Infectious Ribonucleic Acid (RNA) of Japanese B Encephalitis (JBE) Virus: High Yields of RNA and Its Stabilization.* (29672)

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It is commonly known that infectivity associated with extracted RNA is of the order of 0.1% of that of original intact virus (1,2). We have reported(3) that an infectious RNA and JBE virus extracted from infected mouse brains homogenized with sterile distilled water was quite labile for storage. This communication describes the effect of ethylenediaminetetraacetate (EDTA) on the increase of plaque forming units (PFU) and on the stabilization of infectious JBE-RNA for titrating in chick embryo cell monolayers, and also the thermostabilities of JBE-RNA in different molarities of salt solutions.

Materials and methods. Brains of adult mice infected with the Nakayama strain (M-67 and -68) of JBE virus were used. Infectious RNA was extracted from a clarified 10% infected mouse brain suspension which was supernatant fluid after low centrifugation, by means of phenol extraction as described previously(3,4). Homogenizing solutions for the brains and diluents for virus and RNA preparations were respectively indicated in the text. Clarified 10% virus suspension and the RNA preparation obtained from this virus suspension were regarded as 10^{-1} dilution. The virus and RNA assays were carried out in chick embryo cell monolayers using the plaque method described previously(3,4). PFU were counted after 4 days' incubation at 36°C.

Results. Stabilities of intact JBE virus by cations at 50°C. Clarified virus suspensions prepared with sterile distilled water were diluted 2 times with different kinds of salt solutions (Fig. 1) which were made up at twice the final concentration desired. These virus-salt mixtures were heated at 50°C for 30 and 60 minutes in water baths, then the heated tubes were cooled quickly by immersion in an ice bath. Heated virus materials were diluted more than 100 × with physiological saline to exclude particulate matter which might be formed in the heating process. The results obtained in 4 experiments are summarized in Fig. 1, in which 1.0 M Na_2SO_4, 1.5 M $MgSO_4$ and H_2O protected JBE virus from inactivation at 50°C, whereas 0.1 M Na_2SO_4, 0.15 M $MgSO_4$, 0.1 M NaCl and 1.0 M NaCl markedly increased the rate of inactivation of the virus. Therefore, in the case of JBE virus, distilled water and high concentrations of monovalent and divalent cations tested, except NaCl, stabilized the virus to inactivation at 50°C. This finding is, in part, in contrast to the results obtained in the case of enterovirus by others (5,6).

Effect of cations on inactivation of JBE-RNA at 50°C and at 37°C. In the case of JBE-RNA, 1.0 M NaCl was a better stabilizer than 1.0 M Na_2SO_4 and 1.5 M $MgSO_4$ at 50°C (Fig. 2). Treatment of intact virus for 30 minutes with distilled water or 1.5 M $MgSO_4$ did not alter its infectivity, while under the same conditions, there was loss of infectivity of JBE-RNA. When the RNA-salt mixtures were heated at 37°C, the inactivation rates of the RNA which were indicated in Fig. 2 were more emphasized, except that 0.15 M $MgSO_4$ afforded a little protection at 37°C, although it was an ineffective stabilizer for the RNA at 50°C.

Effects of homogenizing solutions for infected mouse brains and of diluents on the infectivity of JBE-RNA. Each of 2 mouse brains infected with JBE virus was individually homogenized in sterile distilled water, 0.01% EDTA in Aq., 0.01% EDTA in M/20 Sorensen buffer (pH 7.8), bentonite 100 μg/ml of Aq., and 1000 μg/ml of Aq. suspension to make a 10% suspension. After low centrifugation, the supernatant fluids were used as virus titrating materials and as

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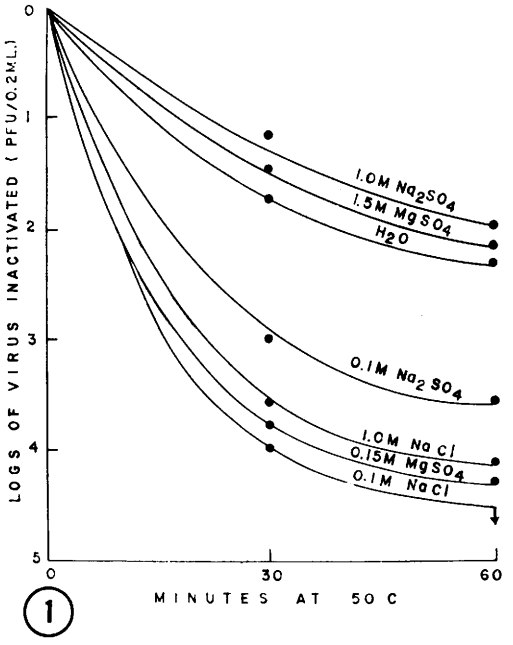
source materials for extraction of infectious RNA. The RNA preparations obtained from these homogenates were diluted with various kinds of diluent (Table I) and RNA infectivities in those preparations were assayed on chick embryo cell monolayers. Results are shown in Table I, in which maximum plaques of the RNA were produced when the RNA was extracted from a brain suspension with 0.01% EDTA/Aq. as well as 0.01% EDTA/buffer and when this RNA preparation was serially diluted with 1.0 M Na_2SO_4/M/20 Sorensen buffer (pH 7.8); a ratio of log PFU of RNA to that of virus was 1.0. On the other hand, bentonite had less effect than that of EDTA on the increase of RNA infectivity. Of course the RNA thus extracted was very sensitive to RNAase treatment, whereas the virus was not sensitive to RNAase. Furthermore, a strict linearity between the number of plaque on cell monolayers and relative RNA concentration thus obtained from EDTA-homogenate was observed.



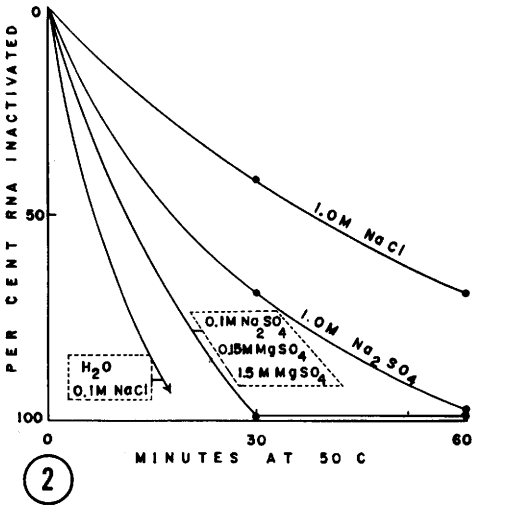


FIG. 1. Stabilities of intact JBE virus by salts at 50 °C.

FIG. 2. Effect of salts on inactivation of JBE-RNA at 50°C.

Effects of suspending solutions on stability of infectious JBE-RNA. Following homogenization of infected brains with distilled water or 0.01% EDTA/buffer, an infectious RNA was freshly extracted, then the RNA was promptly diluted 2 times with an equal volume of the solutions as indicated in Table II. These diluted RNA preparations were stored in a refrigerator (4°C) and in a deep freezer (—20°C), and the infectivities of these materials were measured at various intervals. Results obtained (Table II) indicated that the infectivity of the RNA was very stable when the RNA was suspended in 0.01% EDTA solution and was stored at —20°C,

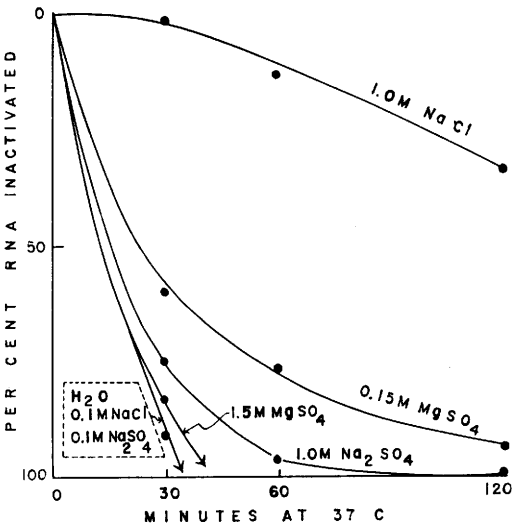


FIG. 3. Effect of salts on inactivation of JBE-RNA at 37°C.

especially when using the RNA which was extracted from the virus suspension made with 0.01% EDTA buffer. The fact that the infectivity of the RNA obtained from water suspension diluted 2 times with 0.02% EDTA/2.0 M NaCl was undetectable after 2 days' storage at 4°C, but detectable at −20°C, and also the fact that the infectivity of the RNA obtained from 0.01% EDTA virus suspension was almost completely main-

TABLE I. Effects of Homogenizing Solutions for Infected Mouse Brains and Diluents on Infectivity of JBE-RNA.

Solution for homogenization	Virus		RNA		
	Diluent	Log PFU*	Diluent†	Log PFU	Virus/RNA
H_2O	Saline	6.6	1.0 M NaCl	3.3	3.3
			1.5 M $MgSO_4$	3.0	3.6
			1.0 M Na_2SO_4	4.4	2.2
			1.0 M Na_2SO_4–.01% EDTA	4.2	2.4
	RNAase treatment‡ Saline	7.5	RNAase treatment 1.0 M Na_2SO_4	0 §	7.5
.01% EDTA/Aq.	Saline	7.0	.1 M NaCl	0	7.0
			1.0 M NaCl	4.4	2.6
			.1 M Na_2SO_4	0	7.0
			1.0 M Na_2SO_4	6.0	1.0
			1.0 M Na_2SO_4–.01% EDTA	5.5	1.5
	RNAase treatment Saline	8.0	RNAase treatment 1.0 M Na_2SO_4	0	8.0
.01% EDTA/buffer	Saline	7.0	1.0 M Na_2SO_4	6.0	1.0
Bentonite 100 μg‖	"	7.0	1.0 M Na_2SO_4	5.3	1.7
" 1000 μg	"	7.0	1.0 M Na_2SO_4	5.5	1.5
			1.0 M Na_2SO_4–.01% EDTA	5.7	1.3

* Log PFU/0.2 ml.
† Salts were dissolved in M/20 Sorensen buffer (pH 7.8).
‡ RNAase treatment: 50 μg/ml for 10 min at 4°C.
§ No plaque was formed at an obtained RNA (referred to 10^{-1}) diluted 10× with 1.0 M Na_2SO_4/buffer.
‖ Suspended in Aq.

TABLE II. Effects of Homogenizing and Suspending Solutions on Stability of Infectious JBE-RNA.

Infected brains homogenized with	RNA diluted 2× with	Storage temp,°C	Storage time (day) 0	2	12
H_2O	H_2O	4	3.0+*	0	0
		−20		0	0
	.02% EDTA Aq.	4	5.0	5.0	4.7
		−20		4.0	3.6
	.02% EDTA buffer	4	5.0	3.6	2.3
		−20		4.3	4.4
	.02% EDTA, 2.0 M NaCl	4	4.6	0	0
		−20		3.7	4.0
.01% EDTA buffer	.02% EDTA buffer	4	6.0	5.7	5.0
		−20		5.4	5.3
	.02% EDTA, 2.0 M NaCl	4	6.0	5.7	5.0
		−20		6.0	6.0

* Log PFU/0.2 ml.

TABLE III. Effects of Organic Salts and Other Treatment on Stability of Infectious JBE-RNA.

RNA + Equal volume of following solutions	Log PFU/0.2 ml		
	0	50°C, 60 min	Log PFU inactivation
H_2O	4.0	0 *	4.0
.2% EDTA	5.6	5.1	.5
.02% EDTA	5.8	5.1	.7
.002% EDTA	5.8	5.0	.8
Sodium benzoate†	5.3	1.0	4.3
" thioglycolate	5.3	3.0	2.3
" acetate	5.0	1.0	4.0
" citrate	5.6	1.0	4.6
" succinate	5.1	1.0	4.1
.2% Formaline	4.8	0	4.8
.02% EDTA + .2% formaline	4.8	3.7	1.1
4 M Guanidin-HCl	4.4	0	4.4
Fluorocarbon treatment‡	4.6	0	4.6
Bentonite 1000 μg/ml§	4.0	0	4.0

* No plaque was formed at an obtained RNA diluted 10× with 1.0 M Na_2SO_4/buffer.

† .02%.

‡ RNA was treated 3 times with an equal volume of fluorocarbon.

§ RNA was extracted from infected mouse brains homogenized with bentonite 1000 μg/ml.

tained under the same conditions have not been explained.

Effects of organic salts and other treatments on stability of infectious JBE-RNA. To determine the effects of various substances and concentrations of EDTA on stabilization of infectious JBE-RNA, the following experiments were performed. First, the effects of other organic salts on the stability of the RNA were tested. The results were demonstrated in Table III, in which other organic salts tested did not stabilize the RNA to the same degree as EDTA, but thioglycolate had a somewhat efficient stabilizing effect. Second, the effects of protein denaturating agents such as formaline and guanidin-HCl, or of protein-removing treatments such as fluorocarbon and bentonite treatments of mouse brain suspension before extraction of RNA, were tested. As shown in Table III, these agents and treatments did not protect the RNA against heat inactivation (Table III).

Discussion. Melnick(5,6) demonstrated that viruses could be divided into 2 groups by stabilization or inactivation in the presence of high concentrations of monovalent and divalent cations; (a) the Mg-stabilized viruses containing RNA and (b) the Mg-inactivated viruses containing DNA. On the other hand, Norman and Veomett(7) reported that raising the molarity of NaCl from 0.12 M to 1.2 M resulted in a 2-fold increase of survival at 60°C of RNA from poliovirus. In the recent communication, stabilization and inactivation of the JBE virus as well as its RNA were tested from a similar viewpoint. The results indicated that high concentration of monovalent or divalent cations and distilled water protected the RNA virus from inactivation by heat, except NaCl which had been known as an effective stabilizer for DNA viruses(6). Thus a difference in stabilizing effect on JBE virus was noted among monovalent cations, but there was no indication that divalent cations had any greater effect than the monovalent. The suggestion then arises that the differences in effect are related to the kind of anion present. This speculation is supported by the data of others(6) which show that NaCl protects some but not all DNA viruses from thermal inactivation indicating that the anion is the determining factor.

It is suggested that the effect of anions on inactivation of virus is associated with viral surface protein, but not viral ribonucleic acid; in other words, virus constitution, because inactivation of virus by heat is due to denaturation of viral protein without inactivating virus nucleic acid(8,9). This suggestion would be supported by the experiments described here, in which a high concentration of monovalent cations protected the JBE-RNA from thermal inactivation at 50°C, whereas a high concentration $MgSO_4$ and H_2O which are effective stabilizers for the virus inactivated the JBE-RNA. However, this conclusion should be confirmed by further experiments testing many kinds of salt.

On the other hand, the facts that infectivity associated with extracted RNA is of the order of 0.1% of that of original intact virus(1,2), and that there are relatively stable infectious RNA such as tobacco mosaic virus as well as enterovirus RNA(10) and an ex-

tremely labile one such as foot-and-mouth disease virus RNA(11) even in the presence of chelating agents, are commonly well known. It might demonstrate that the latter fact would be due to some differences of viral particle constitutions between viruses tested. Furthermore, the fact that EDTA has a marked effect stabilizing the RNA, on the hypothesis that the inactivation of the RNA was caused by heavy-metal contamination of the preparations, has been reported (12,13). However, it was reported that an increase in the plaque count of the poliovirus RNA could not be brought about with the use of sodium EDTA(14). We reported(3) that an infectious JBE-RNA extracted from infected mouse brains homogenized with distilled water was quite labile for storage; the plaque count of a RNA preparation with 81 PFU decreased to 22 at —20°C, to 2 at 4°C, and to 0 at room temperature for 24 hours. However, in this paper, the results obtained indicate that sodium EDTA stabilized the JBE-RNA either when EDTA was used as a homogenizing solution of the infected mouse brain or when it was added to the RNA extracted from virus to which no sodium EDTA had been added, that the plaque count of the JBE-RNA was greatly increased when EDTA was used as a homogenizing solution of starting material and when 1.0 M Na_2SO_4 was used as a diluent of the RNA, and that bentonite which is a specific RNAase inactivator(15) and other protein denaturating or protein removing agents and treatments had no such effects. Therefore, it might be speculated that the lability of JBE-RNA is not due to contamination of protein which contained RNAase but due to heavy-metal contamination of the preparations.

Summary. H_2O, 1.0 M Na_2SO_4, and 1.5 M $MgSO_4$ protected the JBE virus from inactivation at 50°C, whereas 0.1 M and 1.0 M NaCl, 0.1 M Na_2SO_4 as well as 0.15 M $MgSO_4$ inactivated the virus. However, the JBE-RNA was more stable in the presence of a high concentration of NaCl than in H_2O and other salts tested. In addition, when EDTA was used either as a solution for homogenization of infected mouse brains or as a suspending solution of extracted infectious JBE-RNA, especially in the former case, the PFU of the RNA which were measured on chick embryo cell monolayers closely approached that of the virus and the infectivity of the RNA was protected from inactivation.

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