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The Genome of Infectious Bursal Disease Virus Consists of Two Segments of Double-Stranded RNA

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The RNA of infectious bursal disease virus was reexamined in a detailed analysis. It could be established that its genome consists of two segments of double-stranded RNA. The RNA is RNase resistant and has a sedimentation coefficient of 14S and a buoyant density of 1.62 g/ml. The purine/pyrimidine ratio is nearly 1; the guanine plus cytosine content is 55.3%; the T_m is 95.5°C. The molecular weights of the two double-stranded segments were determined to be 2.2×10^6 and 2.5×10^6.

Infectious bursal disease virus (IBDV) is the causative agent of a highly contagious disease of young chickens with severe inflammatory changes in the bursa of Fabricius followed by immunodeficiency (Gumboro disease). In a previous report from this laboratory (11), IBDV was characterized as a nonenveloped particle with a diameter of about 60 nm which has a genome consisting of two segments of RNA. The RNA was found to be only partially resistant to treatment with pancreatic RNase (RNase A), and it has been proposed that it might be mainly single stranded with some back-folded regions. Since the possibility also remained that IBDV RNA is double stranded and that the material sensitive to RNase represents cellular contaminants, further studies were undertaken that focused on a careful purification procedure and further criteria for double-strandedness. Since structural similarities exist between IBDV and the infectious pancreatic necrosis virus (IPNV) of fish (P. Dobos, R. Hallet, D. T. C. Kells, B. J. Hills, H. Becht, and D. Teninges, Abstr. 4th Int. Congr. Virol., The Hague, The Netherlands, P23, p. 335, 1978), this virus was included in our studies for comparative purposes.

MATERIALS AND METHODS

Viruses and cell cultures. Plaque-purified IBDV strain Cu-1 (10) was propagated in chicken embryo fibroblasts grown in roller bottles at a multiplicity of infection of 0.01 PFU/cell. Infected cultures were incubated at 37°C until extensive cytopathic effect had developed, usually after 36 to 48 h.

IPNV (ATCC VR 299) was obtained from P. Dobos, University of Guelph (Canada). It was serially passed at a multiplicity of infection of 0.01 to 0.001 in a rainbow trout gonad cell line (RTG-2, obtained from G. Wizigmann, Grub, W. Germany), which was grown in plastic petri dishes. Extensive cytopathic effect was observed after 2 to 3 days of incubation at 20°C.

Reovirus type 3 (Dearing strain) was grown in L cells and purified as described previously (10).

Fecal material containing calf rotavirus was collected from field cases of neonatal calf diarrhea and was provided by E. Weiss, University of Giessen, W. Germany. The virus was purified by using the method as described in detail for the purification of *Mastomys natalensis* papilloma virus (9).

Isotopic labeling of IBDV and IPNV. Isotopic labeling of IBDV or IPNV with [^{3}H]uridine was greatly facilitated by exhausting the uridine pool of the host cell with *d*-glucosamine (13). Growth medium was removed 1 h before infection and substituted by Earle balanced salt solution containing 30 mM *d*-glucosamine. The cells were infected, and after a 1-h adsorption period the inoculum was replaced by Eagle minimal essential medium with Earle salts containing 2% fetal calf serum and 5 μCi of [^{3}H]uridine per ml. More [^{3}H]uridine was added to the medium 2 and 4 h later to increase its total concentration to 15 μCi/ml. For labeling with ^{32}P, infected cells were washed with phosphate-free Eagle minimal essential medium twice at the end of the adsorption perioid and covered with phosphate-free minimal essential medium containing 2% dialyzed fetal calf serum, supplemented with 100 μCi of [^{32}P]orthophosphate (carrier-free) per ml. Again, [^{32}P]orthophosphate (100 μCi/ml) was added 6 and 12 h later.

Virus purification and preparation of virus nucleic acid. IBDV and IPNV were purified as described (4, 8) with minor modifications. When extensive cytopathic effect had developed in infected cells, they were scraped into the medium, centrifuged at 8,000 rpm for 10 min, and suspended in TNE buffer (0.01 M Tris-hydrochloride, pH 7.5; 0.1 M NaCl; 1 mM EDTA). Cells were disrupted by ultrasonic treatment, and virus was extracted twice with freon 113. Virus in the medium was sedimented in a Beckman type 19 rotor or concentrated with an Amicon hollow-fiber system (H1P100). Isotopically labeled virus was pelleted through a 30% (wt/wt) cushion of sucrose in TNE in an SW27 rotor (3.5 h, 26,000 rpm). This was followed by centrifugation to equilibrium in CsCl gradients with a mean density of 1.33 g/ml (SW50, 20 h,

36,000 rpm) and velocity centrifugation in 10 to 40% (wt/wt) gradients of sucrose in TNE (SW41, 1.5 h, 36,000 rpm). The resulting virus bands were layered over stepwise gradients of CsCl in TNE with densities of 1.30 and 1.40 g/ml and centrifuged for 3 h at 36,000 rpm in an SW41 rotor. Finally, the virus particles were pelleted through a cushion as described above (SW41, 1.5 h, 40,000 rpm).

The virus pellets were suspended in phosphate-buffered saline containing 20 μg of RNase A per ml and kept at 20°C for 20 min for the removal of contaminating RNA not encapsidated into virions. Once again the virus preparations were pelleted through sucrose cushions and suspended in buffer (0.01 M Tris-hydrochloride, pH 8.0; 0.01 M NaCl; 0.01 M EDTA) containing 0.5% (wt/wt) sodium dodecyl sulfate (SDS) and 1 mg of proteinase K per ml as described (4). The samples were incubated at 37°C for 1 h. Thereafter they were either applied directly to polyacrylamide gels or layered onto 5 to 20% (wt/wt) sucrose gradients in TNE containing 0.5% SDS and centrifuged at 20°C for 5.5 h at 40,000 rpm in an SW41 rotor. Fractions containing viral RNA as detected by measuring the acid-insoluble radioactivity in a sample (see below) were pooled, mixed with 2 volumes of ethanol containing 0.4 M LiCl, and precipitated at −20°C without carrier. The resulting pellets were collected by centrifugation, washed with ethanol, and suspended in the appropriate buffer for subsequent analysis.

Sucrose gradient analysis of IBDV and IPNV RNA. IBDV or IPNV RNA labeled with [^{3}H]uridine was mixed with [^{14}C]rRNA from BHK-21 cells (provided by G. Wengler, Giessen) and centrifuged in gradients of 5 to 20% (wt/wt) sucrose in TNE (SW41, 2°C, 7.0 h, 40,000 rpm). Fractions of 0.5 ml were collected, and their radioactivity was determined. The sedimentation rate of the viral RNA was calculated from its position relative to the [^{14}C]rRNA marker in the gradient.

Cesium sulfate gradient analysis of IBDV and IPNV RNA. ^{3}H-labeled IBDV ([^{3}H]IBDV) or [^{3}H]-IPNV RNA was mixed with [^{14}C]rRNA and subjected to isopycnic centrifugation in Cs_2SO_4 gradients in TNE as described (8). The gradients were fractionated, and the radioactivity of each fraction was determined. The refractive indexes were measured with an Abbé refractometer, and Cs_2SO_4 buoyant densities were determined as outlined in the *Handbook of Biochemistry* (16).

RNase sensitivity of IBDV and IPNV RNA. Methods to determine sensitivity of IBDV and IPNV RNA to RNase were essentially the same as described previously (14). Briefly, samples of labeled RNA were mixed with 2× SSC (SSC = 0.15 M NaCl–0.01 M sodium citrate, pH 7.4), 1× SSC, 0.1× SSC, or 0.01× SSC in 0.5-cm-diameter test tubes to give a total volume of 0.1 ml. The sealed tubes were immersed in a water bath of 80°C, slowly cooled to 65°C, and kept at this temperature overnight. For RNA digestion 50 μg of RNase A per ml in the appropriate dilution of SSC was added, and the reaction mixture was kept at 20°C for 20 min. After addition of carrier protein the samples were precipitated and washed twice with 6% (wt/vol) trichloroacetic acid. The precipitates were dissolved in 1 ml of 0.2 M NaOH each, heated for 15 min in a boiling-water bath, transferred with 1 ml of distilled water to counting vessels, and mixed with 10 ml of Mix 76 (12). The samples were counted for 5 min in a Packard Tri-Carb liquid scintillation counter (^{3}H-labeled samples with an external standard).

Melting behavior of IBDV and IPNV RNA. Melting points were measured by determining the temperature at which 50% of the RNA was digested by RNase A. Samples of RNA in 2× SSC, which had been kept at 65°C overnight as described above, were diluted 1:1 with 2% formaldehyde in water and equilibrated for 8 min at various temperatures. Formaldehyde was applied to avoid reannealing after melting (1, 2). One sample, which had been diluted only with water, was heated to 110°C in hot glycerol and chilled rapidly to −30°C. All samples were mixed in an ice bath with the same volume of 4× SSC containing 1 mg of RNase A per ml and were incubated for 20 min at 20°C. After addition of carrier protein the samples were precipitated and processed as described above for the determination of radioactivity that had been rendered acid soluble.

Nucleotide composition of IBDV RNA. An ethanol-precipitated pellet of ^{32}P-labeled IBDV RNA was suspended in 10 μl of 0.3 N KOH, sealed in a capillary tube, and incubated for 20 h at 37°C. The sample was mixed with an equal volume of 0.3 N HCl and spotted onto a Whatman 3MM paper strip. Electrophoresis was carried out at 1,000 V for 3 h in a buffer containing 0.5% pyridine, 5% acetic acid, and 0.5 mM EDTA (pH 3.5). The paper strip was autoradiographed using Ilford X-ray rilm Rapid S, and the spots were cut and counted in Mix 76 (12).

Polyacrylamide gel electrophoresis of viral RNA. The discontinuous SDS-gel system as described by Laemmli (6) was used. The gels were formed as slabs (0.2 by 10 by 12 cm) between glass plates according to the description of Studier (17) or in glass tubes with an internal diameter of 0.6 cm. Samples contained at least 0.50 μg of RNA in 40 to 50 μl, as determined by the optical density at 260 nm. A 5-μl volume of a 75% (wt/vol) sucrose–5% (wt/vol) bromophenol blue tracking dye solution was added to each sample. Electrophoresis was carried out for 20 h at room temperature at a constant voltage of 50 V. The electrophoresis buffer was recirculated using a peristaltic pump and an overflow. Slabs were stained with ethidium bromide (1.0 μg/ml in 0.01 M Tris, pH 8.0) for 30 min and visualized with a short-wave UV transilluminator. Photographs were taken with a Polaroid Land camera. Cylindrical gels containing ^{32}P-labeled RNA were frozen and sliced into 0.1-cm sections, and their radioactivity was determined by Cerenkov counting.

RESULTS

Sedimentation behavior of IBDV RNA. The nucleic acid extracted from purified [^{3}H]-uridine-labeled IBDV by SDS and proteinase K formed one compact peak when analyzed by velocity centrifugation in sucrose gradients (Fig. 1). The sedimentation coefficient was 14S when the 28S, 18S, and 5S rRNA's from BHK-21 cells centrifuged in the same gradient were used as

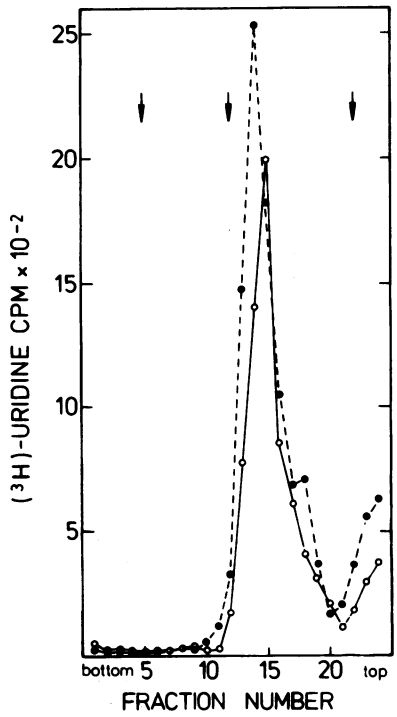

FIG. 1. *Sucrose gradient analysis of IBDV RNA (○——○) and IPNV RNA (●——●). ³H-labeled viral RNA was mixed with [¹⁴C]rRNA from BHK-21 cells, and the samples were layered on top of 5 to 20% (△/△) sucrose gradients in TNE buffer. Centrifugation was for 7 h at 40,000 rpm in an SW41 rotor at 2°C. The positions of the 28S, 18S, and 5S rRNA species are indicated by arrows.*

standards. [³H]uridine-labeled IPNV RNA sedimented faster in the same run, reaching a position between the 14S IBDV RNA and the fast-sedimenting 15S (19) L species of reovirus type 3 RNA (data not shown). Only a little radioactivity remained on top of the gradients, moving more slowly than 5S rRNA. Using the Doty equation (5) which relates the sedimentation velocity of DNA to its molecular weight, the molecular weight of IBDV RNA would be 2.2 × 10^6 and that of IPNV RNA somewhat higher.

Density gradient centrifugation in Cs_2SO_4 gradients. When purified IBD viral RNA was centrifuged to equilibrium in a Cs_2SO_4 gradient, most of the RNA banded as a sharp peak at a density of 1.62 g/ml (Fig. 2) and IPN viral RNA banded at a density of 1.61 g/ml, as did reovirus type 3 RNA (data not shown). [¹⁴C]rRNA from BHK-21 cells centrifuged in the same gradients banded at a density of 1.67 g/ml, as expected for single-stranded RNAs (18).

Sensitivity of IBDV RNA to RNase A. At least 95% of ³²P-labeled IBDV RNA released from purified virions was found to be resistant to hydrolysis by RNase A as judged by trichloroacetic acid precipitation (Table 1). The amount of RNA rendered acid soluble after enzyme treatment was markedly increased at salt concentrations lower than 0.1× SSC. A total of

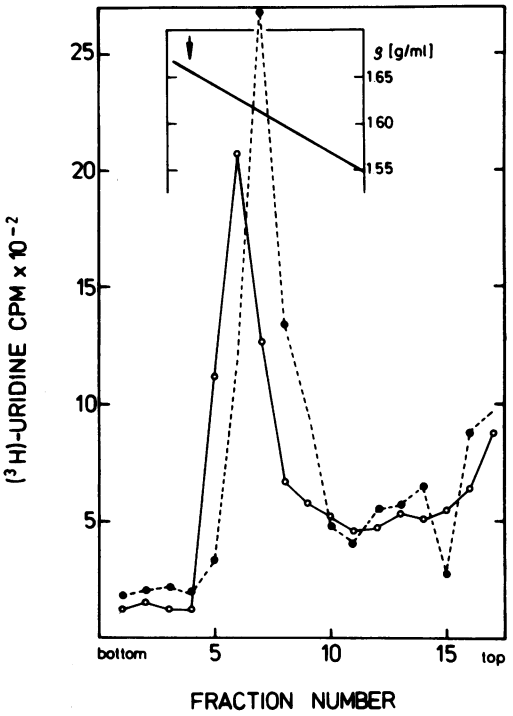

FIG. 2. *Cs_2SO_4 gradient analysis of IBDV RNA (○——○) and IPNV RNA (●- - -●). ³H-labeled viral RNA was mixed with [¹⁴C]rRNA from BHK-21 cells, and the samples were layered on top of Cs_2SO_4 gradients. Centrifugation was for 48 h at 36,000 rpm in an SW50 rotor at 12°C. The position of the rRNA is indicated by an arrow.*

TABLE 1. *Susceptibility of IBDV RNA and IPNV RNA to treatment with RNase A*[a]

SSC concn	RNase A (μg/ml)	Percent acid-insoluble radioactivity after treatment of:	
		[³²P]IBDV RNA	[³H]IPNV RNA
2×	—	100	100
2×	50	95	86
1×	50	89	81
0.1×	50	5	3
0.01×	50	1	1

[a] Samples without RNase A treatment contained the following amounts of acid-insoluble radioactivity (equal to 100%): [³²P]IBDV RNA, 4,267 cpm; [³H]-uridine-labeled IPNV RNA, 4,371 cpm. Incubation was for 20 min at 20°C.

86% of [^{3}H]IPNV RNA resisted RNase A treatment under identical conditions. Changes of the molarity of salts in the reaction mixture had the same consequences as for IBDV RNA.

Melting behavior. The melting behavior of ^{32}P-labeled IBDV RNA in 1× SSC and in the presence of 1% formaldehyde is shown in Fig. 3. The RNA exhibited a sharp melting profile with a T_m of 95.5°C. For IPNV RNA a T_m of 95.0°C was determined (data not shown). Samples of either RNA in 1× SSC without formaldehyde, heated to 110°C and rapidly quenched at −30°C, became fully sensitive to treatment with RNase A. About 92% of both types of nucleic acid was rendered acid soluble by the enzymes.

Nucleotide composition of IBDV RNA. 14S IBDV RNA labeled with [^{32}P]orthophosphate was used to determine the nucleotide composition. The relative amounts of radioactivity obtained for the individual nucleotide species were 27.8% for C, 23.4% for A, 27.5% for G, and 21.3% for U, from a total of 33,929 cpm equal to 100%. The results show that IBDV RNA had pyrimidine-to-purine ratios near unity with a guanine plus cytosine content of 55.3%. The guanine plus cytosine content of IPNV RNA has been determined to be about 54% (3, 8).

Electrophoresis of viral RNA. Purified ^{32}P-labeled IBDV RNA was analyzed by electrophoresis on 7.5% cylindrical polyacrylamide gels. The RNA separated into two distinct bands after electrophoresis for 20 h (Fig. 4). When electrophoresis lasted only 12 h, no radioactivity was found in the gel below these two bands,

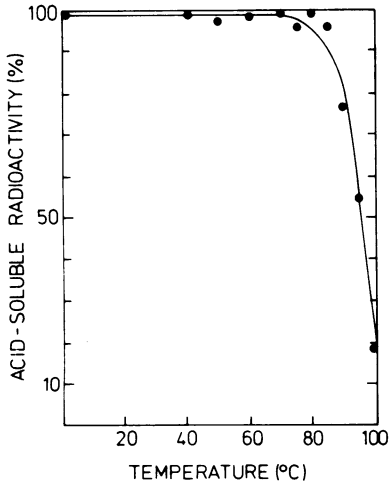

FIG. 3. *Melting behavior of IBDV RNA. The melting point was determined by measuring the temperature at which 50% of [^{32}P]IBDV RNA in 1× SSC became sensitive to RNase A (50 μg/ml, 20 min, 20°C). Radioactivity: 4,800 cpm equals 100%.*

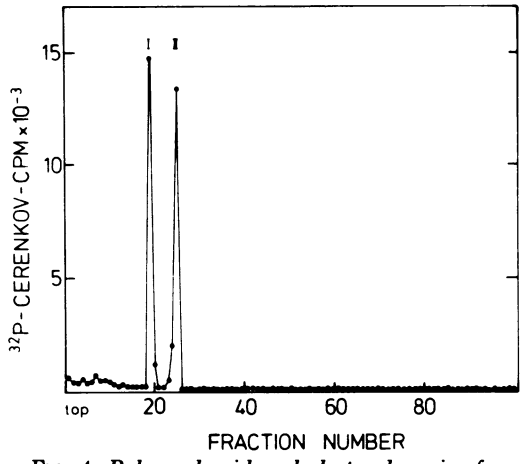

FIG. 4. *Polyacrylamide gel electrophoresis of purified (14S) [^{32}P]IBDV RNA on a cylindrical 7.5% polyacrylamide gel according to the method of Laemmli (6). Electrophoresis was performed at 20°C for 20 h at a constant voltage of 50 V. The gel was frozen and cut in slices, and the radioactivity of each slice was determined by Cerenkov counting.*

indicating that there are no low-molecular-weight oligonucleotides associated with IBDV RNA (data not shown). The relative amount of radioactivity in the two bands was determined by excising the two regions from the gel and extracting them with phenol saturated with 2× SSC after homogenization with a Teflon-coated Dounce homogenizer. The total radioactivity in the component migrating more slowly (component I) was slightly higher than that in the faster band (component II), and its resistance to treatment with RNase A was 99% as compared to 98% for component II. To estimate the molecular weights of the two components, unlabeled RNA was released from virus particles by treatment with SDS and proteinase K and directly applied to a 7.5% polyacrylamide slab gel. Incubation of IBDV in 2 M urea, 1% SDS, and 0.1% 2-mercaptoethanol for 60 min at 60°C, the method commonly used for reovirus RNA (10), proved to give inconsistent results. In a number of previous experiments the amount of IBDV RNA released by SDS and urea was so low that RNA bands were barely visible after gel staining. When the two RNA bands could be identified in a gel containing 6 M urea, their migration proved to be retarded compared to the position of the RNA bands of reovirus. After digestion of IBDV with proteinase K in the presence of SDS, however, reproducible gel patterns were obtained. Furthermore, proteinase K inactivated any RNase that had been added to the sample for the final removal of extraviral RNA. Molecular weights of 2.5 × 10^6 and 2.2 × 10^6 were deter-

mined for the two segments of IBDV RNA from the gel patterns shown in Fig. 5. The 10 segments of reovirus type 3 and the 11 bands of a calf rotavirus were used as standards. Values of 2.45 $\times 10^6$ and 2.2 $\times 10^6$ daltons were obtained for the segments of IPNV RNA.

DISCUSSION

Based on the three criteria of RNase resistance, melting behavior, and nucleotide composition, it could be firmly established that IBDV RNA is double stranded.

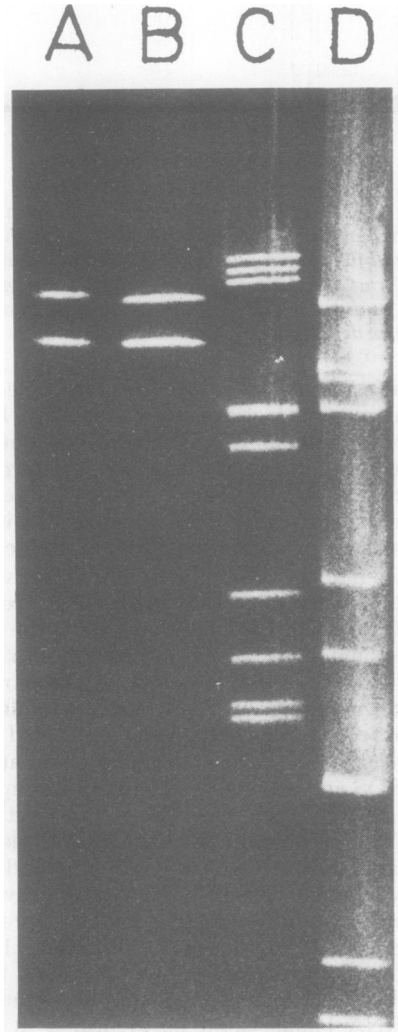

FIG. 5. *Polyacrylamide gel electrophoresis of IBDV RNA (A), IPNV RNA (B), reovirus type 3 RNA (C), and calf rotavirus RNA (D). The RNA was released from purified virions by SDS-proteinase K treatment and applied directly to a 7.5% polyacrylamide gel prepared as described by Laemmli (6). Electrophoresis was performed at 20°C for 20 h at a constant voltage of 50 V. Bands were stained with ethidium bromide and visualized under UV light.*

Since a high proportion of the radioactive label had appeared in cellular material, particular care was taken to exclude RNase-sensitive cellular contaminants as a source of error by repeated steps of gradient centrifugation and pretreatment of the virus with RNase A to remove labeled RNA not encapsidated into virions.

After it had been observed repeatedly that the experimental conditions commonly used for the analysis of reovirus RNA (10, 11) in polyacrylamide gels released IBDV RNA inefficiently from the virion and gave inconsistent results, the size of the two RNA segments was reexamined with a modified gel system. Urea was omitted from the gels, and proteinase K combined with SDS was employed to release the RNA from the virus particles. It could be confirmed that IBDV RNA consists of two segments, the molecular weights of which were determined to be 2.2 $\times 10^6$ and 2.5 $\times 10^6$ in polyacrylamide gels without urea. The migration rates of the two segments in relation to the reovirus standard were much faster in gels without urea. The buffer systems used for gel electrophoresis were without significance, since the results did not differ when the buffers described by Loening (7) or by Laemmli (6) were employed. The decisive factor for the slower migration of IBDV RNA must therefore be the denaturing effect of urea, which influenced the migration rate of both segments of IBDV RNA to a greater extent than the segments of reovirus RNA; in particular, the position of the L species was only insignificantly altered in the urea-containing gel. Regions with less firmly established hydrogen bonds in A/U-rich regions of IBDV RNA might be the reason for this behavior. The values obtained under the least denaturing conditions should be closest to the true molecular weights and are almost identical with the 2.2 $\times 10^6$ and 2.4 $\times 10^6$ determined for IPNV RNA. Double-strandedness must hold true for both segments because the material extracted from either one of the two bands proved to be fully resistant to RNase treatment.

The sensitivity of IBDV RNA to treatment with RNase A was drastically increased beyond a temperature of about 90°C. This fact again underlines its double-stranded nature and enabled us to determine a T_m of 95.5°C. A T_m of 95.0°C, which we determined for IPNV RNA, is almost identical. The differences from the T_m of 89°C published for IPNV RNA (3) are probably due to different experimental conditions.

Finally, the almost equimolar ratios of purines to pyrimidines support again the above conclu-

sions that IBDV RNA is double stranded and that it appears improbable that major amounts of single-stranded RNA are encapsidated in the IBDV virion. One can conclude, therefore, that the genome of IBDV consists of two segments of double-stranded RNA. In view of the structural characteristics of the genome and the capsid (11), it seems justified to place this virus with IPNV and some other virus strains into a separate taxonomic group, as recently proposed (Dobos et al., Abstr. 4th Int. Congr. Virol., P23, p. 335, 1978).

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