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Infectivity of the Ribonucleic Acid Fraction from Mouse Brain Infected with Japanese Encephalitis Virus

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SUMMARY

An infective ribonucleic acid (RNA) fraction was obtained from the microsomal fraction of adult mouse brain infected with the Nakayama strain of Japanese encephalitis virus (JEV). The infectivity, which was assayed on primary cultures of chick embryo cell monolayers, using 1 M $MgSO_4$ as a diluent, differs in many respects from that of intact JEV. The infectivity of the RNA fraction sediments as a single peak and faster than the bulk of non-infections RNA in a sucrose density gradient column and is more stable than JEV in a moderately high temperature range. Established cell lines, which are not suitable as indicators for either JEV or the RNA fraction under an ordinary agar overlay medium, could be used as primary hosts to form infectious centers by incubation with the RNA fraction in suspended cell systems.

INTRODUCTION

The recognition of the central role played by RNA in the process of tabacco mosaic virus infection (Gierer and Schramm, 1956a, b; Fraenkel-Conrat, 1956) has led many investigators to isolate "infectious RNA" from a number of animal viruses also (Colter *et al.*, 1957a, b; Wecker and Schäfer, 1957; Alexander *et al.*, 1958a; Cheng, 1958; Huppert and Sanders, 1958; Brown *et al.*, 1958 Wecker, 1959a, b; Ada and Anderson, 1959; Sprunt *et al.*, 1959, Franklin *et al.*, 1959; Nakamura, 1961) by the phenol method. Studies on molecular aspects of these biologically active RNAs depend upon accurate, simple and reproducible assay procedures and these were achieved by plaque titration in tissue culture systems, using environments different from those for intact viruses (Alexander *et al.*, 1958a b; Holland *et al.*, 1960; Ellem and Colter, 1960a, b; Koch *et al.*, 1960; Mayer and Sokol, 1961; Nakamura and Ueno, 1963). As a first step in the physicochemical study on the RNA of JEV as a biologically active macromolecules, this paper reports on the extraction of an infectious RNA fraction from the microsomal fraction of infected mouse brain, a simple and reproducible plaque assay system for measuring its infectivity and some basic characters of this infectious component.

MATERIALS AND METHODS

1. *Virus*

The Nakayama strain of Japanese encephalitis virus (JEV) was grown in brains of adult mice and infected brains were stored at −70°C and used as virus stock.

2. *Preparation of microsoml fraction*

Infected brains were homogenized in a Waring blendor with 9 volumes of 0.25 M sucrose in 0.02 M phosphate buffer, pH 7.4, and then centrifuged at $700 \times g$ for 10 min. and $8,500 \times g$ for 15 min. to remove nuclear and mitochondrial fractions. The pH was adjusted to 7.8 with $NaHCO_3$ and the supernatant was centrifuged at $90,000 \times g$ for 90 min. The supernatant of this high speed centrifugation was removed and the pellet was resuspended in 0.14 M NaCl in 0.02 M phosphate buffer (PBS), pH 7.4, and recentrifuged at $2,000 \times g$ for 15 min. This supernatant containing JEV was regarded as the microsomal fraction.

3. *Extraction and precipitation of RNA*

Cold phenol extraction of RNA from the microsomal fraction was carried out according to the method of Gierer and Schramm (1956a, b). After four extractions with water-saturated phenol containing 1 mM ethylenediamine tetraacetate (EDTA), the water phase was extracted five times with ethylether to remove phenol. RNA was then precipitated by adding 2 volumes of cold ethanol. The precipitate was collected by centrifugation and dissolved in PBS, pH 7.4, containing 1 mM EDTA and further precipitated twice with ethanol and once more with 1 M NaCl at 0°C. Aliquots were stored at −70°C for several weeks without significant loss of infectivity.

4. *Preparation of JEV*

Infected brains were homogenized with 9 volumes of 0.14 M NaCl in 0.05 M tris buffer (TBS), pH 7.6, containing 100 μg of cystine per ml (Nojima, 1962), and the homogenate was centrifuged at $2,000 \times g$ for 30 min. The supernatnat was treated with protamine sulfate at a final concentration of 1 mg per ml at 0°C for 30 min. to remove cellular components and freed from precipitates by centrifugation ($2,000 \times g$ for 15 min.). Sucrose and bovine plasma albumin (BPA) were added at final concentrations of 10 and 0.2 per cent respectively and then the supernatant was centrifuged at $90,000 \times g$ for 90 min. on a cushion of 40 per cent sucrose solution (0.5 ml per tube in a Spinco No. 40 rotor). The supernatant was removed and the precipitate was resuspended with TBS, pH 7.6, containing 100 μg of cystine and 2 mg of BPA per ml, and centrifuged at $2,000 \times g$ for 30 min. This cycle of differential centrifugation was repeated once more and the supernatant of the final low speed centrifugation was regarded as the partially purified JEV sample, and used as a virus control for the RNA fraction.

All the procedures in methods 2, 3 and 4 were performed in the cold.

5. *Tissue culture*

1) *Primary culture of chick embryo cells*

Chick cells were prepared by the method of Porterfield (1959, 1960) and cultivated according to the method of Inoue *et al.* (1961) adding 0.1 per cent yeast extract to their growth medium. Cell sheets were used after 24 to 36 hours' incubation at 37°C.

2) *Established cells*

Established lines of HeLa S 3, KB and MS cells were kindly supplied by Dr. S. Funahashi of this laboratory and grown as monolayers in large prescription bottles in the same medium as used for chick cells, except that the 10 per cent calf serum was replaced by 15 per cent bovine serum.

6. *Skimmed milk*

Twenty g of defatted skimmed milk (Yukijirushi Brand, Japan) was dissolved in 180 ml of distilled water and centrifuged at 2,000×*g* for 30 min. The supernatant was regarded as 100 per cent skimmed milk and stored at −20°C, after sterilization by autoclaving at +0.5 atm. for 15 min.

7. *Plaque titration*

Primary cultures of chick embryo cell monolayers were used as indicators of the infectivities of the JEV and the RNA fraction. The growth medium was removed and the cell sheets were washed twice with 5 ml of PBS, pH 7.4, and then excess fluid was removed.

1) *Titration of JEV*

The virus sample was diluted serially by ten fold steps with 10 per cent skimmed milk in PBS, pH 8, and 0.2 ml sample of each dilution were inoculated onto and spread over prewashed cell sheets. At least two bottles were used for each sample. After two hours adsorption at 37°C, the cell sheets were covered with agar overlay medium.

2) *Titration of RNA fraction in monolayer systems*

The efficiency of plating (eop) of the infectivity in the RNA fraction was much improved when hypertonic salt diluents were used. To avoid possible aggregation of RNA at high salt concentration, the RNA fraction was first diluted with TBS, pH 7.6 ,containing 1 mM EDTA, and then, just prior to inoculation, mixed with hypertonic salt solutions to give the desired final concentration of the salts. Samples of 0.2 ml of this mixture were inoculated onto and quickly spread over prewashed cell sheets. After a given period of adsorption at room temperature (20 to 25°C), cell sheets were washed twice with 5 ml of PBS, pH 7.4 and immediately overlayed with agar medium. Although Nakamura and Ueno (1963) reported that the best solution for JEV-RNA plaque formation was 1 M NaCl, with 1.5 M $MgSO_4$ next, preliminary experiments showed that the number of plaques formed by a given sample varied more when NaCl was used as diluent than when $MgSO_4$ was used. Therefore $MgSO_4$ was adopted as the diluent for RNA in these experiments. A higher concentration of the salt gave a higher eop as well as a higher degree of cellular damage for a given concentration of RNA and a given time of adsorption. Because results were reproducible, the sensitivity of the titration system was reduced by employing a lower concentration of $MgSO_4$ than that giving the highest eop. The optimal concentration of the salt was not markedly affected by the RNA concentration. The pH of the diluent did not have so much effect as the salt concentration. When $MgSO_4$ solutions at final concentrations of 1.0 to 1.5 M were used, the number of plaques formed increased during a 15 minute adsorption period and then gradually approached a plateau. Then damage to cell sheets became manifest. Thus $MgSO_4$ as the inoculation medium at a final concentration of 1 M in 0.05 M tris buffer, pH 7.6, using a 20 minute adsorption period at room temperature was adopted as the standard conditions for the infectivity tirtation of the RNA fraction in chick cell monolayers. The eop of JEV in this condition is about 10 per cent of that in the standard virus titration method. The infectivity of the RNA fraction assayed by this method is about 0.1 per cent of that of the starting material before phenol extraction assayed by standard virus titration method, as described previously (Igarashi *et al.*, 1963).

3) *Suspended cell system for the RNA fraction*

The method used was essentially that of Ellem and Colter (1960 a, b). The chief difference was that in these experiments chick cells were used as indicators of plaques, while three established cell lines were tested for their capacity as primary hosts to form infective centers by incubation with the RNA fraction. Monolayer cells were detached by incubation with 0.25 per cent trypsin in PBS, and washed twice with Hanks' balanced salt solution by centrifugation and resuspension (300×*g* for 5 min.). Methods of infecting these cells with the RNA fraction and titration of infective centers were the same as those used by Ellem and Colter, except that the diluting medium of infective centers was chick cell growth medium (0.5 per cent lactalbumin hydrolysate and 0.1 per

cent yeast extract in Hanks' solution supplemented with 10 per cent calf serum).

The overlay medium of Hashimoto and Prince (1963) was used for all experiments using plaque titration, with the modification of omitting serum and supplementing the mixture with 5 per cent skimmed milk. Plaques were counted from the third to the seventh day after incubation at 37°C. Titers were expressed as plaque forming units (PFU) per ml.

8. *Hemagglutination of JEV*

Hemagglutination activity (HA) of JEV was determined by Clarke and Casals' method (1958).

9. *Ultraviolet absorption*

The ultraviolet (UV) absorption spectrum was recorded by a Shimadzu automatic recording spectrophotometer, type RS-27, using 10 mm cuvettes.

10. *Sucrose density gradient centrifugation*

A sucrose density gradient ranging from 1.02 to 1.10 g/ml was constructed in a lusteroid tube by overlayering 0.5 ml of 23.2 per cent sucrose with 1.0 ml volumes of 18.8, 14.3, 9.7 and 4.9 per cent (w/w) sucrose dissolved in TBS, pH 7.6, containing 0.01 M sodium citrate. After standing for 2 to 4 hours in a refrigerator, the resulting gradient column was loaded with 0.2 ml of sample and centrifuged in a SW-39 rotor of a Spinco model E or model L ultracentrifuge with refrigeration. The rotor was decelerated without braking. Samples were collected by droplet fractionation from a pinhole pierced at the bottom of the tube. The linearity of the gradient was checked by measuring the refractive index of a part of each fraction using a Hitachi Abbe type refractometer, type PRA-B. UV absorbancy and PFU of RNA samples and HA and PFU of JEV samples were determined after appropriate dilution of each fraction.

11. *Enzymes*

Crystalline ribonuclease (RNase) and deoxyribonuclease (DNase) were products of Sigma Chemical Co. U. S. A. Seven times recrystallized salt free trypsin was kindly supplied by Prof. K. Narita of The Institute for Protein Research, Osaka University. RNase activity was assayed by the method of Kalnitsky *et al.* (1959).

12. *Antiserum*

Hyperimmune rabbit serum against the Nakayama strain of Japanese encephalitis virus was kindly supplied by Dr. A. M. Prince, the former head of the Virus Section of the 406 Medical General Laboratory of the Far East U. S. Army at Sagamihara, Japan.

RESULTS

1. *UV absorption of the RNA fraction*

When diluted with PBS, pH 7.4, the RNA fraction showed the characteristic absorption curve of nucleic acids (Fig. 1). An absorption maximum at 258 mμ and a minimum at 230 to 232 mμ were observed. The ratio of E_{260} to E_{280} was 2.05 (1.96 to 2.10) and that of E_{max} to E_{min} was 1.95 (1.90 to 2.08). Possible contamination of this fraction with protein cannot be ruled out from the results because of the much higher extinction coefficient of nucleic acids than of proteins.

2. *Validity of plaque titration of the RNA fraction on chick cell monolayers*

The RNA fraction extracted from a crude homogenate or the microsomal frac-

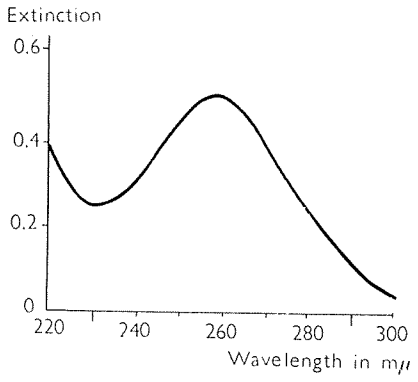

Fig. 1. Ultraviolet Absorption Spectrum of the RNA Fraction

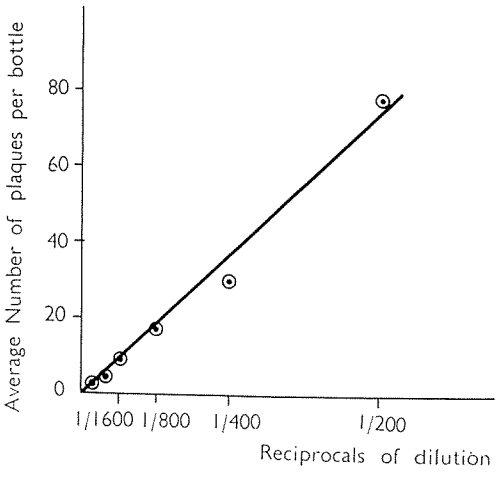

Fig. 2. Relationship between Relative Concentration of RNA and Number of Plaques Formed

tion of normal brains of mice (normal RNA) did not produce any plaques on chick cell sheets, while infective agents could be recovered from plaques formed by the RNA fractions from infected brains (infected RNA). These agents cause the same symptoms as JEV in mice, produce plaques on chick cell sheets indistinguishable from those of JEV and their plaque producing capacity is neutralized by incubation with anti-Nakayama serum. Therefore, the plaques formed by infected RNA were not due to some non-specific lytic substances, but were produced by a specific infective component contained in the infected RNA fraction related to JEV. There is a linear relationship between the reciprocals of the dilution factor of the infected RNA inoculated and the average number of plaques formed on a cell sheet (Fig. 2). When 36 replicate plates of chick cells were inoculated with the same amount of RNA containing a mean of 1.069 plaques, no significant difference between the observed and the expected distribution of the theoretical Poisson distribution was noted, as tested by the chi-square test (for the calculated value of $\chi^2 = 2.854$ with 2 degrees of freedom, the probability is between 0.2 and 0.3, from the table of Fisher and Yates). Reproducibility of the titration system was tested on seven different days using the same RNA sample freshly thawed on the day of inoculation The distribution of the average number of plaques on each day had a mean of 87.3 with a standard error of 10.8 with a range of distribution of 58 to 140.

3. *Differentiation of the infectivity in the RNA fraction from that of JEV*

To exclude the possibility that the infectivity in the RNA fraction might be due to surviving residual virus particles, the following experiments were undertaken.

1) *Inactivation with enzymes*

The infectivity of the RNA fraction was completely destroyed by incubation with RNase, while that of JEV was resistant to this enzyme (Table 1). This sensitivity to RNase is not due to interference on the cell sheets caused by residual RNase carried into the inoculation medium, because even phenol and ether extraction to remove this enzyme after the treatment also failed to produce any plaques, and the infectivity of the RNA fraction decreased with time by incubation with very low levels of RNase (Fig. 3). The infectivity of the RNA fraction was resistant to DNase, and was not affected by trypsin at the enzyme level which can reduce the infectivity of JEV (Table 1).

2) *Protamine sulfate treatment*

Protamine sulfate can be used during JEV purification to remove cellular components without precipitation of JEV. When the RNA fraction was incubated with protamine sulfate at a final concentration of 0.5 mg/ml at 0°C for 15 min. and then centrifuged, no infectivity was detectable in the supernatant. (Table 2).

3) *Ethanol and ether treatment*

The infectivity of the RNA fraction was resistant to ether treatment but was precipitated by 2 volumes of cold ethanol without appreciable loss of infectivity, while JEV was largely inactivated by these treatments (Table 2).

4) *Reaction with immune serum*

On incubation with an equal volume of immune or normal rabbit serum diluted ten fold with PBS, pH 7.8, at 22°C for 30 min., the RNA fraction lost its plaque forming capacity completely, while JEV was inactivated only by immune serum. The sensitivity of the infectivity in the RNA fraction to immune and normal sera can be explained by the inactivation by RNase contained in these

Table 1. Effect of Enzymes on the Infectivities of the RNA Fraction and JEV

Enzymes	Infectivity of the RNA fraction		Infectivity of JEV	
	control	treated	control	treated
RNase	4.6×10^4	<1	6.0×10^4	6.5×10^4
RNase	5.0×10^3	<1	1.8×10^4	1.5×10^4
DNase	3.0×10^2	3.6×10^2	not tested	
DNase	1.7×10^3	1.6×10^3		
Trypsin	1.2×10^4	1.4×10^4	1.5×10^4	0.13×10^4
Trypsin	2.4×10^4	2.4×10^4	6.3×10^3	0.50×10^3

Infectivities are expressed in PFU/ml.
Enzyme treatments were carried out at a concentration of 10 μg of enzyme per ml of PBS, pH 7.4, at 37°C for 15 min. in the cases of RNase and DNase and for 30 min. in the case of trypsin. Controls: PBS only.

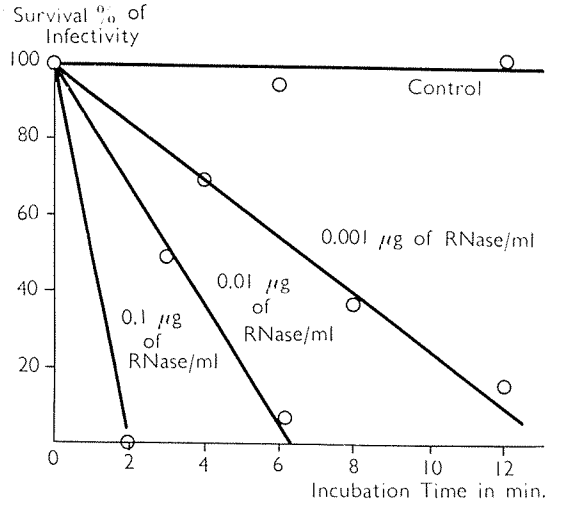

Fig. 3. Inactivation of Infectivity in the RNA Fraction by Ribonuclease
Inactivation at 20°C in PBS, pH 7.4

sera, because all five samples of immune and normal sera exhibited appreciable amount of RNase activity corresponding to 1 to 3 μg/ml of standard RNase. To remove the RNase activity, 1 ml of immune serum diluted four fold with PBS, pH 5.8, was fractionated by passing through Sephadex G 75 column (1.5 cm ø× 50 cm)

Table 2. Effect of Various Treatments on Infectivities of the RNA Fraction and JEV

Treatment	Infectivity of the RNA fraction		Infectivity of JEV	
	control	treated	control	treated
Protamine sulfate	2.5×10^3	<25	1.8×10^4	1.5×10^4
Ether	2.5×10^3	2.6×10^3	5.2×10^6	25
Ethanol ppt.	2.5×10^3	2.1×10^3	5.2×10^6	<25
Fractionated antiserum	1.1×10^4	8.5×10^3	2.4×10^3	50

Infectivities are expressed in PFU/ml.
Protamine sulfate treatment: Supernatant after incubation with 0.5 mg/ml of protamine sulfate in PBS at 0°C for 15 min.
Ether treatment: Waterphase after shaking with an equal volume of ethylether at 0°C.
Ethanol ppt.: The precipitate formed on addition of 2 volumes of cold ethanol was dissolved in PBS and its infectivity was titrated.
Fractionated antiserum: Incubated with an equal volume of antiserum fractionated with Sephadex G 75 column, at 22°C for 30 min. then titrated for infectivity.

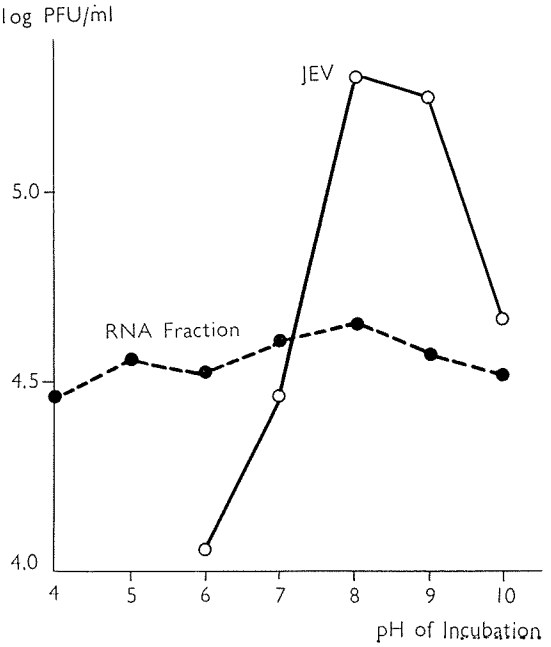

Fig. 4. pH Stability Curves of Infectivity of JEV and the RNA Fraction

Infectivity of JEV
Infectivity of the RNA fraction

equilibrated with this PBS. Three ml of each fraction was collected with a flow rate of 6 ml/hour and absorbancy at 280 mμ was measured. The first fraction that showed appreciable amount of E_{280} was incubated with JEV and/or the RNA fraction after passing through Membrane filter and adjusting pH to 7.8. The result (Table 2) shows that the infectivity of the RNA fraction was not inactivated so much as that of JEV.

5) *pH stability*

Samples of JEV and the RNA fraction were diluted ten fold with 0.14 M NaCl, 0.01 M sodium citrate in 0.02 M of the following buffer solutions: Citrate for pH 4 to 6, phosphate for pH 6 to 8, borate for pH 8 to 9 and bicarbonate for pH 9 to 10. Diluted samples were incubated at 0°C for 60 min., then diluted with TBS, pH 7.6, and titrated for infectivities. The infectivity of JEV showed its maximal stability in the range of pH 8 to 9, as reported by Duffy and Stanley (1945). On the other hand the infectivity of the RNA fraction had almost the same stability over all pH range of this experiment (Fig. 4).

6) *Heat stability*

The RNA fraction and JEV were diluted with PBS. pH 7.6, containing 0.01

M sodium citrate and heated in a water bath at various temperatures for 10 min. Then they were rapidly cooled by dilution and assayed for infectivities. The results (Fig. 5) show the greater stability of the infectivity in the RNA fraction than that of JEV in a moderately high temperature range.

7) *Sedimentation in sucrose gradient columns*

The RNA fraction was sedimented in a sucrose gradient column at 30,000 rpm for 60 min. in parallel with JEV. Its infectivity moved much slower than the infectivity and HA of JEV (Fig. 6).

All these facts support the idea that the infectivity in the RNA fraction was not due to the presence of intact JEV which had survived the extraction procedures.

4. *Homogeneity of the infectivity in the RNA fraction*

0.2 ml of the RNA fraction, having 5×10^3 PFU. was centrifuged in a sucrose gradient column at 37,020 rpm for 3 hours. Four drops of each fraction were collected and diluted with 3.3 ml of TBS. pH 7.6, containing 0.01 M sodium citrate before their UV absorbancies and PFU were determined. The result (Fig. 7) indicates a unimodal distribution of infectivity which sediments faster than the bulk of non-infectious UV-absorbing materials. Absence of infectivity in fractions

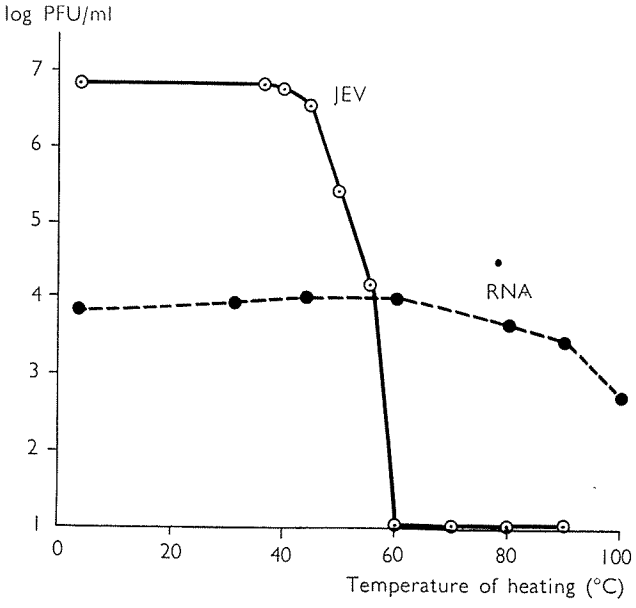

Fig. 5. Temperature Stability Curves of Infectivity of JEV and the RNA Fraction

Medium: 0.14 M NaCl in 0.02 M phosphate buffer, pH 7.6
with 0.01M Sodium citrate

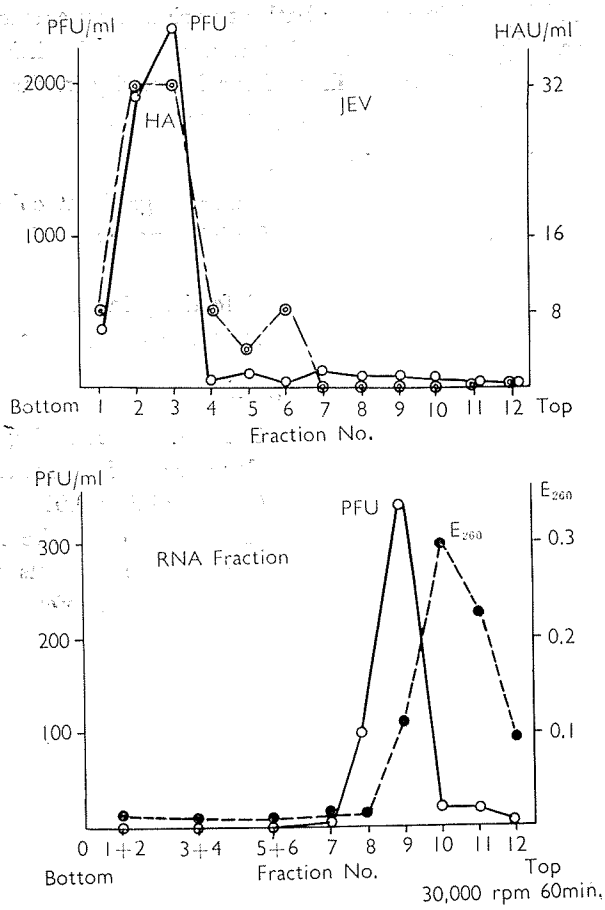

Fig. 6. Sedimentation of Infectivity and Hemagglutination Activity of JEV and UV Absorption and Infectivity of the RNA Fraction in Sucrose Density Gradient Columns

○——○ Infectivity (PFU/ml)
◎—·—◎ Hemagglutinating activity (HA)
●----● UV absorption at 260 mμ (E_{260})

having high UV absorption cannot be explained solely by the inhibiting action of normal RNAs on the infective principle. Approximately three peaks of E_{260} were also observed in the normal RNA fraction centrifuged in parallel with infected RNA. These peaks probably corresponds to microsomal RNAs.

5. *Infectivity of the RNA fraction in suspended cell systems*

Infectivity of the RNA fraction is detectable in this system also. Primary

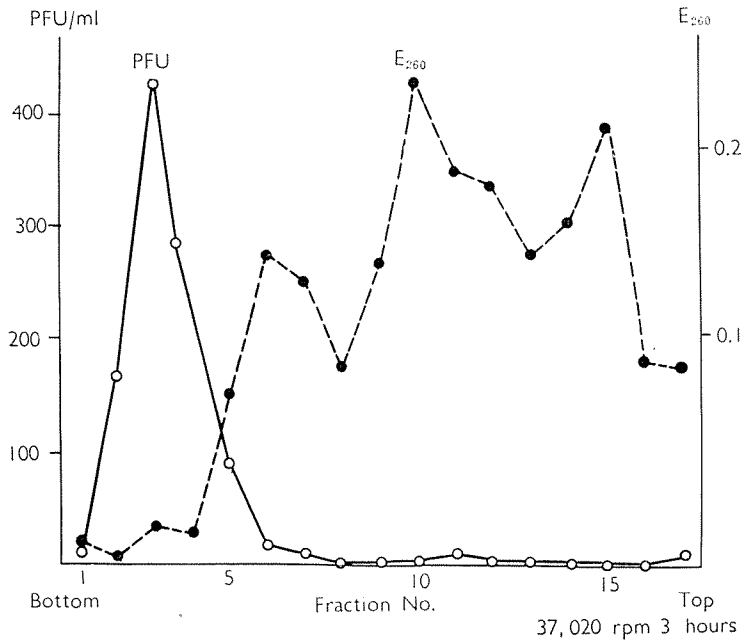

Fig. 7. Distribution of Infectivity and UV Absorption of the RNA Fraction in Sucrose Gradient Columns

Infectivity (PFU/ml)
UV absorption at 260 mμ (E_{260})

host cells detached from monolayers were suspended at a concentration of 10^6 cells /ml in RNA solution dissolved in various concentration of NaCl in 0.02 M phosphate buffer, pH 7.4. Cell-RNA mixtures were incubated at 37°C for a given time, then diluted and titrated for infective centers on chick cell sheets. When chick cells were used as a primary host for RNA, infective center formation followed the course represented in Fig. 8; a concentration of 0.64 M NaCl and 15 minutes' interaction were the optimal conditions in this case. The three established cell lines tested so far (HeLa S3, KB and MS cells) were all able to serve as primary hosts for RNA, but the optimal conditions for cell-RNA interaction and efficiency as a host differed with the type of cell (Table 3).

There was no carry-over of infective centers of RNA to the monolayer indicator of chick cells other than those which had been caught by interaction with primary host cells. This was because, in diluted conditions no infective centers were formed by cell-RNA mixtures and also because RNA diluted in the absence of primary host cells produced no plaques. There is little, if any possibility that the infective components which had simply been adsorbed on the surface of primary host cells were transferred to chick cells to form plaques after the attachment of the primary host cells to the chick cells. This was because, when cell-RNA mixtures were

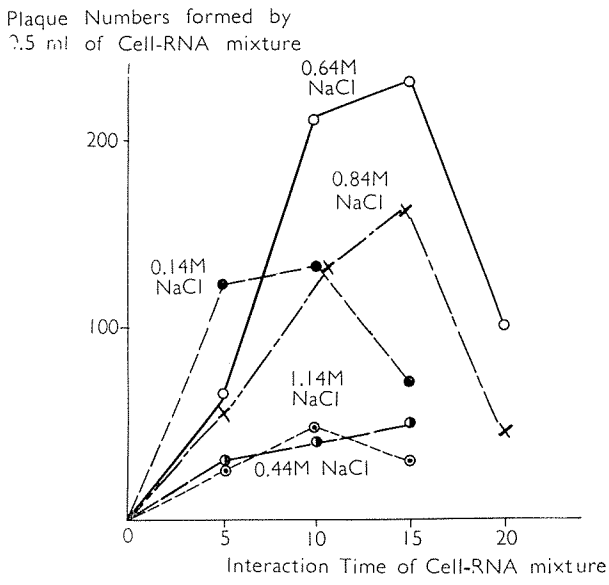

Fig. 8. Time Course of Infective Center Formation in Suspended Chick Cell-RNA System

diluted with medium containing 10 μg of RNase per ml and incubated at 20°C for 30 min. then titrated for infective centers, they formed appreciable amount of plaques comparable to the controls without RNase treatment. Neither HeLa S3, KB or MS cells can serve as indicator monolayers for JEV and the RNA fraction even under hypertonic conditions.

Table 3. Capacities of Established Cell Line as Primary Hosts for the RNA Fraction to Form Infective Centers

Primary hosts for RNA	Optimal conditions for cell-RNA interaction	Plaque numbers formed by 0.5 ml of cell-RNA
Chick cells	0.64 M NaCl, 15 min.	459
Hela S 3 cells	0.07 M NaCl, 20 min.	227
KB cells	0.14 M NaCl, 15 min.	56
MS cells	0.07 M NaCl, 10 min.	60

Cell-RNA mixtures were incubated at 37°C for appropriate period and titrated for infective centers.
Cell concentration: 1×10^6 cells/ml when suspended in RNA.
Experiments with chick cells and HeLa S3 cells were done on the same day, but those with KB and MS cells were performed on the following day.

DISCUSSION

In the case of tobacco mosaic virus RNA, participation of viral protein components in the process of RNA infection has almost been ruled out (Fraenkel-Conrat *et al.*, 1961). But it is not yet the case with JEV-RNA because a pure preparation of JEV has not been obtained. The infectivity in the RNA fraction reported here differs in many respects from that of intact JEV, so that is not due to residual intact virus. The smaller sedimentation coefficient of the infective unit in the RNA fraction suggests that it is smaller than JEV particle. The sensitivity to RNase indicates that at least part of the viral RNA is not protected from the action of this enzyme. No critical points which had the same degree of trypsin sensitivity as in JEV were demonstrated in the infective principle of the RNA fraction. Resistance to ether and ethanol may be due to the absence of lipid components essential for the integrity of the intact JEV. The difference in pH stability and heat stability from that of JEV might be caused by loss of heat and acid labile parts of JEV necessary for its infectivity. Inactivation of the infectivity in the RNA fraction by fractionated immune serum is small when compared with the large inactivation of JEV. It is still uncertain whether the infective component in the RNA fraction is high molecular weight RNA itself or whether it has some chemical components besides RNA. The faster sedimentation of the infectious and RNase-sensitive component than the bulk of cellular non-infectious RNAs observed in a sucrose gradient coincides with the findings in poliovirus RNA that the virus RNA is of a higher molecular weight than cellular RNAs (Holland *et al.*, 1960; Kubinski *et al.*, 1962). However it is possible that a higher sedimentation rate may be due to some heavy contaminating substance. Even so, the apparent unimodal distribution of the infectivity in a sucrose gradient suggests the uniformity of the contaminating substance, *i. e.* all the infective units in the RNA fraction would contain the same amount of contaminants.

Nakamura and Ueno (1963) reported that the infectivity of RNA was more sensitive to heat at 50°C than intact JEV. This is not the case in the presence of chelating agents as reported in this paper. This difference may imply the participation of heavy metal contamination in the process of RNA inactivation, as has been suggested by Colter and Ellem (1961).

The efficiency of plating (eop) of the RNA fraction is a function of environmental conditions as well as the conditions of the host cells. The standard method used in these experiments is by no means the most sensitive one, and a better system would raise the eop close to that of intact virus as in the case of tobacco mosaic virus (Sarker, 1963).

Three established cell lines, which are not suitable as indicators of plaque formation by JEV or its RNA under an ordinary agar overlay, were able to serve as primary hosts for the infected RNA fraction in suspended cell systems. This

agrees with the results of Holland *et al.* (1959a, b) that cells which are naturally not susceptible to poliovirus could be infected with poliovirus RNA and produce virus indistinguishable from the original poliovirus.

ACKNOWLEDGEMENTS

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