

Genome of Sindbis Virus

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³²P-labeled ribonucleic acid (RNA) from purified Sindbis virus was examined for the presence of hidden breaks. Viral RNA was treated with acid at pH 2.9 or with formaldehyde and was analyzed on sucrose gradients or by polyacrylamide gel electrophoresis. The sedimentation pattern and mobility on polyacrylamide gels of the 42S RNA was unaffected by heating and quick cooling and was not altered by denaturing agents such as dimethyl sulfoxide and urea. No evidence that Sindbis RNA is a polyaggregate of fragments was obtained. It is concluded that the genome consists of a continuous length of single-stranded polynucleotide.

Several group A arboviruses are considered to contain a genome which consists of a single-stranded polynucleotide. In the case of Sindbis virus, it exists as 42S infectious ribonucleic acid (RNA) which may be extracted either from purified virions or from the 140S nucleoid present in infected cells (6). The ribonuclease-sensitive infectious RNA of these viruses appears homogeneous on sucrose gradients (7, 15) and moves as a single fraction on polyacrylamide gels (3, 7). RNA from the virion is converted to a species having a lowered sedimentation coefficient upon heating followed by rapid cooling (7, 15). This change in sedimentation coefficient is accompanied by fragmentation and increased mobility on gels (3, 7). Both Sindbis and Western equine encephalomyelitis (WEE) 40S RNA are believed to possess a hydrogen-bonded secondary structure since they undergo hypochromic shift upon heating and appear to be almost completely reannealed upon slow cooling (14, 15). Treatment of 40S WEE RNA with dimethyl sulfoxide (DMSO) or with urea did not result in a change in the sedimentation pattern in sucrose gradient after removal of the reagents (15). However, virion RNA from WEE and Sindbis appeared to cosediment with 28S cell ribosomal RNA (rRNA) or with viral-specific 26S RNA when centrifuged in sucrose gradients prepared in DMSO (5, 15). Sindbis RNA treated with 12 M urea showed a similar electrophoretic mobility to 28S rRNA on gels containing 8 M urea (8). These changes in the biophysical properties of group A arbovirus RNA induced by treatments which are known to break hydrogen bonds have led to speculations about the structure of the viral genome (3, 5, 7, 15). It was suggested that viral RNA may exist in two conformations which sediment differently on sucrose gradients (42 and 26S species) and which

may migrate differently on gels; or the genome may be considered as a polyaggregate of two or more roughly equal-size fragments which sediment at the same rate and migrate at roughly similar mobility on gels.

In this communication we show that if sufficient precautions are taken to extract RNA free of hidden breaks, no fragmentation of the genome is observed after heating or treatment with denaturing agents and that Sindbis virus RNA behaves as an unbroken polynucleotide.

MATERIALS AND METHODS

Chemicals. Acrylamide, *N,N'*-methylenebisacrylamide and *N,N,N',N'*-tetramethylenebisacrylamide were purchased from Eastman Organic Chemicals. Acrylamide and *N',N'*-methylenebisacrylamide were recrystallized from chloroform and acetone, respectively. Carrier-free ³²PO₄ was obtained from the Atomic Energy of Canada Ltd. DMSO and urea were bought from Fisher Scientific Co.

Tissue culture and virus growth. The procedure for the preparation of BHK/21 cell monolayers on Falcon plastic petri dishes and the growth of Sindbis virus have been described (1).

Preparation of ³²P-labeled cells and extraction of RNA. Cell monolayers of BHK/21 cells were washed twice with phosphate-free minimum essential medium (MEM, Grand Island Biological Co.) and were overlaid with medium A: MEM containing 2.5% BHK/21 medium (Grand Island Biological Co.), 1.5% dialyzed calf serum, and 10 μCi of ³²PO₄ per ml. Cell monolayers were incubated at 37 C for 18 hr. RNA was extracted as previously described (6).

Preparation of ³²P-labeled viral RNA. BHK/21 cells were prelabeled with ³²PO₄ as described above and were infected with Sindbis virus at a multiplicity of 40:1. After adsorption, they were overlaid with medium A, and the ³²P-labeled virus was harvested 18 hr later. The medium was clarified at 15,000 rev/min for 15 min at 4 C (Sorvall SS34), and the virus was pelleted from the supernatant fraction at

25,000 rev/min for 1 hr in a Spinco SW25 rotor at 4 C. The virus pellet was suspended in borate saline (BS; 50 mM borate and 120 mM NaCl, pH 9) containing 0.4% bovine serum albumin (BS/BSA) and was clarified at 10,000 rev/min for 10 min in a Spinco SW50 rotor at 4 C. Concentrated virus was further purified by centrifugation on a preformed 5 to 50% potassium tartrate gradient in BS/BSA (11). The virus was detected by radioactivity assay and by a hemagglutination test with goose erythrocytes (4). Fractions containing the virus were pooled, diluted with BS, and pelleted by centrifugation at 25,000 rev/min for 2 hr in a Spinco SW25 rotor at 4 C. The virus pellet was digested with 4% sodium dodecyl sulfate (SDS) and 1% 2-mercaptoethanol in phosphate-buffered saline A (140 mM NaCl, 27 mM KCl,

8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.2) at 37 C for 1 hr. The digested material was then centrifuged on a 10 to 20% sucrose gradient (38,000 rev/min for 3 hr in an SW50 rotor at 21 C) prepared in electrophoresis buffer [EPB; 4 mM tris(hydroxymethyl)aminomethane (Tris), 2 mM sodium acetate, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1% SDS, pH 7.2]. The viral RNA sedimenting at 42S (calculated with respect to cell RNA markers included in the gradient) was precipitated in the cold with 2.5 volumes of absolute ethanol and stored at -20 C.

Acid treatment. ³²P-labeled viral RNA was exposed to 0.2 ml of 5 mM hydrochloric acid to lower the pH to 2.9 for 30 sec (10), followed by 0.15 ml of 10 mM EDTA containing 0.2% SDS. The final pH of the mixture was 5.6. The material was then layered onto

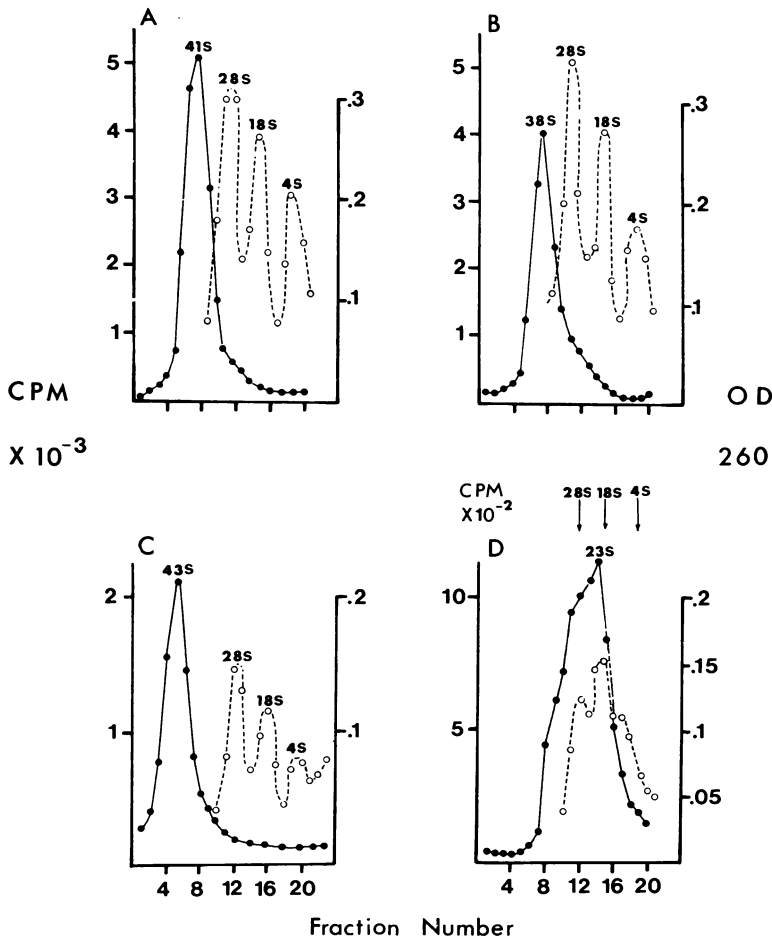


FIG. 1. Test for hidden breaks by the acid treatment. ³²P-labeled viral RNA (●) was centrifuged on 10 to 20% sucrose gradients prepared in EPB (see text) at 45,000 rev/min for 2.25 hr in an SW50 rotor at 21 C. Mammalian cell RNA markers (○) were included in the gradients. A, Viral RNA was dissolved in EPB and centrifuged on a sucrose gradient. B, Viral RNA was treated with 0.2 ml of 5 mM HCl at 21 C for 30 sec (pH 2.9), followed by 0.15 ml of 10 mM EDTA containing 0.2% SDS (final pH was 5.6). It was then centrifuged on a sucrose gradient. C, Viral RNA was treated with 2 μg of ribonuclease per ml in EPB at 0 C for 5 min. SDS (0.1%) and cell RNA markers were added and analyzed on a sucrose gradient. D, Ribonuclease-treated viral RNA was exposed to acid as described for panel B, followed by analysis on a sucrose gradient.

a 10 to 20% sucrose gradient prepared in EPB and centrifuged at 38,000 rev/min for 3 hr in a Spinco SW50 rotor at 21 C. Each fraction (0.2 ml) was added to 10 ml of *p*-dioxane-based scintillator and counted in a liquid scintillation spectrometer.

Formaldehyde treatment. 32 P-labeled viral RNA was treated with 1% formaldehyde in EPB, and then centrifuged on a 10 to 20% sucrose gradient containing 1% formaldehyde at 21 C. The fractions were counted as described for the acid treatment.

Treatment of RNA with DMSO. 32 P-labeled RNA, in 10 μ liters of EPB, was incubated with 0.1 ml of DMSO at room temperature for 15 min. The DMSO was removed by dialysis against 500 ml of EPB at room temperature, and the RNA was examined by polyacrylamide gel electrophoresis.

The technique for sedimentation of RNA in sucrose gradients prepared in DMSO-1 mM EDTA has been described (5).

Treatment of RNA with urea. 32 P-labeled RNA was incubated with 10 M urea prepared in saline A at 37 C for 30 min. The RNA was analyzed by gel electrophoresis.

Polyacrylamide gel electrophoresis. Composite gels (6 cm in length) of 2.5% acrylamide and 0.5% agarose were prepared in EPB by the method of Peacock and Dingman (13). The gels were prerun by electrophoresis in EPB at 50 v for 30 min at 4 C before the RNA sample was added. After RNA analysis the gels were sliced transversely on a plastic fractionating device. Each slice was dried on a planchet and counted in a gas flow counter.

RESULTS

Absence of hidden breaks in the preparation. To investigate the effect of denaturing reagents such as DMSO and urea on viral RNA, it was necessary to show that all the RNA preparations were free of hidden breaks which may have been induced during the extraction procedures. Treatment of viral preparations with acid at pH 2.9 (10) or with formaldehyde (2, 9) followed by re-examination of the RNA preparation on gradients may be used to indicate the absence of hidden breaks. These criteria were applied to Sindbis viral RNA (Fig. 1 and 3). The profile in Fig. 1A shows that Sindbis RNA, prepared as described above, sediments at about 42S when compared to hamster cell RNA. In Fig. 1B it is seen that viral RNA continues to sediment as a single species after the acid treatment.

Viral RNA was then nicked by a limited digestion with pancreatic ribonuclease (2 μ g/ml for 5 min at 0 C). The enzyme action was stopped by the addition of SDS and cell RNA markers. The sample was divided into two parts. One part was treated with acid as described above, and the other part was left untreated. The data in Fig. 1C show that mild ribonuclease treatment did not alter the sedimentation coefficient of the viral RNA. However, a heterogeneous profile was

observed when the ribonuclease-treated material was exposed to acid (Fig. 1D) which indicated that none of the original 42S viral RNA remained intact.

Further evidence of the integrity of the viral RNA was obtained when the mobility of preparations on acrylamide gels was examined after acid treatment at pH 2.9. The profiles in Fig. 2 show that the mobilities of 28S rRNA and Sindbis 42S RNA were unaltered by the treatment. There is no evidence of increased heterogeneity which would have resulted in the production of faster-moving (smaller) RNA fragments.

A similar series of results was obtained when the viral RNA was reacted with 1% formaldehyde (Fig. 3). The 42S viral RNA (Fig. 3A) retained its sedimentation characteristic after treatment with formaldehyde. In this experiment the effect of ribonuclease was more severe than that seen in Fig. 1B, and even before treatment with formaldehyde it was possible to demonstrate the heterogeneity of the viral RNA (Fig. 3C). When the ribonuclease-treated viral RNA was reacted with formaldehyde and re-examined on a sucrose gradient containing 1% formaldehyde (Fig. 3D), the major species of RNA sedimented in the range 18 to 25S.

Effect of heating viral RNA. 32 P-labeled Sindbis

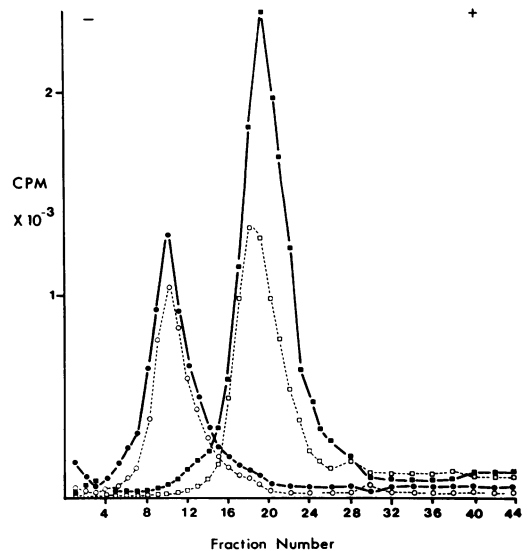


FIG. 2. Polyacrylamide gel electrophoresis of acid-treated RNA. 32 P-labeled viral RNA was treated with acid as described in legend to Fig. 1 and was analyzed by electrophoresis at 4 C on 2.5 to 0.5% acrylamide-agarose composite gels for 50 min at 125 v. Symbols: (○), acid-treated viral RNA; (□), acid-treated 28S rRNA; (●), viral RNA control; (■), 28S rRNA control.

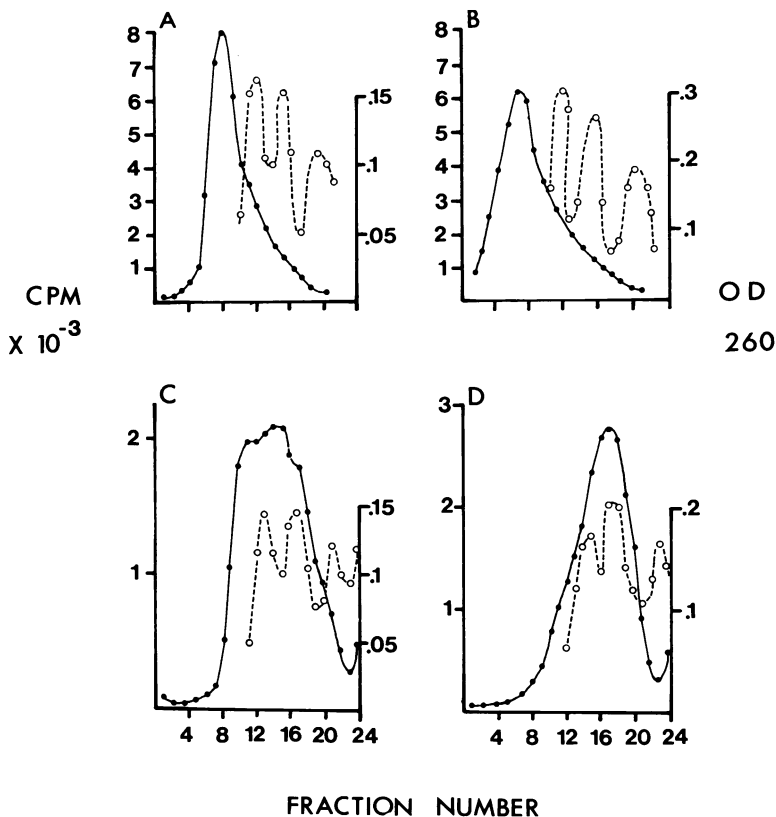


FIG. 3. Treatment of viral RNA with formaldehyde. ^{32}P -labeled viral RNA (●) was centrifuged on 10 to 20% sucrose gradients prepared in EPB (see text) at 45,000 rev/min for 2.25 hr in an SW50 rotor at 21 C. Mammalian-cell RNA markers (○) were included in the gradients. A, Viral RNA was dissolved in EPB and centrifuged on a sucrose gradient. B, Viral RNA was dissolved in EPB containing 1% formaldehyde and analyzed on a gradient containing 1% formaldehyde. C, Viral RNA was treated with 2 μg of ribonuclease per ml in EPB at 0 C for 5 min. SDS (0.1%) and cell RNA markers were added and the mixture was analyzed on a sucrose gradient. D, Ribonuclease-treated viral RNA was made up to 1% with respect to formaldehyde and analyzed on a gradient containing 1% formaldehyde.

RNA was prepared and dissolved in buffers of either low molarity (EPB) or high molarity (standard buffer, STB; 500 mM Tris, 100 mM NaCl, 1 mM EDTA, pH 7.2). The solution was held at 85 C for 3 min in a water bath and then quickly cooled in melting ice. Analysis of the RNA on polyacrylamide gels (Fig. 4) showed that there was no loss of the principal 42S RNA component when break-free RNA was used in the experiment. Previous studies with Sindbis and Semliki Forest RNA treated in this manner (3, 7) indicated that fragmentation of the genome had occurred, and, in the case of Semliki Forest virus RNA, it was thought that several distinct subunits were separated (3). However, it must be concluded from the data in Fig. 4 that the genome does not cleave upon heating.

Effects of DMSO and urea on viral RNA. Sindbis RNA was obtained from ^{32}P -labeled

virions by digestion with SDS and was separated on a sucrose gradient and precipitated. The RNA was subsequently dissolved in EPB and divided into two portions. One was treated with DMSO (final concentration, 91%) and the other was left untreated. The 28S rRNA was similarly treated, and the four samples were examined on 2.5 to 0.5% polyacrylamide-agarose gels. These gels contained a plug of low porosity at the bottom to trap any fast-moving low-molecular-weight product. The profile in Fig. 5A indicates that the mobility of 28S rRNA is virtually unaltered upon treatment with DMSO. The mobility of Sindbis RNA was fractionally increased upon DMSO treatment as a result of the denaturation process, but there was no evidence of fragmentation.

The routine method of extracting viral RNA was by digestion of the virions with SDS and subsequent separation of the viral RNA on a

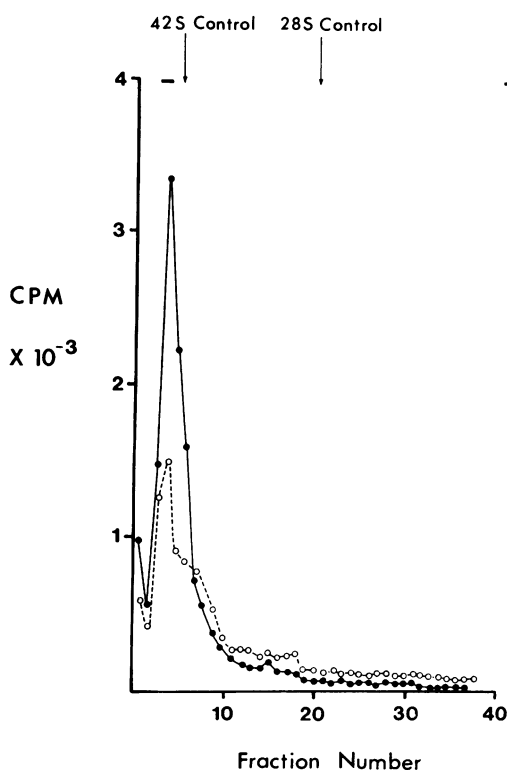


FIG. 4. Effect of heating on viral RNA. ^{32}P -labeled viral RNA, dissolved in either EPB (●) or STB (○) was heated at 85 C for 3 min and quickly cooled in melting ice. It was analyzed by electrophoresis at 4 C on 2.5 to 0.5% polyacrylamide gels at 100 v for 1 hr.

sucrose gradient. When the RNA obtained by this technique was further extracted with phenol to remove traces of protein, similar results were obtained upon DMSO treatment (Fig. 5B). The mobility of the DMSO-treated viral RNA was only fractionally increased by the agent. The sedimentation pattern of arbovirus RNA in sucrose gradients prepared in DMSO has been shown to be similar to that of 28S rRNA (5) or viral-specific 26S RNA (15). These similar patterns were thought to have been due to a breakdown of a polyaggregate 42S RNA in the sucrose-DMSO gradient or to a conformational change in the viral 42S RNA. An experiment was performed to see whether cleavage occurs when Sindbis 42S RNA is fractionated on 99% DMSO-sucrose gradients. The results (Fig. 6A) confirm that the sedimentation pattern of Sindbis RNA is similar to that of 28S rRNA as described previously (5). RNA was taken from the peak fractions on the gradient, precipitated with unlabeled carrier RNA, and applied to polyacrylamide gels. The

data (Fig. 6B) show that the 42S RNA derived from the gradient prepared in 99% DMSO comigrated with untreated 42S RNA and was not cleaved by the treatment.

An essentially similar result was obtained when the electrophoretic pattern of urea-treated viral RNA was compared with that of untreated 42S RNA. In this experiment, viral RNA was incubated with 10 M urea for 30 min at 37 C. Carrier RNA was added, and the mixture was precipitated with ethanol. The RNA was dissolved in EPB and applied to gels (Fig. 7). The profile of the untreated RNA indicates some heterogeneity in the preparation, but the principal component was not eliminated by urea treatment as would have occurred if fragmentation had been brought about by the urea treatment.

Absence of low-molecular-weight RNA in the virion. A model that has been considered for the structure of arbovirus RNA is that the genome consists of two approximately equal-sized fragments of RNA linked together by complementary base pairing with a short piece of oligonucleotide. We investigated this possibility by subjecting Sindbis RNA of high specific activity to gel electrophoresis in a 10% gel with cell 4S RNA used as a marker (Fig. 8). As expected, viral RNA did not penetrate the gel, whereas 4S RNA moved about 42% along its length. Treatment of Sindbis RNA with DMSO did not release a fragment that could be detected under these conditions. We estimate the sensitivity of our technique was such that we would have detected an oligonucleotide 100 nucleotides in length by this technique.

DISCUSSION

The findings reported here indicate that Sindbis RNA which is demonstrably free of hidden breaks does not change its sedimentation behavior on sucrose gradients or its mobility on acrylamide gels when heated and quick-cooled or when treated with agents which denature secondary structure. There appeared to be ample evidence that the genome of the group A arbovirus was cleaved when RNA extracted from the virions was heated in aqueous neutral solutions. This treatment was reported to result in a decrease in sedimentation coefficient of WEE RNA (15), accompanied by an increased mobility on gels in the cases of Semliki Forest RNA (3) and Sindbis RNA (7). Pronounced increased mobility of heated RNA was ascribed to the migration of fragments of the viral RNA formed by chemical cleavage or by melting of hydrogen bonds which hold subunits of the genome in a polyaggregate structure (3, 7). Conformational changes have

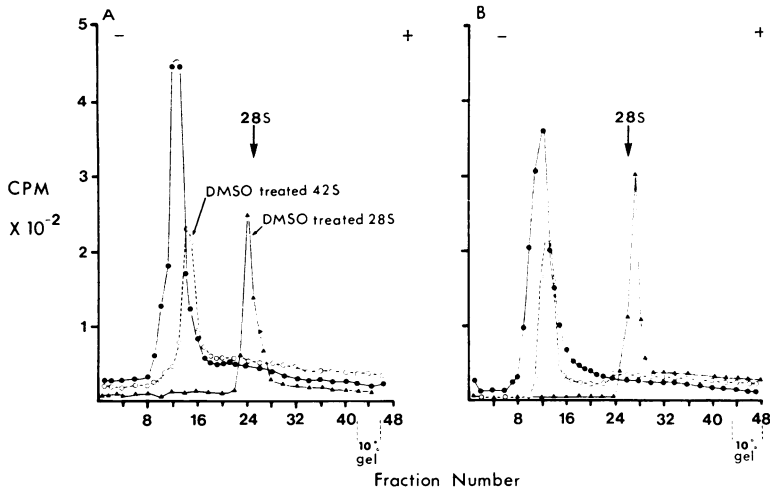


FIG. 5. Effect of DMSO on viral and cellular RNA. A, ^{32}P -labeled viral and cellular 28S rRNA were dissolved in 10 μl of EPB (see text) followed by 0.1 ml of DMSO and incubated at room temperature for 10 min. DMSO was removed by dialysis against EPB, and the RNA was analyzed by electrophoresis at 4 C on 2.5 to 0.5% acrylamide-agarose composite gels at 125 v for 50 min. Symbols: (O) DMSO-treated viral RNA; (\blacktriangle) DMSO-treated 28S rRNA; (\bullet) untreated viral RNA control. Arrow indicates the position of the untreated 28S rRNA control. B, ^{32}P -labeled viral RNA, prepared as outlined in text, was extracted twice with redistilled water-saturated phenol. It was precipitated with 2.5 volumes of absolute ethanol and stored at -20 C . A portion of the precipitate was dissolved in 10 μl of EPB, treated with DMSO, and analyzed by gel electrophoresis as described above. Symbols are as for A.

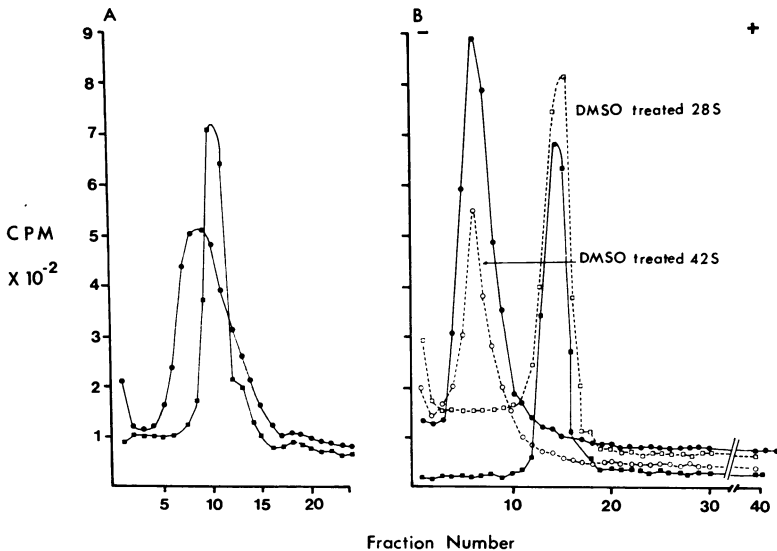


FIG. 6. A, Analysis of viral RNA and 28S rRNA on 99% DMSO gradients. ^{32}P -labeled viral RNA and 28S rRNA were dissolved in 10 μl of EPB (see text), followed by 0.1 ml of DMSO. It was centrifuged at 22 C on a preformed 5 to 20% sucrose gradient prepared in 99% DMSO and 1 mM EDTA at 55,000 rev/min for 19 hr in an SW56 rotor. The viral RNA (\bullet) in fractions 8, 9, and 10 and the 28S rRNA (\blacksquare) in fractions 10 and 11 were precipitated with 2.5 volumes of absolute ethanol and an excess of carrier yeast RNA. B, The RNA precipitates from the DMSO gradient were dissolved in EPB and analyzed at 4 C on 2.5 to 0.5% acrylamide-agarose composite gels at 125 v for 50 min. Symbols: (O) DMSO-treated 42S; (\square) DMSO-treated 28S; (\bullet) untreated 28S.

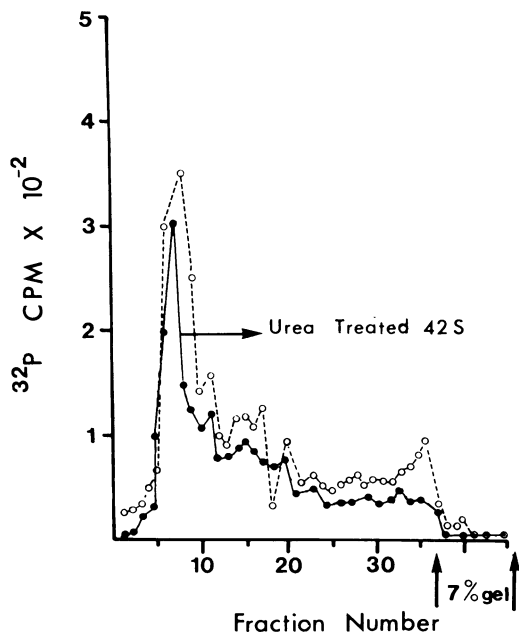


FIG. 7. Effect of urea on viral RNA. ^{32}P -labeled viral RNA was dissolved in EPB (see text) containing 10 M urea and incubated at 37 C for 30 min. It was analyzed on 2.5 to 0.5% acrylamide-agarose composite gels at 100 v for 1 hr. Symbols: (●) urea-treated viral RNA; (○) untreated viral RNA control.

very little effect on the mobility of large fragments of RNA in polyacrylamide gels (12), so the suggestion that the change in sedimentation behavior of WEE RNA from 42 to 26S upon heating is due to an extreme conformation change (15) is not considered feasible. It appears that hidden breaks were present in arboviral RNA previously used for studies of sedimentation behavior and electrophoretic mobility. The data in Fig. 1 demonstrate clearly that nicked RNA sediments on gradients and migrates on gels in a manner indistinguishable from the native molecule, and divergence from the pattern is observed only when ribonuclease action is more extensive (Fig. 3). We show in Fig. 4 that break-free Sindbis RNA is not fragmented when heated either in high (STB)- or low (EPB)-ionic-strength buffers at 85 C. Sindbis RNA melts at 65.5 C (14), and a polyaggregate RNA would have dissociated under the heating conditions used in the experiment.

We found no evidence that denaturing agents such as DMSO and urea led to fragmentation of the genome, which should have been detected by increased mobility on acrylamide gels. Similarly, Sreevalsan et al. (15) found no change in sedimentation coefficient in sucrose gradients when WEE RNA was treated with 75% DMSO and subsequently rerun on gradient in the absence of DMSO. We conclude that RNA extracted from

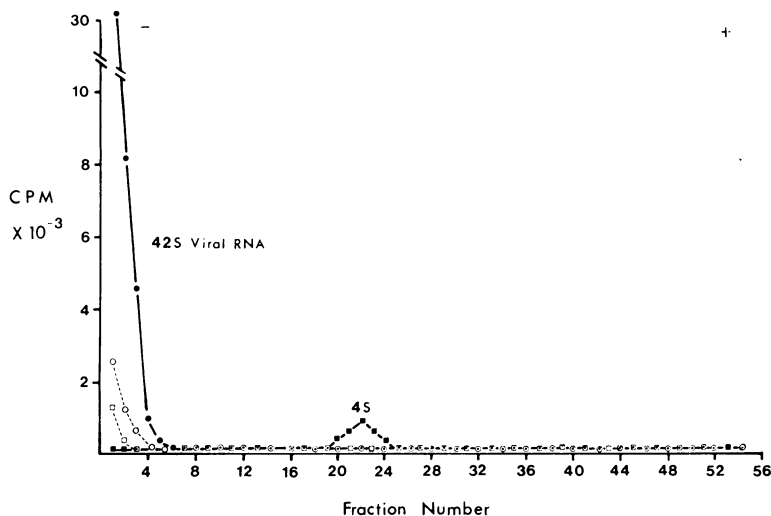


FIG. 8. Absence of small-molecular-weight RNA from the genome of *Sindbis virus*. ^{32}P -labeled viral RNA was dissolved in 10 μl of EPB (see text) followed by 0.1 ml of DMSO and incubated at room temperature for 10 min. It was then layered on 10% polyacrylamide gels (0.25% bisacrylamide), and electrophoresis was carried out at 50 v for 50 min at room temperature. Symbols: (●) viral RNA; (○) 28S RNA; (□) 18S RNA; (■) 4S RNA.

virions is present as a single strand and not made up of subunits joined by hydrogen bonding, since the subunit structure would have been detected in our experiments in which RNA was treated at pH 2.9 or with formaldehyde, DMSO, or urea.

The data in Fig. 6 show that no cleavage occurred when Sindbis RNA was sedimented in sucrose gradients prepared in 99% DMSO-1 mM EDTA since the peak fractions taken from these gradients comigrated with untreated 42S RNA on gels. In light of the foregoing, it is difficult to understand how Sindbis RNA as well as WEE RNA sedimented at rates similar to that of 28S rRNA or viral 26S RNA in sucrose gradients prepared in DMSO (5, 15), where there exists a linear relationship between S_{25} of RNA in 99% DMSO and log molecular weight (17). If this technique is used to determine molecular weight of RNA, extremely precise measurements have to be made since, using the formula given (17), the S_{25} value of RNA having a molecular weight of 1.7×10^6 is 4.44, and an RNA having molecular weight of 4×10^6 has an S_{25} of 5.66. Thus, apparent similarities in sedimentation coefficients observed in sucrose gradients prepared in DMSO between arbovirus RNA and 28S cell RNA (5) may be questioned on technical grounds when the resolution of the method is not known. Factors such as these may help explain the discrepancies noted in estimations of the molecular weight of Sindbis RNA made by this technique (5, 16) as opposed to estimates made by using polyacrylamide gel electrophoresis (3, 8).

The work reported here emphasizes the need to check that RNA used in biophysical studies is break-free. We find no evidence that Sindbis viral RNA exists other than as a continuous polynucleotide strand and consider that previously stated theories about its polyaggregate nature are unfounded.

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