JOURNAL OF VIROLOGY, Oct. 1969, p. 429-438
Vol. 4, No. 4

Printed in U.S.A.

Properties of 42S and 26S Sindbis Viral Ribonucleic Acid Species

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Received for publication 15 May 1969

Two species of ribonuclease-sensitive Sindbis viral ribonucleic acids which sedimented at 42S and 26S were studied. 42S RNA, derived either from virions or from viral nucleoids extracted from infected cultures, was converted by heating to an RNA which sedimented at 26S. The sedimentation patterns of 42S RNA and "derived" 26S RNA were similarly affected in low ionic strength buffers. 42S RNA ran as a homogeneous fraction on polyacrylamide gels; the "derived" 26S RNA as well as "natural" 26S RNA from infected cultures showed similar electrophoretic patterns of heterogeneity. A doubling of 3' polynucleotide termini was observed when 42S RNA was heated. Two possibilities concerning the structure of 42S RNA are considered. (i) It may consist of an aggregate of subunits, joined by means of hydrogen bonds to form a complex molecule. (ii) A heat-labile covalent bond of unknown type may link viral RNA subunits. Although 26S RNA from infected cultures and "derived" 26S RNA from 42S RNA behaved in a similar qualitative manner on gels, their sedimentation characteristics were affected differently in low ionic strength buffers. "Natural" and "derived" 26S RNA appear to consist of a population of fragments. and their behavior in gradients and in gels is probably dictated by the experimental conditions of the analytical methods used.

Three forms of viral ribonucleic acid distinguishable on the basis of their sedimentation characteristics have been extracted from cells infected with group A arboviruses (1, 14, 23, 27). In the case of Sindbis virus these are 42S, 26S, ribonuclease-sensitive and 20S ribonuclease-resistant RNA. Infectious 42S is present in the Sindbis virion and in the 140S viral nucleoid prepared from infected cells. 26S RNA is noninfectious and is extractable from the 65S particle present in infected cells (5). The 40S and 26S RNA forms produced in Western equine encephalitis (WEE) infection have similar base compositions and identical buoyant densities. Both Sindbis and WEE 40S RNA species are believed to possess secondary hydrogen bonded structures, since they undergo hyperchromic shift upon heating and appear to be almost completely reannealed upon slow cooling (25, 28). The 40S WEE RNA can be converted to 26S RNA in vitro by heating and quick cooling as well as by treatment with either dimethyl sulfoxide or 8 M urea. Such denatured RNA lost at least 90% of its infectivity. On the basis of these studies (28), it is suggested that WEE viral RNA may exist in two conformations which sediment differently in sucrose gradients, or the genome may contain an extremely heat-labile portion near the center which is easily broken into two equal pieces (28). Similarly, 42S and 20S Semliki forest virus (SFV) RNA give rise to 26S interjacent RNA after heat denaturation (13).

A decrease in sedimentation rate upon heating or after dimethyl sulfoxide treatment was also observed when RNA extracted from certain tumor viruses was studied (3, 6, 7, 9). The electrophoretic mobility of Rous sarcoma virus (RSV) 62S RNA on polyacrylamide gel before and after melting (37S) was investigated. Untreated 62S RNA appeared as a slow-moving homogeneous peak on the gel, but became heterogeneous with a higher electrophoretic mobility after melting. On the basis of the observed changes in sedimentation coefficients and altered electropherograms upon melting, it was concluded that the 62S form of RSV RNA is an aggregate of smaller RNA's rather than a single polynucleotide which deaggregates upon heating to its smaller components (6). The possible effect of conformational changes on electrophoretic mobility was not considered.

As an approach to further characterize the relationship of 42S and 26S Sindbis viral specific RNA molecules we have investigated their behavior in polyacrylamide gels. Since it is possible that 42S RNA may be an aggregate which

separates upon heating, we have used an enzymatic method to determine whether any increase of 3′ polynucleotide termini is observed after conversion by heat of 42*S* RNA to 26*S* RNA.

MATERIALS AND METHODS

Actinomycin D (AcD) was the gift of Merck & Co., Inc., Rahway, N.J. ^{3}H-uridine (6.7 c/mmole) was obtained from Amersham/Searle Corp., Toronto, Canada and carrier-free $^{32}PO_4$ was obtained from the Atomic Energy of Canada Ltd., Ottawa. Acrylamide and NN′-methylenebisacrylamide and *NNN′N′*-tetramethylethylenediamine were bought from Eastman Organic Chemicals. Snake venom phosphodiesterase, E.C. 3.1.4.1, (three times recrystallized) was purchased from Worthington Biochemical Corp., Freehold, N.J.

Media, cells, and virus. The preparation of high titer Sindbis virus on BHK-21-C13 cell monolayers and the media employed have been described (5, 10).

Preparation of ^{32}P-labeled virus. Cell monolayers were infected for 15 to 20 min at room temperature at a virus-to-cell ratio of 40:1. BHK medium containing ^{32}P (0.3 mc/10^8 cells) was added and the monolayers were incubated 14 to 16 hr at 37 C. After the incubation period, the medium was clarified by centrifugation at 3,000 rev/min (Spinco SW 25:1 rotor) in the cold, and the supernatant fluid was pelleted for 90 min at 25,000 rev/min in the same rotor to sediment the virus. The pellet was resuspended in standard buffer (STB: 100 mM NaCl, 50 mM tris(hydroxymethyl)aminomethane (Tris), 1 mM ethylenediaminetetraacetic acid (EDTA; *p*H 7.2) and clarified at 10,000 rev/min for 10 min with the SW-39 rotor. The supernatant fluid contained the concentrated ^{32}P-labeled virus.

Preparation of ^{3}H-uridine-labeled virus. Cell monolayers were infected at high input multiplicity. After adsorption (20 min), the inoculum was washed off with phosphate-buffered saline (PBS, ref. 8), and the cells were overlaid with medium containing 1 μg/ml AcD and incubated for 3 to 4 hr. ^{3}H-uridine (10 μc/10^6 cells) was added to the medium and the cultures were incubated for an additional 11 hr. The culture medium was processed as for the preparation of ^{32}P-labeled virus.

Preparation of 140S and 65S cytoplasmic particles. The technique has been described (5). Fractions containing the particles were prepared by sucrose rate zonal centrifugation of cytoplasmic extracts obtained by disrupting AcD-treated and ^{3}H-uridine-labeled infected cells. Sedimentation coefficients were estimated by adding unlabeled chick ribosomes (74*S*) to serve as a reference marker (19).

Preparation of ^{32}P-labeled ribosomal RNA. Cell monolayers grown for 48 hr in the presence of $^{32}PO_4$ (0.4 mc/10^8 cells) were washed five times with cold PBS, scraped into Reticulocyte Standard Buffer (RSB, 10 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris, *p*H 7.4) and disrupted with a Dounce homogenizer. The nuclei and debris were removed by centrifuging the homogenate for 10 min at 3,000 rev/min and RNA was extracted from the supernatant fraction.

Extraction of RNA. RNA was extracted from labeled virus, 140*S* and 65*S* cell fractions, and from ^{32}P-labeled ribosomes by a phenol-detergent method at room temperature. Samples were diluted with STB containing enough sodium dodecyl sulfate (SDS) to make the final concentration of SDS 1%. An equal volume of water-saturated, redistilled phenol was added and the preparation was shaken for 5 min. The phases were separated by low-speed centrifugation at room temperature; the upper aqueous phase was removed and reextracted four more times with fresh phenol. The final aqueous phase was washed twice with ether to remove residual phenol, and ether was evaporated by bubbling N_2 through the preparation. In the preparation of viral RNA, unlabeled chick ribosomal RNA was added to serve as a carrier and the total RNA was precipitated twice with four volumes of ice-cold ethyl alcohol. The RNA precipitate was collected by centrifugation (15,000 rev/min, Sorvall SS 34 rotor) in the cold and was resuspended in either STB or in electrophoresis buffer [EPB: 4 mM Tris, 2 mM sodium acetate, 1 mM EDTA (*p*H 7.2), containing 0.2% SDS; ref. 6].

Rate zonal centrifugation of RNA in sucrose gradients. Sucrose solutions used in the preparation of gradients were adsorbed with bentonite (1 mg/ml) to remove possible contaminant ribonuclease (11). Bentonite was removed by centrifugation before forming the sucrose gradients. The various RNA preparations were sedimented in a 5 to 20% sucrose gradient in the appropriate buffer (STB or EPB) at 38,000 rev/min for 2.5 hr with the SW 50 rotor (Spinco). Fractions were collected after puncturing the bottom of the centrifuge tube. In the case of viral RNA preparations, sedimentation coefficients were estimated (19) with the use of added chick ribosomal RNA (28*S* and 16*S*) as reference markers (12, 19). The acid-precipitable radioactivity of each fraction was determined (4). Pooled peak fractions of the various RNA species were used in acrylamide gel electrophoresis studies or in enzyme digestion experiments.

Heat treatment of RNA. RNA samples purified on sucrose gradients were "melted" by heating them in a water bath at 85 C for 3 min in stoppered test tubes. At the end of the heating period the tubes were immediately placed in melting ice for 5 min to prevent reannealing.

Polyacrylamide gel electrophoresis. The methods used were based on published techniques (2, 6, 17). Acrylamide and NN′-methylenebisacrylamide were recrystallized from chloroform and acetone, respectively. The 3% acrylamide gels were prepared by making up a 6% acrylamide solution in double concentrated EPB containing 0.4% SDS and 0.15% NN′-methylenebisacrylamide. One volume of the preparation mixed with an equal volume of initiator solution (0.15 *NNN′N′*-tetramethylethylenediamine and 0.2% ammonium persulfate in distilled water) was deaerated. Acrylamide gel running tubes (0.5 cm in diameter and 7.4 cm long), stoppered in one end, were aligned in an upright position and were filled with gel mixture to the 6-cm level. The mixture was carefully overlaid with EPB to give a flat interface, and the gels were

allowed to polymerize for about 20 min. The tubes were placed in a Shandon analytical disc electrophoresis apparatus. The upper and lower reservoirs were filled with EPB and the gels were prerun at room temperature for 30 min at 50 v. Samples of RNA were applied to the gels with a Pasteur pipette either in STB or in EPB containing 10% sucrose. The volume of sample did not exceed 50 μliters. Electrophoresis was performed for 2.5 hr at room temperature at 50 v. After the run, the gels were fixed in 7% acetic acid for 15 min. They were floated onto a transparent plastic gel cutter which had slits 2 mm apart, and were sliced with a razor blade. When ^{32}P-labeled RNA was subjected to gel electrophoresis, the gel slices were placed on aluminum planchets, dried, and counted. When ^{3}H-labeled RNA was to be counted, the gel slices were placed in the bottom of test tubes and 4 to 5 drops of 30% H_2O_2 were added. The tubes were tightly sealed with rubber stoppers and placed in a water bath at 60 C for 2 to 3 hr (16). After the slices dissolved, 0.5 ml of solubilizer solution was added (Nuclear-Chicago Corp., Des Plaines, Ill.), and the mixture was transferred into scintillation vials containing 5 ml of concentrated Liquifluor (Nuclear-Chicago) for counting.

Digestion of ^{32}P-ribosomal RNA and ^{32}P-Sindbis viral RNA by snake venom phosphodiesterase. For digestion of ^{32}P-ribosomal RNA and ^{32}P-Sindbis viral RNA by snake venom phosphodiesterase (4), all buffers used were treated with bentonite, which was removed prior to experimentation by centrifugation. RNA samples extracted from ^{32}P-labeled virus or from ^{32}P-labeled ribosomes were dissolved in 100 mM Tris, *p*H 8.5 and sedimented in a 5 to 20% sucrose gradient in the same buffer. The peak fractions containing the RNA species under investigation were pooled and recentrifuged in another sucrose gradient to further purify the preparation. To avoid contamination of the viral RNA preparation by ribosomal RNA, unlabeled ribosomal markers were not used in the second centrifugation. Peak radioactive fractions were pooled, and the amount of RNA in the preparation was estimated by optical density measurements at 260 nm (18). Both total and acid-precipitable radioactivity were measured to determine the specific activity of various samples. The acid-soluble radioactivity of the substrates was between 0.1 and 0.3% of the total radioactivity. Venom phosphodiesterase was dissolved in 10 mM $MgCl_2$, 10 mM Tris (*p*H 8.5) to a concentration of 2.5 mg/ml. The enzyme was used at 0.25 mg/0.1 ml or 0.125 mg/0.1 ml in the final incubation mixture. The amount of RNA (substrate) added was varied in preliminary experiments until a concentration was found at which the rate of reaction was independent of enzyme concentration, i.e. doubling the enzyme concentration did not increase the rate of the reaction. Under these conditions, the rate of the reaction was seen to be proportional to the substrate concentration. The final RNA concentrations used were 1 μg of ^{32}P-viral 42*S* RNA (10,000 counts per min) or 5 μg of ^{32}P-ribosomal 28*S* RNA (16,000 counts per min).

The incubation mixture contained 0.1 ml of enzyme preparation, 0.2 ml of an RNA preparation, and Tris-$MgCl_2$ buffer to 0.4 ml total volume. Mixtures were incubated at 37 C in sealed test tubes. When heated RNA samples were used, the *p*H of the RNA samples was adjusted to *p*H 7.1 before the heat treatment. Afterwards the samples were readjusted to *p*H 8.5 for enzyme digestion. Each incubation set contained a control preparation with no enzyme to measure the stability of the RNA preparations for the duration of the experiment. Samples (0.1 ml) were removed at 0, 20, and 40 min after the addition of substrate; they were placed in ice, 0.1 ml of bovine serum albumin (5 ml/ml) was added, and the RNA was precipitated with 0.2 ml of 0.4 N perchloric acid. Acid-precipitated material was pelleted at 10,000 rev/min for 10 min (Spinco, SW 50 rotor). Acid-soluble radioactivity was measured by transferring the supernatant fractions directly into scintillation vials containing a *p*-dioxane based scintillating mixture for counting. Preliminary experiments indicated that the counting efficiency of ^{32}P was virtually 100%; neither the presence of sucrose nor perchloric acid influenced the efficiency of counting of ^{32}P. The acid-insoluble fractions obtained from the incubated mixtures were digested with 0.3 N KOH for 20 hr to measure total acid-soluble nucleotides. The radioactivities of the acid-soluble and acid-insoluble fractions tallied with the total radioactivity of the RNA sample measured at the beginning of each experiment.

RESULTS

Sedimentation characteristics of 26S and 42S RNA. To study the effect of heating on the sedimentation characteristics of 42*S* and 26*S* viral RNA, it was first necessary to demonstrate that each of the two RNA species could be prepared separately from the other. Purified ^{3}H-uridine-labeled virus, as well as ^{3}H-uridine-labeled nucleoids (140*S* particles), was used as a source of 42*S* RNA; 26*S* "natural" RNA was extracted from labeled 65*S* particles obtained from cytoplasmic fractions of infected cells. The data given in Fig. 1A show that viral specific nucleic acid is present in fractions sedimenting at 140*S* and at 65*S*. These fractions were combined and extracted with phenol-SDS at room temperature. Profiles in Fig. 1B illustrate that 42*S* and 26*S* RNA are separated in a gradient.

The effect of a brief heat treatment (3 min at 85 C in STB) on the sedimentation of 42*S* RNA from virions is shown in Fig. 2. The 42*S* RNA was converted to RNA with a sedimentation coefficient of 26*S*. Subsequently this RNA will be referred to as "derived" 26*S* RNA. The profile indicated that the product obtained on heating is more heterogeneous than the original 42*S* RNA, but it is noteworthy that there is no accumulation of radioactivity at the top of the gradient, which would occur if random cleavage of the polynucleotide took place. Similar results were obtained when 40*S* WEE RNA was heated

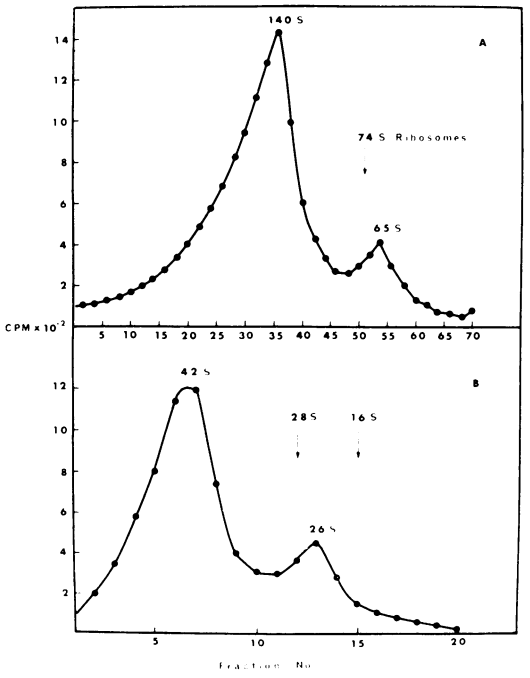

FIG. 1. *Sucrose gradient analysis of (A) cytoplasmic extract from virus-infected, ^{3}H-uridine-labeled cells and (B) RNA extracted from 140S and 65S regions of gradient A. In A, cell monolayers were treated with actinomycin D (1 μg/ml) and simultaneously infected with Sindbis virus. At 3 hr postinfection ^{3}H-uridine (10 μc/10^6 cells) was added, and the incubation was continued for an additional 11 hr. Cells were disrupted in RSB buffer and after centrifugation at 18,000 × g for 15 min, the cytoplasmic extract was layered over a 15 to 30% sucrose gradient prepared in RSB. Chick ribosomes were added as reference markers (74S), and the preparation was centrifuged at 25,000 rev/min for 100 min in a Spinco SW-25.1 rotor. Fractions were assayed for acid-precipitable radioactivity. In B, RNA was extracted with phenol-SDS from the 140S and 65S region of the gradient shown in A, and alcohol precipitated with unlabeled carrier ribosomal RNA. The RNA was resuspended in STB, layered over a 5 to 20% sucrose gradient prepared in STB, and centrifuged at 38,000 rev/min for 1 hr in a Spinco SW-50 rotor. Fractions were assayed for acid-precipitable radioactivity. In all figures showing sucrose gradient analysis, the bottom of the gradient is to the left, and the designations 28S and 16S refer to the optical density (260 nm) peaks of added ribosomal RNA markers.*

(28); 26S RNA is also the product of heat treatment when 42S RNA from Sindbis nucleoids prepared as described in Fig. 1 is heated under the same conditions.

It was found that the melting temperature (T_m) for Sindbis virus RNA was 65.5 C, and the hyperchromic shift observed was reversible when the RNA preparation was slowly cooled (25). Thus extensive reannealing occurred upon cooling melted Sindbis RNA. The sedimentation coefficient of the reannealed viral RNA species was not measured by other workers (25, 28). In an experiment we heated Sindbis 42S RNA to 90 C slowly (0.5 C/min in standard buffer) and let it cool slowly to room temperature. Both samples now sedimented at 26S. Thus, even under reannealing conditions, heat denaturation of 42S RNA was not reversible.

There is no change in the sedimentation coefficient when 26S RNA derived from the 65S particle is heated (Fig. 3), although again there is a slight increase in the breadth of the sedimentation profile.

Recent studies on the thermal stability of BHK-21 cell ribosomal RNA have established that 28S RNA is converted to 18S RNA after brief heating at 95 C in "salt-free solution." The sedimentation coefficient of natural 18S RNA was also reduced after heating (20). We have found that chick ribosomal RNA also undergoes

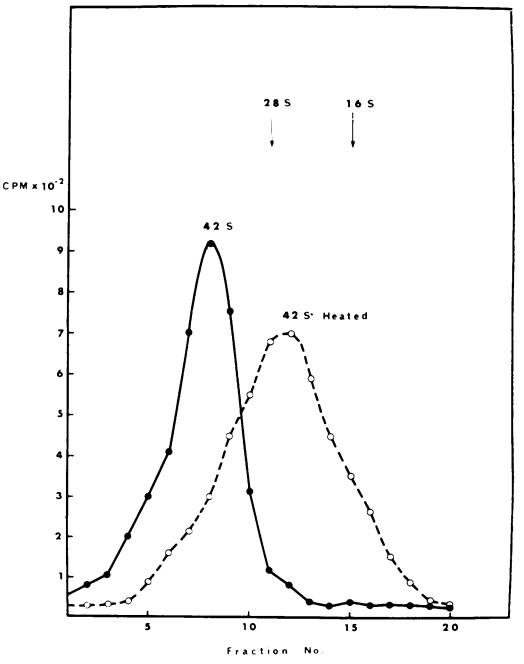

FIG. 2. *Effect of heat treatment on the sedimentation rate of viral RNA. RNA was extracted from ^{3}H-uridine-labeled virus with phenol-SDS. Heated (85 C, 3 min) and unheated samples were analyzed in a 5 to 20% sucrose gradient in STB. Sedimentation was carried out at 38,000 rev/min for 2.5 hr (Spinco SW-50 rotor). Fractions were assayed for acid-precipitable radioactivity.*

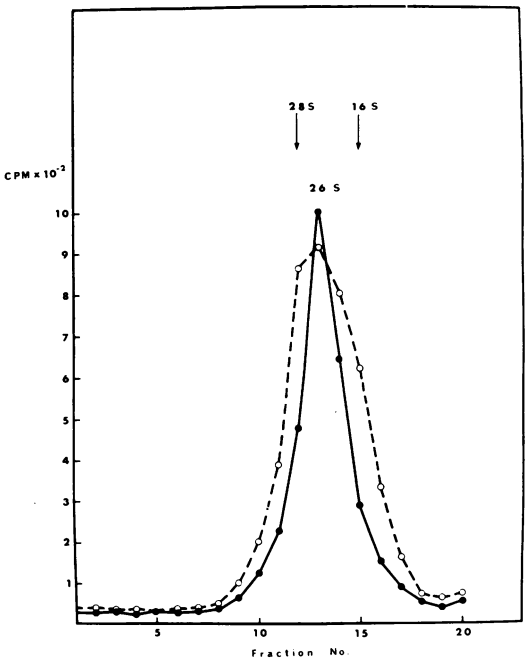

FIG. 3. *Effect of heat treatment on the sedimentation rate of "natural" 26S RNA prepared from a 65S cytoplasmic fraction. RNA was extracted from ³H-uridine-labeled 65S cytoplasmic fraction as in Fig. 1 (B). Heated (85 C, 3 min) and unheated samples were analyzed in a 5 to 20% sucrose gradient in STB by sedimentation (38,000 rev/min, 2.5 hr, SW-50 rotor). Fractions were assayed for acid-precipitable radioactivity.* (●) *Unheated RNA samples.* (○) *Heated RNA sample.*

changes in its sedimentation profile when heated in STB (Fig. 4). Sedimentation coefficients of 28*S*, 16*S*, and 4*S* were observed for untreated ribosomal RNA in standard buffer. Upon heating, the 28*S* zone was eliminated, and, instead, a new zone appeared at 15*S*, and there was a major increase in the amount of material sedimenting in the 4*S* region. There was no evidence that heating led to random nucleotide cleavage, since no fragments less than 4*S* were found after heating. Thus similar patterns of thermal lability were observed when viral 42*S* RNA and ribosomal 28*S* RNA were briefly heated and cooled.

Similar patterns of behavior between ribosomal and viral 42*S* RNA were also observed when the effect of changes in ionic strength on the sedimentation coefficients were studied (Table 1). The sedimentation coefficients of both 28*S* and 16*S* ribosomal RNA were reduced by approximately 19% in sucrose gradients containing low concentrations of salt as compared with their sedimentation characteristics in standard buffer. These changes are believed to result from modification of the conformation of the RNA molecules. It has been suggested that there is a disruption of the hydrogen bonding leading to the loss of secondary and tertiary structure of ribosomal RNA in low salt concentrations (24). Electrostatic force in the internucleotide bond becomes an

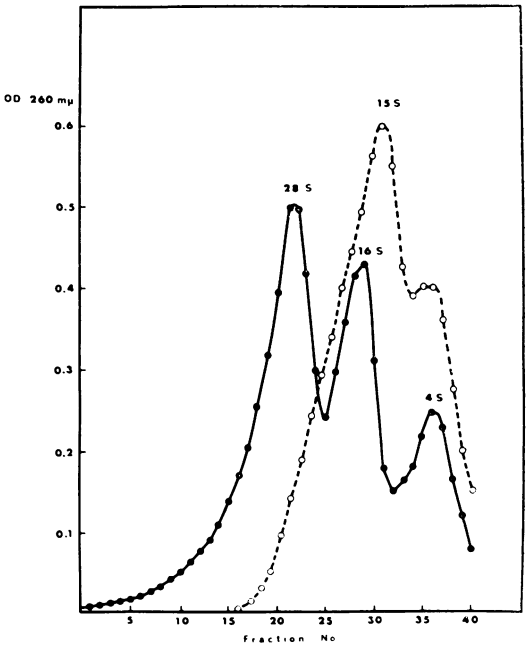

FIG. 4. *Effect of heat treatment on the sedimentation rate of ribosomal RNA. RNA was extracted from a chick ribosome preparation. Heat treatment and sedimentation were as in Fig. 3. Fractions were assayed for optical density at 260 nm.* (○) *Heated samples.* (●) *Unheated samples.*

TABLE 1. *Sedimentation characteristics of ribosomal RNA and viral RNA in low ionic strength buffers*

Sedimentation coefficient in standard buffer	Sedimentation coefficient in electrophoresis buffer	Per cent reduction
28*S* [a] Ribosomal RNA	23	19
16*S* Ribosomal RNA	13	19
42*S* Sindbis RNA	30	28
26*S* RNA derived from 42*S* Sindbis RNA	19	27
26*S* Sindbis RNA from 65*S* particles	23	10

[a] Sedimentation coefficients were obtained by comparing behavior of samples to that of 28*S* ribosomal RNA in standard buffer.

important factor in affecting the conformation at low ionic strength. The 42S viral RNA and "derived" 26S RNA underwent similar reductions of their sedimentation coefficients in low salt sucrose gradients (Table 1), whereas the reduction was much less with "natural" 26S RNA. Thus, there are differences in the conformation of 42S and "natural" 26S viral RNA species.

Acrylamide gel electrophoresis of viral and ribosomal RNA. Gel electrophoresis permits the separation of molecules on the basis of their charge and size. We used 3% gels to compare the electrophoretic mobility profiles of untreated and heated viral 42S and 26S RNA. Parallel gels were loaded with ^{32}P-labeled ribosomal RNA to serve as reference markers for electrophoretic mobility. The effect of heat on 42S RNA is illustrated in Fig. 5 and 6, and on "native" 26S RNA extracted from 65S particles in Fig. 7. The profiles in Fig. 5 illustrate the effect of heat treatment on 42S viral RNA collected from a sucrose gradient which contained the low ionic strength EPB. The unheated 42S viral RNA was seen to migrate as a single homogeneous fraction. Upon heating 42S viral RNA in EPB and applying the sample to a column, the electropherogram illustrates that the original material was converted to a heterogeneous product in which the components moved at higher mobility than the unheated 42S viral RNA. Fig. 6 illustrates the profiles resulting when viral RNA sedimenting at 42S on gradients containing STB was applied to gels. Here the untreated viral RNA appeared to contain a small proportion of fast-running components, so that a single sharp profile was not observed. When heated in STB the 42S RNA was again converted to a heterogeneous population of RNA species. The electropherogram of untreated and heated "native" 26S RNA derived from 65S particles is shown in Fig. 7. Note that the untreated RNA which sediments in gradients as a homogeneous band (Fig. 3) is heterogeneous when examined on the gels. It is striking that there was no major qualitative change in the electrophoretic mobilities of the principle components of 26S RNA after heating to species of RNA possessing slower or faster electrophoretic mobilities.

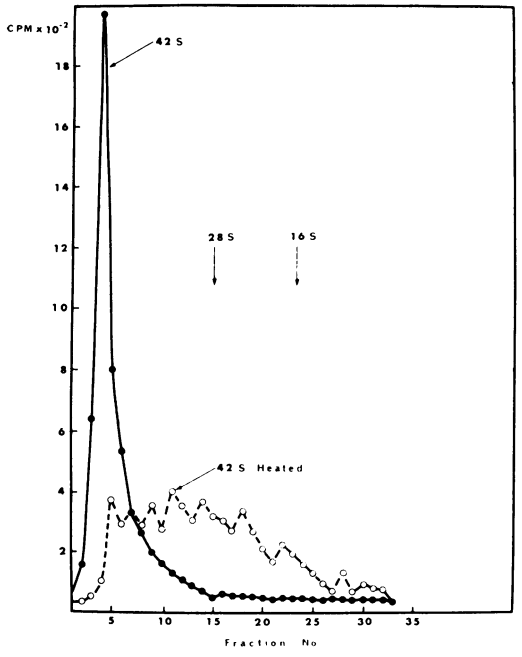

FIG. 5. *Effect of heat treatment on the electrophoretic behavior of viral RNA in polyacrylamide gel. RNA was extracted from ^{32}P-labeled virus with phenol-SDS and dissolved in EPB. Heated (85 C, 3 min) and unheated samples (10 to 30 μliters) were layered onto 3% gel columns (6 cm), and electrophoresis was carried out at room temperature for 2.5 hr (8 v/cm). After fixation in 7% acetic acid (15 min), the gels were sliced and the gel slices were placed on aluminium planchets and air dried. Radioactivity was counted in a gas flow counter. In all figures showing electrophoretic profiles, the cathode is to the left. The arrows indicate the position of ^{32}P-labeled 28S and 16S ribosomal RNA, run in parallel gels, which serve as reference markers.*

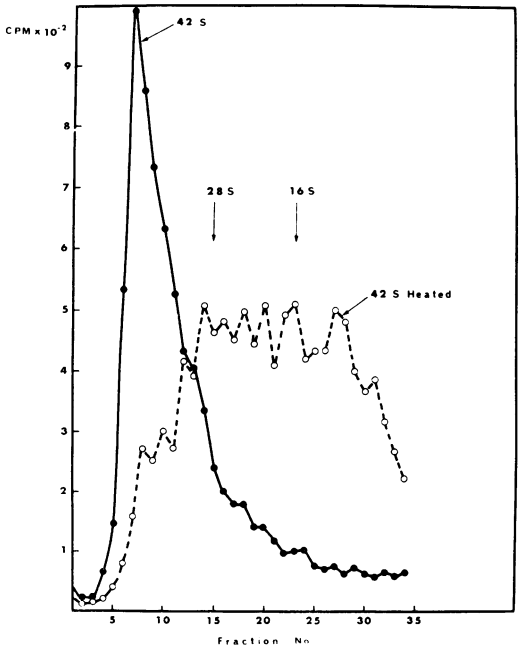

FIG. 6. *Effect of heat treatment on the electrophoretic behavior of viral RNA in polyacrylamide gel. RNA was extracted from ^{32}P-labeled virus with phenol-SDS and dissolved in STB. Conditions of heating and gel electrophoresis were the same as in Fig. 5.*

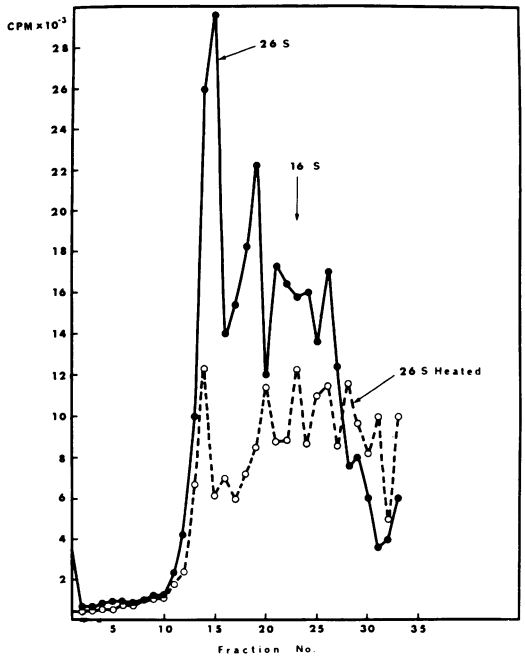

FIG. 7. *Effect of heat treatment on the electrophoretic behavior of "native" 26S RNA prepared from the 65S cytoplasmic fraction. RNA was extracted from ^{3}H-uridine-labeled cytoplasmic fractions as in Fig 1B. Heated (85 C, 3 min) and unheated samples in EPB were layered onto a 6-cm long, 3% gel column, and electrophoresis was carried out at room temperature for 2.5 hr (8 v/cm). After fixation in 7% acetic acid (15 min), the gels were sliced, and the slices were digested with 30% H_2O_2 at 60 C for 2 to 3 hr. Radioactivity was counted in a liquid scintillation counter. (●) Unheated RNA sample. (○) Heated RNA sample.*

Digestion of ribosomal RNA and 42S viral RNA with snake venom phosphodiesterase. The conversion of 42*S* viral RNA to 26*S* RNA by heating and the resulting changes in electrophoretic mobilities can be looked upon either as brought about by extreme conformational changes in RNA structure or to changes which resulted from a deaggregation or a cleavage of the RNA molecule. To help distinguish between these possibilities, viral RNA was treated with exonuclease, snake venom phosphodiesterase. In parallel experiments ribosomal RNA was digested with the enzyme. The enzyme cleaves nucleoside 5′ phosphates from the 3′ end of single- and double-stranded RNA and deoxyribonucleic acid. Incubations were carried out under conditions of enzyme excess, so that doubling the concentration of enzyme had no effect on the rate of hydrolysis of either viral or ribosomal RNA. Thus, all 3′ termini of the RNA species being examined were available to the enzyme at the beginning of the incubation, and the rate of release of acid-soluble nucleotides was proportional to the concentration of polynucleotides. The data in Fig. 8 show the effect of heating on the rate of hydrolysis of 42*S* viral RNA and ribosomal RNA. It will be noted that the rate of release of acid soluble nucleotides was linear with time in all cases. Approximately 4% of the unheated and 8% of heated viral and ribosomal RNA were digested after 40 min of incubation. Thus, heat treatment had made available approximately twice as many 3′ termini in both RNA species.

DISCUSSION

The structural relationships between Sindbis 42*S* viral RNA, the "derived" 26*S* RNA obtained by heating 42*S* RNA, and "natural" 26*S* RNA extracted from 65*S* particles are considered in this discussion. A shift to a lower sedimentation coefficient was demonstrated when solutions of RNA from WEE (28), SFV (13), Rauscher mouse leukemia virus (3), RSV (6), or Avian Myoblastosis virus (9) were heated or when ribosomal RNA from BHK-21 cells was heated (20). These changes in sedimentation coefficients upon heating were considered to result from extreme conformational changes or from cleavage or deaggregation of RNA or a combination of these processes. No measurements of the molecular weights of the derived RNA were reported. The molecular weight of the viral genome in group A arboviruses is not well established. Only in the case of Eastern equine encephalomyelitis have biophysical studies based on determination of sedimentation coefficients and specific viscosities led to an estimated molecular weight of 2×10^6 (30). Thus we find that authors assume that the molecular weight of RNA in group A arboviruses varies from 2×10^6 to 3×10^6 with little direct evidence. An unpublished report puts the molecular weight of Sindbis RNA at 4×10^6 to 5×10^6 (29). Knowledge of the molecular weight of the viral genome is important in attempting to evaluate the relationship between Sindbis 42*S* RNA and the conversion product, "derived" 26*S* RNA. Since no other RNA is found in gradients (Fig. 2, ref. 28), it has been suggested that one heat-labile cleavage point exists near the center of the viral genome. The assumption requires evidence that no major conformation changes occur in the secondary structures of 42*S* and "derived" 26*S* RNA. In favor of this, the melting curves of Sindbis and WEE RNA are known to be reversible (25, 28). If major differences were to occur in the proportion of hydrogen bonds upon reannealing, it is unlikely that the melting curve would be identical to the reannealing curve. Further information on the conformation of viral RNA molecules is provided in our

studies on the effect of changes in ionic strength on the sedimentation coefficient (Table 1). Both 42*S* viral RNA and "derived" 26*S* RNA underwent a similar reduction in sedimentation coefficient when transferred to low ionic strength buffer (29%), whereas ribosomal 28*S* RNA and 16*S* RNA each were reduced by 19%. We interpret these similar reductions of viral RNA on the one hand and ribosomal RNA on the other hand as indicating that 42*S* viral RNA and "derived" 26*S* RNA molecules are relaxed to a similar extent at the lower molarity just as are 28*S* ribosomal RNA and 16*S* ribosomal RNA (24). The reduction of sedimentation coefficient of "natural" 26*S* RNA in low ionic strength buffer was only 10%, which suggests that its conformation is different from that of "derived" 26*S* RNA. It is also possible that "natural" and "derived" 26*S* RNA represent different aggregates of fragments of RNA, so that the sedimentation coefficient which represents an average distribution is different in the two instances.

Upon heating, chick ribosomal 28*S* RNA was converted to 15*S* RNA (Fig. 4). A similar result was observed when BHK 28*S* RNA was heated in "salt free solutions" (20). At *p*H 9, *E. coli* 23*S* ribosomal RNA is converted to 16*S*, and this conversion is accompanied by an increase in the number of periodate reactive groups (21). It was suggested that 23*S* RNA may possess two periodate oxidizable groups per molecule, one of which is masked in the intact molecule but is freed and available for periodate oxidation by mild alkaline treatment of the RNA. Recently it has been found that 28*S* RNA from animal cells is a complex between a large molecule (nominally 28*S*) and a small molecule (nominally 7*S*). The 7*S* fragment is released by treatments designed to break hydrogen bonds (22). Our experiments on the action of phosphodiesterase on 42*S* viral RNA demonstrate an analogous behavior of viral 42*S* and ribosomal 28*S* RNA. Under conditions of enzyme excess, the proportionate release of acid-soluble nucleotides from viral and ribosomal RNA species was almost identical. The conformation of liver ribosomal RNA does not affect the affinity of venom phosphodiesterase for its substrate. Thus, similar rates of hydrolysis were observed when RNA was dissolved in distilled water or in 0.1 M sodium chloride (15). Upon heating, we observed a doubling in the concentration of 3′ termini with both viral and ribosomal RNA (Fig. 8). This is consistent with the interpretation that both viral 42*S* RNA and ribosomal 28*S* RNA consist of a complex of two subunits or a multiple of two subunits. In the case of ribosomal RNA, these were nominal 28*S* and 7*S* molecules. In the untreated condition, one of the two 3′ termini was

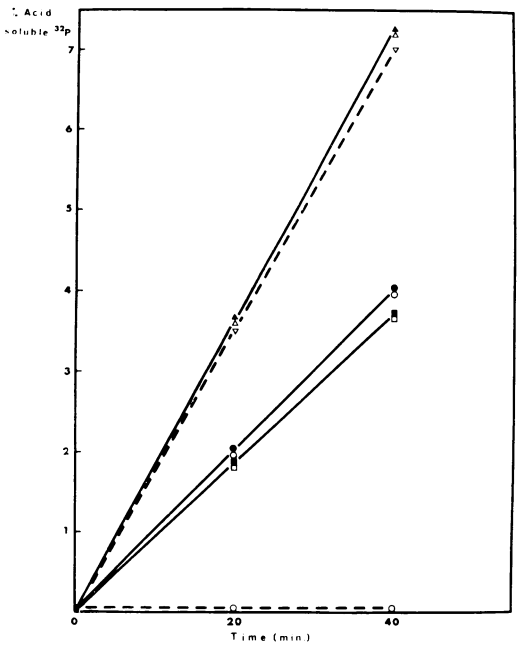

FIG. 8. *Rate of release of acid-soluble* ^{32}P *from labeled 42S viral RNA and 28S ribosomal RNA, before and after heating, by snake venom phosphodiesterase. Heated (85 C, 3 min) and unheated* ^{32}P*-labeled viral 42S and ribosomal 28S RNA was digested with SVP at 37 C for 40 min. At intervals samples were removed from the incubation mixture, and the enzyme reaction stopped by placing the samples in an ice bath. Beef serum albumin (5 mg/ml) was added to serve as carrier and macromolecular RNA was precipitated by the addition of ice cold perchloric acid. The preparation was centrifuged at 10,000 rev/min for 10 min (Spinco SW-50 rotor), and the acid-soluble radioactivity in the supernatant fraction was counted in a liquid scintillation counter. Symbols:* ●, *with solid line, 42S RNA and 0.25 mg of SVP;* ○, *with solid line, 42S RNA and 0.125 mg of SVP;* ■, *28S RNA and 0.25 mg of SVP;* □, *28S RNA and 0.125 mg of SVP;* ▲, *42S RNA heated and 0.25 mg of SVP;* △ *with solid line, 42S heated and 0.125 mg of SVP;* △ *with broken line, 28S RNA heated and 0.25 mg of SVP;* ○ *with broken line, either 42S or 28S RNA and buffer without SVP (control).*

available to the diesterase and the second was masked. Upon heating, the second 3′ terminus became available for digestion. The size of the subunits of 42*S* RNA released upon heating is not known. However, because the products of heating migrated more rapidly on acrylamide gels than unheated 42*S* RNA, it is likely that deaggregation or cleavage occurred.

Two possibilities concerning the structure of 42*S* RNA are considered. (i) The subunits may represent an aggregate, joined by means of hydro-

gen bonds to form a complex; (ii) a heat-labile covalent bond of unknown type may link viral RNA subunits. The first possibility would account for the results of the enzyme digestion experiment only if unheated RNA had a conformation which resulted in a masking of one-half of the 3′ termini within the complex. Upon heating, the subunits would separate, and the second half of the 3′ termini would be available for digestion. If there is no masking of 3′ termini and the subunits overlap, phosphodiesterase would have digested the untreated viral RNA at the same rate as the heated RNA, since the enzyme digests both single-stranded and double-stranded polynucleotide chains. We assume here that snake venom phosphodiesterase has the same order of affinity for both single- and double-stranded RNA. If, however, the enzyme has a markedly lower affinity for double-stranded RNA than for single-stranded RNA, it is conceivable that heating may unfold a terminal region of 42*S* RNA and result in an increased reaction rate with the enzyme. The second suggestion (ii) would only apply if there were no masking of 3′ termini in unheated 42*S* RNA. In this case it is unlikely that the linkage between the subunits is a 3′ to 5′ phosphodiester, since this type of bond would be unaffected by the heating conditions. Thus, although the enzyme experiments indicate a doubling of 3′ polynucleotide termini upon heating viral RNA, it is not possible to distinguish between the two postulated structures, because it is not known whether under the experimental conditions the conformation of RNA denied access to the enzyme.

Sindbis 42*S* RNA moved as a single fraction in polyacrylamide gels. After heating, either in buffers of moderate ionic strength (100 to 150 mM) or in electrophoresis buffer (approximately 7 mM), 42*S* viral RNA was converted to heterogeneous RNA species (Fig. 5 and 6). This behavior is similar to that observed with RNA from RSV, Rauscher mouse leukemia, and avian myoblastosis viruses but different from TMV or NDV RNA which are unaffected by heat (3, 6, 7). "Derived" 26*S* RNA which is homogeneous in sucrose gradient is seen as a heterogeneous population in polyacrylamide gels. Whether this heterogeneity represents species of RNA differing in both charge and size is not clear, since no information on the effect of conformation on the electrophoretic mobilities of these viral RNA species is available. The electropherograms of "derived" 26*S* RNA (Fig. 5 and 6) and "natural" 26*S* RNA (Fig. 7) bear striking qualitative similarities. Both are heterogeneous and the pattern is basically unaltered upon heating "natural" 26*S* RNA. We suggest that "natural" 26*S* RNA derived from the 65*S* particle represents a population of RNA molecules which may differ in size and shape or in both of these properties. The appearance of such a population may of course have been due to artifacts induced in the extraction process. This is unlikely, since the methods of extraction were identical to those which permitted a homogeneous pattern to be observed when 42*S* RNA from virions was electrophoresed. It is unlikely that electrophoresis per se degraded 26*S* RNA. In support of this, it has been shown that when a peak fraction of Qβ RNA was reelectrophoresed, 95% of the radioactivity appeared in a peak of similar mobility (2).

Both 26*S* RNA and 42*S* RNA are readily demonstrated in cells infected with group A arboviruses, but it is not yet established whether 26*S* RNA is a precursor of 42*S* RNA. The 26*S* RNA was the sole ribonuclease-sensitive RNA detected in cells briefly pulsed (3 min) with ^{3}H-uridine at 6 hr postinfection with WEE virus. Longer pulses showed the label also entered 40*S* WEE RNA (26). Similar results are reported for Semliki Forest virus infected cells (12). Successful pulse chase experiments have not been reported so the possibility remains that 26*S* RNA is not the precursor of 42*S* viral RNA. At present, we consider "natural" 26*S* RNA and 26*S* RNA "derived" from 42*S* viral RNA as separate entities, although their behavior in the sedimentation experiments and on polyacrylamide gels after heating indicates a basic similarity in size and structure.

ACKNOWLEDGMENT

This work was supported by a grant from the Medical Research Council of Canada.

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