The Structure of Infectious Pancreatic Necrosis Virus RNA

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SUMMARY

The RNA from infectious pancreatic necrosis virus has been purified and had a sedimentation velocity of 14S on sucrose gradients, a buoyant density of 1.60 g/ml in Cs2SO4 and pyrimidine to purine ratios near unity. The RNA had the appearance of a linear double stranded molecule with an average length of 0.92 μm and a standard deviation of 0.07 μm when observed under the electron microscope using the Kleinschmidt protein film technique. This would correspond to a mol. wt. of 2.4 × 10^6. The RNase A resistance of IPN virus RNA exhibited a marked salt dependence; it was 92% resistant in 0.1 M NaCl, but only 9%, or less, in 0.01 M NaCl. The RNA was resistant to denaturation by boiling at NaCl concentrations of 0.04 M or higher, but did denature at lower concentrations. Polyacrylamide gel electrophoresis of the RNA indicated that two RNA species were present and the standard deviation of lengths in the electron microscope indicated that they could not differ by more than 4 × 10^5 in mol. wt.

INTRODUCTION

Infectious pancreatic necrosis (IPN) virus of salmonids has been shown to cause a highly contagious disease with high mortality in young salmonids (Wolf et al. 1960). The survivors of this disease remain carriers of the virus and can spread the disease agent into the water (Wolf, Quimby & Bradford, 1963; Yamamoto, 1975).

Classification of the virus has had a turbulent history. Initially it was classified as a picornavirus based upon the structure and size of the capsid (reported to be 18 nm; Malsberger & Cerini, 1963). This was substantiated by the discovery that the nucleic acid was RNA and that the RNA appeared to be single stranded (Malsberger & Cerini, 1965; Nicholson, 1971). More recently IPN virus has been found to be larger than reported previously (Moss & Gravel, 1969) and in fact seemed to be close to the size of reovirus, but did not contain the double capsid structure common to viruses of that group (Kelly & Loh, 1972).

Two conflicting studies on the nature of IPN virus RNA have been reported. Kelly & Loh (1972) found IPN virus RNA to be 16S during sucrose gradient sedimentation, to be sensitive to pancreatic RNase (RNase A), and to have a nucleotide composition which would indicate a single stranded RNA. In contrast, Cohen, Ponsard & Scherrer (1973) found the RNA to be 14S on sucrose gradients, to be resistant to RNase A, and to have a nucleotide composition which indicated a double stranded RNA. The conflicting data suggested that IPN virus RNA might be a combination of the two models—a single stranded RNA with a great deal of double stranded character.
The results of this study, in agreement with those of Cohen et al. (1973), indicate that IPN virus RNA consists of a linear double stranded molecule of mol. wt. about $2.4 \times 10^6$. This conclusion is based on observation of the RNA under the electron microscope utilizing the protein film technique, irreversible denaturation of the RNA with a combination of heat and low salt, and pyrimidine to purine ratios near unity. In addition IPN virus RNA was shown by polyacrylamide gel electrophoresis and electron microscopy to be composed of 2 species of different mol. wt.

METHODS

Growth and labelling of IPN virus. IPN virus was an RTG-2 tissue culture passaged strain (ATCC-VR 299) and was the kind gift of Dr K. Wolf (Eastern Fish Disease Laboratory, Kearnsville, W. Va., U.S.A.). Chinook salmon embryo cell line (CHSE-214) used for the growth of the virus was the kind gift of Dr J. L. Fryer (Oregon State University, Corvallis, Oregon). The cells were grown at 22°C in Roux bottles in minimal essential medium (MEM, GIBCO, Grand Island, N.Y., U.S.A.) with 0.22% sodium bicarbonate and 5% foetal calf serum (GIBCO).

The virus was plaque purified three consecutive times prior to use. A virus inoculum was prepared by resuspending each discrete plaque in 1 ml of culture medium and it was then stored at 4°C. For preparation of a working stock of virus, 0.1 ml of the inoculum was added to a 3 oz prescription bottle containing a confluent monolayer of CHSE-214 cells. Incubation of cells after inoculation was at 18°C, and maximum c.p.e. appeared after 3 days. This became the stock virus, and a fresh stock was prepared for each experiment. The stock contained about 3 to 4 x 10^8 p.f.u./ml.

For infection and radioactive labelling, the stock virus was diluted 10-fold with MEM plus 5% foetal calf serum (FCS) and 3 ml was added to each Roux bottle after removal of the old medium. Adsorption was allowed to proceed at room temperature for 1 h; then 50 ml of MEM plus 5% FCS was added to each bottle. Radioactivity was added at this time; either 3H-guanine (Amersham/Searle, 6.8 Ci/mmol) at a concentration of 2 μCi/ml or 32P (New England Nuclear, carrier free) at a concentration of 20 μCi/ml. For 32P-labelling, phosphate free MEM and dialysed FCS were used. Incubation was at 18°C for 18 to 24 h, at which time maximum c.p.e. was evident.

Virus purification. The cells were harvested by centrifugation, and were resuspended in 2 ml of TNE buffer (0.01 M-tris-HCl, 0.1 M-NaCl, 1 mM-EDTA, pH 7.5). An equal volume of Freon TF113 (Dupont Canada, Maitland, Ontario) was added, and the mixture vortexed vigorously for 5 min at room temperature. After centrifugation at 2000 rev/min the upper aqueous phase was removed and stored at 0°C prior to CsCl gradient sedimentation (see below).

The medium that remained after removal of the cells was made 2.2% in NaCl and 5% in polyethylene glycol (PEG, J. T. Baker Chemicals, Philadelphia, N.J., mol. wt. 15000 to 20000) and was stirred at 4°C for 2 to 4 h. Precipitated material was collected by centrifugation at 8500 rev/min for 1 h in a Sorvall RC 2-B centrifuge using the GSA rotor (Ivan Sorvall Inc., Norwalk, Conn., U.S.A.). The supernatant fluid was removed and the pellet was resuspended in 2 ml TNE buffer.

The Freon-extracted and PEG-concentrated virus samples were layered over a step-wise CsCl gradient consisting of 1.5 ml 40% CsCl, 1.0 ml 30% CsCl and 0.5 ml 20% CsCl. Centrifugation was for 18 h at 4°C in a Beckman Ultracentrifuge (Model L2-65B) using the SW 50.1 rotor at 35000 rev/min. The virus band was withdrawn by tube puncture from the side.
Infectious pancreatic necrosis RNA

The samples were pooled and dialysed for 1 h against TNE buffer to remove the CsCl. The sample was then layered over a sucrose gradient of 15 to 30% sucrose in TNE buffer and centrifuged in the Spinco SW 40 Ti rotor at 30,000 rev/min for 1.5 h at 4°C. The virus band was collected by dripping from the bottom of the tube. The sucrose fractions containing virus were layered over a second CsCl gradient and centrifuged for 3 to 5 h at 35,000 rev/min at 4°C, in the Spinco SW 50.1 rotor. The virus band was again withdrawn and dialysed against TNE buffer overnight at 4°C.

Density of IPN virus. CsCl gradients were prepared as above and the virus samples layered on top. Mineral oil was added to fill the tube to the top. The gradient was centrifuged for 20 h in the Spinco SW 50.1 rotor at 35,000 rev/min at 4°C. Drops were collected from the bottom and kept on ice until the refractive index (RI) was taken using an Erma refractometer (Erma Optical Works, Tokyo, Japan). The RI was corrected for the contribution of solutes in the TNE buffer by subtraction of 0.002 units from each reading (this was the difference between TNE and distilled water). The fractions were then counted directly for radioactivity in 5 ml of Bray’s scintillation fluid (Bray, 1960) in an Isocap 300 liquid scintillation counter (Nuclear Chicago, Chicago, Ill., U.S.A.).

Extraction of IPN RNA from virions. Pronase (Calbiochem, La Jolla, California) was stored at −20°C as a stock solution of 5 mg/ml in TNE buffer after pre-incubation at 37°C for 30 min. It was added to IPN virions to a final concentration of 500 μg/ml. Sodium N-lauryl sarcosinate or SDS (Sigma Chemical Co., St Louis, Mo., U.S.A.) was added to a final concentration of 1% and the sample was incubated at 43°C for 1.5 h. The sample was then frozen at −20°C and was used either without further treatment or, more often, after phenol extraction. Liquified phenol (Fisher Scientific Company, Fairlawn, N.J., U.S.A.) was neutralized with 1 N-NaOH and washed with three vol. of TNE buffer. An equal vol. of treated phenol was added to the IPN virus RNA sample and the mixture was vortexed for 10 min. The sample was centrifuged at 20,000 rev/min for 5 min, and the aqueous supernatant fluid was extracted with phenol twice more. The RNA was dialysed against TNE buffer for 2 h or, alternatively, was precipitated with 3 vol. of ethanol at −20°C and the pellet was resuspended in TNE buffer.

RNase sensitivity of IPN virus RNA. IPN virus RNA was purified by a Cs₂SO₄ gradient. Samples were diluted into various concentrations of TNE as indicated in the appropriate figure. RNase A (Calbiochem, La Jolla, California), EC. 2.7.7.16, was added to a final concentration of 2 μg/ml. Incubation was for 30 min at 37°C. Yeast RNA was added as carrier for precipitation with an excess of 10% trichloroacetic acid (TCA). Samples were filtered with Whatman GF/A glass fibre filters and the filters were dried and counted in toluene-POP-PPO scintillation fluid.

Sucrose gradient analysis of IPN virus RNA. Sucrose (Fisher Scientific Co., Fairlawn, N.J., U.S.A.) gradient solutions of 5% and 20% were prepared in TNE buffer. SDS was present in the sucrose solutions at a final concentration of 1%. Centrifugation was for 3 h at 45,000 rev/min in the Spinco SW 50.1 rotor at 22°C. Drops were collected from the bottom and radioactivity was counted directly in Bray’s scintillation fluid (Bray, 1960).

Caesium sulphate analysis of IPN virus RNA. Phenol extracted IPN virus RNA was mixed with Escherichia coli 16S and 23S rRNAs and the sample was layered over a 2 ml step-wise gradient consisting of 1 ml Cs₂SO₄ at 1.75 g/ml and 1 ml Cs₂SO₄ at 1.40 g/ml in TNE buffer. Mineral oil was used to fill the polyallomer tube (Beckman). The gradient was centrifuged for 43 h at 35,000 rev/min in the Spinco SW 50.1 rotor at 4°C. Drops were collected from the bottom of the tube and the RI was taken with an Erma refractometer and the readings
were collected for TNE buffer as above. Cs₂SO₄ buoyant densities of the fractions were determined using the equation:

\[
\text{density} = \frac{RI - 1.2646}{0.073}
\]

Carrier yeast RNA was added and each fraction was precipitated with an excess of 10% TCA. The precipitate was collected on Whatman GF/A glass fibre filters. The filters were dried and counted in toluene-POPOP-PPO scintillation fluid.

**Nucleotide composition of IPN virus RNA.** ³²P-labelled IPN virions or RNA (purified by phenol extraction and Cs₂SO₄ gradient centrifugation) were precipitated with 3 vol. of ethanol. The pellets were resuspended in 20 µl 0.5 N-NaOH and incubated at 43 °C for varying lengths of time. A spreading solution was prepared by adding together 0.06 ml of cytochrome c (1 mg/ml in distilled water), 0.03 ml isopropanol (0.1%) and 0.2 ml of 2 M-ammonium acetate, pH 8.0. The virus solution and the spreading solution were mixed in the ratio of 1:1 and the mixture was spread on distilled water in a Teflon trough. The film was picked up on 200 mesh copper grids coated with carbon strengthened formvar. The film was dehydrated in absolute ethanol and air dried. The grids were shadowed with palladium using a Denton DV 502 vacuum evaporator (Denton Vacuum Inc., Cherry Hill, N.J., U.S.A.) with continuous rotation at an angle of 7° to the object. Photographs were taken with a Philips EM 200 electron microscope at instrumental magnification from 6500 to 11 200 times on Kodak 35 mm fine grain positive film. The RNA molecules were printed on photographic paper at final magnification from 33 000 to 56 200 times to permit length measurements using a Keuffel and Esser map measurer. All RNA molecules measured for the frequency distribution were from photographs at a final magnification of 55 200 times.

**Polyacrylamide gel electrophoresis of IPN virus RNA.** Purification of the acrylamide and the buffer system used for electrophoresis were according to the method of Loening (1967). The gels were 2.5% acrylamide with a ratio of acrylamide to bisacrylamide of 19:1. Acrylamide and bisacrylamide were purchased from Matheson, Coleman and Bell (Norwood, Ohio, U.S.A.). The gels were cast in a slab gel apparatus of dimensions 28 cm x 16 cm x 1.5 mm (Hoefer Scientific Instruments, San Francisco, Ca., U.S.A.).

³²P-IPN virus RNA was released from purified virions by pronase and SDS and was phenol extracted 3 times and precipitated with ethanol. It was resuspended in TNE buffer and a sample added to electrophoresis buffer which included bromophenol blue and 50% glycerol. Electrophoresis was for 20 min at 200 V followed by 12 h at 100 V. The gel was dried under vacuum in slab gel drier model SE540 (Hoefer Scientific Instruments, San Francisco, Ca.) and was autoradiographed for 1 day using Kodak No-Screen medical X-ray film (NS54T).

**Preparation of ¹⁴C-labelled E. coli RNA.** E. coli B cells were grown overnight in 100 ml minimal medium in the presence of 0.01 µCi/ml of ¹⁴C-uracil (Amersham/Searle, 54.9 mCi/
Infectious pancreatic necrosis RNA

Fig. 1. Isopycnic sedimentation of IPN virions in CsCl. $^{32}$P-IPN virions were purified by centrifugation in two CsCl gradients. The virus was layered on top of a 3 ml CsCl gradient from 40 % to 20 % CsCl in TNE buffer. Centrifugation was for 20 h at 35000 rev/min in a Spinco SW 50.1 rotor at 4 °C.

RESULTS

Purification and properties of IPN virus

It was found that IPN virus at optimum input multiplicity of infection completed its replication cycle and gave c.p.e. in 10 h. At our standard harvest time of 20 to 24 h post infection one half of the infectivity remained cell associated and the other half was released into the medium. Our total recovery of purified virus from both places by the procedures outlined in Methods was 80 to 100 % of the initial infectivity. The sucrose gradient was found to be an important step in the purification of virus – only 5 to 20 % of the radioactivity in the CsCl virus band migrated with the virus band in sucrose.

The morphology of IPN virions negatively stained with 3 % phosphotungstate and viewed with the electron microscope was identical to that reported previously (Kelly & Loh, 1972;
**Fig. 2.** Resistance of IPN virus RNA to RNase A as a function of NaCl concentration. $^3$H-guanine labelled IPN virus RNA was purified by phenol extraction and centrifugation in a Cs$_2$SO$_4$ gradient. Samples were diluted to the indicated NaCl concentration as in Methods, and RNase A was added at 2 $\mu$g/ml. Incubation was for 30 min at 37 °C; 1118 radioactive cts/min equals 100%.

**Fig. 3.** Heat denaturation of IPN virus RNA as a function of NaCl concentration. $^{32}$P-labelled IPN virus RNA was purified by phenol extraction and centrifugation in a Cs$_2$SO$_4$ gradient. Samples were diluted into the appropriate salt concentration and held in a boiling water-steam bath at 96 °C for the indicated times. The tubes were quick-cooled on ice water and the samples were made up to 0.1 M-NaCl as described in Methods. RNase was added at 2 $\mu$g/ml and incubation was for 30 min at 37°C; 2020 radioactive cts/min equals 100%. The points are the average of duplicate results: ○—○, not boiled, no RNase treatment; ●—●, not boiled, with RNase treatment; △—△, boiled 5 min, with RNase treatment; ▲—▲, boiled 15 min, with RNase treatment.
Infectious pancreatic necrosis RNA

**Fig. 4.** Sucrose gradient analysis of IPN virus RNA. \(^{3}H\)-labelled IPN virus RNA was mixed with \(^{14}C\)-labelled *E. coli* 23, 16 and 4S RNAs and the sample was layered on top of a 5 to 20 \% sucrose gradient in TNE buffer containing 1 \% SDS. Centrifugation was for 3 h at 45000 rev/min in a Spinco SW 50.1 rotor at 22°C. ● – ●, \(^{3}H\); ○ – – ○, \(^{14}C\).

Cohen *et al.* 1973). The diam. of the capsid was found to be 74 nm when compared to latex particles of 88 nm on the same photograph. There was no evidence of a double capsid structure.

IPN virus banded at a density of 1.33 g/ml when centrifuged to equilibrium in CsCl (Fig. 1). The infectivity of IPN virus was also present at this position, and when the CsCl band was extracted during purification it contained over 10\(^{10}\) p.f.u./ml. The electron microscope showed over 90 \% intact virions with a few empty capsids (N. Chang, personal communication).

**RNase sensitivity of IPN virus RNA**

IPN virus RNA was released from purified virions by pronase and sarcosinate treatment. The RNA was phenol extracted three times, and was purified on Cs\(_{2}\)SO\(_{4}\). The Cs\(_{2}\)SO\(_{4}\) peak was collected and dialysed against TNE buffer for 2 h at 4°C.

IPN virus RNA purified in this manner was found to be greater than 90 \% resistant to RNase A as judged by TCA precipitation (Fig. 2). Sucrose gradient analysis of this RNA after RNase treatment indicated that the RNA no longer migrated at the normal 14S position but was in a broad peak centred around 10S (data not shown). The resistance of IPN virus RNA to RNase A was found to be salt dependent. In one experiment it decreased from an initial resistance of 92 \% in 0.1 M-NaCl to 9 \% in 0.01 M-NaCl (Fig. 2). This behaviour suggested that IPN virus RNA was a double stranded molecule, but was also consistent with a single stranded RNA with internal double stranded regions (hairpin model).
Fig. 5. Cs₂SO₄ gradient analysis of IPN virus RNA. ³H-labelled IPN virus RNA was purified by phenol extraction. It was mixed with ¹⁴C-labelled E. coli 23S and 16S RNAs and the mixture was layered on top of a Cs₂SO₄ gradient. Centrifugation was for 43 h at 35000 rev/min in a Spinco SW 50.1 rotor at 4°C. ●—●, ³H; ○—○, ¹⁴C; Δ—Δ, density.

Another aspect of a double stranded molecule would be that it should undergo an irreversible denaturation in response to heat. Fig. 3 illustrates an experiment designed to demonstrate such a denaturation of IPN virus RNA. IPN virus RNA was diluted 100-fold into varying dilutions of TNE buffer and the samples were boiled for 5 min, 15 min, or not at all. The NaCl concentration in each tube was then restored to 0.1 M, and RNase A was added to a final concentration of 2 µg/ml. The results in Fig. 3 show that IPN virus RNA did irreversibly denature with boiling, but only at salt concentrations below 0.04 M-NaCl.

**IPN virus RNA on sucrose gradients**

IPN virus RNA migrated as 14S when compared to E. coli rRNA markers added to the same gradient (Fig. 4). This was true whether the RNA was used directly following a pronase and sarcosinate digestion of the virions, or whether the RNA was phenol extracted first. The peak on the sucrose gradient was as compact as the marker peaks, indicating that only one size class of molecules was present.

**IPN virus RNA on Cs₂SO₄ gradients**

IPN virus RNA was subjected to isopycnic centrifugation in a Cs₂SO₄ gradient. The RNA was layered over a step-wise gradient of Cs₂SO₄ from 1.75 to 1.40 g/ml in TNE buffer. The virus RNA banded at a density of 1.60 g/ml, while E. coli rRNA banded at a density of 1.66 g/ml in the same gradient (Fig. 5). A fraction of the IPN virus RNA appeared to band at the single stranded position, but this was variable.
Infectious pancreatic necrosis RNA

Fig. 6. Electron micrographs of IPN virus RNA. (a) IPN virus RNA (average length 0.92 μm). (b) IPN virus RNA plus a molecule of infectious canine laryngotracheitis (adenovirus) DNA (average length 10.4 μm).
electron microscope; 151 molecules were measured three times and the average plotted as a histogram.

Table 1. Nucleotide composition of IPN virus RNA

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<td>27.2</td>
<td>24.0</td>
<td>1.03</td>
<td>0.93</td>
<td>0.98</td>
<td>53.7</td>
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* Average of a single determination of 2 different preparations.
† Average of 3 determinations of a single preparation.

Electron microscopy of IPN virus RNA

Purified IPN virions were treated with pronase and diluted into the spreading solution as described in Methods. The sample was spread on the hypophase either as quickly as possible or after varying incubation times at 43 °C. When the virus sample was omitted from the spreading solution there was no evidence of nucleic acid on the grid. If pronase was omitted there was also no nucleic acid visible on the grid. Thus the IPN preparation was not contaminated with exogenous double stranded nucleic acid. The pronase was found to effect the release of some of the virus RNA as quickly as the sample could be spread.

The RNA from IPN virions was found to be in the form of a short linear double strand (Fig. 6). Sixteen photographs were selected and all of the RNA molecules were measured using a map tracer. Each molecule was measured 3 times and the average was plotted as a frequency distribution (Fig. 7). The average length of the total 151 molecules measured was 0.92 μm with a standard deviation of 0.07 μm. The distribution further suggested that two distinct RNA species were present.

Nucleotide composition of IPN virus RNA

Intact ³²P-labelled IPN virions or IPN virus RNA purified on a Cs₂SO₄ gradient were used to determine nucleotide composition as in Methods. Table 1 shows that IPN virus RNA from either sample had pyrimidine to purine ratios near unity with a G+C content of 54%.
**Polyacrylamide gel electrophoresis of IPN virus RNA**

$^{32}$P-labelled IPN virus RNA was subjected to polyacrylamide gel electrophoresis (Fig. 8). The RNA separated into two well defined bands. In 12 gels run on 3 different RNA preparations there was no indication of the presence of any other bands. When cylindrical gels were run and the slices counted for radioactivity, equal amounts of $^{32}$P were present in each of the two peaks.

**DISCUSSION**

Cohen *et al.* (1973) have presented evidence that IPN virus contains double stranded RNA. Their evidence was RNase resistance, heat denaturation, Cs$_2$SO$_4$ buoyant density, and pyrimidine to purine ratios equal to unity. This was in contrast to an earlier report by Kelly & Loh (1972) that IPN virus RNA was single stranded by the same criteria. Similar experiments reported in this paper substantiate that IPN virus RNA is double stranded. The RNA was found to be resistant to pancreatic RNase A at high NaCl concentrations but became sensitive at low concentrations. The RNA exhibited a denaturation in response to heat, but only at NaCl concentrations of 0.02 M or less. Even after 15 min of boiling, IPN virus RNA in higher concentrations of NaCl did not denature, indicating that longer boiling times were unlikely to effect a denaturation. The RNA was found to have a density of 1.60 g/ml in Cs$_2$SO$_4$, close to the 1.615 value reported by Cohen *et al.* (1973). The nucleotide composition determined here by electrophoresis is almost identical to that reported by Cohen *et al.* (1973) using the method of thin layer chromatography.

We have found that purification of the virus by velocity sedimentation was crucial to remove small mol. wt. material that contaminated the IPN virus region of a CsCl gradient. Characteristically only about 10% of the radioactivity contained in the virus band from the first CsCl gradient moved with the virions in the subsequent sucrose gradient. The rest remained at the top of the gradient. The sucrose step can be substituted with a second CsCl gradient provided the virus is centrifuged for short periods (3 h) so that the majority of the purification is due to velocity sedimentation. In our purification we have preferred to use a CsCl gradient followed by a sucrose gradient followed by another CsCl gradient.
The visualization of IPN virus RNA under the electron microscope has provided another piece of direct evidence that the RNA is double stranded. Single stranded molecules would have appeared as collapsed 'bushes' with this technique. Thus the fact that IPN virus RNA appeared as a uniform linear strand with a width equal to that of DNA (Fig. 6) indicated that the RNA was double stranded. Polyacrylamide gel electrophoresis indicated that two RNA species were present in IPN virions, and the frequency distribution of RNA lengths under the electron microscope seemed to substantiate this, although too few molecules have yet been measured to be certain. The average length of all the RNA molecules measured was 0.92 μm with a standard deviation of 0.07 μm. Since the spread in the frequency distribution may reflect the difference in the length of the two RNA species, we estimate that this difference is not likely to be more than 0.15 μm.

It is possible to estimate the mol. wt. of IPN virus RNA by the techniques employed in this study. Using the Doty equation which relates the sedimentation velocity of DNA to its mol. wt. (Doty, McGill & Rice, 1958), IPN virus 14S RNA would be 2.2 × 10⁶. The length of the RNA in the electron micrographs also gives an estimate of the mol. wt. Gomatos & Stoeckenius (1964) have proposed a mass-to-length ratio of 2.39 × 10⁸ daltons/μm for double stranded reovirus RNA. From this it can be calculated that the 0.92 μm average length of IPN virus RNA corresponds to 2.2 ± 0.2 × 10⁶ daltons. A serious drawback to this method of determining the mol. wt. is given by Davis, Simon & Davidson (1971). They pointed out that the length of nucleic acid molecules could vary depending upon the experimental conditions of spreading, and it is best to include internal standards when estimating mol. wt. To this end we have included an adenovirus DNA in some of our experiments. The adenovirus DNA was measured to be 10.4 μm in length, and has a mol. wt. of 23 × 10⁶. This would correspond to a mass-to-length ratio of 2.21 × 10⁶ daltons/μm for DNA. It is doubtful if this figure can be directly applied to RNA, since at least two differences between RNA and DNA may necessitate a correction. One is the difference between the average mol. wt. of a deoxyribonucleotide residue versus a ribonucleotide residue, and the other is the distance between nucleotide pairs in a DNA helix (0.34 nm for DNA and 0.3 nm for RNA; Langridge & Gomatos, 1963). These two effects would increase the mass-to-length ratio of RNA to 1.18 times that of DNA, thus giving double stranded RNA a value of 2.6 × 10⁶ daltons/μm.

If this figure is used, the IPN virus RNA averages 2.4 ± 0.2 × 10⁶ daltons. This result is in good agreement with that obtained by the previous method.

IPN virus RNA has been shown to be double stranded and to consist of two mol. wt. species. From the standard deviation of the lengths observed in the electron microscope it appears that these two RNAs cannot differ by more than 4 × 10⁵ daltons. The exact mol. wt. of IPN virus RNA is still uncertain, since the two methods of calculation have given slightly different values. We are inclined to trust the use of the internal standard, even though it is DNA, and assign an average mol. wt. of 2.4 × 10⁶. If the two highest peaks on the frequency distribution represent the actual lengths of the two RNA species, then the lengths of IPN virus RNA are 0.91 and 0.96 μm. This would correspond to 2.4 × 10⁶ and 2.5 × 10⁶ daltons of RNA using the internal DNA standard as reference. It is unlikely that the peak at 0.85 μm represented a third RNA species since gel electrophoresis showed only two RNA bands with the ³²P-radioactivity distributed equally between them. These values are somewhat lower than the previous estimate of 2.55 to 2.85 × 10⁶ daltons for the IPN virus RNA based upon comparison with reovirus RNA during gel electrophoresis (Cohen et al. 1973).

Several attempts were made to observe the early stages in the release of RNA molecules from IPN virus during pronase treatment but the release appeared to be all or nothing. At no time was the formation of doughnut-like or spider-like structures observed as has been
described for reovirus (Kavenoff, Talcove & Mudd, 1975). The present analysis of IPN virus RNA has made it appear likely that this virus belongs to a group separate from the Reoviridae.

The size and number of RNA molecules present in IPN virus RNA is similar to that found in some of the fungal viruses. However, the fungal virions are 40 nm in diam. compared to 74 nm for IPN virus (Wood & Bozarth, 1972; Bevan, Herring and Mitchell, 1973; Herring & Bevan, 1974; Buck & Ratti, 1975).

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