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Physicochemical Properties of RNA of Salmonid Hematopoietic Necrosis Virus[1] (Oregon Strain) (38161)

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The Oregon strain of infectious hematopoietic necrosis virus (IHNV) was isolated from diseased sockeye salmon (*Oncorhynchus nerka*) fingerlings in an Oregon fish hatchery in 1958 (1). This isolate is antigenically closely related to two other viruses isolated from salmonid fishes, the chinook salmon virus (CSV) and the virus originally named IHNV which was isolated in 1967 from moribund *Salmo gairdneri* (2, 3). This relationship together with reports that these viruses produce similar cytopathic effects and have almost identical bullet-shaped morphologies (4, 5) support the conclusion that they are three variants of the same agent and that they belong to the rhabdovirus group (4, 5). Wingfield *et al.* reported that IHNV contained RNA and essential lipids (1). The presence of RNA in IHNV was determined presumptively from the observation that 5-bromodeoxyuridine failed to inhibit replication of the virus. The experiments described here confirm the identification of the viral nucleic acid as RNA and define some of its physicochemical properties.

Materials and Methods. Cell cultures. IHNV was propagated and assayed in cell lines SSE-30 and CHSE-214 derived from whole embryos of sockeye and chinook (*Oncorhynchus tshawytscha*) salmon, respectively (3, 6). Both cell lines were maintained as monolayer cultures at 18° in Eagle's minimal essential medium (MEM) (7) supplemented with 10% agamma calf serum (Hyland Laboratories), 100 units/ml of penicillin, 100 ng/ml of streptomycin, and 25 units/ml of mycostatin.

Virus. The stock IHNV used in these experiments had been propagated in sockeye salmon cell cultures, and the resulting infectious culture fluids stored at −60° until used (1). Stock virus contained 3.0×10^6 plaque forming units (PFU)/ml.

Virus infectivity assays. IHNV was assayed by either the end-point dilution or plaque assay method. Both techniques are described elsewhere (1, 3). The end-point dilution method was performed by inoculating tenfold dilutions of virus suspension into small Pyrex tubes containing 2- to 5-day-old cell monolayers. After incubating the stoppered tubes at 18° for at least 7 days, they were examined for cytopathic effects (CPE) and the $TCID_{50}$ calculated by the method of Reed and Muench (8). The plaque assay procedure was employed in these experiments only as a means of determining the PFU/ml in the stock virus suspensions.

Determinations of virus density. IHNV used in density experiments was propagated in SSE-30 cells. Cell cultures were infected with approximately 0.0015 $TCID_{50}$ units per cell, and the culture fluids were harvested after incubation at 18° for 3–5 days, at which time the cultures showed extensive CPE. The cell monolayer was not removed. The infectious culture fluids were subjected to a differential centrifugation cycle consisting of a low-speed step at 2200*g* for 30 min and a high speed step at 54,000*g* for 60 min in a Spinco no. 30 rotor. After suspending the virus pellets in 2 ml of 0.05 *M* Tris buffer, pH 7.0, the suspension was layered onto a 30 ml, 10–60% sucrose density gradient, prepared with the same buffer, and centrifuged at 59,000*g* for 16 hr, in a Spinco SW 25.1 rotor. Fractions of 110 drops were collected from the bottom of the tube, and aliquots were removed from

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each fraction for infectivity assays and from every third fraction for sucrose density determinations. Infectivity was assayed by the end-point dilution procedure and sucrose density was determined from refractive index measurements using a standard conversion table.

Characterization of viral nucleic acid. Both unlabeled and ^{32}P-labeled IHNV were used as sources of viral nucleic acid in experiments designed to establish the nucleic acid type, and whether it was single- or double-stranded.

Unlabeled virus was propagated and differentially centrifuged as described above for virus density determinations. The virus pellet from the high speed centrifugation was suspended in 0.05 *M* Tris buffer, pH 7.0, containing 0.001 *M* $MgCl_2$ and 30 ng/ml each of ribonuclease and deoxyribonuclease. After incubation at 25° for 1 hr, 2 ml of the nuclease treated virus was layered on a 30 ml, 10–55% sucrose gradient and centrifuged at 59,000*g* in a Spinco SW 25.1 rotor for 2 hr. One-milliliter fractions were collected from the bottom of the centrifuge tube, and the fractions constituting the virus band were pooled, diluted about tenfold with TBE buffer (0.05 *M* Tris, with 0.001 *M* EDTA, pH 7.5), and centrifuged at 54,000*g* in a Spinco no. 30 rotor for 1 hr. The pellet was resuspended in 25 ml of TBE buffer, dialyzed for 24 hr at 4° against 1 liter of the buffer, with two changes, and repelleted.

The nucleic acid was extracted from the purified, unlabeled IHNV by treatment with 0.5 *N* perchloric acid at 70° for 1 hr (9). The extract was tested for its absorption properties between 230 and 290 nm, its RNA content by the orcinol reaction (10) and for the presence of DNA by the diphenylamine reaction (11).

^{32}P-labeled IHNV was produced in either SSE-30 or CHSE-214 cell cultures in the presence of MEM containing 10% of the normal amount of monobasic sodium phosphate, 0.4% bovine serum albumin (Fraction V, Nutritional Biochemicals), the normal amounts of antibiotics, and 22 μCi/ml of carrier-free ^{32}P-phosphoric acid (International Chemical and Nuclear Corporation). Cultures were incubated at 18° for 24–40 hr, at which time an additional 22 μCi/ml of ^{32}P-phosphoric acid was added, and the cultures allowed to incubate for another 50–70 hr until 70–90% CPE was observed.

Labeled virus was purified as described for unlabeled virus, except the low-speed step of the differential centrifugation cycle was changed to 8700*g* for 10 min in order to eliminate more cellular debris. Sucrose gradient fractions containing the virus band with a sucrose density of 1.14 g/cm^3 were pooled, diluted with 2 vol of TS buffer (0.01 *M* Tris buffer with 0.148 *M* NaCl, pH 8.8), and lysed in the presence of 0.5% sodium dodecylsulfate (SDS) at room temperature for 10 min. Deproteinization of the lysed virus was performed by two extractions at 4° with equal volumes of TS buffer-saturated phenol. Nucleic acid in the final aqueous phase was precipitated with 2 vol of 95% ethanol and 0.1 vol of 20% sodium acetate, and stored at −20° for 4–5 hr. The precipitate was pelleted, and dissolved in TML buffer (0.01 *M* Tris with 0.001 *M* $MgCl_2$ pH 7.4).

In order to further purify the viral nucleic acid and to establish whether it was RNA or DNA, the nucleic acid in TML buffer was mixed with a sufficient volume of saturated cesium sulfate to give a density of 1.60 g/cm^3 and a gradient was formed by centrifugation in a Spinco SW-50 rotor at 131,000*g* for 60 hr at 22°. Fractions were collected from the bottom and aliquots were removed from every fifth fraction for determination of refractive index. Each fraction was then diluted to 1 ml with TML buffer, the absorbance at 260 nm was measured, and an aliquot was removed from each fraction for estimation of trichloracetic acid (TCA) precipitable radioactivity.

Fractions from the cesium sulfate gradient comprising the peak of ^{32}P-radioactivity were pooled, and the nucleotide composition of the viral RNA determined by anion exchange chromatography. The procedure used is described elsewhere (12). Briefly, ^{32}P-labeled viral RNA was mixed with unlabeled *E. coli* RNA, hydrolyzed with 0.3 *N* potassium hydroxide, and the pH adjusted to 9.5 with formic acid; the samples were loaded onto Dowex 1 × 8 (100–200 mesh, formate form, Bio-Rad Laboratories) columns. Fractions eluted from each column were analyzed for absorbance at 260 nm and total ^{32}P-radioactivity. Nucleotide composition was calculated

by totaling the ^{32}P-counts per minute (cpm) in the four nucleotide peaks and dividing the cpm in each peak by this total value.

The sedimentation properties of viral RNA and its sensitivity to ribonuclease treatment were examined by layering 0.2 ml of ^{32}P-labeled RNA onto a 5 ml, 10–30% glycerol gradient, centrifuging at 179,000*g* for 2.5 hr in a Spinco SW 65 rotor, collecting the fractions, and treating an aliquot of each fraction with ribonuclease. The portion of a fraction that was to be nuclease treated was diluted with TSE buffer (0.01 *M* Tris, with 0.1 *M* NaCl and 0.001 *M* EDTA, pH 7.4) containing 20 ng/ml ribonuclease and incubated at 30° for 40 min. Nuclease treated and untreated portions of each fraction were assayed for TCA precipitable radioactivity in the same manner.

Results and Discussion. IHNV was purified by a procedure consisting of differential centrifugation, nuclease treatment, and sucrose gradient centrifugation. Because IHNV preparations sedimented as three bands, in the sucrose gradients, each containing some infectivity, it was necessary to further characterize these bands by determining their densities. Three separate density determinations were made, and the result of one is shown in Fig. 1. In each determination the predominant peak of virus infectivity was located at a sucrose density of 1.16 g/cm^3. This density is the same as the values reported for vesicular stomatitis virus (13) and rabies virus (14). The lower band had a density of approximately 1.20 g/cm^3 and may have consisted of host cell debris complexed with virus as in the case of rabies virus (15). The trace amount of infectivity detected in the upper band may have been due to virus adsorbed to cellular material and serum proteins (14). Virus used as the source of nucleic acid was recovered from the gradient fractions constituting the peak of infectivity banding at densities between 1.14 and 1.17 g/cm^3.

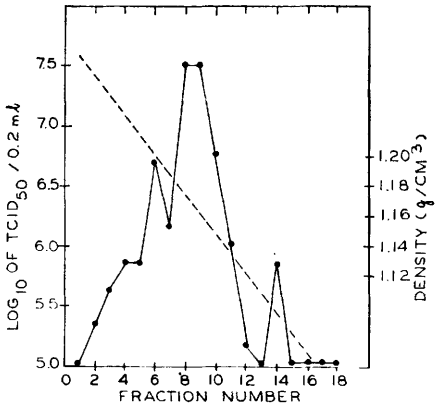

FIG. 1. Isopycnic centrifugation of IHNV in a 10–60% linear sucrose gradient at 59,000*g* for 16 hr in a Spinco SW 25.1 rotor. Log_{10} $TCID_{50}$ (●—●—●—●—●); density (----------).

TABLE I. Spectrophotometric Analyses of IHNV Nucleic Acid[a]

Experiment no.	ng/ml in Viral extracts[b] RNA	DNA	Absorbance Ratio (260/280 nm)
1	3.48	0	1.25
2	2.93	0.20	1.37
3	7.82	0.40	1.27

[a] Data represent analyses of three samples of viral nucleic acid from three different lots of purified virus.

[b] Nucleic acid was extracted from virus suspensions containing between 3×10^7 and 5×10^8 $TCID_{50}$s by treatment with hot 0.5 *N* perchloric acid. Concentrations of RNA and DNA were estimated by the orcinol and diphenylamine reactions, respectively.

Nucleic acid extracted from purified, unlabeled virus with hot perchloric acid was analyzed by the orcinol and diphenylamine reactions for the presence of RNA and DNA, respectively (Table I). Concentrations of RNA in the extracts were at least 14 times higher than those of DNA. The trace amounts of DNA were thought to be the results of nonspecific reactions between the diphenylamine test reagents and viral proteins (16), and/or contamination of viral nucleic acid with host cell DNA.

The above results, indicating that the nucleic acid of IHNV is RNA were confirmed, using ^{32}P-labeled virus. Figure 2 demonstrates a close correspondence between virus infectivity and radioactivity after rate-zonal centrifugation of differentially centrifuged virus in a sucrose gradient. Fractions constituting the virus band at a sucrose density of 1.14 g/cm^3, were pooled, the virus lysed, and the viral nucleic acid deproteinized by the SDS-

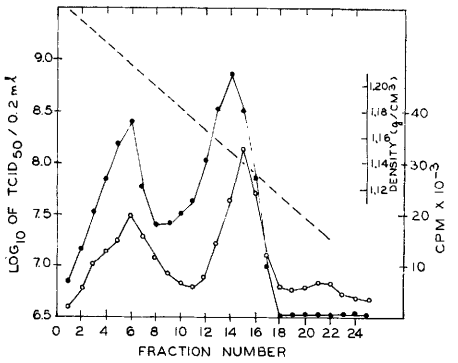

FIG. 2. Centrifugation of ^{32}P-labeled IHNV in a 10–55% sucrose gradient for 2 hr at 59,000g in a Spinco SW 25.1 rotor. TCA precipitable ^{32}P-cpm (●—●—●—●—●); density (———); Log_{10} $TCID_{50}$ (O—O—O—O—O).

phenol procedure. The resulting nucleic acid was centrifuged to equilibrium in a cesium sulfate density gradient (Fig. 3). There was no significant ^{32}P radioactivity in the region of the gradient where DNA should have banded (1.4–1.5 g/cm^3); however, in the region of the gradient where RNA should band, there was a pronounced peak of radioactivity, corresponding to a density of 1.59 g/cm^3.

These data leave no doubt that the nucleic acid of IHNV is RNA. Its density has an interpretive advantage, because host cell RNA had a density of about 1.65 g/cm^3 and there is a very small amount of ^{32}P radioactivity in this region of the gradient in Fig. 3, suggesting that the IHNV RNA preparation contained very little host-cell RNA.

^{32}P-labeled IHNV RNA recovered from cesium sulfate gradients was used in nucleotide composition analyses in order to establish whether the RNA was double- or single-stranded and to learn more about its properties. The average values from four such analyses of two different lots of RNA expressed as mole percent, with the standard deviations, were as follows: cytidylic acid, 25.4 ± 0.9%; adenylic acid, 22.5 ± 1.2%; uridylic acid, 27.7 ± 0.5%; and guanylic acid, 24.2 ± 1.6%. The marked difference between the adenylic and uridylic acid values is strong evidence for the single-stranded nature of the viral RNA.

Another method of determining if an RNA molecule is double- or single-stranded depends upon its response to treatment with pancreatic ribonuclease. ^{32}P-labeled IHNV RNA was sedimented in a 10–30% glycerol gradient in order to determine its sedimentation properties and its susceptibility to the ribonuclease treatment. The RNA sedimented in a very heterogeneous manner as a broad peak with the greatest radioactivity in the 26 S region of the gradient, and a pronounced shoulder at approximately 37 S. This pattern might result if the RNA was complexed with protein, or if it had undergone considerable degradation. The fractions collected from the above gradient were divided into two sets of equal volume. One set was used for determination of TCA-precipitable radioactivity and yielded the pattern described above. The other set was treated with ribonuclease and the TCA-precipitable radioactivity was then determined. Following the enzyme treatment, this activity was reduced in all fractions by 85–97%. The degree by which the viral RNA was hydrolyzed by ribonuclease confirms its single stranded character.

Summary. Infectious hematopoietic necrosis virus (IHNV) (Oregon strain) was propagated in salmonid cell lines and purified by differential and density gradient centrifugation. The virus had a density in sucrose gradients of 1.16 g/cm^3. The nucleic acid extracted from purified virus was shown to be RNA as determined by chemical analysis, by its density in a cesium sulfate gradient, and

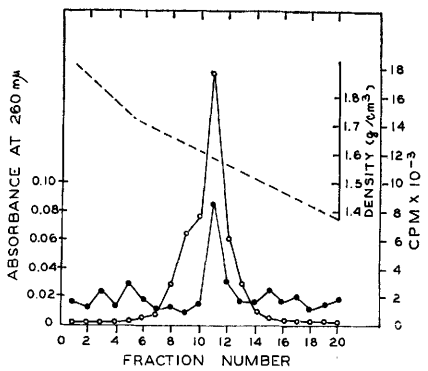

FIG. 3. Centrifugation of ^{32}P-labeled IHNV nucleic acid in cesium sulfate, with starting density 1.60 g/cm^3, for 60 hr at 131,000g in a Spinco SW 50 rotor at 22°C. TCA precipitable ^{32}P-cpm (O—O—O—O—O). Absorbance at 260 μm (●—●—●—●—●); density (————).

by its sensitivity to ribonuclease. The latter property, together with a nonequimolar ratio of adenylic to uridylic acids, indicate its single stranded nature.

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