

# Characterization of Bluetongue Virus Ribonucleic Acid

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An improved purification procedure yielded bluetongue virus free from any single-stranded ribonucleic acid (RNA) component. Double-stranded RNA obtained from purified virus or isolated from infected cells was fractionated into 5 components by means of sucrose gradient sedimentation analysis, and into 10 components by electrophoresis on polyacrylamide gels. The size of these components vary from  $0.5 \times 10^6$  to  $2.8 \times 10^6$  daltons, with a total molecular weight estimate of about  $1.5 \times 10^7$  for the viral nucleic acid. The denaturation of the genome and separation of the resulting fragments are also discussed.

A striking similarity has been found between the nucleic acid moieties of bluetongue virus (BTV) and reovirus, although the virions differ in size and morphology. Both genomes consist of double-stranded ribonucleic acid (RNA) which has been found, after isolation, to consist of a number of segments of reproducible size. In the case of reovirus, the presence of 10 double-stranded segments was demonstrated in fractionation experiments utilizing polyacrylamide gel electrophoresis (12, 20), whereas electron microscopy studies using the Kleinschmidt technique indicated 9 to 11 fragments (15). In addition, a single-stranded, adenine-rich component has been found (1, 11).

Previous studies on BTV-RNA indicated the presence of at least three size groups of segments, by the use of sucrose gradient sedimentation for fractionation (16).

In the experiments reported here, the presence or absence of a single-stranded RNA component in BTV was investigated, and the double-stranded viral RNA was subjected to further purification and characterization.

## MATERIALS AND METHODS

**Buffers.** The STE buffer consisted of 0.1 M NaCl, 0.005 M tris(hydroxymethyl)aminomethane (Tris), and 0.001 M ethylenediaminetetraacetic acid (EDTA), pH 7.4; the SSC buffer consisted of 0.15 M NaCl and 0.015 M sodium citrate, pH 7.2.

**Virus.** BTV serotype 10 has been used throughout this work. Methods for the production of virus in BHK-21 cell cultures and for plaque titrations in L-cells have been described (4).

Reovirus type 1 was obtained from N. F. Stanley, Melbourne, Australia, grown in BHK-21 cells, and

purified according to the method of Bellamy et al. (1).

**Purification of BTV.** The following modifications have been introduced into the procedure described previously (16). After extraction with Tween 80 and ether, sucrose was added to the aqueous phase containing the virus to a final concentration of about 4%, and the salt concentration was adjusted to 0.1 M. It was then layered over 2 ml of a 40% sucrose solution in STE, and the virus was pelleted through this layer for 2 hr at  $102,000 \times g$ . The pellets were dissolved in a small volume of 0.002 M Tris-hydrochloride at pH 8.0 and finally purified by centrifugation through a 10 to 30% sucrose gradient in the same buffer at  $78,000 \times g$  for 70 min. These modifications are based on the observation that purified BTV form aggregates in 0.1 M buffer, with disaggregation at a salt concentration of 0.002 M. Gradients were fractionated in a model D fractionator (Instrumentation Specialties Co., Inc., Lincoln, Neb.) and assayed for virus activity by means of plaque titrations; the fractions containing virus were pooled for phenol extraction or for pelleting of the virus if required.

**Phenol extraction.** The procedure described by Scherrer and Darnell (10) was followed for the isolation of RNA from infected cells. In the case of purified virus, the pooled fractions were dialyzed against STE buffer, and sodium dodecyl sulfate was added to a concentration of 1%. Deproteinization with phenol was carried out at pH 7.4 at room temperature.

**Isolation of double-stranded BTV-RNA from infected cells.** Cells were harvested about 20 hr after infection and were collected by low-speed centrifugation. A cytoplasmic extract was prepared according to the method of Bellamy et al. (1), and the extract was deproteinized with phenol. After precipitation of the total RNA with two volumes of cold ethyl alcohol, transfer and double-stranded RNA were selectively dissolved in 1 M NaCl. After dialysis against STE, the double-stranded RNA was finally purified by

means of chromatography on a methylated albumin-kieselguhr (MAK) column (18). The double-stranded RNA peak, eluted at 0.7 M in the NaCl gradient, was pooled, precipitated overnight in two volumes of cold ethyl alcohol, and dissolved in  $0.1 \times$  SSC.

**MAK column chromatography.** MAK columns were prepared according to the simplified method described by Osawa and Sibatani (9). Elution was carried out with a linear gradient of 0.2 to 1.2 M NaCl buffered to pH 6.7 with Tris-hydrochloride.

**Density gradient sedimentation analysis.** Purified virus was disrupted by the addition of 0.5 M urea and 0.1 M sodium acetate, and its RNA was analyzed in sucrose density gradients containing 0.1% sodium dodecyl sulfate (SDS) by the method of Bellamy et al. (1).

Analysis of isolated RNA was carried out in gradients prepared from ribonuclease-free sucrose in STE buffer containing 0.025 M EDTA. Centrifugation was routinely carried out for 5 hr at  $200,000 \times g$  in a Spinco SW50 rotor. Gradients were fractionated in an Isco model D gradient fractionator.

**Polyacrylamide gel electrophoresis.** Electrophoresis on polyacrylamide gels was carried out by the method of Loening (7), with the modification that 0.1% SDS was added to the buffer and ethylene diacrylate (0.2%), was used as cross-linking agent. Gels (3%), prepared in SDS-containing Loening's buffer, were used routinely, and separation was normally for 4 hr, at 100 v in 10-cm gels at room temperature. The same buffer was used in the electrode chambers. To resolve the high-molecular-weight components, the gel concentration was increased to 3.6% and the time was extended to 7 hr. Gels were cut into 1- or 2-mm slices as required, dissolved in 0.2 ml of 1 M piperidine, and counted in Kinard's (6) scintillator solution.

**Thermal denaturation.** Melting curves were determined by heating the samples in the heating attachment of a DK-2A spectrophotometer (Beckman Instruments Co., Inc., Fullerton, Calif.) in glass-stoppered cuvettes.

**Radioactive precursors.** The  $^{14}\text{C}$ -uridine,  $^3\text{H}$ -uridine and  $^{32}\text{P}$ , as orthophosphate, were obtained from the Radiochemical Centre, Amersham, England.

**Radioactivity assays.** All determinations of radioactivity were made by scintillation counting in a Packard Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). Aqueous samples were counted in Bray's scintillator (2) and gel slices in Kinard's solution (6). The  $^{32}\text{P}$ -containing samples were counted without scintillator, utilizing Cerenkov radiation.

## RESULTS

**Purity of the isolated virus.** Earlier studies provided evidence for a single-stranded RNA component present in varying amounts in the double-stranded RNA preparations isolated from purified BTV (16). The similarity of the BTV genome to that of reovirus naturally led to the speculation that this component might be equivalent to the single-stranded, adenine-rich, 2S material reported in reovirus (1, 11). The irregularity of

the amount present in various preparations, however, indicated the possibility of this component being a contaminant. Subsequent modification of the purification procedure (see Materials and Methods) resulted in many preparations containing no single-stranded RNA, thus strengthening this view. The possibility still existed, however, that such a low-molecular-weight fragment could have been lost during the isolation of viral RNA. The fact that  $^3\text{H}$ -uridine was normally used for labeling viral RNA might have been a contributory factor in view of its low concentration in the adenine-rich component. To resolve this question, it was decided to label BTV with  $^{32}\text{P}$  and to liberate the RNA from purified virus directly onto sucrose gradients.

Although the modified purification procedure resulted in better yields of virus with a higher degree of purity, two virus bands were still commonly encountered in the sucrose gradients constituting the final purification step. After  $^{32}\text{P}$ -labeling and purification, the two bands were collected separately, the virus was solubilized by adding urea and sodium acetate, and the RNA was fractionated by centrifugation in 15 to 30% sucrose gradients containing 0.1% SDS (1). Results are shown in Fig. 1. Band B, the faster-sedimenting band always present, contained only high-molecular-weight material equivalent to the 8 to 16S, double-stranded components previously demonstrated. Band A, which was absent in some experiments and present in vari-

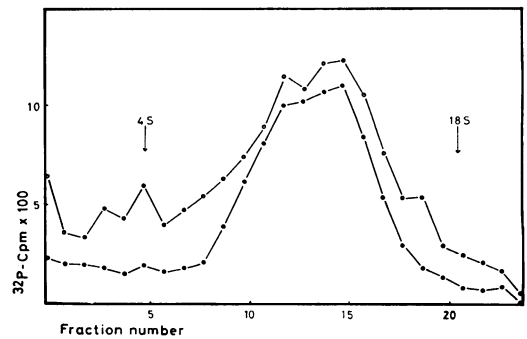


FIG. 1. Sucrose density gradient sedimentation analysis of fractions A and B obtained during purification of  $^{32}\text{P}$ -labeled BTV. Virus was solubilized with urea and acetate and layered directly onto 15 to 30% ribonuclease-free sucrose gradients in STE buffer. Gradients were centrifuged for 5 hr at  $200,000 \times g$  and fractionated as described; the total number of counts in each fraction was determined. Sedimentation is from left to right. The positions of  $^{14}\text{C}$ -uridine-labeled ribosomal RNA from BHK-21 cells, added as markers, are indicated by arrows. Symbols:  $\circ$ , fraction A;  $\bullet$ , fraction B.

able amounts in others, contained, in addition, some ribonuclease-sensitive material in the 2 to 4S region. This material was not available in sufficient quantities to allow a base analysis.

Various tests were performed on the two fractions to exclude the possibility that the second band represented contamination with another related virus or serotype of BTV and to see whether any characteristic difference could be found which would explain the presence of two bands.

Negatively stained preparations examined in an electron microscope did not reveal any morphological difference. Both bands contained similar numbers of empty and full particles.

Antisera were produced against both A and B virus in rabbits, and complement fixation as well as cross-neutralization tests were performed as previously described (3). The same single BTV type 10-specific antibody was found in the antiserum, and no neutralization of other serotypes could be demonstrated (P. G. Howell, *personal communication*).

RNA isolated from both fractions was fractionated on a MAK column and by polyacrylamide gel electrophoresis. No difference between A and B could be detected by either method. Both preparations eluted from the MAK column at a salt concentration of 0.68 to 0.7 M, and both yielded the standard electrophoretic pattern for double-stranded RNA shown in Fig. 3. Other data collected during this investigation are summarized in Table 1.

It is obvious that the main differences between the two bands are the variation in buoyant density, the considerably higher specific infectivity of the faster-sedimenting B band, and a large

difference in sedimentation velocity. The differences in density and specific infectivity are compatible with the evidence that the A band contains some additional low-molecular-weight RNA, as revealed by density gradient sedimentation analysis. It is more difficult to explain the difference in sedimentation value. One can only speculate that the RNA contaminant might be "tailing" from the spherical virus particles, causing a considerable increase of the frictional coefficient.

In the absence of any evidence to the contrary, it is concluded that band B represents purified virus free from the single-stranded, 2 to 4S RNA component found in previous preparations, which represents a contamination and forms no essential part of the BTV genome.

**Fractionation of double-stranded RNA by sedimentation in sucrose gradients.** The fractionation of the BTV genome into fragments of three size groups by means of sucrose gradient sedimentation analysis has been described (16). The resolution obtained, however, was never as good as in the case of the reovirus genome (12, 20). In an attempt to improve the resolution by extended centrifugation and by collection of smaller fractions, it was found that the loss of resolution was caused by the presence of more than three peaks. These peaks could be partly resolved by using the improved technique mentioned. The fractionation pattern obtained is illustrated in Fig. 2. The pattern obtained with reovirus RNA is included for

TABLE 1. Comparative data obtained for bands A and B found during purification of BTV by zonal centrifugation in sucrose gradients

Characteristic	Band A	Band B
Approximate sedimentation constant <sup>a</sup> .....	520 S	750 S
Buoyant density <sup>b</sup> .....	1.39	1.38
Specific radioactivity <sup>c</sup> .....	7,620	8,500
Specific infectivity <sup>d</sup> .....	2.1 × 10 <sup>9</sup>	4.0 × 10 <sup>10</sup>

<sup>a</sup> Calculated according to the method of McEwen (8).

<sup>b</sup> Determined by means of equilibrium centrifugation in CsCl density gradients.

<sup>c</sup> Expressed as counts per minute per absorbancy unit at 260 nm.

<sup>d</sup> Expressed as plaque-forming units per milliliter per absorbancy unit at 260 nm, determined by the plaque assay previously described (16).

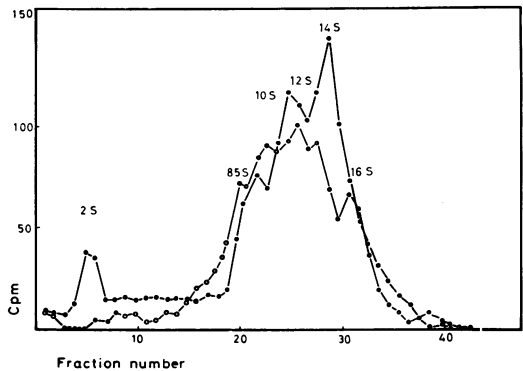


FIG. 2. Fractionation pattern of BTV-RNA on sucrose gradients compared to that obtained for reovirus RNA. Both viruses were labeled with <sup>14</sup>C-uridine (specific activity, 510 mc/mm) at a final concentration of 0.01 μc/ml. BTV was purified as described. Reovirus was purified by the method of Bellamy et al. (1). Both virus preparations were solubilized with urea and acetate, and the liberated RNA was fractionated in sucrose gradients as described in the legend to Fig. 1. Sedimentation values were calculated from the relative positions of cellular RNA markers indicated in Fig. 1. Symbols: ○, BTV; ●, reovirus.

comparison. Five peaks are resolved with approximate sedimentation constants of 8.5, 10, 12, 14, and 15.5S. Sedimentation values are based on the sedimentation rates of cellular transfer RNA and 18S ribosomal RNA, prepared from BHK-21 cells and added as markers. Calculated on the basis of the relationship  $S = 0.0882 \times \text{molecular weight}^{0.346}$ , deduced by Studier for double-stranded DNA (14), these sedimentation constants correspond to values of  $0.5 \times 10^6$ ,  $0.8 \times 10^6$ ,  $1.5 \times 10^6$ ,  $2.3 \times 10^6$ , and  $3.0 \times 10^6$  daltons, respectively, for the molecular weights of the fragments.

**Polyacrylamide gel electrophoresis of the BTV genome.** Tritium-labeled, double-stranded RNA isolated from purified BTV was resolved into 10 components by means of electrophoresis in 3.6% polyacrylamide gels. These components, numbered 1 to 10, are shown in Fig. 3. The fractionation pattern is similar, but not identical, to that obtained for reovirus (12). The 10 peaks are not as clearly grouped into three size categories as in the case of reovirus, mainly because the largest and smallest components, possessing the lowest and highest rates of migration, respectively, are well separated from their neighbors. The general impression given by the pattern is a grouping into five categories rather than three. This would correlate well with the separation of BTV-RNA into five components in sucrose gradients.

To obtain a more detailed comparison of the migration rates of the components derived from reovirus and BTV, tritium-labeled RNA derived from both were fractionated under strictly identical

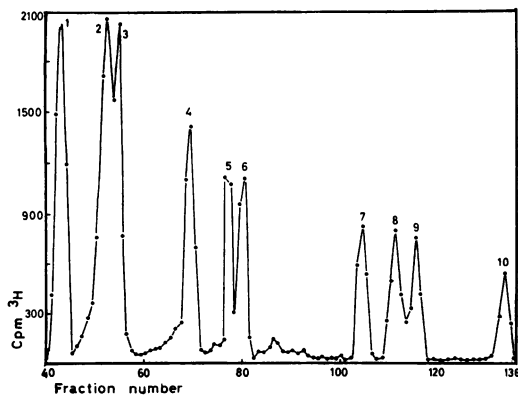


FIG. 3. Fractionation of double-stranded RNA derived from tritium-labeled BTV by means of electrophoresis in 3.6% polyacrylamide gels containing 0.1% SDS. Virus was labeled with  $^3\text{H}$ -uridine (specific activity, 1.5 c/mm) at a final concentration of 1.0  $\mu\text{g/ml}$ . Migration is from left to right. Electrophoresis was carried out for 7 hr at 25 C at a voltage gradient of 10v/cm. Gels were cut into 1-mm slices.

conditions. Electrophoresis was carried out in 3% gels, 10 cm long, for 4 hr at 10 v/cm. Under these conditions, reovirus RNA is separated into five components only, the first two peaks corresponding to the unresolved large and medium size groups, the small group being resolved into three components. The BTV-RNA pattern, in contrast, is resolved into nine components which are clearly discernible in Fig. 4.

The double-stranded RNA from reovirus has been reasonably well characterized, and the molecular weights of the main components have been determined by several authors. Therefore, the average molecular weights (1, 12, 19) of the five peaks resolved in Fig. 4A ( $2.5 \times 10^6$ ,  $1.4 \times 10^6$ ,  $0.92 \times 10^6$ ,  $0.76 \times 10^6$ , and  $0.63 \times 10^6$  daltons, respectively) were plotted against the distances they migrated to obtain a reference curve. A linear relationship was obtained (Fig. 5).

The molecular weights of the BTV-RNA components in Fig. 4B were then determined from

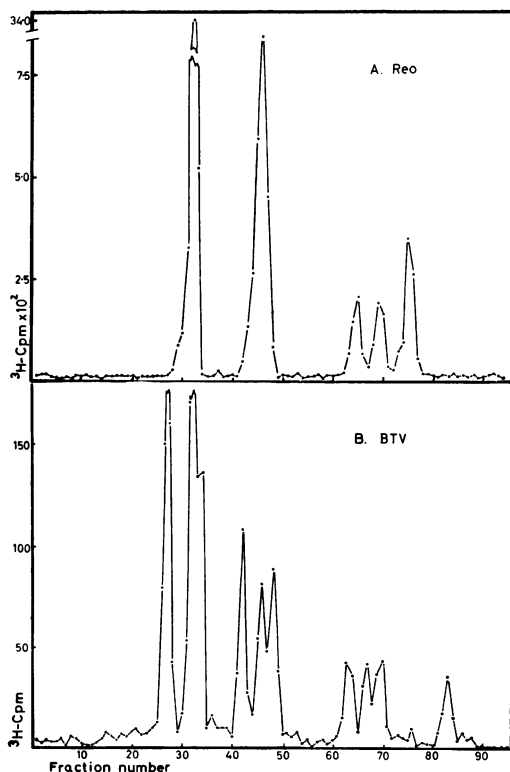


FIG. 4. Comparison of the electrophoretic patterns of (A) reovirus RNA and (B) BTV-RNA. Purified tritium-labeled virus was solubilized with urea and acetate and layered directly onto 3% gels containing SDS. Time for separation was 5 hr; otherwise, conditions were the same as for Fig. 3.

this plot on the basis of their relative electrophoretic mobilities. The values thus obtained for peaks 1 to 10 are shown in Table 2. As a further test for the validity of these figures, an analysis was made of the distribution of radioactivity among the peaks in Fig. 3. The total counts per minute for all peaks was divided by the total estimated molecular weight of  $1.5 \times 10^7$ , and the resultant figure was multiplied by the estimated molecular weight of each peak to obtain the estimated counts per minute for that peak. These values are compared with the observed counts per minute in Table 2. The good agreement between estimated and observed counts suggests that the molecular weight figures are reasonably accurate.

**Melting behavior.** Identical thermal denaturation curves were obtained with the double-stranded RNA isolated from highly purified BTV and with that isolated from infected cells. The first denaturation step observed in previous prepa-

rations (16) was no longer present. As expected,  $T_m$  values were dependent on the ionic strength and pH of the solution (Table 3). An interesting observation was the effect of Tris on the melting point of the RNA. At all concentrations, the sample in the Tris buffer had a  $T_m$  value about 4 C higher than that of an equivalent sample in SSC. As all buffers were prepared with deionized water, this phenomenon cannot be explained in terms of divalent ions forming complexes with the citrate-containing SSC. The absolute  $T_m$  values varied within a range of about 2 C in different experiments. To ensure comparability of the data in Table 3, one RNA preparation was used, after dilution in the various buffers, for all determinations. The  $T_m$  values in SSC are fairly similar to those reported for reovirus RNA at different ionic strengths (1). The hyperchromic increase after melting also showed some variation reflecting the degree of purity of the preparation, but was usually about 30 to 36%.

Rapid cooling in an ice bath after denaturation invariably led to about 50% renaturation, as judged by the decrease in absorption (Table 3). A second cycle of thermal denaturation resulted in reversible melting at a much lower temperature of about 40 to 60 C, indicating that this decrease in absorption on cooling represents the folding of the single strands and is not true renaturation (Fig. 6).

**Denaturation with chemical agents.** Attempts to fractionate the single-stranded components, obtained by thermal denaturation, by means of sucrose density gradient sedimentation or poly-

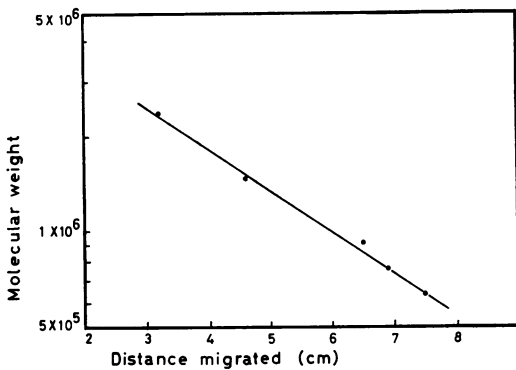


FIG. 5. Relationship between molecular weight of reovirus type 1 components and their electrophoretic mobility in 3% gels.

TABLE 2. Molecular weight of, and distribution of radioactivity in, the electrophoretic components of BTV-RNA in Fig. 4B

Peak	Distance migrated (mm)	Estimated molecular weight	Estimated counts/min <sup>a</sup>	Observed counts/min
1	27.0	$2.8 \times 10^6$	3,320	3,760
2	32.0	$2.4 \times 10^6$		
3	33.5	$2.2 \times 10^6$	5,460	5,490
4	42.0	$1.7 \times 10^6$		
5	46.0	$1.5 \times 10^6$	2,020	1,920
6	48.0	$1.4 \times 10^6$	1,780	1,700
7	63.5	$0.90 \times 10^6$	1,660	1,510
8	67.0	$0.80 \times 10^6$	1,060	1,040
9	69.5	$0.74 \times 10^6$	950	910
10	83.0	$0.49 \times 10^6$	880	860
			580	620

<sup>a</sup> For calculation, see text.

TABLE 3. Thermal denaturation data for BTV-RNA in various buffers of different ionic strength and pH values

Buffer	Concn	pH	$T_m$	Per cent hyperchromicity	
				After melting	After cooling in ice
SSC.....	0.165	7.4	95.0	33.0	17.0
Tris/hydrochloride.....	0.2	7.4	99.0	34.0	18.0
0.1 × SSC.....	0.0165	7.4	80.0	30.0	16.0
Tris/hydrochloride.....	0.02	7.4	83.5	36.0	19.0
0.01 × SSC.....	0.00165	7.4	74.0	34.0	18.0
0.01 × SSC <sup>a</sup> .....		7.4	58.0	46.0	45.0
Tris/hydrochloride.....	0.002	7.2	78.5	34.0	18.0
Tris/hydrochloride.....	0.002	9.0	75.0	38.0	23.0

<sup>a</sup> Plus 8% formaldehyde.

acrylamide gel electrophoresis invariably failed due to partial degradation of the single strands. Various chemical agents have been employed by other workers to reduce the temperature of denaturation, such as formamide, formaldehyde, and dimethyl sulfoxide (DMSO). DMSO has been used extensively for the denaturation of reovirus RNA (1, 5, 19). Under identical conditions, however, denaturation of both BTV-RNA and reovirus RNA was usually incomplete in our hands, and partial renaturation was encountered after precipitation of the denatured material from the DMSO. Therefore, no satisfactory fractional tion pattern could be obtained after DMSO treatment.

Formaldehyde at a concentration of 8% induced complete denaturation at a temperature of 60 C and an ionic strength of 0.002 M. No degradation and no folding of the single strands were encountered after formaldehyde treatment. Fractionation of the denatured material by sucrose gradient sedimentation yielded the pattern shown in Fig. 7, in which it is compared with that of normal double-stranded RNA. Approximate *S* values for the single-stranded RNA fragments are 11.0, 13.0, 16.5, 19.5, and 22.5, corresponding to molecular weights of  $0.24 \times 10^6$ ,  $0.34 \times 10^6$ ,  $0.56 \times 10^6$ ,  $0.8 \times 10^6$ , and  $1.1 \times 10^6$ , respectively, according to the empirical relation molecular weight =  $1,150 \times S^{2.1}$  derived by Spirin (13). These values are all slightly less than half of the equivalent molecular weights derived for the double-stranded fragments. This discrepancy is probably caused by the absence of secondary structure in the denatured strands due to the formaldehyde treatment, which would tend to lower the sedimentation rates. No separation of formaldehyde-

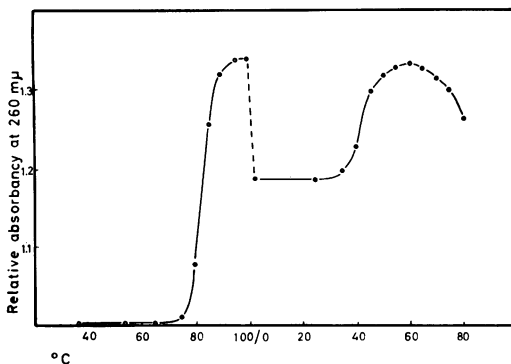


FIG. 6. Thermal denaturation profile of BTV-RNA. The RNA sample, in 0.02 M Tris-hydrochloride buffer at pH 7.2, was heated to 98 C, cooled quickly by immersion in a salt-ice bath, heated again to 60 C, and allowed to cool down, recording the absorption at 260 nm at regular intervals.

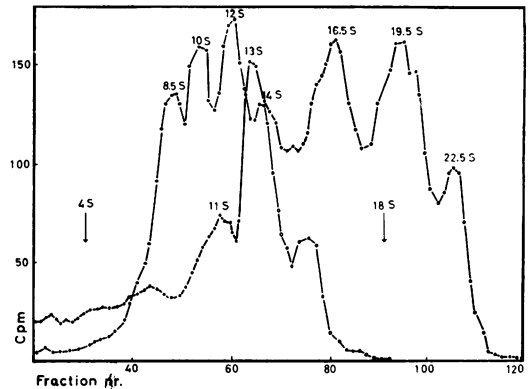


FIG. 7. Sucrose gradient sedimentation analysis of native and formaldehyde denatured BTV-RNA. Samples were centrifuged at  $200,000 \times g$  for 5 hr in 15 to 30% gradients of ribonuclease-free sucrose. Symbols:  $\circ$ , untreated double-stranded RNA;  $\bullet$ , same preparation of RNA after heating for 10 min at 60 C in  $0.01 \times$  SSC buffer containing 8% formaldehyde.

denatured fragments could be obtained by gel electrophoresis under our standard conditions.

## DISCUSSION

The basic principle of construction of the double-stranded RNA genomes of BTV and reovirus is obviously very similar, but there are some finer points of distinction. The single-stranded component found in partly purified BTV, and thought to be equivalent to the adenine-rich component of reovirus, was shown to be absent from highly purified BTV. Further work is needed to ascertain whether the presence or absence of a single-stranded component constitutes a real difference between the two viruses.

Fractionation of the double-stranded RNA by means of polyacrylamide gel electrophoresis reveals the presence of ten segments in the genomes of both viruses. Differences in the electrophoretic patterns, reflecting differences in electrophoretic mobilities of the components concerned, indicate slight variations in the molecular sizes of the segments. Because of the reproducible variations in the electrophoretic patterns obtained for different serotypes of reovirus (12), it is perhaps not surprising that the serologically unrelated BT virus and reovirus are still more dissimilar in this respect.

The different size distribution of the segments is also reflected in the patterns of separation obtained in sucrose gradient sedimentation analysis. In contrast to the distinct distribution of components into three size categories in the case of reovirus, five poorly resolved peaks are found for BTV. This reproducible poor resolution of BTV-RNA on sucrose gradients throws some doubt on

the accuracy of the molecular-weight estimations derived by comparison with ribosomal RNA markers. However, molecular weights determined from gels by comparison of the electrophoretic mobilities of BTV components with those of reovirus segments agree closely with the values obtained by sedimentation analysis. The values are also consistent with the distribution of radioactivity among the peaks. The molecular weight estimates for components 1 to 10 given in Table 2 can therefore be accepted with reasonable confidence.

A comparison of the molecular-weight estimates for BTV-RNA components obtained by the 2 methods moreover clearly reveals a correlation between the 5 peaks resolved in sedimentation analysis and the 10 components resolved in gel electrophoresis. The 15.5S peak obviously corresponds to component 1, the 14S peak to components 2 and 3, 12S to 4, 5, and 6, 10S to 7, 8 and 9, and 8.5S to component 10.

The significance of the segmented structure of the genomes of the double-stranded RNA viruses remains to be elucidated. It does seem to be a common characteristic of this viral group, however, although it is not yet clear whether the segmentation is a result of the isolation procedure. African horsesickness virus also has recently been shown to possess a similar double-stranded genome, separable into at least six components by gel electrophoresis (R. A. Oellermann *et al.*, *in press*). The fact that other viruses of animal, plant, and insect hosts are now known to possess a similar structure and to belong to a large group of related viruses (17) adds new interest to the investigation of the biological functions of the various parts of such a complex genome.

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