Size and structure of the genome of infectious pancreatic necrosis virus

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ABSTRACT

The genome of infectious pancreatic necrosis virus consists of two segments of dsRNA, in equimolar amounts, with molecular weights of $2.5 \times 10^6$ and $2.3 \times 10^6$ daltons, as determined by polyacrylamide gel electrophoresis and autoradiography. The viral RNA was resistant to ribonuclease, and in sucrose gradient it co-sedimented at 14S with RNase resistant RNA from virus infected cells. Upon denaturation in 98% formamide, the viral genome sedimented at 24S in formamide sucrose gradient and became sensitive to RNase. Denatured 24S viral RNA did not revert to its undenatured 14S form upon re-centrifugation in aqueous sucrose gradient (0.1 M NaCl), but co-sedimented with the denatured large size class of reovirus 25S RNA. The same results were obtained if the native viral RNA was pre-treated with ribonuclease before denaturation, indicating the absence of exposed single stranded regions in the viral genome. Since infectious pancreatic necrosis virus contains only two dsRNA segments it does not belong to the family Reoviridae and may represent a new group of viruses.

INTRODUCTION

Infectious pancreatic necrosis (IPN) virus is the causal agent of a highly contagious and destructive disease of young hatchery-reared trout (1). The virus can be grown in tissue culture in a variety of established fish cell lines at an optimal temperature of 22-24°C (2). It replicates in the cytoplasm of the host cell and studies with metabolic inhibitors indicated that the viral genome is RNA (2). Electron microscopic observations of purified virus revealed structures similar in size and shape to reoviruses but lacking the characteristic double capsid of the latter (3,4).

Considerable controversy exists concerning the nature of the viral genome; whether it is segmented (5) or unsegmented (3), double stranded (4) or single stranded RNA (3). Nicholson (5), on the basis of cytochemical and autoradiographic studies of infected cells, suggested that the genome of IPN virus is ssRNA. Kelly and Loh (3), using biochemical and biophysical methods of characterizing the viral RNA, found that its base composition, susceptibility to ribonuclease, and resistance to thermal denaturation indicated a ss RNA structure; one, however, that was soluble in 2M LiCl and showed a sedimentation behaviour (between 0.1M and 0.001 M NaCl).
independent of ionic strength in sucrose gradients. Since these last two attributes are characteristic of double stranded polynucleotides, the authors concluded that the viral genome is single stranded with a high degree of secondary structure of unknown nature. By polyacrylamide gel electrophoresis they showed IPN virus RNA to be a single (non-segmented) polynucleotide with a molecular weight of $3.2 \times 10^6$ daltons.

In contrast to these findings, Cohen et al. (4) reported that IPN virus contains dsRNA, based on the following criteria; base composition, resistance to ribonuclease, sharp melting curve with a $T_m$ of 89°C in hypotonic buffer, low buoyant density in Cs$_2$SO$_4$ ($p=1.615$), increased electrophoretic mobility in acrylamide gels after melting and sedimentation behaviour in sucrose gradients which was independent of ionic strength. Using 2.4% acrylamide-agarose gels they resolved the viral RNA into three peaks corresponding to double stranded molecules ranging in size from $2.85 \times 10^5$ to $2.55 \times 10^6$ daltons. Both groups of investigators (4,6) agree that the IPN virion contains three size classes of polypeptides (large, medium and small, with molecular weight ranges of 80-120,000, 65-50,000 and 35-30,000 daltons respectively) although the total number of polypeptides reported by the two groups was not identical.

The purpose of the investigation reported here was to resolve the controversy concerning the nature and molecular weight of viral RNA in order to define the relationship between the genome segments and the size of virus specific polypeptides.

To determine the number of segments and molecular weight of IPN virus RNA, we employed 5 and 7% acrylamide slab gel electrophoresis with internal markers (reovirus and Q6 phage RHA) followed by autoradiography.

When melting the viral RNA we refrained from using high temperatures to avoid thermal scission (7), and instead, denatured the virus genome in 98% formamide at 50°C or in 90% dimethylsulphoxide (DMSO), these methods have been shown to separate dsRNA molecules completely (8,20). The melted RNA was analysed in sucrose gradients under both, denaturing and undenaturing conditions.

The results reported here indicate that the genome of IPN virus consists of two segments of (large size class) dsRNA, which place the virus amongst the dsRNA viruses. However, since IPN virus contains only two RNA segments instead of 10-15 pieces found in other animal viruses with dsRNA genomes, it cannot be included in the family Reoviridae (9) and may represent a new virus group.
MATERIALS AND METHODS

Rainbow trout gonad cell line, RTG-2 (10) and fathead minnow (FHM) cells (11) were propagated as monolayers at 25°C in Corning tissue culture flasks. The growth medium consisted of Eagle minimum essential medium (MEM) with Earle balanced salt solution supplemented with 10% fetal calf serum and 40 μg/ml Gentamycin.

Virus

The virus used in this study was the reference strain of IPN, VR299 obtained from the American Type Culture Collection. It was serially passed at low input multiplicity of infection (MOI) (0.01-0.001 p.f.u. per cell), or alternately, low passage stock virus was prepared that was no more than 2-3 passages away from the reference stock.

Reovirus type 3 was provided by Dr. A. Graham (McGill University, Montreal), and dsRNA extracted from φ6 bacteriophage was the generous gift of Dr. A.L. Vidover and J.L. Van Etten of the University of Nebraska.

Preparation of 32P labelled virus

To label the virus with $^{32}$P, the growth medium was removed from confluent monolayers and enough phosphate-free MEM was added to cover the cells. Carrier-free orthophosphate $^{32}$P was then added to give a concentration of 10 μCi/ml. The cells were pre-incubated with $^{32}$P at 22°C for 24 hrs. in order to increase the specific activity of the nucleotide pool. IPN virus was added at an input MOI of 0.1 p.f.u. per cell and the cultures were incubated until advanced cytopathogenic effect (cpe) was observed.

Preparation of $^3$H-uridine labelled RNA from infected cells

RTG-2 cells were grown in 6 cm plastic tissue culture plates. When the cell layers were confluent, the medium was removed and IPN virus was added at an input MOI of 30-40 p.f.u. per cell. After a one hour adsorption period, the inoculum was removed and the cells were incubated in growth medium for 6 hours. At this time the medium was replaced with serum-free MEM containing 5 μCi/ml of $^3$H-uridine (45 Ci/m mole) and incubation continued for an additional 3 hours. The medium was removed, the monolayers washed with Earle's balanced salt solution and digested with proteinase K (1 mg/ml) in proteinase K (PK) buffer (0.01 M Tris, 0.01 M NaCl, 0.01 M EDTA, 0.5% sodium dodecyl sulfate, (SDS) pH 8.0). After bringing the concentration of NaCl to 0.5 M, the RNA was precipitated with ice-cold ethanol and the DNA was spooled out immediately with a glass rod. Since proteinase K co-precipitates with RNA in ethanol (12), it was removed by phenol extraction (13).
Virus purification

A modified method of Cohen et al. (4) was used to purify IPN virus. After the development of extensive c.p.e. in the infected cultures, the cells were scraped off the culture vessel into the culture medium and disrupted by sonication (Biosonik III, maximum setting, 3 x 30 sec. in an ice bath). The virus was concentrated by centrifugation at 90,000 g for 2 hrs. at 5°C in a Beckman (L2-65B) preparative ultracentrifuge. The supernatant was discarded and the pellet was resuspended by sonication in 1 vol. of the TNE buffer (0.01 M tris, 0.1 M NaCl 0.001 M EDTA, pH 7.1) and extracted in a Virtis homogenizer for 2 minutes at half speed with a half volume of Freon 113. Alternatively, Freon extraction was performed by Dounce homogenization. The mixture was centrifuged in the cold at 10,000 g for 10 minutes to separate the phases and the organic phase was then re-extracted with an equal volume of TNE buffer. Freon-extracted virus was pelleted by ultracentrifugation at 90,000 g for 2 hrs. and the pellet was resuspended by sonication in 5 ml of phosphate buffered saline which contained 3 mM Mg** and no Ca**. The enzymes DNase and RNase were added to a concentration of 60 μg/ml and 20 μg/ml respectively and the preparation was incubated at 37°C for 30 minutes. The nuclease-treated, semipurified virus suspension was then sedimented through a 10% (w/v) sucrose column in TNE buffer for 4 hrs. at 90,000 g at 5°C. The virus pellet was resuspended by sonication in TNE buffer, CsCl was added to a density of 1.32 g/cm³ and the preparation was then centrifuged at 5°C for 15 hrs. at 150,000 g. The gradient was fractioned from the bottom, 200 μl fractions were collected. A 5 μl sample from each fraction was removed to determine the acid precipitable radioactivity. These samples were dispensed into 0.5 ml of TNE buffer containing 200 μg of bovine serum albumin and an equal volume of 10% ice cold trichloroacetic acid (TCA) was added. After visible precipitation of the albumin, the samples were filtered through Whatman GF1A glass fiber filters, washed with ethanol to remove the TCA, dried and placed in scintillation vials. Radioactivity was counted in a toluene based scintillation cocktail (0.4% w/v PPO and 0.01% w/v POPOP) using a Packard Tricarb liquid scintillation counter. The buoyant densities of representative fractions were calculated from their refractive index measurements (14). When fractions representing infectious virus (ρ = 1.33 g/cm³) were pooled, diluted with TNE buffer and pelleted through a second 10% (v/v) of sucrose column at 90,000 g for 4 hrs., 99% of the radioactivity was sedimented in the virus pellet.
RNA extraction

The virus was dissolved in proteinase K buffer containing 1 mg of proteinase K per ml and left overnight at room temperature. The viral RNA was separated from the enzyme and from digested capsid material by layering the digest onto a 5-20% w/v sucrose gradient (in TNE buffer) containing 0.5% SDS and centrifuging it at 20°C for 3 hrs. at 150,000 g in an SW50.1 rotor. The gradient was fractionated as described above and fractions containing the labelled viral RNA was detected by measuring the acid insoluble radioactivity of a 2 μl sample from each fraction. The RNA was precipitated at -20°C (without carrier) with 2 volumes of ethanol containing 0.4 M LiCl. Portions of the RNA were recovered by centrifugation at 100,000 g for 1 hr. and resuspended in the appropriate buffer for subsequent analysis.

Polyacrylamide gel electrophoresis of viral RNA

The method of Reddy and Black (15) was used to analyze labelled IPN virus RNA on 7.5% and 5% acrylamide gels. This involved the use of two slightly different buffer systems in the gel and in the electrophoresis tanks which permitted the complete separation of all 12 components of Wound Tumor Virus RNA and all 10 components of reovirus RNA. The gels were made up in 0.04 M PTE buffer (0.04 M sodium phosphate, pH 7.0, 0.015 M Tris, 0.002 M EDTA, final pH 7.5-7.6) containing 5 or 7.5% acrylamide and 0.1% Bis. To 10 ml of this solution 0.1 ml of 10% (w/v) ammonium persulfate was added, and the solution was degassed by exposure to vacuum. Ten microliter of TEMED was added, the gels were poured into glass tubes stoppered at one end and were overlaid with 20 μl of water. Alternatively, slab gels (0.1 x 20 x 10 cm) were poured and the sample wells were formed by the insertion of a plexiglass well-making comb.

The electrophoresis buffer was that of Loening's (16)(0.36 M Tris, 0.03 M NaH₂PO₄ and 0.001 M EDTA, pH 7.7). RNA samples were dissolved in sterile distilled water containing 1% SDS, 8% sucrose and a trace of bromophenol blue. Usually 5-20 μl of the RNA solution was layered on top of the gel, with a microsyringe, under a column of electrophoresis buffer. Electrophoresis was carried out at room temperature for 24 hrs. at 50 V. The electrophoresis buffer was recirculated using a peristaltic pump and an overflow.

After the run, the gels were fixed at room temperature in 0.4 M acetate buffer, pH 4.7, for 20 minutes followed by staining for 30 minutes in the same buffer containing 0.1% toluidine blue 0. Destaining was accomplished
with several changes of deionized water. This method always completely separated reovirus RNA segments $L_1$ and $L_2$, which are often difficult to resolve in other gel systems. Cylindrical gels were sliced longitudinally with a gel slicer, and the slices as well as slab gels were dried under vacuum and placed on Kodak X-ray film for autoradiography for 1-7 days, depending on the amount of radioactivity applied to the gels. Molecular weights were determined by superimposing the autoradiograph on the stained marker RNA gel pattern and comparing the electrophoretic mobilities of RNA's of unknown molecular weight to those of marker RNA's.

**RNAase digestion**

RNA samples were tested for ribonuclease sensitivity in 2 x SSC (SSC: 0.15 M NaCl, 0.015 M sodium citrate, pH 7.4) using 5 µg/ml of pancreatic ribonuclease A at 37°C for 30 minutes.

**Denaturation of IPN virus RNA**

Alcohol-precipitated RNA pellet was dried under vacuum and 0.2 ml of deionized 98% formamide was added. The preparation was placed in a 50°C waterbath for 30 minutes. This treatment has been shown to separate dsRNA completely (8) and the strands remain separated when the solution is allowed to cool to room temperature. An equal volume of L-cell ribosomal RNA (in 0.003 M EDTA, 0.005 M tris-HCl, pH 7.4) was added and the sample was layered onto a 5-25% (w/v) sucrose gradient in 70% formamide containing 0.003 M EDTA and 0.003 M tris-HCl, pH 7.4. The gradient was centrifuged for 24 hrs. at 25°C using the SW40 rotor of a Beckman preparative ultracentrifuge at 190,000 g (39,000 rpm). The optical density profile of the gradient was measured at 270 nm to locate the ribosomal RNA markers and the viral RNA was detected by measuring the acid precipitable radioactivity in the gradient fractions.

**Enzymes, Chemicals, Isotopes**

Eagle MEM, Earle balanced salt solution, and fetal calf serum (virus and mycoplasma screened) were purchased from Grand Island Biological Co.; Gentamicin from Microbiological Associates; pancreatic RNase and DNase from Sigma Chemicals Ltd., sodium dodecyl sulfate, specially pure and proteinase K from B.D.H. Ltd.; 99% formamide from Matheson, Colman and Bell, Inc.; RP/R-2 Royal X-omat film from Kodak. Electrophoresis grade acrylamide, N, N'-methylene-biscarylamide (BIS) and N,N,N',N'-tetramethylethylene diamine (TEMED) was purchased from Bio-Rad Laboratories. Tritiated uridine (5,6-3H) specific activity 45 Ci/m mole, (32P)-orthophosphoric acid (carrier free) 2, 5-diphenyloxazole (PPO), and p-bis-(2-(5-phenyloxazoly1)) benzene (POPOP) 1908
were the products of New England Nuclear Corp.

RESULTS

Preparation of radiochemically pure $^{32}$P labelled virus

The first objective was to produce highly-labelled purified virus. Initial attempts to achieve this were hampered by a number of factors characteristic of IPN replication, notably: 1) relatively low virus yield (3-500 p.f.u./cell) even when cells were infected at a very low multiplicity to avoid the formation of defective-interfering particles; 2) most of the progeny virus remained cell-associated and had to be extracted with Freon which is at best 70-80% efficient (4); 3) IPN infection does not inhibit host RNA and protein synthesis (5) and, therefore, nucleic acid precursors are not incorporated preferentially into virus-specific nucleic acids in infected cells; 4) even though IPN replicates in the cytoplasm (1,2), virus replication is severely inhibited by even low doses of actinomycin D (e.g. 0.1 µg/ml of Act D resulted in a 95% reduction in virus yield (17) which therefore cannot be used to inhibit host RNA synthesis without inhibiting viral RNA synthesis as well.

Preliminary experiments indicated that IPN virus with high specific radioactivity could be obtained only by pre-incubating the host cells for a day in phosphate free medium containing 10 Ci/ml of $^{32}$P before infecting with the virus.

To eliminated labelled nucleic acids not encapsidated into IPN virions, the Freon-extracted virus concentrate was digested with DNase and RNase and sedimented through a 20 ml (10% v/w) sucrose column in the ultracentrifuge. Under these conditions the nucleases and their low molecular weight labelled digestion products remained near the top of the sucrose column. When the resuspended virus pellet was subjected to isopycnic gradient centrifugation in CsCl there was little radioactivity in the gradient that was not associated with the infectious virus band (buoyant density = 1.33 g/cm$^3$) Fig. 1. Fractions containing the virus were pooled, diluted in TNE buffer and resedimented through a second 10% sucrose column to free the virus from CsCl and to eliminate residual extravirion label.

The final virus pellet, when resuspended in 1 ml of TNE buffer, had an OD$_{260}$ of 0.2 and represented about $1.2 \times 10^5$ p.f.u. per ml. It contained $1.2 \times 10^5$ cpm of acid precipitable radioactivity.

Isolation of IPN RNA with proteinase K in the presence of SDS

The complete recovery of intact virus nucleic acid, during RNA extraction, is a prerequisite of any subsequent analysis of the virus genome.
Figure 1. Isopycnic sedimentation of $^{32}$P-labelled purified IPN virus in CsCl. Fractions 7-9 were pooled and pelleted through a 10% v/v sucrose column to eliminate residual extravirion label.

Since selective entrapment of viral RNA may occur in the phenol-buffer interface (4) resulting in incomplete RNA recovery, we did not use phenol for RNA extraction but digested purified $^{32}$P-labelled virus with proteinase K in the presence of SDS. It has been shown that proteinase K treatment is superior to phenol extraction for the isolation of undegraded polysomal RNA from HeLa cells (18). Proteinase K activity was stimulated in the presence of SDS which denatured the substrate, making it more accessible to the enzyme (12). The combination of proteinase K and SDS completely and irreversibly eliminated ribonuclease (12) and the danger of selective entrapment of a portion of the IPN RNA, which may occur during phenol extraction (4), was eliminated.

To extract IPN virus RNA, the purified final virus pellet was dissolved in PK buffer containing 0.5% SDS and 1 mg/ml of proteinase K. Overnight incubation at room temperature completely digested the capsid proteins since no acid precipitable radioactivity could be recovered when $^{3}$H-leucine labelled IPN virus was so treated (data not shown).

Elimination of proteinase K after digestion was achieved by sucrose gradient centrifugation of the RNA which left the enzyme in the top gradient.
fractions.

Analysis of RNA by gradient centrifugation and resistance to ribonuclease

Purified $^{32P}$-labelled IPN virus RNA was analyzed in SDS-sucrose gradients using $^3H$-labelled reovirus RNA and 28S and 18S L cell rRNA as sedimentation markers. The virus RNA sedimented at 14S, only slightly behind the 15S large size class reovirus RNA (Fig. 2). As reported by other investigators (3,4), we also found that the ionic strength of the gradient buffer did not influence the sedimentation behaviour of IPN RNA (data not shown).

![Sedimentation profile](image)

Figure 2: Sedimentation profile of $^{32P}$-labelled IPN virus RNA (●); and $^3H$-uridine labelled reovirus RNA (○) in 5-20% sucrose-SDS gradients. The optical density peaks of unlabelled L-cell rRNA and tRNA are indicated by arrows. The letters L, M, S signify the large, medium and small size classes of reovirus RNA.

The sedimentation profile of the viral RNA remained unchanged when exposed to 5 µg/ml of ribonuclease for 30 min at 37°C in 2 x SSC buffer prior to gradient sedimentation (Fig. 3). It was of interest to determine if similar size RNase-resistant RNA could be isolated from infected cells at the time of maximum rate of virus-specific RNA synthesis. According to previous reports, intracellular RNA pulse-labelled with $H^3$-uridine between 8-11 hrs. post-infection, was completely digested by ribonuclease (5,17).
ribonuclease-resistant RNA, virus-infected cell cultures were pulse-labelled with \(^3\text{H}\)-uridine for 3 hrs. at 0, 3, 6, 9 and 12 hrs. post-infection. The RNA was extracted and each sample was dissolved in 2 ml 2 x SSC. Half of each sample was treated with RNase (5 \(\mu\)g/ml) for 30 min at 37\(^\circ\)C together with \(^32\text{P}\)-labelled virion RNA and unlabelled ribosomal RNA. The untreated controls and half of each ribonuclease-treated sample were precipitated with cold 10% TCA for acid-insoluble radioactivity determinations (Table 1). The other half of the nuclease-treated sample was analyzed by sucrose gradient centrifugation.

### Table 1

<table>
<thead>
<tr>
<th>Time of pulse hr. post inf.</th>
<th>Total acid-insoluble (^3\text{H}) cpm</th>
<th>RNase resistant cpm</th>
<th>% of total RNase resistant cpm</th>
</tr>
</thead>
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<tr>
<td>0 - 3</td>
<td>97,100</td>
<td>2,250</td>
<td>2.30</td>
</tr>
<tr>
<td>3 - 6</td>
<td>174,900</td>
<td>8,500</td>
<td>4.86</td>
</tr>
<tr>
<td>6 - 9</td>
<td>178,200</td>
<td>12,700</td>
<td>7.12</td>
</tr>
<tr>
<td>9 - 12</td>
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<td>5.04</td>
</tr>
<tr>
<td>12 - 15</td>
<td>69,000</td>
<td>4,400</td>
<td>6.30</td>
</tr>
</tbody>
</table>

The data presented in Table 1 show that maximum rate of RNA synthesis occurs between 6-9 hrs. post infection in virus-infected cells and that this RNA population contains the highest proportion (6-8%) of ribonuclease-resistant RNA.

Figure 3 indicates that virion RNA and most of the intracellular RNase-resistant RNA co-sedimented as 14S RNA in the sucrose gradient. Little acid insoluble radioactivity containing \(^32\text{P}\) is left at the top of the gradient, however, some of the intracellular \(^3\text{H}\)-uridine labelled ribonuclease resistant RNA is of low molecular weight, thus the 14S RNA represents only 4-5% of the total acid insoluble radioactivity.

**Electrophoresis of viral RNA in acrylamide gels**

Previous analyses of IPN virus RNA in polyacrylamide cylindrical gels indicated that the virus genome was single stranded and unsegmented (3), while others found that the virus RNA was made up of 3 segments of dsRNA slightly larger than the large size (L) class of reovirus RNA (2.85-2.55 x 10^6)(4). In both cases soft (2.4-3%) acrylamide-agarose gels were used and electrophoretic mobility was determined by cross slicing the gels followed by counting the radioactivity in the slices (3, 4).

To improve the resolution, we used 5 and 7.5% acrylamide for electro-
Figure 3: Sedimentation profile of RNase treated $^{32}$P-labelled IPN virus RNA (•---•) and $^3$H-uridine labelled RNA extracted from IPN virus-infected cells after a 3 hr. pulse from 6-9 hrs. post infection (o---o); and unlabelled L-cell rRNA( ). The 28S, 18S and 4S marker RNAs, represented by arrows, were centrifuged in a parallel gradient.

Electrophoresis followed by autoradiography. $^{32}$P-labelled IPN virus RNA was analyzed for different time periods on 5% acrylamide slab gels, together with reovirus RNA and $^3$H phage RNA (Fig. 4). All 10 segments of reovirus RNA were clearly resolved under these conditions. When IPN virus RNA was subjected to electrophoresis for only 12 hours, the viral genome was near the top of the gel and no radioactivity was found below these two bands indicating that there are no low molecular weight oligonucleotides associated with IPN virus comparable to the adenine rich low molecular weight RNA found in reovirus. IPN RNA could not be resolved to more than two bands even under conditions where the medium and small class reovirus RNA pieces ran out of the gel at the bottom and the 3 large segments were well separated. When labelled RNA bands were sliced out of the gels and the amount of radioactivity in each band was measured in a liquid scintillation counter, the relative amount of radioactivity in each band was proportional to its molecular weight, i.e. band A contained slightly higher amounts of radioactivity than band B.
Figure 4: Acrylamide gel electrophoresis of $^{32}$P-labelled IPN virus RNA (I); unlabelled reovirus RNA (R) and unlabelled φ6 phage RNA (φ) in 5% slab gel for different time intervals. The photograph of the stained gel pattern was superimposed on the autoradiogram to determine the relative electrophoretic mobility of IPN virus RNA. The molecular weight marker values indicated on the left are those of the three φ6 phage RNA segments.

A similar electrophoretic profile was observed when $^{32}$P-labelled IPN virus RNA was analyzed together with reovirus and φ6 phage RNA in 7.5% cylindrical gels. The data presented in Figure 5 shows that the $L_1$ segment of reovirus RNA and the 2.8 x 10$^6$ dalton molecular weight segment of φ6 phage RNA co-migrated, resulting in an extra-heavy band. The two IPN virus RNA segments migrated between the $L_2$ segment of reovirus RNA and the smallest of the three φ6 phage RNA segments (mol. wt. 2.2 x 10$^6$).

When the relative electrophoretic mobility of IPN RNA was plotted against those of the marker RNA's, the molecular weights of IPN virus RNA
Figure 5: Acrylamide gel electrophoresis of $^{32}$P-labelled IPN virus RNA together with unlabelled reovirus RNA and Ø6 phage RNA in 7.5% cylindrical acrylamide gel. The left column shows the stained marker RNA bands; the right column is an autoradiogram of the same gel. (Band A: 3300 cpm, Band B: 2950 cpm).

Segments A and B were found to be $2.5 \times 10^6$ and $2.3 \times 10^6$ daltons respectively.

The same results were observed when IPN virus was grown in FHM cell culture instead of RTG-2 cells or when a new IPN virus stock was obtained from the American Type Culture Collection and the $^{32}$P-labelled purified virus was only two passages away from the reference virus stock.

Melting of IPN RNA

Denaturation of dsRNA results in increased electrophoretic mobility and sedimentation rate with a concomitant loss of resistance to ribonuclease. Single stranded RNA with a high helical content will only demonstrate increased electrophoretic mobility upon denaturation if the nucleotide
chain was nicked during preparation at exposed loop regions (19). Such RNA, however, will sediment more slowly than its native undenatured form (19). An increase in electrophoretic mobility upon denaturation thus does not indicate unequivocally that the undenatured RNA is double stranded. On the other hand, an increase in sedimentation rate after melting indicates the transition of the nucleic acid from a "rigid" double stranded structure to a flexible random coil (20).

We analyzed the sedimentation behaviour of IPN virus RNA after denaturation in 98% deionized formamide (50°C for 30 min.) under conditions where the secondary configuration of ssRNA is eliminated. The denatured viral RNA was centrifuged in a 5-25% (w/v) sucrose gradient made up in 70% buffered formamide. The sedimentation profile of rRNA markers and \(^{32}\)P labelled RNA is shown in Figure 6. Whereas before denaturation IPN virus RNA sedimented more slowly than 18S rRNA (Figure 2), after melting it sedimented just behind the 28S rRNA with a relative sedimentation constant of 24S. The denatured IPN virus genome was susceptible to RNase in 2xSSC buffer (Table 2).

The viral RNA could also be denatured by 90% DMSO according to the method used to melt reovirus dsRNA (20). When the denaturing agent was
Table 2

<table>
<thead>
<tr>
<th>Buffer</th>
<th>RNase µg/ml</th>
<th>Time of incubation at 37°C (min)</th>
<th>Percent acid insoluble radioactivity (b) after RNase treatment of:</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>IPN RDN IPN RDN 14S 15-10S dennatured dennatured (c) RDN 25-12S</td>
</tr>
<tr>
<td>2 x SSC</td>
<td>-</td>
<td>30</td>
<td>100 100 100 100 100 100</td>
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<tr>
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<td>5</td>
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<tr>
<td>1 x SSC</td>
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<td>60</td>
<td>85 98 - -</td>
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<tr>
<td>0.1 x SSC</td>
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<td>2 4 - - -</td>
</tr>
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<td>0.01 x SSC</td>
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</tr>
<tr>
<td>1 x SSC</td>
<td>100.0</td>
<td>30</td>
<td>85 90 - - -</td>
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</table>

(a) 3H-uridine labeled RNA was extracted from CsCl gradient purified virus and centrifuged in sucrose gradients as described in Methods, except that proteinase K was removed by phenol extraction and the gradients did not contain SDS. Fractions, containing 14S RNA in the case of IPN virus or 15-10S RNA in the case of reovirus, were pooled, the RNA precipitated with ethanol, washed and dissolved in 2 x SSC. Five microliter portions were used in each sample; these contained the following amounts of radioactivity: reovirus RNA 14,100 cpm; IPN virus RNA 12,900 cpm; denatured reovirus RNA 2,700 cpm; denatured IPN virus RNA 3,900 cpm; rRNA 40,000 cpm.

(b) Values represent mean of duplicate samples from two different virus preparations.

(c) denatured IPN RNA (24S) and reovirus RNA (25-12S) was recovered from the appropriate gradient fractions of Figure 7 precipitated with ethanol, washed and resuspended in 2xSSC; one half served as control the other half was digested with RNase.

removed and the RNA recovered by ethanol precipitation and centrifuged on an ordinary sucrose gradient (containing 0.1 M NaCl) it co-sedimented with the large size (25S) segments of similarly denatured reovirus RNA. The removal of the denaturing agent did not cause the 24S RNA to revert to its original 14S RNase resistant form, nor did it allow the denatured 25-12S reovirus RNA to revert to its 15-10S RNase resistant double stranded configuration. This treatment rendered both IPN and reovirus RNA sensitive
to ribonuclease (data not shown and Table 2).

In order to detect the presence of exposed single stranded "loop" regions in the virus genome, IPN virus RNA and reovirus RNA were treated with ribonuclease for ten minutes (1xSSC, 1 μg/ml RNase, 37°C); the ribonuclease was removed by phenol extraction, the RNA precipitated with ethanol, denatured with DMSO, re-precipitated and analysed in sucrose gradients. Even after this treatment denatured IPN virus RNA sedimented at 24S (Figure 7) and was sensitive to ribonuclease (Table 2).

Figure 7: The effect of DMSO on reovirus RNA and IPN virus RNA that had been exposed to ribonuclease before denaturation. Isotopically labeled viral RNA was incubated with RNase as described in the text; the ribonuclease was removed by phenol extraction, the RNA was denatured with DMSO using the method of Bellamy et al. (20) and analysed in 5-20% sucrose gradients. Denatured reovirus RNA • • ; denatured IPN virus RNA ••••; and native 14S IPN virus RNA (marked by arrow) were run in parallel gradients, each containing rRNA markers (indicated by arrows). The letters L,M,S signify the large, medium and small size classes of reovirus RNA.

DISCUSSION

The results presented here demonstrate that the genome of IPN virus consists of two segments of double-stranded RNA. These segments are of the large size class, a designation used to group together the high, medium, and low molecular weight reovirus RNA segments. There is no evidence that IPN virus contains small oligonucleotides comparable to the adenine-rich, low molecular weight ssRNA found in purified reovirus.
Since different investigators have reported conflicting results concerning the strandness and the number of RNA segments that make up the virus genome, we attempted to resolve the controversy by using different methodology in the analysis of IPN virus RNA. Extravirion nucleic acids were eliminated from $^{32}$P labelled semi-purified virus concentrates by digestion with DNase and ribonuclease A. Repeated sedimentation through sucrose columns in combination with isopycnic banding in CsCl gradient eliminated extravirion label to the extent that 99% of radioactivity was pelleted with the virus in the final preparation.

Because of the use of nucleolytic enzymes in the virus purification it was imperative to extract the viral RNA under conditions where traces of ribonuclease would be irreversibly eliminated yet the complete recovery of undegraded viral RNA was ensured. Treatment of the purified virus pellet with proteinase K, in combination with SDS, achieved this goal. Viral capsid proteins as well as any traces of nucleases were completely digested by proteinase K, resulting in complete RNA recovery. Because the enzyme co-precipitates with RNA in ethanol (12), the separation of the labelled viral RNA from proteinase K was achieved by sucrose gradient centrifugation. All of the labelled RNA sedimented as a single 1 Us component and was recovered from gradient fractions by ethanol precipitation.

When the viral RNA was analyzed by acrylamide gel electrophoresis in 5 and 7.5% gels followed by autoradiography, it formed sharp bands characteristic of dsRNA. The two virus RNA segments migrated faster than the large size class of reovirus RNA but slower than the smallest segment of T6 phage RNA (mol. wt. 2.2 x 10$^6$). The gel-buffer system used in combination with autoradiography resulted in very high resolution and completely separated reovirus RNA segments L$_1$ and L$_2$ which are difficult to resolve under the usual electrophoretic conditions (15). Contrary to the date presented by Cohen et al. (16), who showed that the viral RNA was made up of 3 segments, we could not resolve IPN virus RNA into more than two bands. Even after electrophoresis in 5 and 7.5% gels for 2 - 2.5 days there was no indication of either segment A or B separating into two components. Furthermore, when the radioactivity in each band was quantitated, the relative amount of label in each was proportional to the molecular weight i.e., band A contained only 6-10% more radioactivity than band B, reflecting the difference in molecular weight between the two segments. If any one of these segments was a doublet, it would have contained twice as much radioactivity as the other. These results did not change when the virus was purified from reference stock.
instead of serially passed working-stock or from virus grown in FRM cells instead of RTG-2 cells. The fact that IPN RNA clearly entered 5 and 7.5% gels (without the use of urea) also indicate that it is double stranded, ssRNA of this size does not enter gels containing 5% or more acrylamide (21). The discrepancy between the results presented here and those of Cohen et al. (4) concerning the number of RNA segments may be attributed to technique. They used 2.5% acrylamide-agarose gels followed by cross slicing and counting the radioactivity in the solubilized gel slices. This method gave low resolution since the three reovirus RNA marker segments of L1, L2 and L3 were represented by a single peak of radioactivity.

With respect to the ribonuclease sensitivity of IPN RNA, our results agree with those of Cohen et al. (4). Contrary to the report of Kelly and Loh (3), we have found the viral RNA to be resistant to ribonuclease. The data in Table 2 indicate that IPN virus RNA was slightly more sensitive to RNase than reovirus RNA, however, after the initial loss of about 15% of radioactivity (1xSSC 30 min RNase treatment) the viral genome was not digestible any further by using as much as 100 μg/ml of ribonuclease. At high enzyme concentrations we found reovirus RNA more resistant to RNase than that reported by Bellamy et al. (20). In order to rule out the possible protective effect of traces of magnesium ions (although we used freshly deionized, double distilled water to make up the 2xSSC buffer which was subsequently autoclaved) we included labeled RNA in the RNase sensitivity test. Over 90% of this ssRNA was digested by RNase in 2xSSC buffer. When ribonuclease treated IPN RNA was analysed in sucrose gradient (Fig. 3) there was a slight increase in the proportion of radioactivity found at the top of the gradient; this may indicated that the viral dsRNA contains short single stranded "tail" regions, similar to φO phage dsRNA (22). Ribonuclease treatment did not alter the sedimentation of 14S IPN virus RNA (Fig. 3) and in 5% acrylamide gels it co-migrated with viral RNA that has not been exposed to ribonuclease (unpublished data and Cohen, personal communication).

It was of interest to determine the proportion of 14S dsRNA in virus-infected cells at the time when the rate of RNA synthesis was at a maximum. In reovirus-infected cells the RNase-resistant dsRNA made up 40% of total RNA when measured at times later than 9 hrs. post-infection (23). These cells showed characteristic green cytoplasmic inclusion bodies when stained with acridine orange (24). In contrast, Kelly and Loh (17) and Nicholson (5) found that the cytoplasmic inclusion bodies of IPN infected cells stained red after treatment with acridine orange. Both found the inclusions
digestible by RNase at enzyme concentrations of 10C ug/ml (17) and 0.2 ug/ml (5). Furthermore, both investigators found a great increase in the rate of RNA synthesis in virus-infected cells between 7 and 12 hrs. post infection, and they found that over 80% of this RNA was ribonuclease sensitive (5, 17). On this basis the authors suggested that IPN RNA was probably single-stranded. In neither case was the remaining ribonuclease-resistant fraction characterized. Our data show that when virus-infected cells were labelled with H-uridine from 9-12 hrs. post-infection and the extracted RNA was treated with ribonuclease in 2 x SSC, 92-95% of the label became acid soluble. When the RNase-resistant fraction was analyzed on sucrose gradients, together with 32P-labelled ribonuclease-treated virion RNA, the majority of the RNase-resistant intracellular RNA co-sedimented with the 14S virus RNA. This very small proportion of 14S dsRNA in infected cells (4-5%) is probably not enough to show up as orthochromatically (green) staining cytoplasmic inclusion bodies after acridine orange staining, and it would be expected to be lost in the "background" level of radioactivity in the quantitative studies of Nicholson (5), and Kelly and Loh (17). This low level of intracellular RNase resistant RNA (which may also contain RNA replicative forms) could be due to the efficiency of infection and input MOI used. Indeed, using young (24 hours) FHM cellcultures and high MOI, approximately 25% of the intracellular RNA was found to be resistant to ribonuclease at 9 hours post-infection (Cohen, personal communication).

It was hoped that denaturation studies would help to reinforce the conclusion that IPN virus RNA is double stranded. Previously, such experiments resulted in conflicting reports. Kelly and Loh (3) found no change in the electrophoretic mobility of virus RNA after heating (100°C) and quick cooling. Cohen et al. (4), on the other hand, found that after heating (100°C) and rapide cooling of viral RNA preparations the denatured RNA migrated about twice as fast as native RNA when analyzed in acrylamide gels. However, an increase in electrophoretic mobility after melting does not necessarily prove that the RNA is double stranded, for this can also occur if an exposed "loop" region, of a highly hydrogen bonded ssRNA is nicked during preparation. The exposure of such hidden breaks after denaturation of the RNA is well documented (25). Such nicked ssRNA after denaturation will sediment more slowly than native RNA in sucrose gradients and migrate faster in acrylamide gels (19). On the other hand, true dsRNA, such as the reovirus genome, will show an increased mobility in acrylamide gels after melting, and it will also sediment faster in sucrose gradient...
than its native form, indicating a change from a rigid double helix to that of a flexible random coil. At the same time the RNA becomes completely sensitive to ribonuclease regardless of the sodium ion concentration used.

The relative sedimentation rate of IPN virus RNA was measured before and after denaturation. In order to avoid thermal degradation of RNA during denaturation, formamide was used at a relatively low temperature (50°C) to break hydrogen bonds and the RNA was analyzed under conditions that prevented the reformation of any secondary structure. It has been shown by Pinder et al. (8) that dsRNA of P. cyaneofulvum (Tm 100°C) was completely denatured in 98% formamide at 50°C and reannealing did not occur when the preparation was allowed to cool to room temperature. For this reason we used 98% formamide at 50°C to denature IPN virus RNA, followed by sucrose density gradient centrifugation in 70% formamide or by electrophoresis in formamide acrylamide gels. The relative rate of sedimentation of the denatured viral RNA increased to 21S compared to its native 14S form. This was similar to the change in sedimentation behaviour observed with 15S reovirus large size dsRNA which sedimented at 24S RNA after denaturation (20). The relative electrophoretic mobility of denatured IPN RNA in formamide acrylamide gels also indicated a molecular weight of 1.25 x 10^6 when compared to TMV RNA, 28S and 18S rRNA and denatured reovirus RNA (unpublished data).

It may be argued that denatured IPN virus RNA sediments at 21S only because the denaturing agent (formamide, Fig. 6) is present in the gradient and does not allow the melted RNA to return to its original 14S configuration. In order to determine the validity of this argument, IPN virus RNA and reovirus RNA were denatured by DMSO, recovered from the denaturing agent by ethanol precipitation and analysed in ordinary sucrose gradients in which native RNA sedimented at 14S. Both IPN virus RNA and the large size class reovirus RNA sedimented (at about 24S) ahead of the 18S rRNA marker. Furthermore, both of these RNA species were sensitive to ribonuclease (Table 2).

The same results were obtained if the native viral RNA was pre-treated with ribonuclease before denaturation indicating the absence of exposed single stranded regions in the viral genome (Fig. 7).

The data presented in this paper as well as some of those reported by others (3, 4) indicate that IPN virus RNA is double stranded. The results could apply to a ssRNA structure only if it was in a completely backfolded hairpin configuration with an exposed single stranded loop in the
middle of the polynucleotide chain, which was always "nicked" in one place during extraction. Although it is highly unlikely that this is the case, the possibility cannot be excluded.

On the basis of these results concerning the nature of the virus genome, and on the basis of electron microscopic studies of purified virus previously published (3, 4), IPN virus appears to be related to other reo-like viruses. However, all other animal or plant viruses with dsRNA genomes contain 10-15 segments in 3 size-classes (9). Only the virus-like particles in Penicillium stoloniferum contain a single class of dsRNA of two segments, therefore IPN virus cannot be placed in the family reoviridae (26). Purified IPN virus is made up of three size classes of polypeptides as determined by gel electrophoresis (4, 6) and only the largest of these can be a primary gene product generated by the translation of genome length 24S mRNA (27). It is interesting to speculate as to how the medium and small size class polypeptides may arise since they do not seem to be produced by post-translational cleavage of large precursor proteins (Dobos, unpublished results). The 24S genome length mRNA (27) may contain initiation sites for translation similar to the genomes of RNA bacteriophages (28) or subgenomic size mRNAs may be transcribed from the viral RNA as in the case of VSV mRNA synthesis (29) or post-transcriptional cleavage of the large 24S mRNA may occur as in some of the adenovirus specific mRNA's (30). Whichever may be the case, the mechanisms of virus-specific RNA and protein synthesis promises to be as unique as the nature of the virus genome itself.

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REFERENCES

Nucleic Acids Research
