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Replication of Western Equine Encephalomyelitis Virus

I. Some Chemical and Physical Characteristics of Viral Ribonucleic Acid[1]

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The ribonucleic acid (RNA) from Western equine encephalomyelitis (WEE) virions sedimented through sucrose gradients with a sedimentation coefficient of 40*S*. Another viral RNA which was always associated with infected cells possessed a sedimentation coefficient of 26*S*. Both 40*S* and 26*S* RNA had identical base compositions and densities. The 40*S* RNA displayed a hyperchromic effect when heated with a T_m of 57.5 C. When 40*S* RNA was heated at 90 C and cooled rapidly, it sedimented with a coefficient of 26*S*. Dialysis of 40*S* RNA against distilled water changed its sedimentation coefficient to 26*S*. The presence of 8 M urea or 50% dimethyl sulfoxide in the gradients also altered the sedimentation rate of 40*S* RNA to 26*S*. In the latter case, the 26*S* RNA retained 10% of the infectivity originally added as 40*S* RNA. Dialysis of 26*S* RNA against 0.5 M NaCl or 0.05 M acetate buffer at *p*H 4.0 altered it so that about 50% of the radioactivity sedimented with a coefficient of 40*S*. Chromatography on methylated albumin-kieselguhr columns failed to separate 40*S* RNA from 26*S* RNA. Viral RNA either exists in two conformations which sediment differently in sucrose or contains an extremely labile portion near the center and is easily broken into two equal pieces.

We reported previously that three distinct types of viral ribonucleic acid (RNA) were obtained from chick embryo cells infected with Western equine encephalomyelitis (WEE) virus (12). These three forms of RNA were differentiated by their sedimentation coefficients in sucrose gradients, i.e., 40*S*, 26*S*, and 15*S*, respectively. None of the three types of RNA was resistant to ribonuclease. The RNA sedimenting as 40*S* accounted for 99% of the total infectivity.

Species of viral RNA with similar properties have been reported to be associated with the replication of Sindbis and Semliki Forest viruses, group A arboviruses (2, 5). RNA with characteristics similar to those of the 26*S* type has been found in hamster kidney cells infected with foot-and-mouth disease virus, in *Escherichia coli* infected with MS2 phage, and in encephalocarditis virus-infected ascites tumor cells (1, 3, 8).

This communication deals with the possibility that the 40*S* and 26*S* types of WEE virus RNA differ only in secondary structure.

MATERIALS AND METHODS

Cells and virus. Primary cultures of chick embryo cells were prepared and grown in Eagle's medium with 3% calf serum. The source and preparation of WEE virus were as already described (12).

The methods used to infect the cells and to label the viral RNA were identical to those described previously (12).

Chemicals and isotopes. Actinomycin D was generously provided by Merck, Sharp, and Dohme, West Point, Pa. The drug was used at a concentration of 5 μg/ml. Tritiated and ^{14}C-labeled uridine were purchased from Nuclear-Chicago Corp., Des Plaines, Ill. ^{32}P in the orthophosphate form was obtained from New England Nuclear Corp., Boston, Mass. Dimethyl sulfoxide (DMSO), spectral quality, was obtained from Matheson, Coleman, and Bell, East Rutherford, N.J. An Ansitron liquid scintillation counter was used to determine radioactivity.

Sucrose velocity gradient analysis. A 0.5-ml sample of the RNA was placed on a 4.5-ml sucrose gradient (5 to 20% sucrose) in a buffer solution (STE) con-

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taining 0.1 M NaCl, 0.001 M ethylenediaminetetraacetate (EDTA), and 0.05 M tris(hydroxymethyl)-aminomethane (Tris), *p*H 7.4. The gradients were centrifuged at 40,000 rev/min for 2.5 hr at 12 C in a Spinco SW50 rotor. After centrifugation, approximately 0.2-ml samples were collected. The RNA was precipitated by adding 0.1 mg of bovine serum albumin and 0.2 ml of 10% trichloroacetic acid. The precipitates were collected on membrane filters (Millipore Corp., Bedford, Mass.) and were washed three times with 10% trichloroacetic acid. The membranes were then dried, and the dried filter was counted in 10 ml of 2,5-diphenyloxazole-1,4-bis(5-phenyloxazo-2-yl)benzene (PPO-POPOP) with toluene in an Ansitron scintillation counter. RNA for preparative purposes was centrifuged through a 5 to 20% sucrose gradient in a Spinco SW25.1 rotor.

Isopycnic centrifugation in cesium sulfate solution. Cs_2SO_4, optical grade, was obtained from S. H. Cohen, Yonkers, N.Y. Solutions of Cs_2SO_4 in STE ($\rho = 1.60$) containing not more than 50.0 μg of RNA in a volume of 5 ml were centrifuged at 33,000 rev/min for 120 hr in a Spinco model L centrifuge with a SW39 rotor at 5 C. Addition of RNA at concentrations higher than 50 μg to cesium sulfate caused the RNA to be precipitated. The bottom of the centrifuge tube was punctured, fractions were collected, and the refractive index and radioactivity of each fraction were determined. Density was calculated from the refractive index, according to the method of Vinograd and Hearst (14).

Isolation and purification of viral RNA. RNA was isolated from either infected cells or virus by extraction with phenol and sodium dodecyl sulfate (SDS) at 60 C (9). Both labeled 40*S* and 26*S* viral RNA were prepared as follows. The labeled RNA obtained by phenol extraction was precipitated with alcohol, and the precipitate was dissolved in 0.01 M acetate buffer (*p*H 5.0) containing 20 μg of polyvinyl sulfate per ml. Unlabeled mouse cell ribosomal RNA was added (1 mg/ml) as a carrier. An equal volume of 4 M lithium chloride was added, and the RNA was allowed to precipitate at −20 C. overnight. The precipitate was recovered by centrifugation at 27,000 × *g* for 10 min. The precipitate was dissolved in STE buffer, and the RNA was centrifuged on sucrose velocity gradients (5 to 20%). Fractions were collected, and the radioactivity of samples was determined. Fractions containing the peaks of radioactivity were pooled separately, and the RNA was precipitated with 2 volumes of alcohol. The precipitated RNA was dissolved in STE buffer and tested for homogeneity by sucrose velocity gradient analysis. Samples of the RNA were then placed in screw-capped vials and stored at −70 C.

Base composition of viral RNA. Forty-eight-hour-old primary chick embryo cells were starved for phosphate by incubating them with phosphate-free Eagle's medium for 8 hr. The cultures were incubated with 3 ml of Eagle's medium containing 5 μg of actinomycin D per ml for 2 hr and then infected with virus. The cultures, after infection, received Eagle's medium with 10 μc of carrier-free ^{32}P-orthophosphate per ml. The cultures were harvested after 12 hr of incubation, and the RNA was extracted and purified. The species of viral RNA isolated according to the methods described above were mixed with 1 mg of mouse ribosomal RNA as carrier, dissolved in 0.3 N KOH, and hydrolyzed for 18 hr at 37 C. The hydrolysate was processed according to the method of Katz and Coomb (7). The nucleotides were separated by chromatography on Dowex columns as described by Katz and Coomb. The base composition was determined by the relative amounts of isotope in each nucleotide.

Chromatography on methylated albumin columns. Chromatography on methylated albumin columns was done according to the method described by Sueoka and Cheng (13). Columns containing 5 mg of methylated albumin adsorbed to 2 g of kieselguhr were used. The elution of the RNA was carried out by use of a linear gradient of NaCl produced by mixing 88 ml of 0.4 M NaCl solution with 88 ml of 1.3 M NaCl solution, both containing 0.05 M phosphate buffer (*p*H 6.90). The concentration of NaCl in the fractions was determined from the refractive index of the fractions.

Concentration and partial purification of labeled WEE virus. WEE virus containing ^{3}H-uridine in its RNA was prepared by infecting several chick embryo cell cultures which had been incubated previously with actinomycin D. The infected cultures were incubated with Eagle's medium containing 2.5 μc of ^{3}H-uridine per ml and 1% dialyzed calf serum. The supernatant medium containing the mature virus particles released from infected cells was harvested 12 hr after infection. The virus in the fluid was precipitated with ammonium sulfate (50% final saturation) at 4 C and at *p*H 7.3, since preliminary experiments showed that the infectivity of WEE virus was destroyed during precipitation with ammonium sulfate at *p*H below or above 7.3 (D. Hill, *personal communication*). The precipitate was collected by centrifugation in a refrigerated Sorvall centrifuge at 11,700 × *g* and suspended in 1 to 2% of the original fluid volume in 0.05 M borate buffer (*p*H 9.0) containing 0.12 M NaCl (borate saline). The ammonium sulfate was removed by dialysis against borate saline for 12 hr at 4 C. The recovery of virus achieved by this step was 100% when measured in terms of infectivity. The labeled virus concentrate (25 ml) was overlaid onto 5 ml of a solution of Cs_2SO_4 and sucrose (15% Cs_2SO_4 + 60% sucrose in 0.01 M Tris buffer, *p*H 9.0, with 0.12 M NaCl) in a 34-ml centrifuge tube. This formed a dense interface upon which the virus collects. The mixture was centrifuged at 22,500 rev/min for 2.5 hr at 4 C in a Spinco SW25.1 head. Fractions of 1 ml were collected, assayed for infectivity, and counted for radioactivity. Single fractions, which contained both the maximal infectivity and radioactivity, from each of several similar preparations were pooled and dialyzed against borate saline. The nondialyzable portion was layered onto a 5 to 35% sucrose gradient (in borate saline) and centrifuged at 22,500 rev/min for 2 hr in a SW25.1 head. Fractions (1 ml) were collected and tested for infectivity and radioactivity. The fraction containing both the maximal infectivity and radioactivity represented the partially purified preparation

of virus. The overall recovery in terms of infectivity and radioactivity was 60%.

RESULTS

The three types of viral RNA reported in the previous experiments (12) had been obtained from suspensions of infected cells which included the medium and, therefore, mature WEE virus particles. We wished to determine which type or types of RNA are present in mature virus particles.

Tritium-labeled virus was concentrated and purified as described in Materials and Methods. After the second centrifugation, which was on a 5 to 35% sucrose gradient, the fraction which contained the maximal radioactivity and infectivity was selected. It was dialyzed against STE buffer, and the nondialyzable portion was extracted with phenol and SDS at 60 C, as described above. The RNA obtained was analyzed by centrifugation through a sucrose velocity gradient (Fig. 1). The distribution of the label in the gradient showed the presence of a well-defined peak with a sedimentation coefficient of 40S. There was, however, a large trail of radioactivity behind the main peak of the label. We have determined in other experiments that the above trailing of radioactivity was not due to the methods of extraction and centrifugation used. The significance of the presence of this heterogeneity in the viral RNA from the virions is not clear at present. It was deduced that the 26S and 15S types of RNA must be associated with the cells, since most of the RNA present in the virion was of the 40S type. To prove this, the following experiment was done.

Several cultures of chick embryo cells were incubated with actinomycin D and then infected with WEE virus as described earlier. Eagle's medium containing ^{3}H-labeled uridine (10 μc per culture) was added to the cultures, and they were incubated for 12 hr and harvested as follows. One group of cultures was harvested by scraping the cells into the overlying medium. The medium only was removed from another group of cultures and pooled. It should represent mainly free mature virus released from the cells. After the medium was removed, the remaining cells were washed once with cold saline, scraped into cold Eagle's medium, centrifuged, and suspended in fresh medium. RNA was extracted by the phenol-SDS technique at 60 C from (i) infected cells and medium, (ii) infected cells alone, and (iii) medium alone. The nucleic acids obtained were analyzed by sucrose gradient velocity centrifugation (Fig. 2). All three types of virus-specific RNA were present in the nucleic acid from infected cells and medium (Fig. 2a). The nucleic

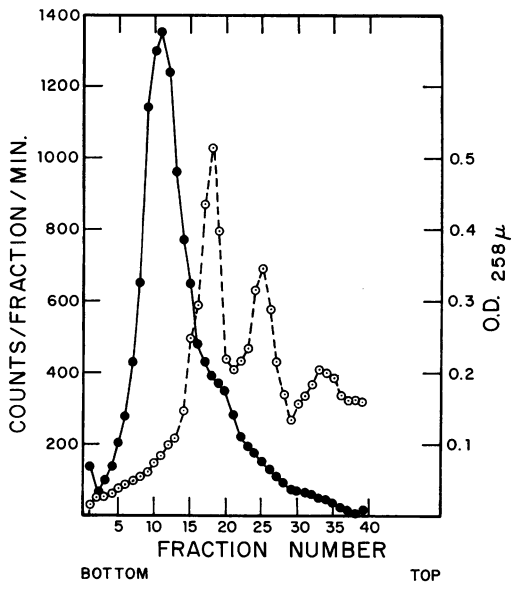

FIG. 1. *Sucrose gradient profile of RNA obtained from ^{3}H-uridine-labeled WEE virus. The details of preparing partially purified virus are described in Materials and Methods. RNA from unlabeled chick embryo cells was added to the gradient as sedimentation marker. Symbols:* ○, *optical density;* ●, *radioactivity.*

acids from infected cells (Fig. 2b) contained 26S and 15S RNA and a small amount of 40S RNA. The RNA from the medium containing the released virus sedimented as 40S RNA (Fig. 2c) and contained neither 26S nor 15S RNA. This was expected, since it is known that WEE virus is released into the medium soon after it is formed inside the cell. The previous experiment had shown that the RNA found in mature virus particles was the 40S type. Since the 40S RNA contains most of the infectivity and is found in mature virus particles, it is assumed to represent the final form of the RNA necessary for the formation of virus. We attempted to elucidate further the nature and function of the 26S type of viral RNA.

Base composition of the viral RNA. Purified samples of ^{32}P-labeled 40S and 26S viral RNA were prepared by the procedures outlined earlier. The base composition of each type of RNA was determined. The results (Table 1) show that base compositions of both types of RNA were essentially the same. Both types of RNA possessed a higher adenine content than the other three bases. This indicated that the 26S type of RNA is not a complementary strand of the 40S RNA, since the expected composition of such a strand should include a high uracil content.

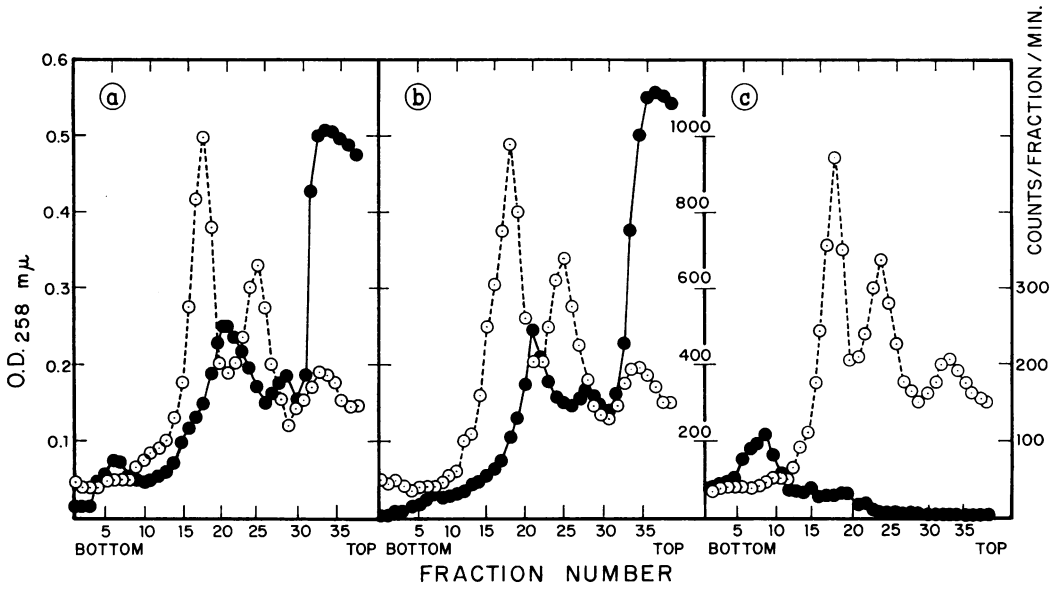

FIG. 2. *Sucrose gradient centrifugation of ^{3}H-uridine-labeled RNA from WEE virus-infected cells. (a) RNA obtained from infected cells and medium; (b) RNA obtained from infected cells only; (c) RNA obtained from medium. RNA from unlabeled chick embryo cells added as sedimentation markers. Symbols:* ○, *optical density;* ●, *radioactivity.*

TABLE 1. *Base ratio analysis of viral RNA*[a]

Base	40S RNA	26S RNA
Cytosine	25.3 ± 0.2	24.2 ± 0.9
Adenine	29.6 ± 0.8	29.2 ± 1.2
Uracil	22.7 ± 0.3	22.3 ± 0.4
Guanine	22.3 ± 0.9	24.3 ± 1.7

[a] All values given are the mean and the standard deviation of the mean calculated from four separate determinations on three different preparations of each type of viral RNA. The methods of preparation and purification of the ^{32}P-labeled viral RNA are described in Materials and Methods. The base ratios were determined by the method of Katz and Coomb (7).

Buoyant density of the 40S and 26S RNA. Purified tritium-labeled 40S RNA and ^{14}C-labeled 26S RNA were mixed and centrifuged in cesium sulfate as described above (Fig. 3). Both the ^{3}H-labeled and ^{14}C-labeled RNA formed only one peak with a density of 1.66. As expected from their base compositions, the buoyant densities of the two kinds of RNA are the same.

From the results described so far, the following conclusions can be drawn. The viral RNA in mature virus particles is of the 40S type. Infected cells contain, in addition to a small amount of the 40S type, both the 26S and 15S type of RNA. The 40S and 26S types of RNA possess the same

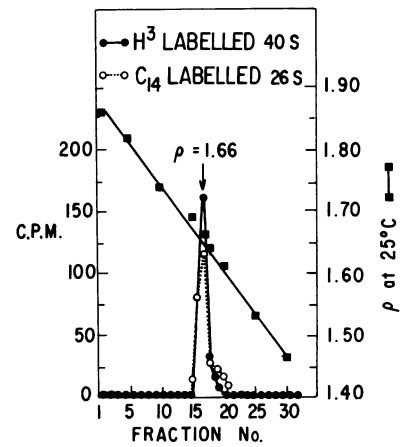

FIG. 3. *Equilibrium centrifugation of 40S and 26S viral RNA in Cs_2SO_4. Purified ^{3}H-labeled 40S RNA and ^{14}C-labeled 26S RNA were combined and then mixed with cesium sulfate solution as described in the text. The bottom of the gradient is to the left of the figure.*

chemical composition and density, though they have different sedimentation rates in sucrose gradients. The apparent difference in the sedimentation rates of two molecular species of RNA possessing similar chemical compositions has to be based on either their size and molecular weights or their conformations in solution. The

following experiments were therefore performed to decide between these possibilities.

Hyperchromicity of 40S RNA. Sprecher-Goldberger (11) reported recently that Sindbis virus RNA probably possesses some secondary structure. Sindbis virus RNA displayed a hyperchromic effect on heating; thus, Sprecher-Goldberger concluded that Sindbis virus RNA possessed more secondary structure than the RNA of many other viruses. Therefore, the hyperchromic effect of the 40S RNA on WEE virus was studied. The 28S ribosomal RNA from L (mouse) cells was included for comparison. Inability to prepare a sufficient amount of purified 26S type of viral RNA prevented our studying its hyperchromic effect. The hyperchromicity of the samples at 258 mμ was recorded continuously, by use of a Beckman-Gilford spectrophotometer. The system was heated by circulating glycerol from a Haake water bath through the inner set of Beckman Dual Thermospacers. The temperature of the samples in the cuvettes was recorded directly by a thermocouple. The temperature was raised slowly (0.5 C at a time) and kept at each temperature until there was no further increase in the optical density of the RNA at 258 mμ. The RNA was cooled to room temperature by switching off the water bath.

Figure 4 shows the results of one such experiment. The optical density increased 32% when WEE virus RNA was heated, and increased sharply between 55 and 60 C. The T_m of the 40S RNA was calculated to be 57.5 C. In contrast, the optical density gradually increased about 15% when mouse ribosomal RNA was heated. Both the RNA samples regained their original optical density on cooling, which demonstrated that the change in absorbance was not due to hydrolysis of the RNA during heating. If hyperchromicity of single-stranded RNA molecules results from the transition of the bases to a less orderly relationship, WEE virus RNA must possess more secondary structure than mouse ribosomal RNA and may even exist as a highly coiled structure.

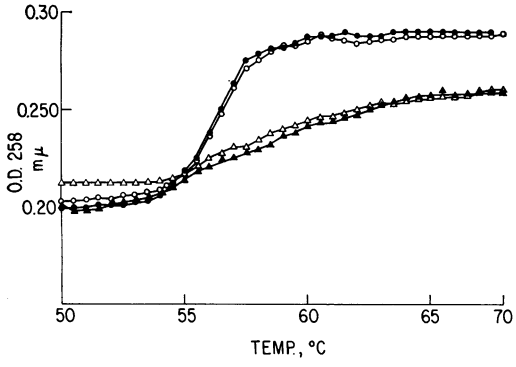

FIG. 4. *Influence of temperature on the optical density of 40S viral RNA and 28S ribosomal RNA. Approximately 10 μg of both types of RNA was dissolved in 1 ml of 1 × SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). The samples were heated and cooled as described in Materials and Methods. Absorbance at 258 mμ was continuously recorded. Symbols: 40S viral RNA; ○, 40S viral RNA, slow cooled; ▲, 28S ribosomal RNA; △, 28S ribosomal RNA, slow cooled.*

It was thus interesting to see what effect thermal denaturation had on the sedimentation rate of 40S RNA. Therefore, 40S RNA was heated at 90 C for 5 min and quickly cooled. The sedimentation rates of both heated and unheated 40S RNA were compared (Fig. 5). The sedimentation rate of the 40S RNA was reduced to 26S, and almost 99% of the infectivity was lost. It is noteworthy that there was no significant breakdown of the RNA into low molecular weight RNA.

Elution profile of 40S and 26S RNA from a methylated albumin-kieselguhr (MAK) column. Chromatography of RNA on MAK columns purportedly separates the molecules according to size and base composition. Since the base ratio analyses of the 40S and 26S RNA showed that they possessed identical compositions, it was ex-

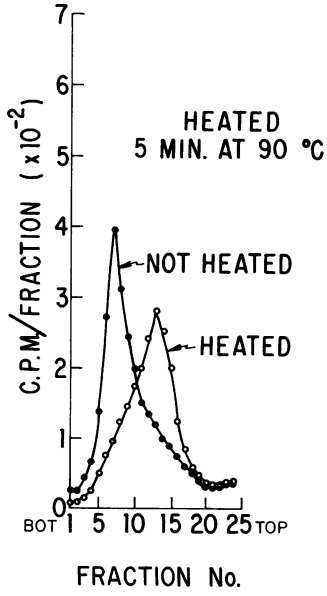

FIG. 5. *Effect of heat on the sedimentation rate of 40S RNA. ^{3}H-labeled 40S viral RNA in STE buffer was heated at 90 C for 5 min and quickly cooled in ice water for 3 min. Both heated and unheated 40S RNA were layered on separate 5 to 20% sucrose gradients in STE buffer. Centrifugation was at 40,000 rev/min in an SW50 rotor for 2.5 hr at 4 C.*

pected that they would be separated on an MAK column if they differed in size. A mixture of ^{14}C-uridine-labeled 40S and ^{3}H-uridine-labeled 26S RNA was added to and eluted from an MAK column. As shown in Fig. 6, both types of RNA eluted from the column at the same concentration of NaCl (viz., 0.96 M). Double-stranded RNA (20S) from WEE-virus-infected cells eluted from the MAK column at 0.74 M sodium chloride. Also, the elution profile of 40S RNA when thermally denatured at 90 C for 5 min was essentially the same as that of native 40S RNA, except that the peak was broader. Chromatography on a MAK column failed to demonstrate any difference between 40S and 26S viral RNA, whether the latter was newly formed or denatured 40S RNA. The previous experiment demonstrated that 40S RNA, on denaturation, sedimented as 26S RNA on a sucrose gradient. This experiment suggests that the native 40S and 26S RNA may not differ in size or molecular weight.

Spirin (10) proposed that in low to moderate ionic strength solvents RNA may possess an asymmetric rodlike structure. These rodlike structures apparently could be transformed to highly compact coils by increasing the salt concentration. The sedimentation profiles of ribosomal RNA and single-stranded viral RNA depend upon the ionic concentration of the solvents used (4). Therefore, 40S and 26S RNA were exposed to conditions of low ionic strength to see whether such treatment influenced their sedimentation rates. Each type of RNA was dialyzed for 16 hr against 1,000 volumes of sterile distilled water containing 20 μg of polyvinyl sulfate per ml. The resulting preparations of RNA were sedimented on a sucrose gradient prepared in STE buffer. Control preparations of 40S and 26S RNA were also sedimented in similar sucrose gradients. As shown in Fig. 7, dialysis against distilled water altered the 40S RNA so that it sedimented with a coefficient of 26S, whereas the sedimentation rate of the 26S RNA was unchanged. The decrease in the sedimentation rate of the 40S RNA appears not to be due to any nonspecific breakage because, under identical conditions, 26S RNA was unaffected. Also, all the radioactivity was accounted for as high molecular weight RNA. After treatment at very low ionic strength and centrifugation at normal ionic strength, the 40S and 26S RNA possess identical rates of sedimentation.

The effect of a high ionic concentration was, however, different. ^{3}H-labeled 26S RNA was dialyzed against 1,000 volumes of 0.5 M NaCl in 0.05 M acetate buffer (*p*H 6.0) for 16 hr. It was found (Fig. 8b) that the sedimentation rate of

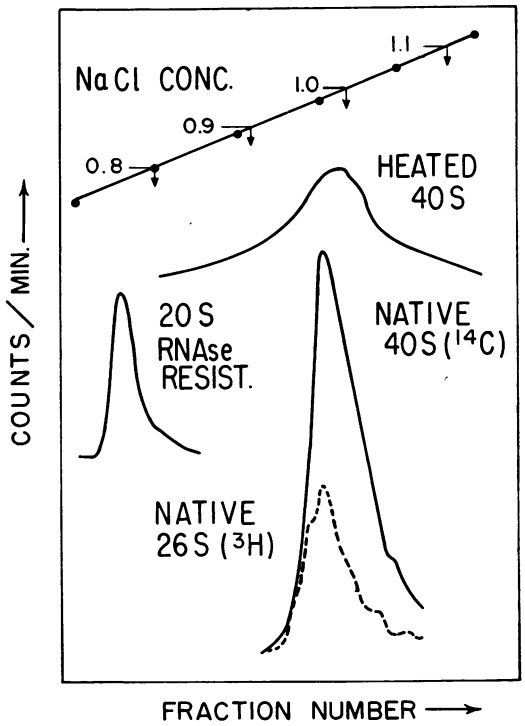

FIG. 6. *Chromatographic analysis of viral RNA preparations by use of an MAK column. The figure summarizes the pattern of elution of various types of viral RNA. ^{3}H-labeled 26S and ^{14}C-labeled 40S RNA were co-chromatographed on an MAK column. In a separate experiment, ^{3}H-labeled 40S RNA was heated at 90 C for 5 min, quickly cooled, and then co-chromatographed with unheated ^{14}C-labeled 40S RNA. In another experiment, ^{3}H-labeled 20S RNA (ribonuclease-resistant viral RNA obtained from WEE virus-infected cells) prepared according to the method of Franklin (4) was chromatographed on an MAK column. The details of technique of chromatography are described in the text.*

the dialyzed RNA was greater than that of the undialyzed RNA. Most of the label sedimented as a broad peak. The distribution of the label shows that both 40S and 26S types of molecules were present. Dialysis of the 26S RNA against 0.15 M NaCl did not alter its sedimentation rate when the *p*H was above 4.0. However, 26S RNA was partially converted to 40S RNA when the 26S RNA was dialyzed against 1,000 volumes of 0.05 M acetate buffer (*p*H 4.0), even when the buffer contained 0.15 M NaCl (Fig. 8b). Here, 50% of the total labeled RNA sedimented as 40S. Thus, under low *p*H or with high ionic concentrations of solvents, the sedimentation rate of the 26S RNA was increased. The infectivity of 26S RNA did not increase under these conditions. The

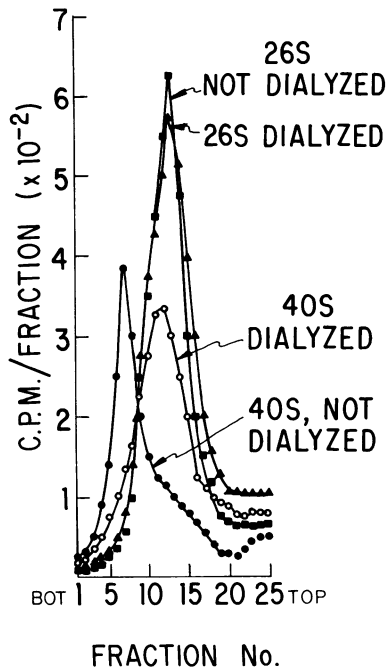

FIG. 7. *Effect of dialysis of 40S and 26S viral RNA against distilled water. ^{3}H-labeled 40S and 26S RNA were dialyzed separately against 1,000 volumes of sterile distilled water for 16 hr. The dialyzed RNA samples were then analyzed on 5 to 20% sucrose gradients in STE buffer. Centrifugation was at 40,000 rev/min in an SW50 rotor for 2.5 hr at 4 C.*

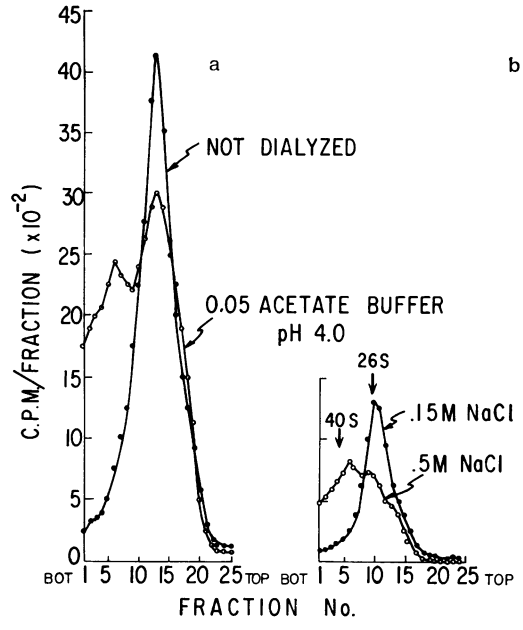

FIG. 8. *Effect of high concentration of NaCl and low hydrogen ion concentration on the sedimentation rate of 26S RNA. Purified 26S RNA obtained from infected cells was dialyzed for 16 hr against (a) 0.05 M acetate buffer with 0.15 M NaCl at pH 4.0 and (b) 0.05 M acetate buffer at pH 6.0 containing 0.5 M NaCl. Samples of the dialyzed viral RNA were layered on separate 5 to 20% sucrose gradients in STE buffer. Centrifugation was at 40,000 rev/min in an SW50 rotor for 2.5 hr at 4 C.*

apparent increase in the sedimentation rate of 26*S* RNA in high ionic solvent was not due to nonspecific aggregation, because this effect was found not to depend on the concentration of RNA.

Mild denaturing agents like DMSO and formamide have been used to disrupt the secondary structure of deoxyribonucleic acids and double-stranded RNA (6). Incubation of poliovirus double-stranded RNA with DMSO converts it into single-stranded RNA. The denaturation is mild, since the infectivity was unaltered. Denaturation by DMSO can be carried out at low temperatures, thus minimizing the chances of nonspecific hydrolysis. Both 40*S* and 26*S* types of viral RNA were incubated with DMSO by the method of Katz and Penman (6). ^{3}H-labeled 40*S* and 26*S* RNA (0.5 ml) were incubated separately with 3 volumes of DMSO at 37 C for 18 min. Unlabeled mouse ribosomal RNA (500 μg) was added, and the RNA was precipitated with 2 volumes of alcohol, dissolved in 0.5 ml of STE buffer, and incubated at 37 C for 15 min to prevent aggregation. The sedimentation rates of the two types of treated viral RNA were compared with those of the two types of untreated RNA. Incubation with DMSO did not alter the sedimentation rate of either 40*S* or 26*S* RNA. The infectivity of 40*S* RNA after incubation with DMSO decreased by only about 20%. Apparently DMSO caused no irreversible denaturation under the above conditions.

The possibility of reversible denaturation by DMSO was then considered. Attempts were made to determine the sedimentation rates of the two types of RNA in the presence of DMSO. Samples of ^{3}H-uridine-labeled 40*S* and ^{14}C-uridine-labeled 26*S* viral RNA were mixed with an equal volume of DMSO. Portions of this mixture were then centrifuged on a sucrose gradient (5 to 20%) in STE buffer containing 50% DMSO. Unlabeled mouse RNA was treated similarly, and the sedimentation rates of the RNA preparations were determined in the presence of 50% DMSO. Figure 9a shows the results of one such experiment. The tritium-labeled 40*S* RNA and the

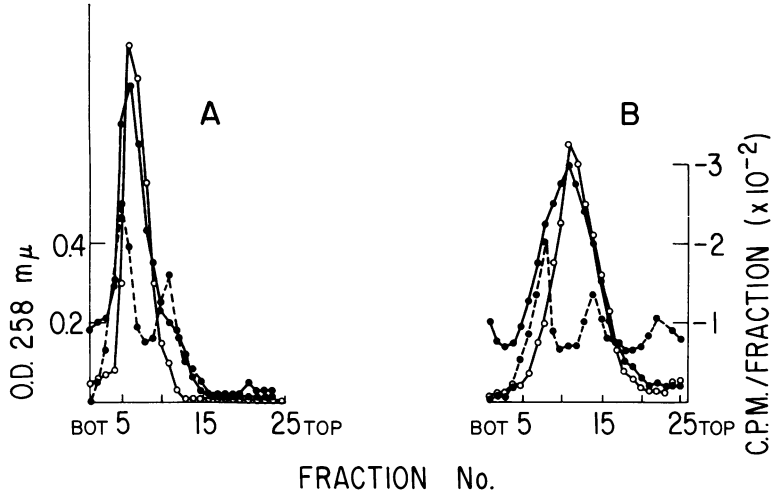

FIG. 9. *Centrifugation of viral RNA on sucrose gradients containing DMSO or urea. A 0.5-ml amount of a mixture of ^{3}H-labeled 40S and ^{14}C-labeled 26S RNA was layered on 4.5 ml of a 5 to 20% sucrose gradient in STE buffer containing 50% DMSO (A) or 8 M urea (B). Unlabeled RNA from chick embryo cells was centrifuged on a separate sucrose gradient containing DMSO or urea. Centrifugation was at 37,000 rev/min for 12 hr at 4 C. Symbols: ●, ^{3}H-labeled 40S RNA; ○, ^{14}C-labeled 26S RNA; ●—●, optical density at 258 mμ of unlabeled chick cell RNA.*

^{14}C-labeled 26S RNA sedimented at the same rates under these conditions. Comparison with the mouse ribosomal RNA under similar conditions showed that both types of viral RNA had a sedimentation coefficient of 26S. The peak fraction was titrated for infectious RNA. About 10% of the infectivity added was recovered. It should be mentioned here that, under the conditions of treatment with DMSO described, the double-stranded RNA of WEE virus was unaffected.

Essentially similar results were achieved when 8 M urea instead of DMSO was used as a denaturing agent (Fig. 9b). Removal of the urea after incubation of the RNA resulted in no alteration of their sedimentation rates. However, sedimentation of the RNA in the presence of 8 M urea showed that the 40S and 26S RNA sedimented at essentially the same rate. It should be pointed out that the peak of the 40S RNA was much broader, and it had a large leading component. This is probably due to incomplete denaturation of the 40S RNA. The infectivity of the RNA obtained under these conditions was not tested.

DISCUSSION

The problem we are dealing with is whether the 26S RNA results from a change in conformation or only represents part of the genome of WEE virus. Considerable evidence favors the first alternative. The RNA obtained from the virus particle sedimented in a 5 to 20% sucrose gradient as a 40S molecule. Upon heating, the ultraviolet absorption of the 40S RNA underwent a sharp transition similar to that found for deoxyribonucleic acid. Heating at 90 C for 5 min, dialysis against distilled water, and centrifugation in sucrose gradients containing either 50% dimethyl sulfoxide or 8 M urea changed the sedimentation properties of the RNA so that it sedimented as 26S RNA. The change from 40S to 26S RNA was not a result of random hydrolysis of the RNA, as the counts in the radioactive material were quantitatively converted, and there was no sign of radioactivity at the top of the gradient. Chromatography on MAK columns failed to separate 40S viral RNA and 26S RNA regardless of whether the latter was obtained as it occurred naturally in infected cells or "transformed" by heating. Since the naturally occurring 26S RNA and 40S RNA had the same base ratios and densities, they might have been expected to be separated by chromatography on MAK if they had different molecular weights. Indeed, control experiments showed that we could easily discern double-stranded RNA and slightly degraded 40S RNA (40S RNA plus T1 ribonuclease; 0.005 μg/ml for 5 min at 0 C) on the MAK columns used.

Under the conditions tested, 26S RNA was only partially converted to 40S RNA. Dialysis of naturally occurring 26S RNA against 0.5 M NaCl or at low *p*H brought about changes so that about 50% of the counts sedimented as 40S RNA.

Since the changes were independent of RNA concentration, simple aggregation is unlikely.

Infectivity of the RNA was measured in a number of experiments. It can be stated with certainty that RNA molecules which sediment with a coefficient of 26*S* are infectious; these include those occurring naturally (12), as well as those obtained from 40*S* RNA (in the presence of 50% DMSO). However, 26*S* RNA contained far less infectivity than 40*S* RNA. In one experiment, the peak fraction of 26*S* RNA from a gradient containing 50% dimethyl sulfoxide contained 10% of the infectivity of the added 40*S* RNA. Incubation of 40*S* RNA in 86% DMSO at 37 C for 18 min, a condition known to promote the separation of double-stranded RNA, failed to alter 40*S* RNA and reduced its infectivity by only about 20%. We were unable to demonstrate any increase in the amount of infectivity of 26*S* RNA when it was exposed to those treatments which favored formation of 40*S* RNA.

Kinetic experiments with WEE (*unpublished data*), Sindbis (2), and Semliki Forest (5) viruses show that 26*S* RNA is labeled more rapidly than 40*S* RNA. However, a number of attempts to demonstrate a precursor role for 26*S* RNA have failed. On the basis of the data on the infectivity of the 26*S* RNA, one must assume that it contains a complete genome of WEE virus. However, the role of the naturally occurring 26*S* RNA in WEE virus replication has yet to be understood.

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LITERATURE CITED

1. Brown, F., and B. Cartwright. 1964. Virus-specific ribonucleic acid in baby hamster-kidney cells infected with foot and mouth disease virus. Nature **204**:855–856.
2. Burge, W. B., and E. R. Pfefferkorn. 1967. Temperature-sensitive mutants of Sindbis virus: biochemical correlates of complementation. J. Virol. **1**:956–962.
3. Delgarno, L. E., E. M. Martin, S. L. Lin, and T. S. Work. 1966. Characterization of the products formed by the RNA polymerases of cell infection with encephalomyocarditis virus. J. Mol. Biol. **15**:77–91.
4. Franklin, R. M. 1967. Replication of bacteriophage RNA: some physical properties of single-stranded, double-stranded, and branched viral RNA. J. Virol. **1**:64–75.
5. Friedman, R. M., H. B. Levy, and W. B. Carter. 1966. Replication of Semliki Forest virus. Three forms of viral RNA produced during infection. Proc. Natl. Acad. Sci. U.S. **56**:440–446.
6. Katz, L., and S. Penman. 1966. The solvent denaturation of double-stranded RNA from poliovirus infected Hela cells. Biochem. Biophys. Res. Commun. **23**:557–560.
7. Katz, S., and D. G. Coomb. 1963. A new method for the determination of the base composition of ribonucleic acid. J. Biol. Chem. **238**:3065–3067.
8. Kelly, R. B., J. L. Gould, and R. L. Sinsheimer. 1965. The replication of bacteriophage MS2. IV. RNA components specifically associated with infection. J. Mol. Biol. **11**:562–575.
9. Scherrer, K., and J. E. Darnell. 1962. Sedimentation characteristics of rapidly labeled RNA from Hela cells. Biochem. Biophys. Res. Commun. **7**:486–490.
10. Spirin, A. S. 1962. Some problems concerning the macromolecular structure of ribonucleic acids. Progr. Nucleic Acid Res. **1**:301–345.
11. Sprecher-Goldberger, S. 1964. The effect of ionic strength and slow cooling on thermolability of infectious ribonucleic acids. Arch. Ges. Virusforsch. **14**:268–271.
12. Sreevalsan, T., and R. Z. Lockart, Jr. 1966. Heterogeneous RNA's occurring during the replication of Western equine encephalomyelitis virus. Proc. Natl. Acad. Sci. U.S. **55**:974–981.
13. Sueoka, N., and T. Cheng. 1962. Fractionation of nucleic acids with the methylated albumin column. J. Mol. Biol. **4**:161–172.
14. Vinograd, J., and J. E. Hearst. 1962. Equilibrium sedimentation of macromolecules and viruses in a density gradient. Progr. Chem. Org. Nat. Prod. **20**:372–422.