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Sedimentation Characteristics of Japanese Encephalitis Virus Ribonucleic Acid

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SUMMARY

The sedimentation coefficient of the infective ribonucleic acid (RNA) of Japanese encephalitis virus (JEV) was determined by assaying its biological activity. In a cesium sulfate density gradient JEV-RNA showed the same buoyant density as poliovirus (PV) RNA. However JEV-RNA had a larger sedimentation coefficient (46S) than PV-RNA. No components other than mature JEV particles could be demonstrated to yield infective RNA by the cold phenol method.

INTRODUCTION

Little is known about the RNA of arthropod-borne viruses such as JEV, compared with the information on those of small ether-resistant clathroviruses such as PV. Using an assay method for the infective RNA fraction of JEV-infected mouse brain (Igarashi *et al.*, 1963a, b), the basic biophysical characters of JEV-RNA have been studied, especially in comparison with those of PV-RNA, using chick cells and HeLa cells as specific biological indicators of JEV-RNA and PV-RNA respectively.

MATERIALS AND METHODS

1. *Viruses*

The Nakayama strain of Japanese encephalitis virus (JEV) grown in adult mouse brain, and the MEF-1 strain of type II poliovirus (PV) grown in HeLa S3 cells were used. The latter was kindly supplied by Dr. M. Takahashi of this Institute.

2. *Preparation of virus samples*

a) *JEV*

Infected brains stored at —70 °C were homogenized in a Waring blendor with 9 volumes of 0.02 M Tris-HCl, pH 7.6, containing 100 μg/ml of cystine and 0.14 M NaCl (TCS), and then spun down at 8,500 $\times$ *g* for 15 min. The supernatant (S_1) was centrifuged at 90,000 $\times$ *g* for 90 min. in the presence of 0.25 M sucrose. The pellet of this high speed centrifugation was resuspended in TCS containing 2 mg/ml of bovine plasma albumin (TCS-BPA), and recentrifuged at 8,500 $\times$ *g* for 15 min. This cycle of differential centrifugation was repeated once more and the final supernatant (U_2) was used as the source for RNA extraction.

b) *PV*

HeLa S3 cells originally supplied by Dr. S. Funahashi of this laboratory, were grown as monolayers in large culture bottles with 15 per cent bovine serum in YLH medium (0.5 per cent lactalbumin hydrolyzate and 0.1 per cent yeast extract in Hanks' balanced salt solution). Cells were detached by

trypsin digestion and washed twice with YLH by centrifugation. The sedimented cells were resuspended in the PV sample at an input multiplicity of 10 PFU/cell and incubated at 37 °C for 15 min. for virus adsorption. Then infected cells were collected by centrifugation and resuspended in YLH at a concentration of 10^6 cells/ml. After 6 hours' incubation at 37 °C with shaking (20 strokes/min.), the infected cells were collected and used for RNA extraction.

3. *Extraction of RNA*

The cold phenol extraction procedure of Gierer and Schramm (1956 a, b) was used, followed by two successive precipitations with two volumes of cold ethanol and one precipitation with 1 M NaCl at 0 °C. RNA precipitates were dissolved in 0.1 M NaCl, contiaining 10 mM ethylenediamine tetraacetate (EDTA), pH 6.8, and stored at —70 °C.

4. *Infectivity titrations*

Infectivities of JEV and JEV-RNA were assayed on primary cultures of chick embryo cell monolayers (CE cells) as described previously (Igarashi *et al.*, 1963a, b). Those of PV and PV-RNA were determined on HeLa S3 cell monolayers by the method of Holland *et al.*, (1960) with the slight modification of omititing preculture in the presence of human serm. In hypertonic $MgSO_4$ diluent, JEV-RNA forms plaques on CE cells but not on HeLa cells and PV-RNA forms plaques on HeLa cells but not on CE cells, so that CE and HeLa cell sheets act as specific indicators for JEV-RNA and PV-RNA, respectively. Titers are expressed here as plaque forming units (PFU) per ml.

5. *Sucrose density gradient centrifugation*

Scurose columns of 5.8 ml volume were overlayed with 0.2 ml of sample and centrifuged in a SW-39 rotor of a Spinco model L ultracentrifuge with refrigeration. For U_2 and S_1 fractions, a sucrose gradient of 5-23 per cent in TCS-BPA, and one hour's centrifugation at 30,000 rpm were used. For RNA fractions a 5-20 per cent sucrose gradient in 2 mM EDTA, pH 6.8, containing various concentration of NaCl (0-0.2 M), and 2.5 hours' centrifugation at 37,000 rpm were used. Fractions were collected by the droplet method.

6. *Cesium sulfate equilibrium density gradient centrifugation*

This procedure was performed according to the method of Doi and Spiegelman (1963), with slight modifications. Cesium sulfate of 99.5 per cent purity was obtained from Mitsuwa Chemical Co. Osaka, Japan. A concentracted aqueous solution of this material was treated with activated charcoal to remove impurities. The optical density at 260 mμ (E_{260}) of this stock solution ($\rho=1.9$) was about 0.1. The densities (ρ) were determined by the refractive indices (n_D^{25}) observed for the NaD line at 25 °C using a Hitachi Abbe type refractometer, type PRA-B, and applying the following formula of Hearst and Vinograd (1961), without correction for the temperature during centrifugation.

$$n_D^{25}=0.0730\ \rho_4^{25}+1.2646$$

Three ml of RNA solution in Cs_2SO_4 at an initial density of 1.64 containing 10 mM EDTA, pH 6.8, was overlayed with 2 ml of liquid paraffin and centrifuged at 33,000 rpm for 24 hours in a SW-39 rotor of a Spinco model L ultracentrifuge with refrigeration. About 0.15 ml of each fraction was collected by the droplet method and analyzed for PFU, n_D^{25} and E_{260}

7. *Determination of the sedimentation coefficient by measurement of biological activity.*

The sedimentation coefficient (s) for JEV-RNA was determined by the procedure of Yphantis and Waugh (1956b) using PFU as a marker of the biological activity. A sample of about 0.6 ml of JEV-RNA in a solution of 0.1 M NaCl containing 10 mM EDTA at pH 6.8, was centrifuged in a movable partition cell at a maximum rotor speed of 33,450 rpm or 37,020 rpm using an AN-A rotor of a Spinco model E ultracentrifuge. The rotor speed was checked every minute to evaluate the angular

velocity (ω) at time t. The final average supernatant PFU above the partition plate (C_T) and the initial PFU of the sample before centrifugation (C_0) were determined by plaque titration. The value of s was calculated according to Cheng's modification (1961) of Yphantis and Waugh's formula (1956a).

$$s = -\frac{1}{2\Sigma\omega^2 \Delta t} \ln \left[\frac{r_o^2}{r_p^2} + \frac{C_T}{C_0}\left(1 - \frac{r_o^2}{r_p^2}\right)\right]$$

Where r_0 and r_p are the distances from the center of rotation to the mensicus and the resting position of the separation plate, which were estimated from the photographic plates taken during centrifugation at maximum speed and at 2,000 rpm after the return of the separation plate.

8. *Hemagglutinating activity (HA) of JEV*

This was determined by the method of Clarke and Casals (1958).

9. *Ultraviolet absorption*

Ultraviolet (UV) absorption at 260 mμ (E_{260}) was determined with a Carl Zeiss spectrophotometer, type M4Q III, using a cuvett of 10 mm light path.

RESULTS

1. *Origin of infective JEV-RNA*

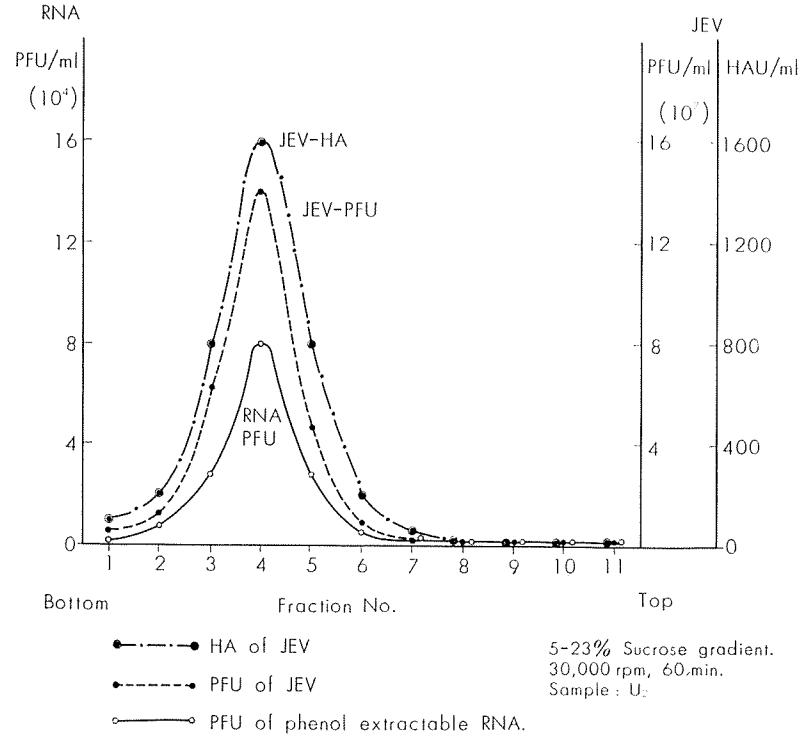

Fig. 1. Distribution of JEV-HA, JEV-PFU and Phenol-extractable RNA-PFU in a Sucrose Density Gradient Column.

U_2 was separated by sucrose density gradient centrifugation and each fraction was assayed for JEV-PFU and HA. Then the fractions were extracted with phenol and ether to assay RNA-PFU. It was found that the component which yielded infective RNA by phenol extraction sedimented as a single peak at the same rate as the peak of JEV-PFU and HA (Fig. 1). There was 80 per cent recovery of JEV-PFU and almost 100 per cent recovery of HA and phenol-extractable RNA-PFU. The phenol-extractable RNA-PFU was completely destroyed by incubation with ribonuclease (1 μg/ml, at 0°C for 15 min.). The only component of S_1 which sedimented at the same rate as JEV-PFU was found to yield infective JEV-RNA on treatment with cold phenol. These facts imply that almost all the infectivity in the RNA fraction obtained from U_2 or S_1 by the phenol method was derived from mature, hemagglutinating JEV particles, although this does not exclude the existence of free JEV-RNA which might be inactivated by tissue nucleases during the preparation procedures.

2. *Sedimentation coefficient of JEV-RNA*

The infective component in the RNA fraction from JEV-infected mouse brain sediments as a single peak and faster than the cellular RNA in a sucrose density gradient (Igarashi *et al.*, 1963b). Now the sedimentation coefficient for this infective component extracted from U_2 was determined by Yphantis and Waugh's method, which can be used for impure materials even when they were at low concentration. Nine successive experiments were performed in two days and the results (Table 1) gave a mean value for $s_{20,w}$ of 46.28 S with a standard error of 3.43 S. This value is much higher than those of cellular ribosomal RNA and even higher than that of PV-RNA. To exclude the possibility that there was inactivation during the sedimentation experiments which gave rise to a falsely large s value, the following experiments were undertaken.

Table 1. Sedimentation Coefficient of JEV-RNA

Experimental number	Maximal rotor speed (rpm)	C_T/C_0	$S_{20,w}$ in S
R-11	33,450	0.496	42.3
R-12	33,450	0.416	46.6
R-13	33,450	0.238	62.6
R-14	37,020	0.346	31.9
R-15	37,020	0.291	41.8
R-16	37,020	0.153	45.3
R-17	37,020	0.031	37.5
R-18	37,020	0.167	46.2
R-19	33,450	0.147	62.4
Average ± S. E.			46.28 ±3.43

3. *Simultaneous sedimentation of JEV-RNA and PV-RNA*

Infective JEV-RNA obtained from U_2 was mixed with PV-RNA and an excess of HeLa cell RNA was added as a UV marker. The mixture was centrifuged in a sucrose density gradient column. Each fraction was assayed for its JEV-RNA-PFU on CE cells, and for PV-RNA-PFU on HeLa cells as well as E_{260}. Although

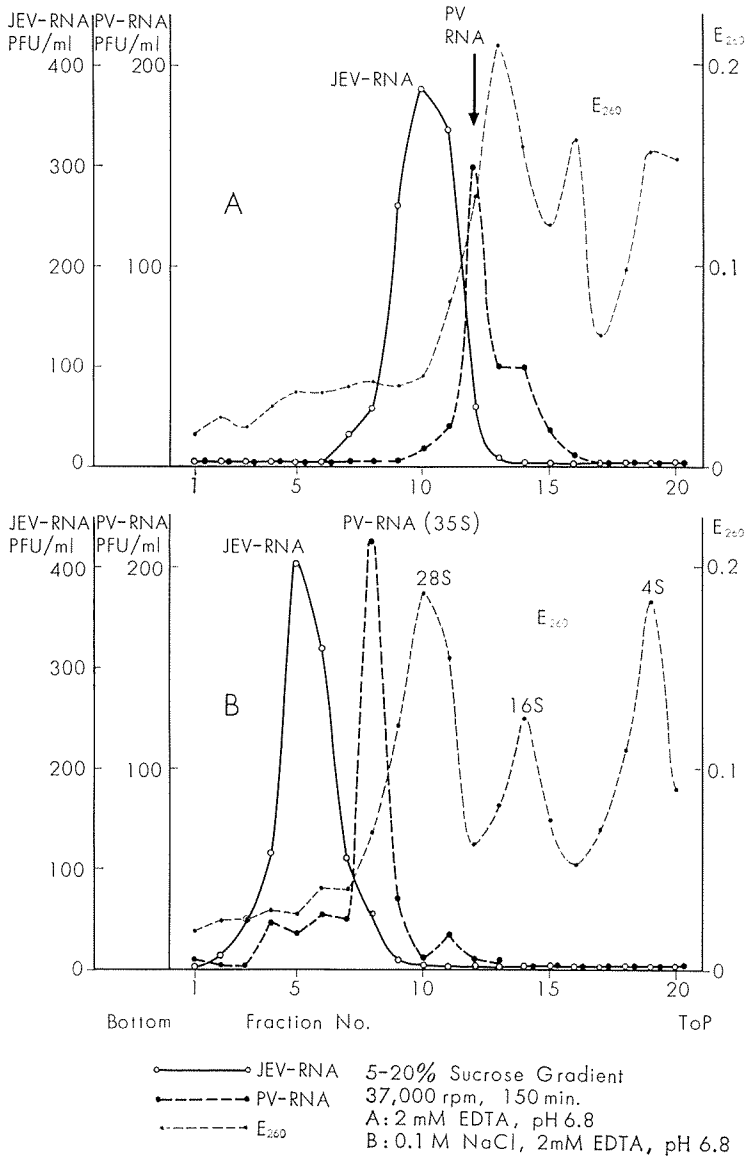

Fig. 2. Sedimentation of a Mixture of JEV-RNA, PV-RNA and HeLa Cell RNA in Sucrose Gradients.

the sedimentation velocity of JEV-RNA is strongly affected by the salt concentration of the medium (Fig. 2), JEV-RNA sedimented much faster than 28 S and 16 S RNA of HeLa cells and faster even than PV-RNA in every salt concentration tested. The recovery of infective RNA was 70-90 per cent. By comparing with the peaks of 28 S and 16 S ribosomal RNA and PV-RNA (35 S), the *s* value of JEV-RNA is estimated as 46.8 S from Fig. 2, which supports the value obtained in the preceding experiment. When a sample of JEV-RNA prepared from S_1 without ethanol and NaCl precipitation, or an extensively dialyzed RNA fraction from partially purified JEV was sedimented in sucrose gradients in parallel with the mixed sample used in the above experiment, the peaks of JEV-RNA in these samples were observed in almost the same position on the sucrose column. Moreover the coexistence of JEV-RNA and an excess of HeLa cell RNA was not shown to affect the sedimentation behavior of PV-RNA in a sucrose density gradient column.

4. *Buoyant density in cesium sulfate*

To exclude the possibility that some heavy contaminants might cause JEV-

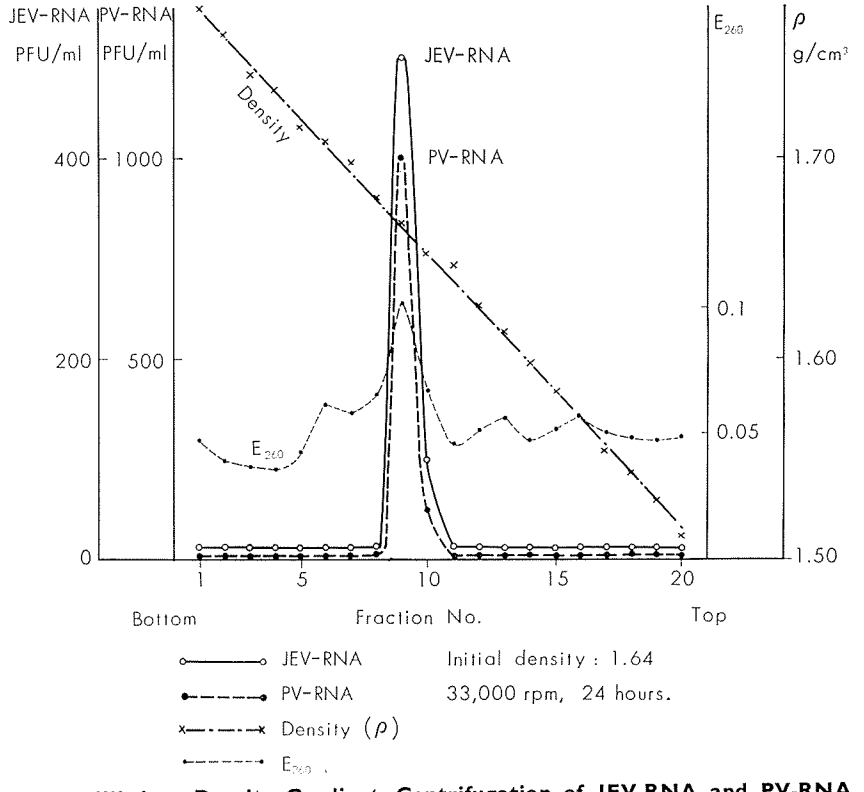

Fig. 3. Equilibrium Density Gradient Centrifugation of JEV-RNA and PV-RNA in Cesium Sulfate

RNA to have a large s value, the buoyant density in Cs_2SO_4 was determined. A mixture of JEV-RNA from U_2 and PV-RNA was separated by Cs_2SO_4 equilibrium density gradient centrifugation, and each fraction was assayed for its JEV-RNA-PFU on CE cells and for PV-RNA-PFU on HeLa cells as well as for its n_D^{25} and E_{260}. The single peak of JEV-RNA was found in the same fraction as the peak of PV-RNA and E_{260}, and the density of that fraction was estimated as 1.666-1.668 g/cm^3 (Fig. 3). The samples containing only JEV-RNA or only PV-RNA which were run parallel with the mixed sample also gave the same buoyant density for JEV-RNA and PV-RNA. Thus the infective JEV-RNA component has the same buoyant density as PV-RNA and cellular RNA, and very probably is itself RNA of high molecular weight.

DISCUSSION

The infective RNA extracted with cold phenol from western equine encephalitis virus (WEE) or eastern equine encephalitis virus (EEE) infected tissues is derived from immature virus particles which sediment slower than the mature virus (Wecker, 1960, Wecker and Richter, 1962). This is not the case for JEV, where most of the infective RNA seems to be derived from mature JEV particles, although the existence of free JEV-RNA which could be inactivated by tissue nucleases cannot be denied. The facts that JEV-RNA has the same buoyant density in Cs_2SO_4 as PV-RNA and that the sedimentation velocity of JEV-RNA varies with the salt concentration of the medium, support the idea that the infective component in the JEV-RNA preparation is itself RNA of high molecular weight. The value of 46 S for the $s_{20,w}$ of JEV-RNA estimated in this experiment is larger than the values of 27.5 S for mouse encephalitis virus RNA (Hausen and Schäfer, 1962), 35 S for PV-RNA (Darnell, 1962), 37 S for encephalomyocarditis (EMC) virus RNA (Burness *et al.*, 1963) and foot-and-mouth disease virus RNA (Strohmaier and Mussgay, 1959a, b). It is rather similar to the value of 50 S estimated for WEE or EEE virus RNA (Wecker and Richter, 1962), and 48 S of Mengovirus RNA (Tobey, 1964). If the empirical formula of Spirin (1961) is valid, the molecular

$$M = 1550 \times s^{2.1}$$

weight of JEV-RNA is calculated as 4.86×10^6. It might be an over-simplification to assign viral RNA a molecular weight of 2-3 $\times 10^6$ irrespective of the type of virus concerned. At present, it has not been possible to find a slow-sedimenting ribonuclease-resistant infective component in the JEV-RNA preparation which would suggest a replicative form of viral RNA such as was reported in the case of EMC virus RNA (Montagnier and Sanders, 1963) and PV-RNA (Baltimore *et al.*, 1964). It may be that a replicative form accumulates at a special phase of viral replication but this is hard to check because of the intracerebral growth of JEV. Another possibility is that the replicative form cannot reproduce in CE cells as suggested for an RNA phage—E. coli system (Kaerner and Hoffmann-

Berling, 1964). A further possibility is that JEV-RNA has a quite different replicative process from PV or EMC virus RNA. These possibilities must be examined in a more refined study using a tissue culture-JEV system.

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