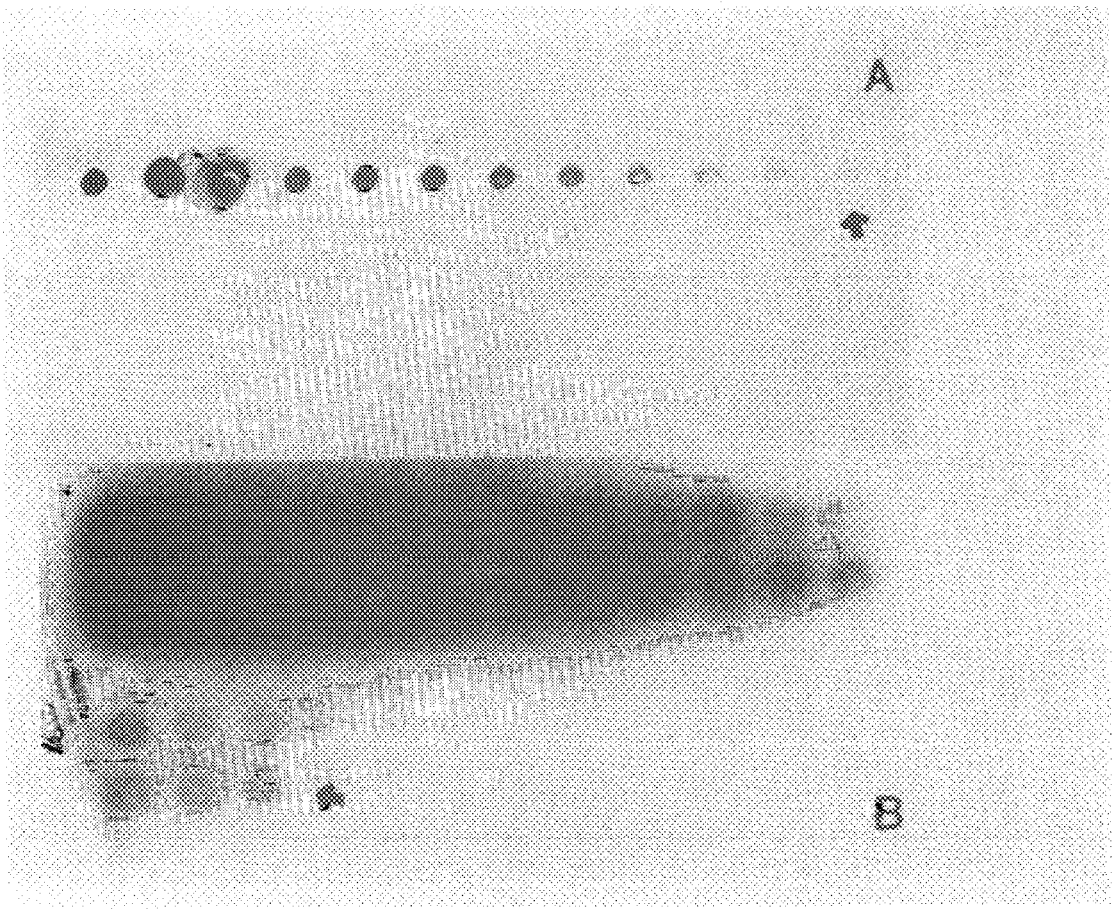
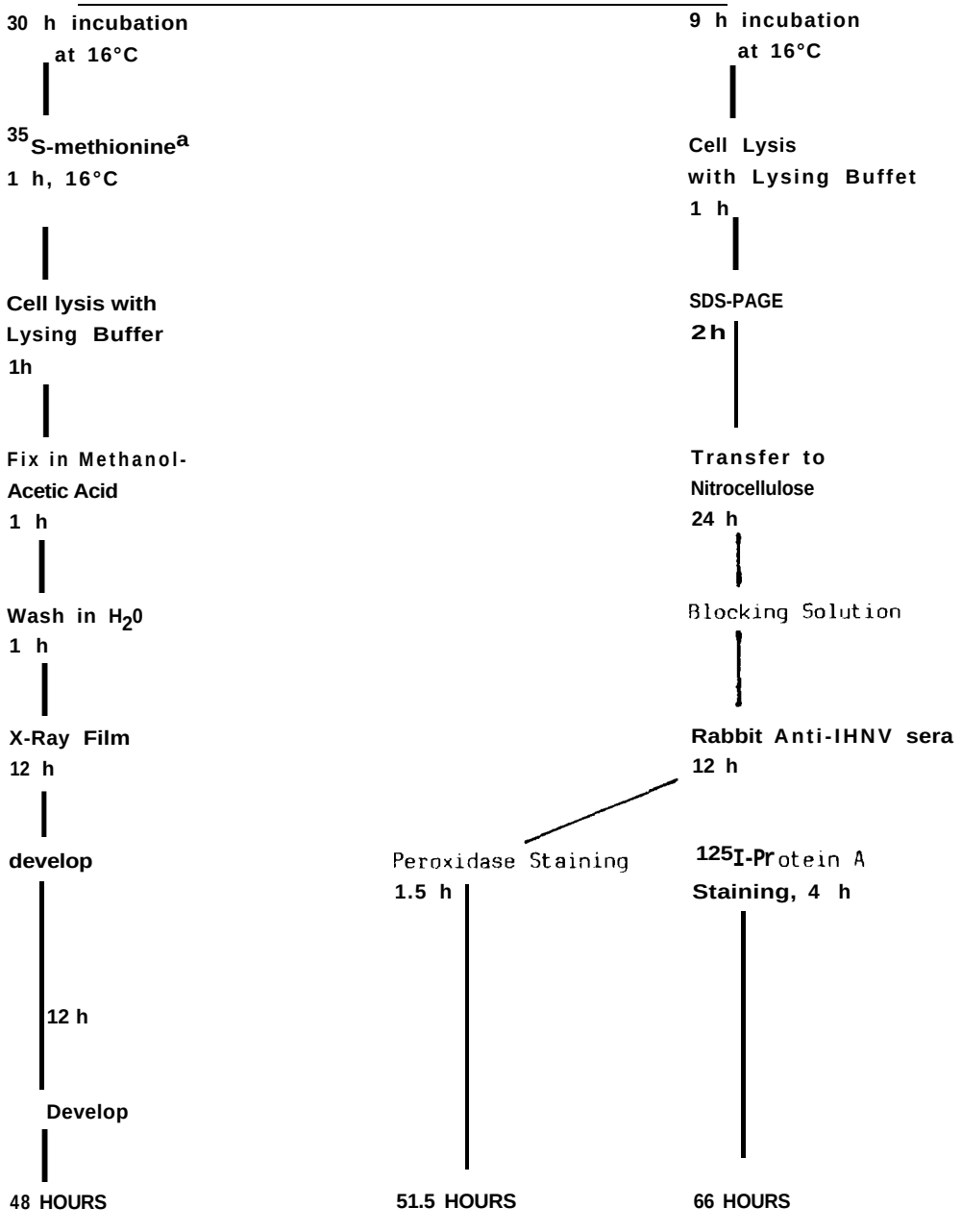

A
B

Figure 2. A comparison of the time required to complete each assay for IHN virus. A detailed account of each assay is described in the Materials and Methods.

TIME REQUIREMENTS FOR ASSAYS

IHNV INFECTED CELLS



RADIOACTIVE LABELING	IMMUNO-PEROXIDASE PROTEIN BLOT	^{125}I-PROTEIN A PROTEIN BLOT

[a] ^{35}S-methionine labeling as described by Leong et al., 1981

Figure 3. A comparison of protein blots developed with ^{125}I-Prot. A and with Per-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 horse radish peroxidase-conjugated goat anti-rabbit gamma globulin sera. B. Protein bands were developed with ^{125}I-labeled Protein A.

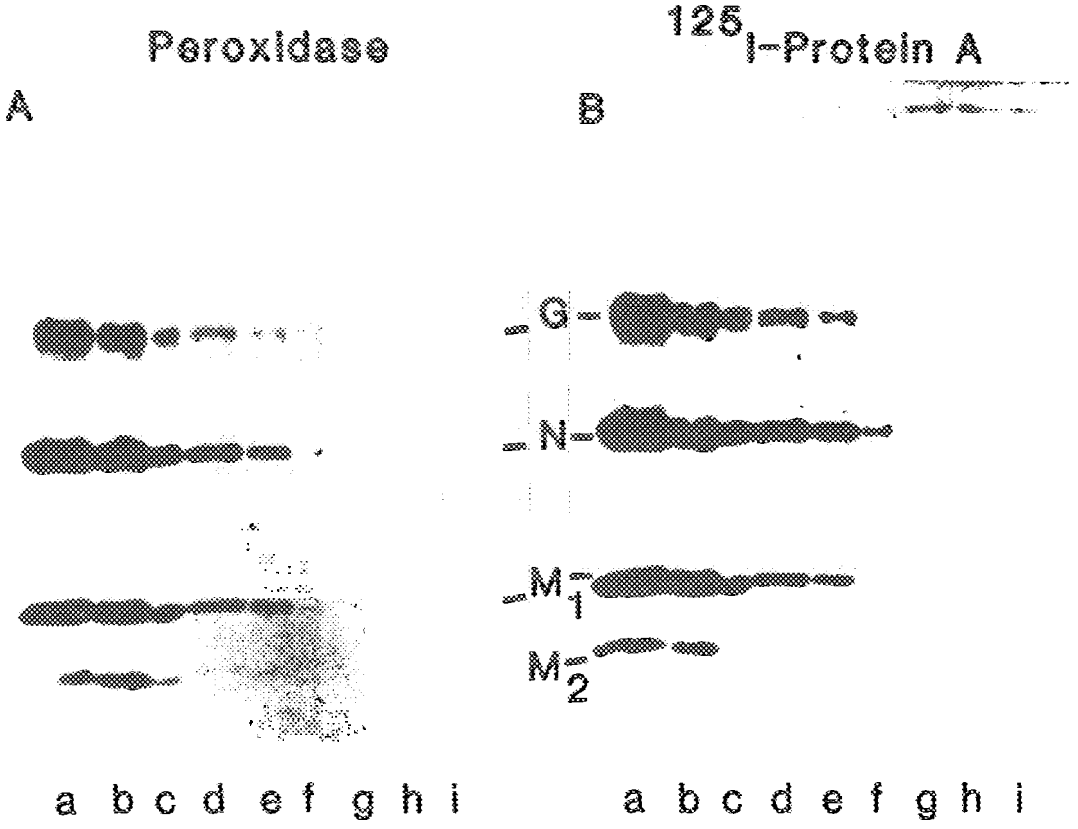

Peroxidase
^{125}I-Protein A
A
B
G
N
M_1
M_2
a b c d e f g h i
a b c d e f g h i

Figure 4. A comparison of different strains of IHN virus with protein blots developed with Per-anti-rab. IgG and with ^{125}I-Prot. A. Lysates of cells infected with different strains of IHN virus were subjected to SDS-PAGE as described. Protein blots were prepared from the gel and reacted with rabbit anti-IHN virus sera. The immune complexes were localized with either Per-anti-rab. IgG (A) or ^{125}I-Prot. A (B). Each lane contained a different IHN virus 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.

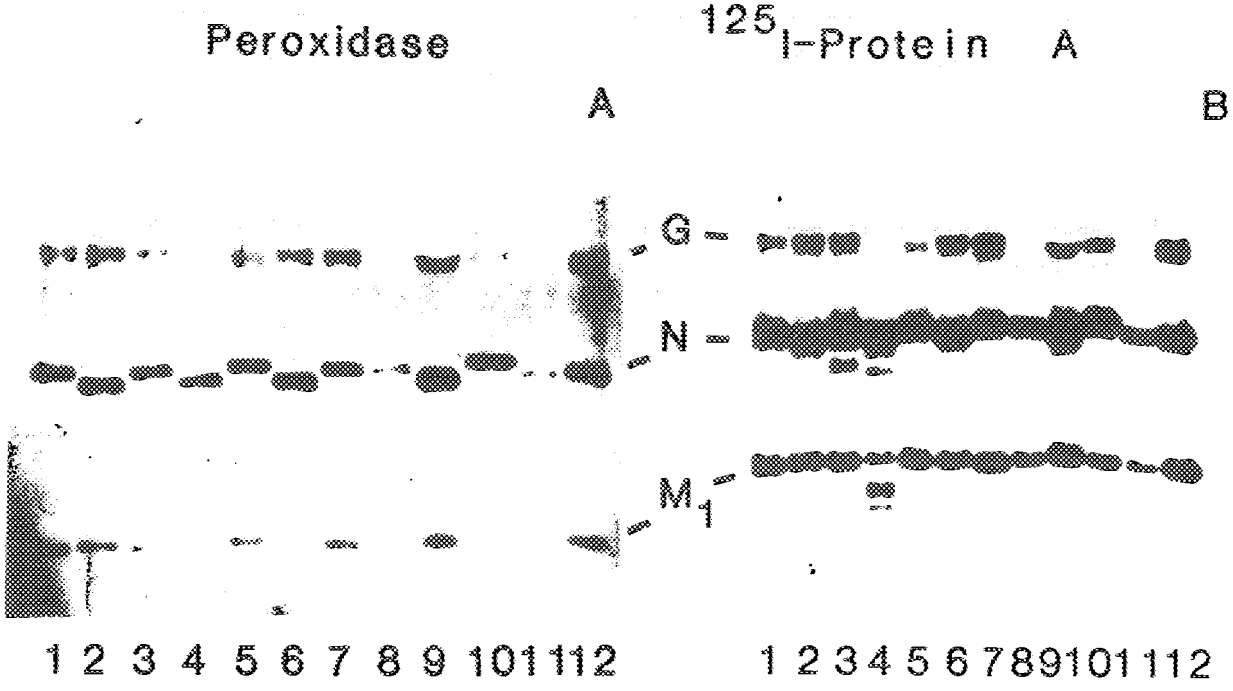

Peroxidase
125I-Protein A
A
B
G
N
M1
1 2 3 4 5 6 7 8 9 10 11 12
1 2 3 4 5 6 7 8 9 10 11 12

Table 1. A Comparison of Antibody Titer by Solid Phase Direct Binding and Virus Neutralization Assays.

Antibody Lot	(A) 50% Plaque Neutralization		(B) Solid Phase Direct Binding	Relative Activity[a]
1	1:250	^{125}I-Prot.A	1: 32,768	131
2	1:32	Per-anti-rab. IgG	1:4,096	128

[a]A comparative measure of each lot of sera for binding vs. plaque neutralization activity, (B)/(A).

Table 2. A Comparison of Diagnostic Methods for IHNV.

Method	Level of Detection	Time Required for Assay
Virus Neutralization	0.007 ng[a]	7-13 days
Radiolabeling	<1.000 ng[b]	48.0 hours
Per-anti-rab. IgG	10.000 ng[c]	51.5 hours
^{125}I-Prot.A	10.000 ng[c]	66.0 hours

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

[b]The level of detection was determined by firs developing the qel with a silver stain. The minimum protein concentration detected with a silver stain was 1 nq. However, ^{35}S-methionine labeling produced virus bands where no protein band was found in a silver stain of the same gel.

[c]The level of detection was determined in the experiment shown in Figure 38. The amount, 10 nq, 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 minimun level of detection of ^{125}-I-Protein A binding.

III. Synthesis of the Structural Proteins of Infectious Hematopoietic Necrosis Virus

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Journals for publication: Journal of General Virology

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_1 and M_2 for the envelope proteins. The glycoprotein nature of the virion procein G has been confirmed by specific labeling of the G protein with ^{3}H-glucosamine and two phosphoproteins have been identified, N and M_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_1, and 17,000 to 20,000 for M_2. These variations in the reported size of the virion proteins suggested that the variations might result from strain differences in the IHN virus 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 IHN virus 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, IHN virus infection results in the inhibition of cellular protein sythesis. 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 (Oncorhynchus tchawytscha) embryo cells (CHSE-214) were obtained from J. L. Fryer, Dept. Microbiology, Oregon State University, Corvallis, Oregon. The epithelioma papillosum cyprini (EPC) cells were obtained from D. Mulcahy, National Fisheries Research Center, Seattle, Washington. Both cells were grown in minimum essential media (MEM) in Earle's salts (Autopow MEM, Flow Laboratories) supplemented with new-born bovine serum (Grand Island Biologicals Company) to 10% glutamine to 10 mM, penicillin to 100 units/ml, and streptomycin to 100 µg/ml.

Infectious hematopoietic necrosis virus (IHNV) 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 EPC cells. For preparation of large quantities of unlabeled and radioactively labeled virus, the virus grown in CHSE-214 cells. No different 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 only three to five times.

Virus Purification

The virus propagated in CHSE-214 cells on 150 cm^2 plastic tissue culture flasks (Corning). The 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 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 STE buffer (0.15 M NaCl 0.01 M Tris-HCl, pH 8.3, 0.01 M EDTA, pH 7.0) 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 SW50.1 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% of 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 in Tris buffer. 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% tricholoroacetic 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 TCID50/cell in 0.1 ml of MEM supplemented with 2% dialyzed fetal bovine serum (Grand Islan d 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_2HPO_6, 0.0015 M KH_2PO_4, 0.0027 K KCl). The cells were lysed with 25µl of SDS-lysis buffer (0.5 M urea, 2% Nondet P-40, 5% 2-mercaptoethanol). The lysates were stored at -7O°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 unlabeled 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 virion 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 l-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 IHN 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 1). Five virion proteins were identified with estimated 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 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 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 3.57×10^6 (Kurath and Leong, unpublished data).

In determining the virion ratio of RNA to protein, a suspension of purified virus labeled with (3H) uridine containing 0.48 mg of protein per ml, and a total of 8.36 x 10^5 cpm was used. After extraction, the purified RNA had a specific activity of 3.6 x 10^4 cpm per µg 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 of 21:l. 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 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 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 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 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 5A). These two forms, G_2 for the lower band (65,000) and Gl 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 G_1 and G_2 appeared and after 5 and 10 min of label, G_2 was the dominant glycoprotein being synthesized in the cell (Figure 6A). However, at 30 min, the amount of radioactivity appeared equally distributed in both bands as labeled Gl protein accumulated in the cell. By 60 min, the Gl 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 6B). A 5 min exposure to the labeled precursor resulted in a predominant G_2 band. After a chase of 20 min with excess unlabeled methionine, the predominantly labeled band was G_1. These results do suggest that G_2 is synthesized immediately and then is further glycosylated to form G_1. 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 M_2 proteins were observed. The rates of synthesis for G and M_1 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 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 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 ata 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 (Gl and G2); 40,500 (N); 22,500 (Ml); 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 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, M_1, and M_2 molecules per virion for rabies is reported as 79 (L), 1723 (G_1 and G_2), 1975 (N), 402 (M_1), and 1156 (M_2) (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 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.

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.

Table 1. Estimated number and molecular weight of IHN virus proteins

Protein Species	Mol. St. (x 10^3)[a]	Percentage of Total Virus Proteins		Mol. Wt. of Protein per Virion (x10^6)[d]		Number of Molecules per Virion[e]	
		Incorporation[c]	Stained[c]	Incorp.	Stained	Incorp.	Stained
L	150.0	8.6	8.1	6.5	6.1	43	40
G	67.0	14.9	26.1	11.1	19.5	166	290
N	40.5	41.4	30.4	31.0	22.7	766	560
M_1	22.5	8.1	15.5	6.1	11.6	270	514
M_2	17.0	27.0	19.9	20.3	14.9	1,192	874

[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 ther molecular wt.

Infection of salmon cells with IHN virus 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.

It is clear that there are two species of virion glysoproteins, 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 6B) suggests that G_2 is synthesized and chased into the Gl 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 G2 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 hours after infection. At h-7 hours after infection, the membrane proteins, M_1 and M_2, can be identified in the autoradiograms. The two forms of the glycoprotein, G_1 and G_2 are found at 9-10 hours 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 and it constitutes a very small portion of the total protein synthesis in the infected cells. However, L protein accumulates faster than the other virion proteins, indicating that L is not turned over as quickly. These experiments show that IHN virus proteins are synthesized independently. Virus production begins at 12-14 hours after infection (Leong et al., 1981).

It appears that IHN virus 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 vivo. These mRNA species correspond appropriately in sizes to the RNA species expected for each virion protein.

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LEGENDS

Fig. 1. A plot of the relative migration of IHNV structural proteins, L, G, N, M, 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).

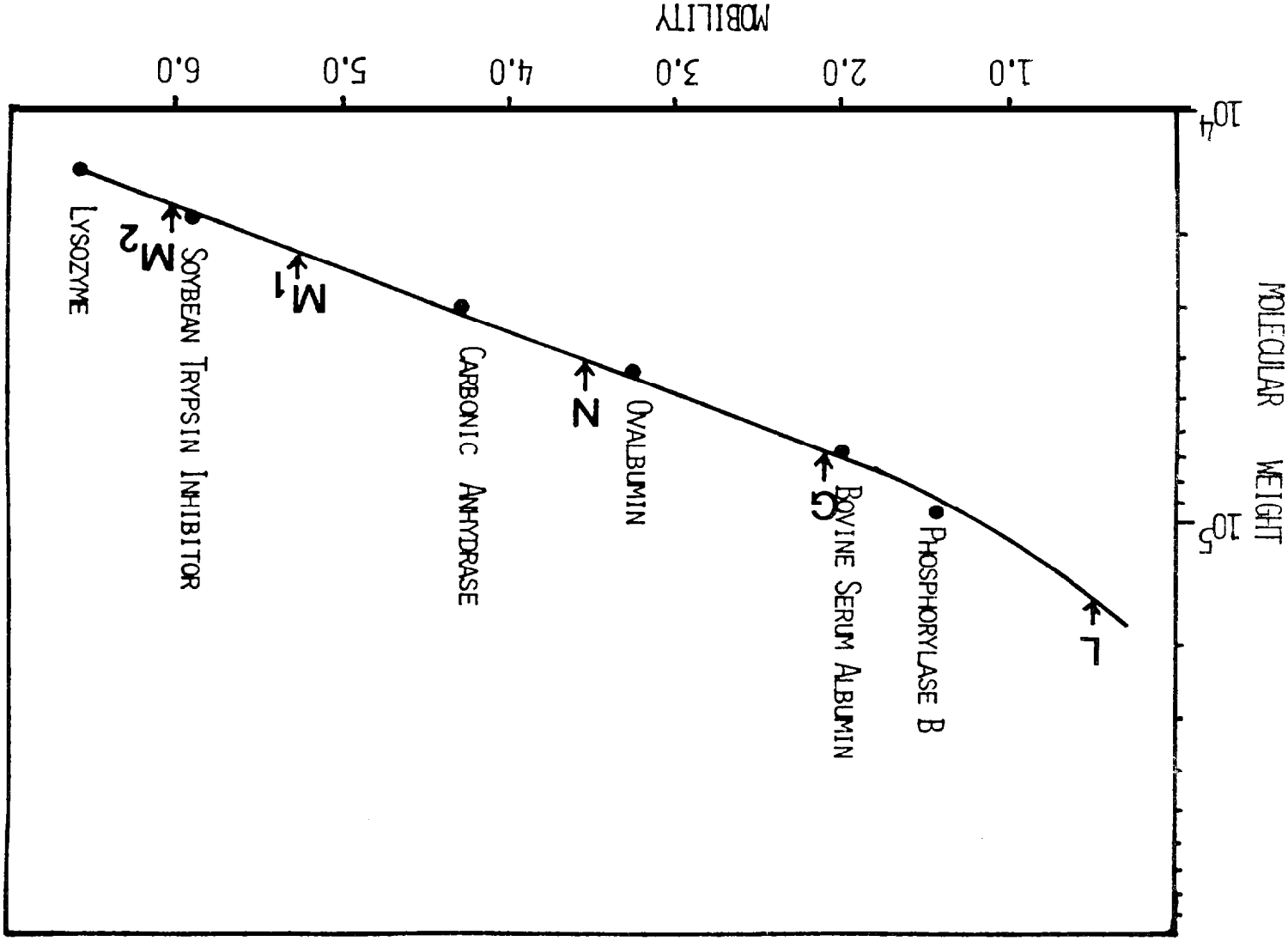

MOLECULAR WEIGHT
10^4
10^5
MOBILITY
1,0
2,0
3,0
4,0
5,0
6,0
PHOSPHORYLASE B
BOVINE SERUM ALBUMIN
OVALBUMIN
CARBONIC ANHYDRASE
SOYBEAN TRYPSIN INHIBITOR
LYSOZYME
M1
M2

Fig. 2. Densitometer tracings of SDS-polyacrylamide gels of purified IHNV virus stained with Coomassie blue, broken line, or silver nitrate, solid line.

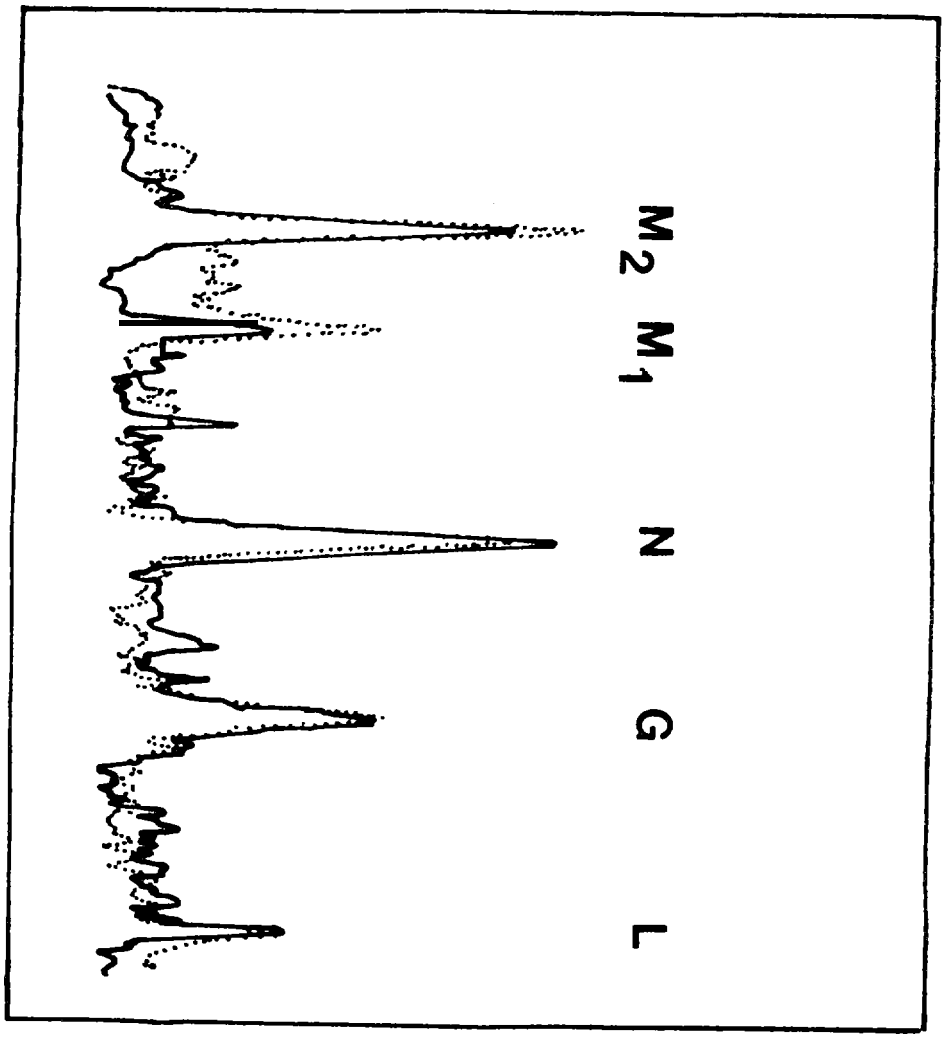
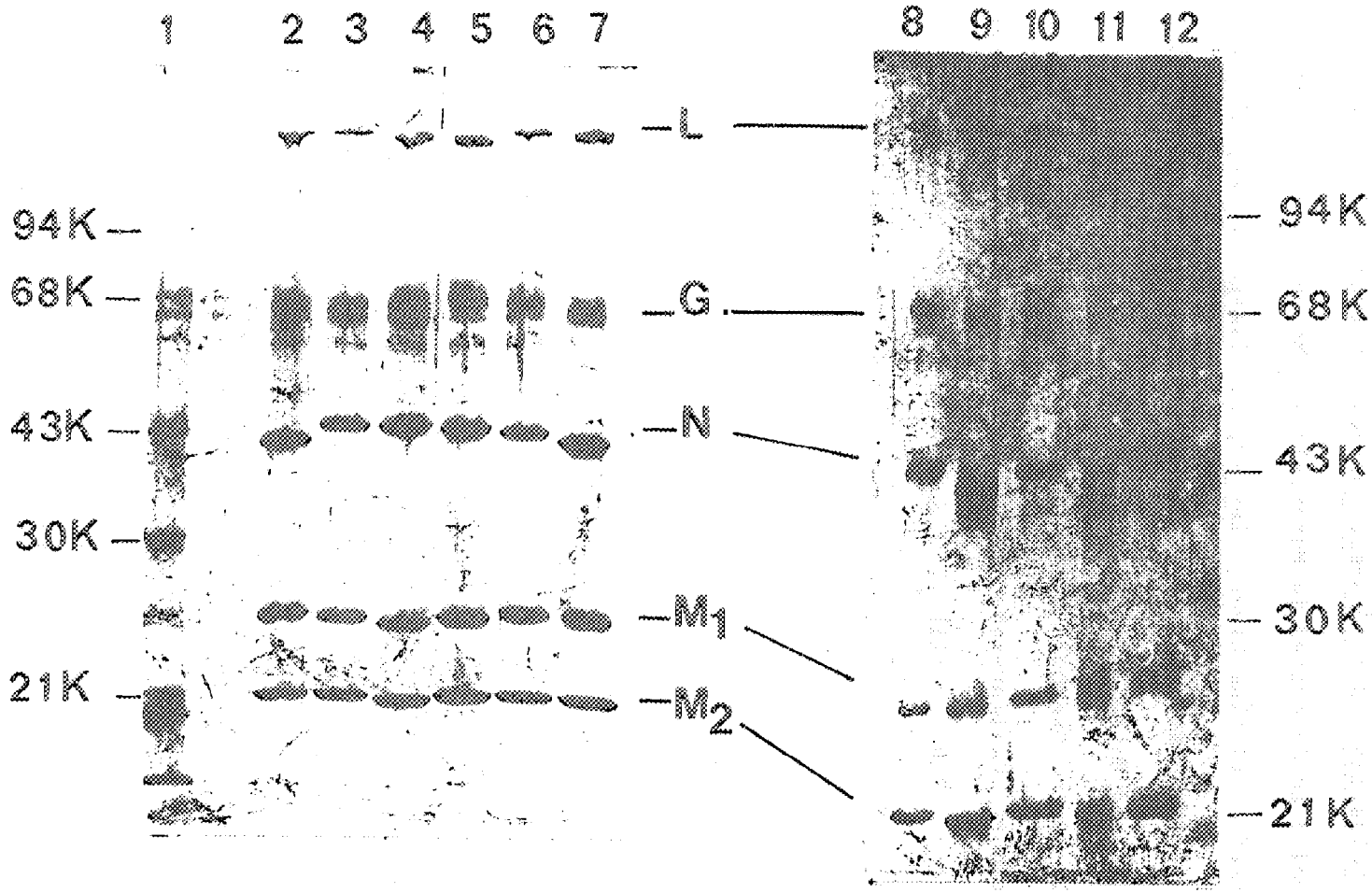

M2 M1 N G L

Fig. 3. Electrophoretic profile of different strains of IHN virus. Purified IHN 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).


1
2
3
4
5
6
7
8
9
10
11
12
L
G
N
M1
M2
94K
68K
43K
30K
21K
94K
68K
43K
30K
21K

100

Fig. 4. A comparison of eleven different strains of IHN 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 (Tamagas 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).

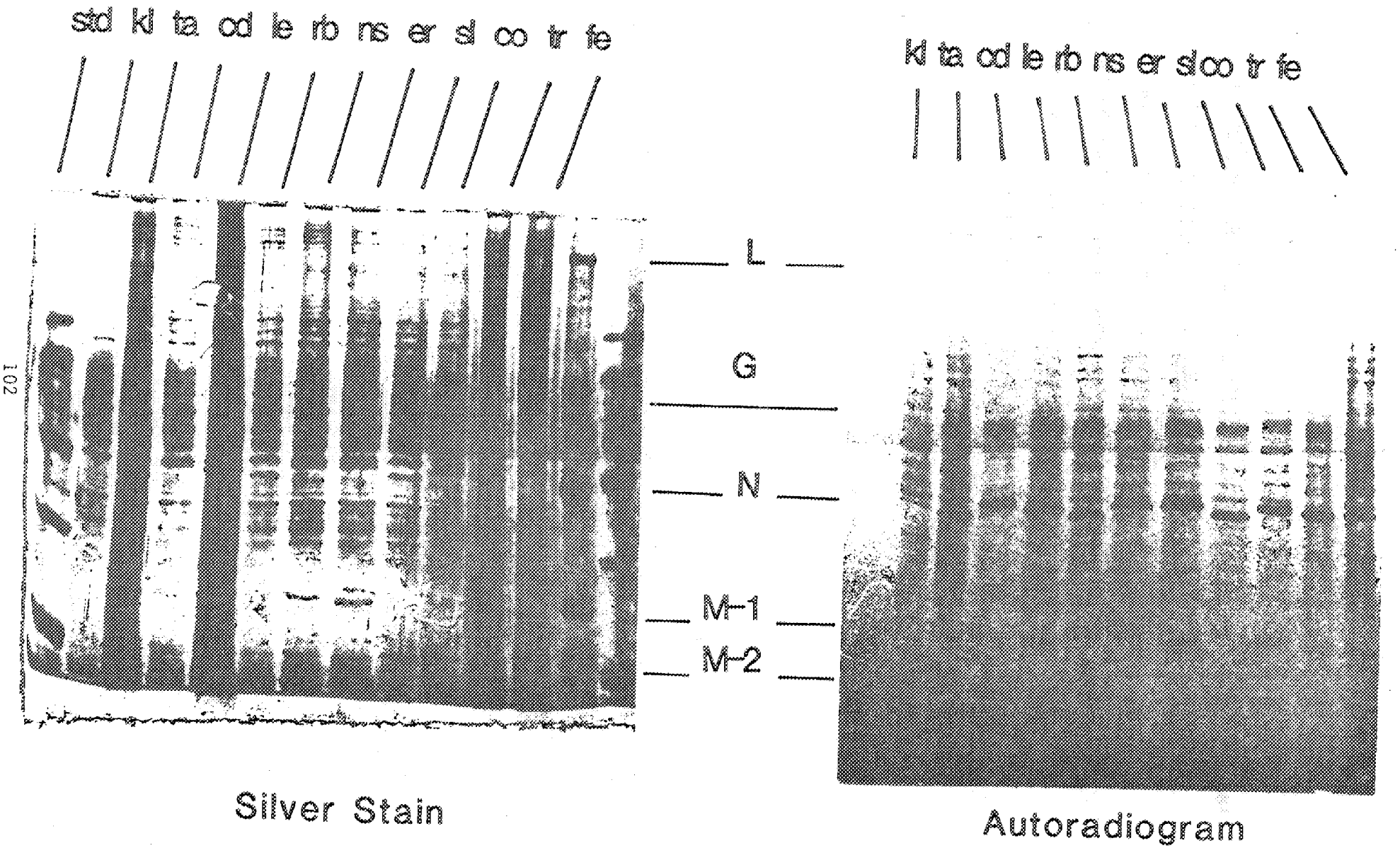

std kl ta cd le rb ns er sl co tr fe
L
G
N
M-1
M-2
Silver Stain
kl ta cd le rb ns er sl co tr fe
Autoradiogram

Fig. 5. Gel electropherogram of IHN virus proteins synthesized in CHSE-214 cells at different times after infection. Infected cells were exposed to ^{35}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.

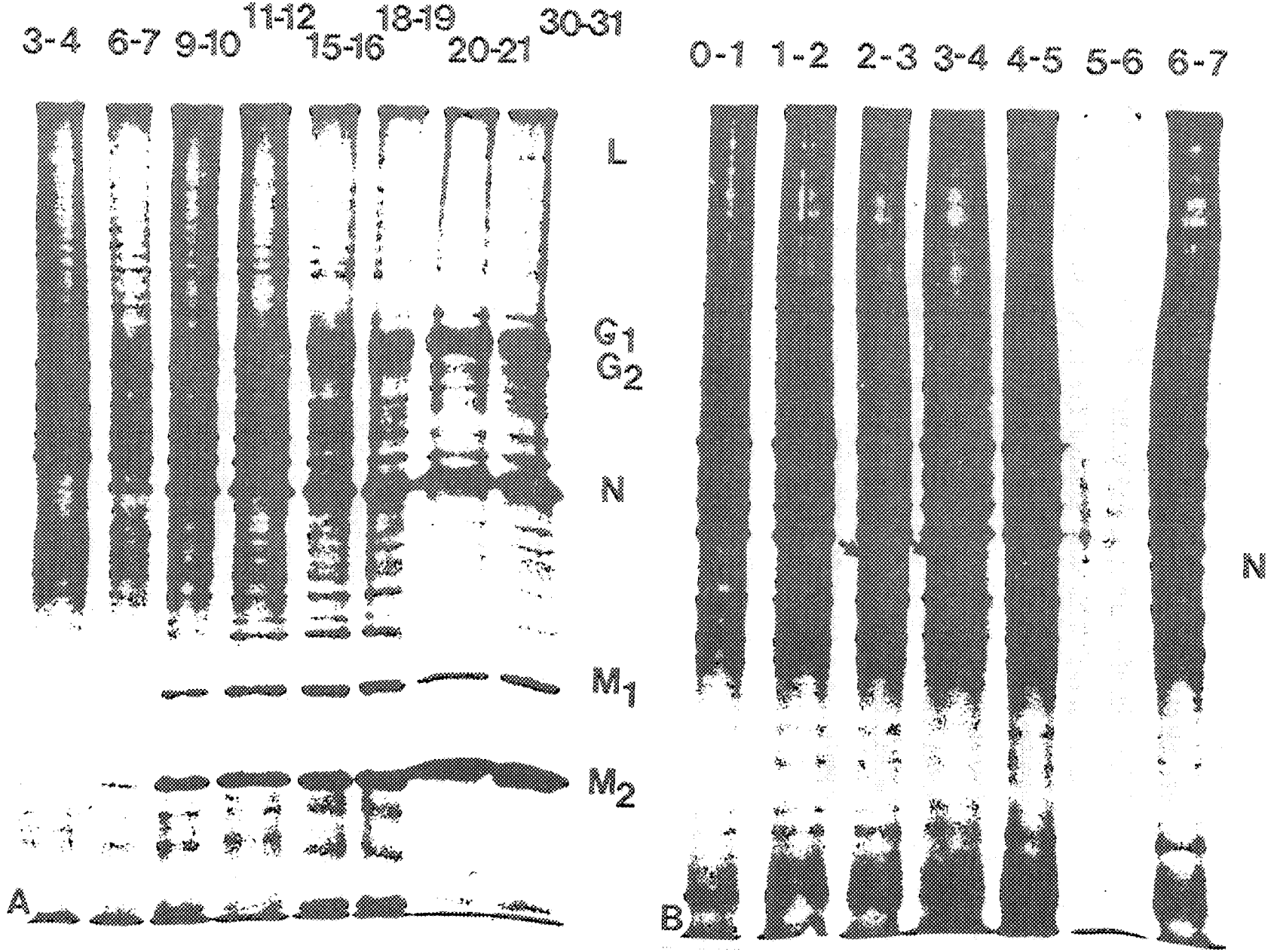

3-4
6-7
9-10
11-12
15-16
18-19
20-21
30-31
L
G1
G2
N
M1
M2
A
0-1
1-2
2-3
3-4
4-5
5-6
6-7
N
B

Fig. 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.

1'
5'
10'
30'
60'
5'
5'
20'
5'
40'
5'
60'
5'
120'
L
G1
G2
N
M1
M2
B
A

IV. The Classification of Infectious Hematopoietic Necrosis Virus by Comparing Virion Protein Pattern.

Authors: Y. L. Hsu, H. Mark Engelking, and J. C. Leong.

Journal Publication: Journal of General Virology, to be submitted.

INTRODUCTION

Infectious hematopoietic necrosis virus (IHNV) is the causative agent of an acute disease in 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 in the Pacific Northwest and Alaska, British Columbia and in the chinook salmon of the Sacramento River system in California (Amend et al., 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, personal communication). Despite the prevalence of this disease in salmon and trout in the Northwest, there has been no comparative study of the different isolates of IHN virus. 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 5 or more types. An analysis of the geographic distribution of these IHNV isolates among several 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 tschawytscha) embryo cells (CHSE-214) were obtained from Dr. J. L. Fryer, Department of Microbiology, Oregon State University, Corvallis, Oregon. Epithelioma papillosum 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 pg/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. 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 cm^2 plastic tissue culture flasks (Corning). The 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 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 mm^2 petri dishes (Corning) were washed twice with MEM and were either mock-infected or infected with an MOI of 5-10 $TCID_{50}$/cell (tissue culture infection dose for 50% infectivity) of IHNV in 0.1 ml of MEM supplemented with 2% dialyzed fetal bovine serum (GlBCO) (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 exhihited 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 ^{35}S-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 aonolayers washed twice with 1.0 ml of phosphate buffered saline (PBS) 0.135 M NaCl, 0.008 M Na_2HP0_4, 0.0015 M KH_2P0_4, 0.0027 M KCl). The cells were lysed with 100-200 µl of SDS-lysis buffer (9.5 M urea, 2% Nondet P-40, 5% 2-mercaptoethanol) and the lysate stored at -70°C in cryotuhes (NUNC). For electrophoresis, the

lysates were mixed with SDS and glycerol to give final concentrations of 2.5% SDS, 10% glycerol, 1% nonidet 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 in Tris buffer. 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 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. 1 and 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 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. 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. 1, Table 3). Since that time, the Type 2 virus has appeared at Skamania (SK), Mossyrock (MO), Minto Pond (IQ), Gnat Creek (GN), Beaver Creek (BCl), and Little White Salmon (LS) hatcheries in the lower Columbia River basin (Fig. 1, Table 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-l, HA-2) in Idaho (Figs. 1, 2, 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 (COl) in California are grouped together as Type 3 viruses (Figs. 3, 4, 7). The N protein of

these strains migrate with an apparent molecular weight of 43,500 SDS-polyacrylamide gels (Fig. 3 and 4).

Viruses grouped under Type 4 were some of the isolates from Coleman Hatchery (PO2 and C03) in California (Fig. 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, C02, 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. 1). In contrast, CD has an N protein which migrates even more slowly than that of Type 3 (Fig. 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 2, 3, 4 and 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 2). Type 2 and Type 3 viruses have never been isolated from sockeye salmon (Tables 3 and 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 4, Fig. 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, 1370). Type 4 and 5 of IHN virus were isolated from chinook and sockeye (Table 5).

Differentiation of IHNV and IPNV by SDS-PAGE

The utility of the detection method was further demonstrated by comparing the intracellular THN-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. 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. 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 IHN 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. 6) were all due to Type 2 IHNV (Fig. 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 3 and Fig. 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. 4). There is also a difference between N proteins of DW and DW2 (Fig. 1, 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.

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Legends

Fig. 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 RBl (Round Butte Hatchery), DW (Dworshak), PA (Pahsimeroi Hatchery), NI (Niagra Springs), EN (Entiate), LE (Lewis River), SK (Skamania), MO (Minto Pond), MP (Mossyrock Hatchery), GN (Gnat Creek), BCl (Beaver Creek), LS (Little White Salmon).

RB1
DW
PA
NI
EN
LE
SK
MO
MP
GN
BC1
LS
F
G1/2
N
M1
M2

Fig. 2. An autoradiogram of Type 1 IHNV and the IHNV isolated in Idaho (Type 2). Infectected 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 (Skamamia), GN (Gnat Creek), RBl (Round Butte Hatchery).

RB1
DW
PA
NI
CS
HA1
HA2
RR
SK
GN
RB1
L
G1, 2
N
M1
M2

Fig. 3. A comparison of IHN 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).

BC2
ER
PA
GN
NS
SK
TR
FE
L
G1,2
N
M1
M2

Fig. 4. A comparison of IHN 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 B), TR (Trinity River), RBl (Round Butte Hatchery), CD2 (Cedar River 2), CD (Cedar River), TR (Trinity River), SV (Sacramento River), CO3 (Coleman River 3), CO2 (Coleman River 2), Elk River), ER2 (Elk River 2).

CO
SV
CO3
TR
L
G
N
M1
M2
RB1
CD2
CD
TR
SV
CO3
CO2
ER
ER2
L
G
N
M1
M2
DW2
CO2
ER
L
G
N

Fig. 5. The differences in virion protein patterns between infectious pancreatic necrosis virus and IHNV infected cells were labeled as described in Materials and Methods. The lanes are marked IPN 1 (Canada 1), IPN2 (Canada), IPN3 (Canada). WR (White River) CNTL (control uninfected cells), RBl (Round Butte 1).

IPN1
IPN2
IPN3
WR
CNTL
RB1
VP1
VP2
VP3
VP4
L
G
N
M2

Fig. 6. The locations where IHNV was isolated from fish in Columbia River basin.

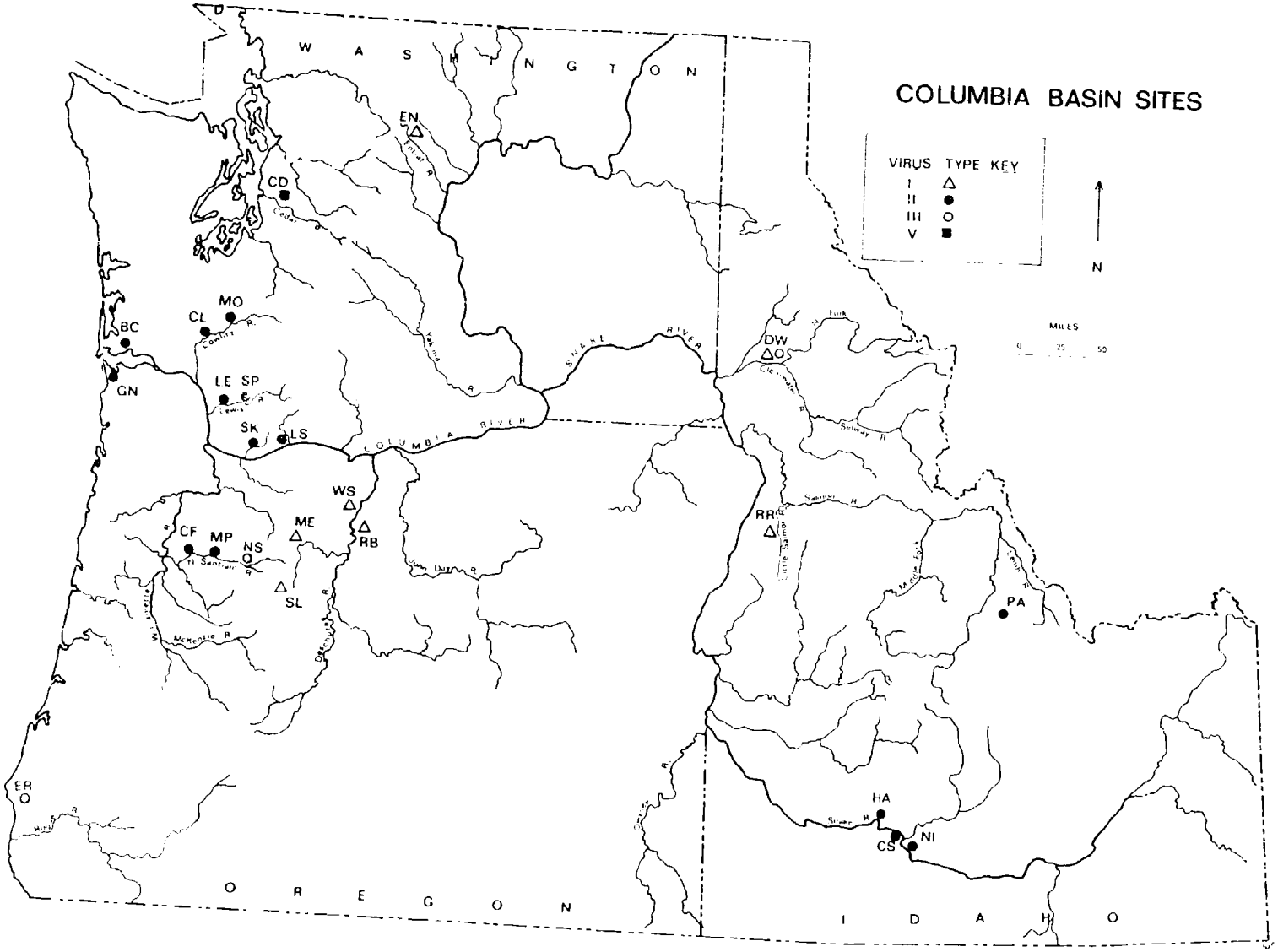
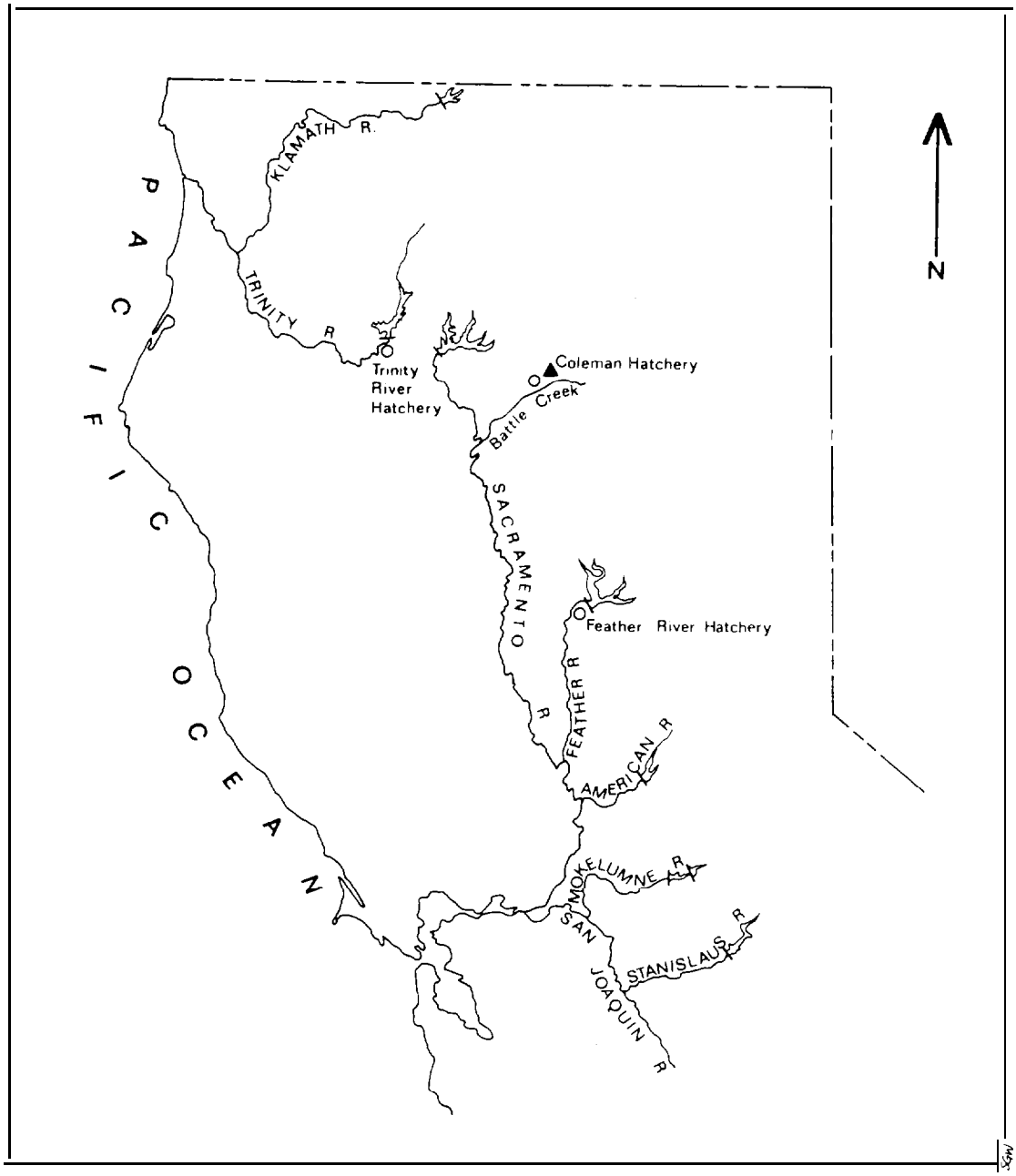

COLUMBIA BASIN SITES
VIRUS TYPE KEY
I
II
III
V
N
MILES
0
25
50
WASHINGTON
OREGON
IDAHO
COLUMBIA RIVER
SNAKE RIVER
EN
CD
MO
CL
BC
GN
LE
SP
SK
LS
WS
ME
RB
CF
MP
NS
SL
DW
RR
PA
HA
CS
NI
ER

Fig. 7. The locations of IHNV was isolated from fish in Northern California.

NORTHERN CALIFORNIA SITES



○ **TYPE III VIRUS**

▲ **TYPE IV VIRUS**

0 24 48

MILES

Table 1. Summary of Infectious Hematopoietic Necrosis Virus strains isolated from Northwest America.

Location	Code	Host Name	Type
Washington			
Beaver Creek	BC1	StW	2
Beaver Creek	BC2	Ct	2
Cedar River	CD	SS	5
Cedar River	CD2	ChF	5
Cowlitz Trout Hatchery	CL2	StW	2
Cowlitz Trout Hatchery	CL3	StS	2
Cowliltz Trout Hatchery	CL4	StS	2
Cowlitz Trout Hatchery	CL5	StW	2
Cowlitz Trout Hatchery	CL6	Ct	2
Entiat	EN	ST	1
Lewis River	LE	CH	2
Lake Quinalt	LQ	SS	1
Little White Salmon	LS	ShS	2
Mossyrock Hatchery	MO	StW	2
Skamania	SK	StS	2
Speelyai Hatchery	SP	ChF	2
White River	WR	SS	I
Oregon			
Cascade Fisheries	CF	Rb	2
Elk River	ER	ChF	3
Elk River	ER2	ChF	3
Gnat Creek	GN	StS	2
Metolius River	ME	K	1
Metolius River	ME2	K	1
Metolius River	ME3	K	1
Minto Pond	MP	ChS	2
Nan Scott Lake	NS	Rb	3
Round Butte Hatchery	RB1	StS	1
Round Butte Hatchery	RB2	StS	1
Round Butte Hatchery	RB3	StS	1
Round Butte Hatchery	RB4	StS	1
Round Butte Hatchery	RB5	StS	1
Round Butte Hatchery	RB6	StS	1
Suttle Lake	SL	K	1
Warm Spring	WS	StS	1

California

Coleman River	CO	ChS	3
Coleman River	CO2	CH	4
Coleman River	CO3	CH	4

Table III. 1 (continued)

Feather River	FE	CH	3
Sacramento River	SV	CH	3
Trinity River	TR	ChS	3

Idaho

Clear Sprinqs	CS	Rb	2
Dworshak	DW	ChS	1
Dworshak	DW2	StS	3
Hagerman River	HA1	Rb	2
Hagerman River	HA2	Rb	2
Hagerman River	HA5	Rb	2
Hagerman River	HA6	Rb	2
Niagra Springs	NI	StS	2
Pahsimeroi Hatchery	PA	StS	2
Rapid River	RR	ChS	1

Alaska

Karluk River	KL	SS	5
Karluk River	KL2	CH	5
Lake Auke	LA	ss	1
Lake Nerka	LN	ss	1
Tamgas Creek	TA1	PK	1
Tamgas Creek	TA2	ss	1
Tamgas Creek	TA3	ss	1

Canada

Boweran Lake	BO	-	1
Cultus Lake	CU	SS	1
Cowichan Lake	CW1	SS	1
Cowichan Lake	CW2	SS	
Cowichan Lake	CW2	SS	1
Fofar Creek	FO	SS	
Gates Creek	GC	SS	1
Great Central Lake	GL	SS	
Great Central Lake	GL2	SS	1
Robertson Creek	RO1	SS	1
Robertson Creek	RO2	SS	1
Sproat River	SRL	SS	1
Sproat River	SR2	SS	1

Weaver Creek	WC1	SS	1
Weaver Creek	WC2	SS	1

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 2. Different species of fish that Type 1 of Iinfectious Hematopoietic Necrosis virus was isolated from.

Code[a]	Host Name	State or Country	Stage of Development	Date of Isolation
ME	kokanee	OR[b]	adult	1977
ME2	kokanee	OR	adult	1978
ME3	kokanee	OR	adult	1981
RBl	steelhead	OR	adult	1975
RB2	steelhead	OR	adult	1981
RB3	steelhead	OR	adult	1982
RB4	steelhead	OR	yearling	1983
SL	kokanee	OR	juvenile	1980
ws	steelhead	OR	adult	1980
EN	steelhead	OR		1982
TA1	pink salmon	AK[c]	juvenile	1981
TA2	sockeye	AK	juvenile	1981
TA3	sockeye	AK	juvenile	1981
LA	sockeye	AK	adult	1979
LN	sockeye	AK	adult	1979
DW	Chinook	ID[d]	adult	1980
RR	Chinook	ID	adult	1982
BO	Chinook	CAN[e]		1975
cu	sockeye	CAN	juvenile	1978
CW1	sockeye	CAN	adult	1978
cw2	sockeye	CAN	adult	1978
cW3	sockeye	CAN	adult	
FO	sockeye	CAN	adult	1975
GC	sockeye	CAN		1975
GL1	sockeye	CAN	adult	1974
GL2	sockeye	CAN	adult	1974
R01	sockeye	CAN	adult	-
R02	sockeye	CAN	adult	
SRl	sockeye	CAN	adult	1974
SR2	sockeye	CAN	adult	1974
WC1	sockeye	CAN	juvenile	1980
WC2	sockeye	CAN	adult	1976

[a]Code as described in Table 1.
[b]OR = Oregon
[c]AK = Alaska
[d]ID = Idaho
[e]CAN = Canada

Table 3. Different species of fish that Type 2 IHN virus was isolated from.

Code[a]	Host Name	State	Stage of Development	Date of Isolation
BCl	steelhead, winter	WA	juvenile	1981
BC2	cutthroat trout	WA[c]	adults	1982
CL2	steelhead, winter	WA	adults	1982
CL3	steelhead, summer	WA	juvenile	1982
CL4	steelhead, summer	WA	adult	1981
CL5	steelhead, winter	WA	adult	1981
CL6	cutthroat trout	WA	adult	1981
cs	rainbow trout	ID[d]	adult	1982
GN	steelhead	OR[b]	juvenile	1981
HA1	rainbow trout	ID	adult	1978
HA2	rainbow trout	ID	adult	1978
HA3	rainbow trout	ID	juvenile	1983
HA4	rainbow trout	ID	juvenile	1983
HA5	rainbow trout	ID	adult	1978
HA6	rainbow trout	ID	adult	1979
LE	Chinook	WA	juvenile	1980
LS	Chinook	WA	adult	1981
MO	steelhead	WA	juvenile	1981
MP	Chinook	OR	adult	1981
NI	steelhead, summer	ID	adult	1978
PA	steelhead	ID	adult	1980
SK	steelhead	WA	juvenile	1981

[a]Code as described in Table 1
[b]OR = Oregon
[c]WA = Washington
[d]ID = Idaho
[e]AK = Alaska

Table 4. Different species of fish that Type 3 Infectious Hematopoietic Necrosis virus was isolated from.

Code[a]	Host Name	State	Stage of Development	Date of Isolation
ER	Chinook	OR[b]	juvenile	1979
ER2	Chinook	OR	juvenile[e]	1977
FE	Chinook	CA[c]	juvenile	1977
sv	Chinook	CA	adult	1979
TR	Chinook	CA	juvenile	1980
NS	rainbow trout	OR	juvenile	1971
co	Chinook	CA	juvenile	1980

[a]Code as described in Table 1
[b]OR = Oregon
[c]CA = California

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

Code[a]	Host Name	State	Stage of Development	Date of Isolation
C02[a]	Chinook	CA[c]	juvenile	1980
C03[a]	Chinook	CA	juvenile	1979
CD[b]	sockeye	WA[d]	juvenile	1980
CD2[b]	Chinook	WA	juvenile	1981
KL[b]	sockeye	AK[e]	juvenile	1979
KL2[b]	Chinook	AK	juvenile	1978, 1979

[a]belongs to Type 4
[b]belongs to Type 5
[c]CA = California
[d]WA = Washington
[e]AK = Alaska