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STUDIES ON CHIKUNGUNYA VIRUS.
III. INFECTIVE RIBONUCLEIC ACID FROM PARTIALLY PURIFIED VIRUS: ITS BIOLOGICAL ASSAY AND SOME OF ITS BASIC CHARACTERISTICS[1]

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SUMMARY The ribonucleic acid (RNA) fraction was extracted by the phenol method from partially purified Chikungunya virus material prepared from infected suckling mouse brains. The infectivity (PFU) of the RNA was assayed by the plaque method using hypertonic 2 M $MgSO_4$ as an RNA diluent. The ratio of virus-PFU to RNA-PFU was in the order of 10^4–10^5. The RNA-PFU was distinguishable from virus-PFU in several respects. The sedimentation coefficient of Chikungunya virus RNA is the same as that of Japanese encephalitis virus RNA (46S) when analyzed by sucrose density gradient centrifugation. The infective RNA is derived not only from complete virus particles but also from some other particles with slightly different sedimentation properties from that of complete virus in sucrose density gradient columns.

INTRODUCTION

Since RNA of tobacco mosaic virus was shown to be infectious to the host (GIERER and SCHRAMM, 1956a, b, FRAENKEL-CONRAT, 1956), many attempts have been made to isolate infectious RNA also from many animal viruses, as reported in the reviews by COLTER and ELLEM (1961) and SCHAFFER and SCHWERDT (1964) including several arthropod-borne viruses. In the case of Chikungunya virus, a member of group A arthropod-borne viruses, however, this problem has not yet been solved. To obtain precise information on the virus particle itself and also on the process of virus multiplication in the host cells, an attempt was made to extract an infective RNA from partially purified Chikungunya virus material and to assay its biological activity in a tissue culture cell system. This paper reports results on this together with some basic characteristics of the infective RNA of Chikungunya virus.

1 This work was performed by cooperation between Thailand and Japan under the Colombo plan.

MATERIALS AND METHODS

1. *Virus*

Chikungunya virus (ChV), African strain, at the 174th passage in suckling mouse brain (SMB) and the Nakayama strain of Japanese encephalitis virus (JEV) were kindly supplied by Dr. Sompop Ahandrik of the Virus Research Institute, Bangkok, Thailand. The virus strains were used after 2 more passages in SMB.

2. *Preparation of virus materials*

a) ChV

The partially purified ChV material was obtained by the procedures described previously (IGARASHI *et al.*, 1967) from a 10% homogenate of infected SMB stored at −70°C. After ultracentrifugation, the pellet (P_6) was suspended in 0.05 M tris-HCl, 0.14 M NaCl, 1mM EDTA, pH 7.6, (TVS), and used for extraction of RNA.

b) JEV

Stock SMB infected with JEV was homogenized with TVS to give a 10% suspension, which was centrifuged at $8,000 \times g$ for 15 min., and the supernatant (S_1) was used for extraction of RNA.

3. *Extraction of RNA*

Virus materials were extracted twice by shaking with water-saturated phenol containing 1 mM EDTA, with or without 0.2% sodium deoxycholate. After centrifugation the water phase was extracted three times with ethylether to remove phenol. In some cases RNA was precipitated by addition of 2 volumes of cold ethanol.

4. *Tissue culture*

An established cell line of African green monkey kidney (VERO) was supplied from Dr. Oya's laboratory, National Institute of Health of Japan, at the 184th passage level. The cell line was transferred as described previously (IGARASHI and TUCHINDA, 1967) and was used up to the 280th passage level.

A primary culture of chick embryo cells was prepared by the method of Porterfield (1960), and was grown in 2 ounce rubber-stoppered prescription bottles with YLE (0.1% yeast extract, 0.5% lactalbumin hydrolyzate and 0.55% glucose in Earle's balanced salt solution with antibiotics and phenol red) supplemented with 10% bovine serum. The cell monolyaers were used after 24 hour's incubation at 37°C.

5. *Infectivity titration*

Infectivity of ChV was assayed by the plaque method on VERO cell monolayers as described previously (IGARASHI and TUCHINDA, 1967). For RNA titration, cell monolayers were washed twice with 4 ml of PBS pH, 7.4, before RNA inoculation. Specimens of RNA were diluted with hypertonic $MgSO_4$ solution in TVS and 0.2 ml samples of diluted RNA were inoculated onto VERO cell sheets, using 2 bottles for each dilution. The RNA was allowed to be adsorbed at room temperature for a given length of time with occasional spreading. At the end of the adsorption period, the cell sheets were washed twice with 4 ml of PBS and 5 ml of first overlay medium, which was the same as for ChV titration, was added to the cell sheets. After solidification of the agar, the bottles were inverted and incubated at 37°C for 2 days. Then they were stained with neutral red as in the case with ChV. Plaques were counted on the third and fourth days. The titer was expressed as plaque forming units (PFU) per ml.

In the case of JEV-RNA, the PFU was assayed as described previously (IGARASHI *et al.*, 1963).

6. *Hemagglutination (HA) and hemagglutination-inhibition (HI)*

The HA activity of ChV and the HI titer of antiserum were determined by the method of CLARKE and CASALS (1958).

7. *Ultraviolet (UV) absorption*

The UV absorption was measured in a Karl-Zeiss spectrophotometer type M4QIII, using a cuvette of 1 cm lightpath.

8. *Sucrose density gradient centrifugation*

Sucrose columns of 4.5 or 4.8 ml volume in TVS were constructed in lusteroid tubes by the layering method. They were overlayed with 0.5 or 0.2 ml of sample and centrifuged in a RPS-40 rotor of a Hitachi model 55 P-2 ultracentrifuge at 4°C. The rotor was stopped without braking and fractions were collected from the bottoms of the tubes.

9. *Enzymes*

Crystalline ribonuclease (RNase) and deoxyribonuclease (DNase) were products of Worthington Biochemical Cooperation, U.S.A. Seven times recrystallized trypsin was kindly supplied by Dr. Narita of the Institute for Protein Research, Osaka University.

10. *Antiserum*

Hyperimmune mouse serum against ChV was obtained by 4 successive intraperitoneal imminizations of adult mice, starting with 0.1 ml of 10^{-3} dilution of infected SMB homogenate followed by two injections of 0.1 ml of 10^{-2} dilution and then 0.1 ml of 10^{-1} dilution, with one week intervals. Bloods were collected 2 weeks after the final immunization, and pooled and after clotting, serum was separated by centrifugation and stored at −20°C. Before experiments, one ml of the serum was passed through a Sephadex G-100 column (1.5 cm × 60 cm) equilibrated with PBS, pH 6.4. Three ml of each fraction were collected and protein content was measured by LOWRY's method (1951) and also the HI titer against ChV. The first fraction that showed an appreciable HI titer and a relatively low protein content was used as fractionated anti-ChV globulin in experiments.

RESULTS

1. *Plaque titration of ChV-RNA*

The RNA fraction prepared from the ChV sample did not produced any plaques when inoculated on VERO cell sheets under the same conditions as for ChV. But when diluted with hypertonic $MgSO_4$ solution it produced plaques on VERO cells as in the case with poliovirus RNA (HOLLAND *et al.*, 1960). Fig. 1 shows the effect of the $MgSO_4$ concentration on the efficiency of plating (EOP) of RNA. In this experiment ChV-RNA was diluted tenfold with various concentrations of hypertonic $MgSO_4$ and inoculated on VERO cell sheets. It was allowed to be adsorbed for 30 min. at room temperature. The EOP of infective RNA was markedly affected by the molarity of $MgSO_4$. Hypertonic NaCl solution was found to be inferior to $MgSO_4$ with respect to RNA-EOP. Next the optimal time of adsorption for ChV-RNA was studied. An aliquot of ChV-RNA was diluted tenfold with 2.2 M $MgSO_4$ and inoculated onto many replicate VERO cell sheets, and allowed to be adsorbed at room temperature for a various lengths of time. Then the cell sheets were washed and overlayed. In this way an adsorption curve of infective ChV-RNA on VERO cells was obtained (Fig. 2). The curve approaches a plateau after 35–40 min. of adsorption.

Thus the standard method of assay of infective ChV-RNA adopted was to dilute RNA samples tenfold with 2.2 M $MgSO_4$ just before

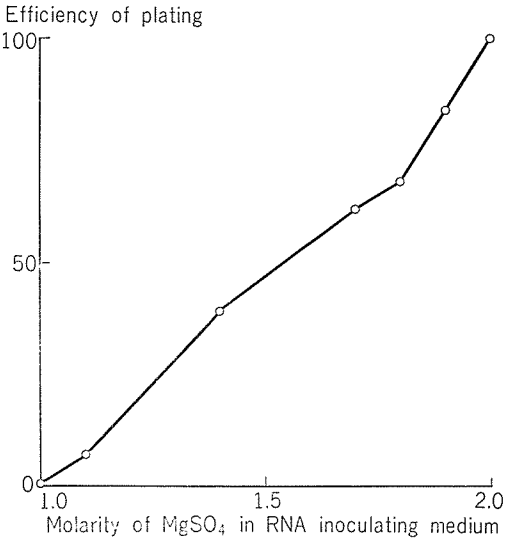

FIGURE 1 Effect of Hypertonic $MgSO_4$ Solution on Plating Efficiency of Infective RNA.

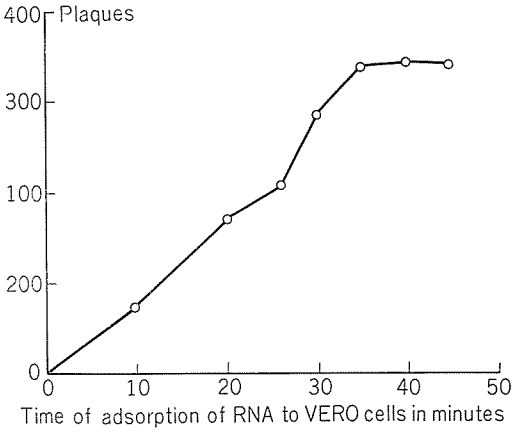

FIGURE 2 Effect of Time of Adsorption of RNA on Plating Efficiency.

inoculation and allow 40 min. for adsorption at room temperature before washing and overlaying. Under these conditions there is a linear relationship between the relative concentration of RNA and the plaque number with under 150 plaques per bottle (Fig. 3). Infective agents were recovered from plaques produced by the RNA and neutralized by incubation with anti-ChV serum. Thus this method can be used as a biological assay system for measuring infective ChV-RNA.

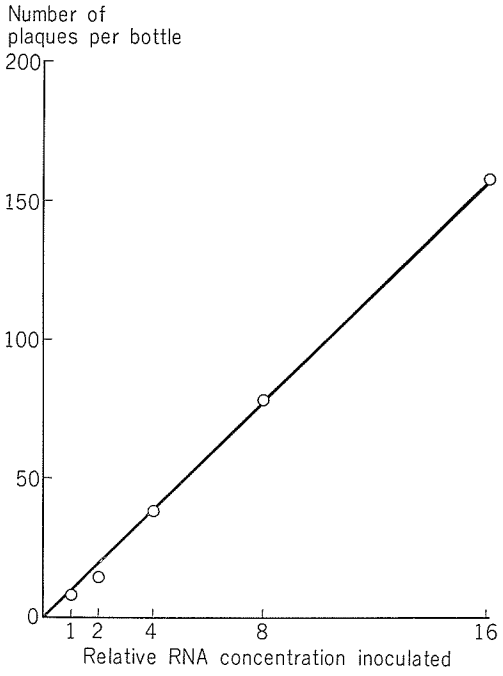

FIGURE 3 Relationship between RNA Concentration and Plaque Number of VERO Cells.

2. *Effect of temperature and sodium deoxycholate on the extraction efficiency of infective ChV-RNA*

In the case of Western equine encephalitis (WEE) virus, infectious RNA can be isolated from both purified virus and virus precursors by hot phenol extraction while cold phenol extraction yields infectious RNA from virus precursors only (WECKER, 1959, WECKER and RICHTER, 1962). Sodium deoxycholate was shown to be effective in liberating infective RNA from murray valley encephalitis virus (ANDERSON and ADA, 1959) and Eastern equine encephalitis virus (RICHTER and WECKER, 1963). Accordingly the effect of extraction temperature and addition of sodium deoxycholate on the efficiency of infectious ChV-RNA extraction were examined. The result is shown in Table 1. The titer of infectious RNA did not depend greatly on the extraction temperature, and was little affected by addition of sodium deoxycholate. Thus as a routine method, phenol extraction was carried out at room temperature (28°C) without addition of sodium deoxycholate. The ratio of ChV-PFU to RNA-PFU was in the order of 10^4–10^5. The UV absorption spectrum showed a typical pattern of nucleic acids with E_{max} at 260 mμ and E_{min} at 230 mμ. Ratios of E_{max}/E_{min} and E_{260}/E_{280} were calculated to be 2.05–2.00.

TABLE 1 *Effect of Extraction Temperature and Sodium Deoxycholate on the Titer of Infective RNA*

Temperature of extraction (°C)	Titer of extracted RNA (10^4 PFU/ml)	
	No addition	Sodium deoxycholate (0.2%) added
4	2.7	0.7
28	3.6	3.5
48	2.6	4.0

Original virus titer: 8.0×10^8 PFU/ml

3. *Evidence that the infectivity in the RNA fraction was not due to residual virus activity*

Although ChV is sensitive to treatment with organic solvents and ChV has a different EOP from ChV-RNA as tested in isotonic and hypertonic systems, the possibility that the infectivity in the RNA was due to residual virus particles had to be disproved, because RNA-PFU is far less than ChV-PFU. For

TABLE 2 *Inactivation of Chikungunya Virus and its Infective RNA by Enzymes and Antiserum*

	Infective RNA No. 1	Infective RNA No. 2	Chikungunya virus
Control	4.5×10^3	5.0×10^3	1.3×10^4
RNase, 0.1 μg/ml, 1 min.	<25	<25	1.2×10^4
DNase, 1 μg/ml, 15 min.	4.2×10^3	5.4×10^3	1.1×10^4
Trypsin, 1 μg/ml, 15 min.	4.2×10^3	5.0×10^3	0.8×10^4
Anti-ChV globulin × 10 diluted, 30 min.	3.2×10^3	4.6×10^3	0.1×10^4

Incubation at room temperature (28°C)
Medium: TVS, Control: TVS only

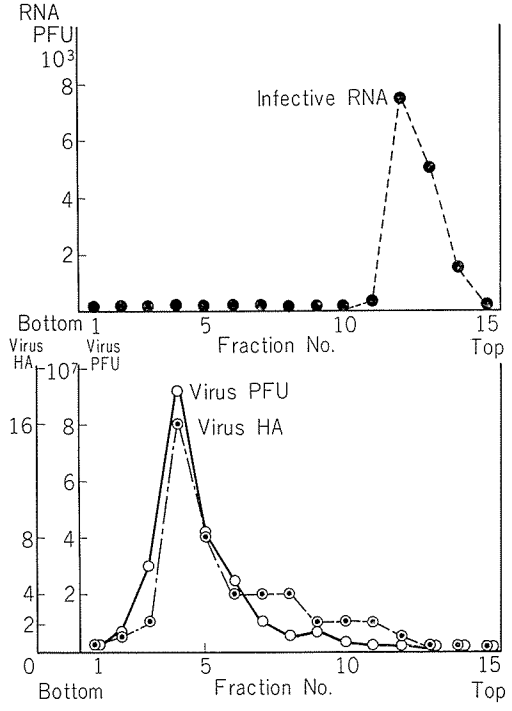

FIGURE 4 Sedimentation of Chikungunya Virus and its Infective RNA in a Sucrose Gradient Column.
○———○ Virus PFU 5—30% sucrose gradient
◉— — —◉ Virus HA 30,000 rpm, 90 min.
●------● RNA PFU

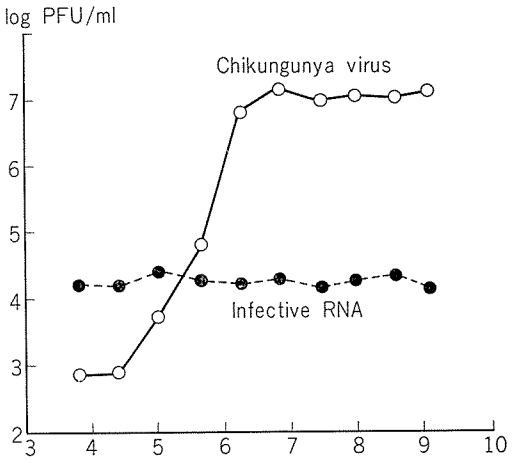

FIGURE 5 pH stability of Chikungunya Virus and its Infective RNA.
pH of incubation medium
○———○ PFU of Chikungunya Virus
●------● PFU of Chikungunya infective RNA
Incubation at room temperature for 1 hour
Medium: 0.14 M NaCl, 1 mM EDTA, 0.05 M buffer solution (pH 3.8–5.7; acetate, pH 5.7–8.0; phosphate, and pH 8.0–9.1; tris-HCl).

this purpose the effects of incubation of ChV and ChV-RNA with various enzymes and anti-ChV globulin was tested. The results obtained (Table 2) show that ChV-RNA-PFU was completely destroyed by RNase but that was resistant to DNase, trypsin and anti-ChV globulin. ChV was resistant to RNase but inactivated by anti-ChV globulin. This result was supported by the difference in sedimentation velocities in sucrose gradient columns (Fig. 4) and the differences in pH stabilities and heat stabilities (Figs. 5 and 6). RNA-PFU sediments much slower than ChV-PFU, and the former has a wider pH stability range and is more stable to heat than ChV-PFU. Thus it can be concluded that the infectivity of the RNA fraction was not due to residual ChV activity.

4. *Sedimentation of infectious ChV-RNA*

The approximate sedimentation coefficient

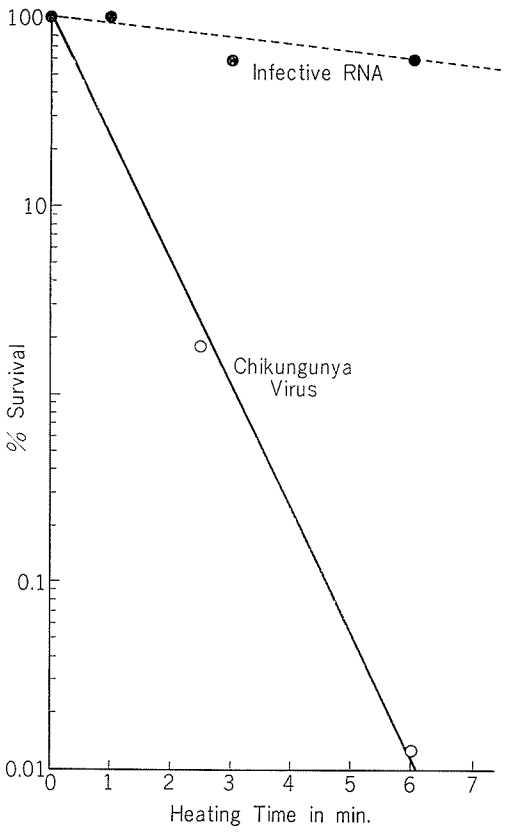

FIGURE 6 Heat Inactivation of Chikungunya Virus and Its Infective RNA at 56°C in TVS.

(*s*) of ChV-RNA was measured. A mixture of ChV-RNA and JEV-RNA was centrifged through a sucrose gradient column and then fractionated and titrated for infectivity on VERO cell, and chick embryo cell monolayers, respectively. The infectivities of these two kinds of RNA are readily distinguishable with these two types of indicator cells. The results in Fig. 7 show that infective ChV-RNA sediments at the same velocity as infective JEV-RNA. When ChV-RNA and JEV-RNA were centrifuged separately, the infectivities of these RNA were observed at the same position as that of the mixed sample. thus the *s*-value of ChV-RNA seems to be the same as that of JEV-RNA. This was estimated as 46S in a separate experiment (IGARASHI *et al.*, 1964).

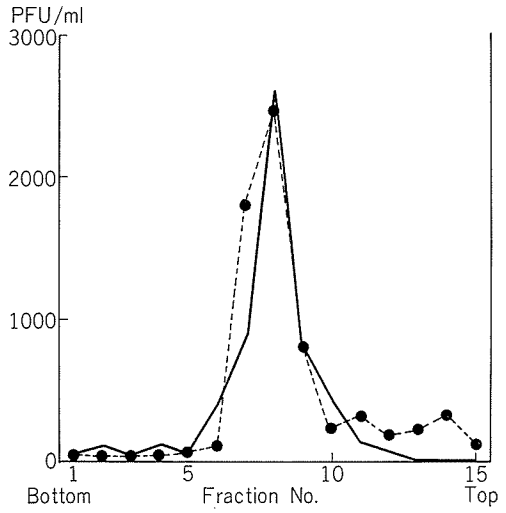

FIGURE 7 Simultaneous sedimentation of Chikungunya Virus RNA and Japanese Encephalitis Virus RNA in a Sucrose Gradient Column.
●------● ChV RNA 5—20% sucrose gradient
——— JEV RNA 0.15 M NaCl, 0.05 M tris, 1 mM EDTA, pH 7.6,
rotor temperature: 4°C 37,000 rpm, 150 min.

5. *Origin of infectious ChV-RNA*

Studies were made to see whether infective ChV-RNA was derived from complete ChV particles or from some other particles, as in the case of WEE virus (WECKER and RICHTER, 1962). The partially purified P_6 fraction was fractionated by sucrose density gradient centrifugation and the ChV-PFU and HA of each fraction were measured. Then RNA was extracted from each fraction by the routine method and assayed for RNA-PFU. The result (Fig. 8) indicates that the infective RNA peak coincides with the ChV-PFU peak, but the distribution of infective RNA is a little broader. This was clearer when a cruder sample (S_3, protamine and carbon treated supernatant) was analyzed in the similar way (Fig. 9). The infective RNA seems to be derived not only from complete virus particles but also from some other particles with a slightly different sedimentation velocity from complete virus.

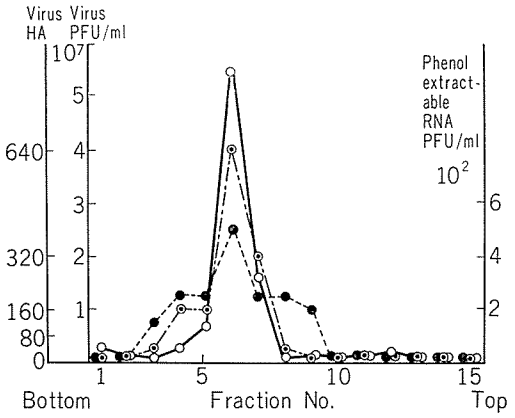

FIGURE 8 Distribution of Chikungunya Virus Activity: Virus PFU, Virus HA and Phenol-extractable RNA-PFU, in a Sucrose Gradient Column.
○———○ Virus PFU 10—35% sucrose gradient
◉– – –◉ Virus HA 30,000 rpm, 90 min.
Sample P_6
●------● PFU of phenol-extractable RNA

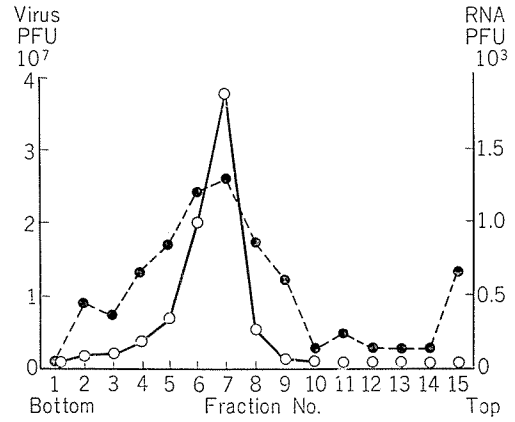

FIGURE 9 Distribution of Infectivity for Chikungunya Virus and Phenol-extractable RNA on Sucrose Density Gradient Centrifugation.
○———○ PFU of Chikungunya Virus 10—35% sucrose
30,000 rpm. 90 min.
●------● PFU of phenol-extracted RNA Sample: S_3

DISCUSSION

The RNA fraction prepared from partially purified Chikungunya virus material was shown to be infectious, and its infectivity was assayed in a tissue culture system under standard conditions. Although the RNA fraction had a low titer of infectivity (10^{-4}—10^{-5} of that of the virus), it was clearly distinguishable from virus-PFU in many respects, so that the RNA-PFU was not due to residual virus-PFU. It is interesting that the yield of infective RNA did not depend greatly on the temperature of extraction. This is different from the case with WEE (WECKER, 1959, WECKER and RICHTER, 1962). This fact, together with the relatively low titer of infective RNA, may be because special structural properties of ChV are somewhat different from those of WEE.

Infective ChV-RNA was shown to be deived not only from complete virus but also from some other particles with slightly different physical properties, but no distinct peak of precursor particles yielding infective RNA could be obtained with cold phenol as in the case with WEE. However, it may be possible to obtain such a peak with different kinds of gradient columns and different conditions of centrifugation or RNA extraction.

The *s*-value of ChV-RNA was shown to be the same as that of JEV-RNA by sucrose density gradient analysis, though these two viruses belong to different groups of arthropod-borne viruses. Semliki Forest virus RNA has been reported to have an *s*-value of 45S (MÉCS *et al.*, 1967) and WEE-RNA has an *s*-value of 50S (WECKER and RICHTER, 1962). Thus the RNA of arthropod-borne viruses seem to have larger *s*-values than picornavirus RNA, but these points can only be confirmed by more refined and extensive researches.

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