the methicillin-sensitive ones (MIC $\leq$ 6.25 μg/ml). It is concluded that the level of resistance to penicillin G in *Staph. albus* is the result of both the activity of the penicillinase it produces and the level of its "inherent" or natural resistance, the latter being higher for the methicillin-resistant than for the methicillin-sensitive cells.

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A Diluent of Infectious Ribonucleic Acid of Japanese B Encephalitis Virus For Experiments with Chick Embryo Cell Monolayers.* (28565)

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Optimal conditions for extraction of infectious ribonucleic acid (RNA) of Japanese B encephalitis (JBE) virus and for plaque formation in chick embryo cell monolayers have been described(1), in which maximum number of plaques was formed when 1.0 M NaCl solution was used for a diluent of infectious JBE RNA. This report concerns a more efficient diluent than 1.0 M NaCl which was found in recent experiments.

Materials and methods. Brains of mice infected with the Nakayama strain (M-67 and -68) of JBE virus were homogenized with sterile distilled water. This suspension was used as a starting material. Infectious RNA was freshly extracted from this suspension just before each experiment by means of phenol extraction according to the methods of Gierer and Schramm(2) and Colter *et al.* (3) as modified by Nakamura and Ueno(1). Details of the procedure and extraction efficiency for RNA in relation to the starting virus have been given(1). That the infectivity of extracted RNA was not due to whole virus was demonstrated by the use of purified RNAase(1); this extracted RNA was very sensitive to RNAase treatment and showed a u.v. adsorption pattern characteristic of RNA, whereas the virus was not sensitive to RNAase. Chick embryo cell monolayers were prepared by seeding each bottle with 4 ml of cells obtained by trypsinization of 9-day-old chick embryos. The cells were suspended in 0.5% lactalbumin hydrolysate, 10% inactivated bovine serum, and 0.002% phenol red in Hanks' balanced salt solution (BSS) with antibiotics. After overnight incubation at 37°C, the cultures were used for assay of virus and RNA infectivities according to the plaque method of Porterfield(4). The cell monolayers were washed with phosphate buf-

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TABLE I. Effects of Diluents of JBE Virus and Its RNA on Plaque Formation in Chick Embryo Cell Monolayers.

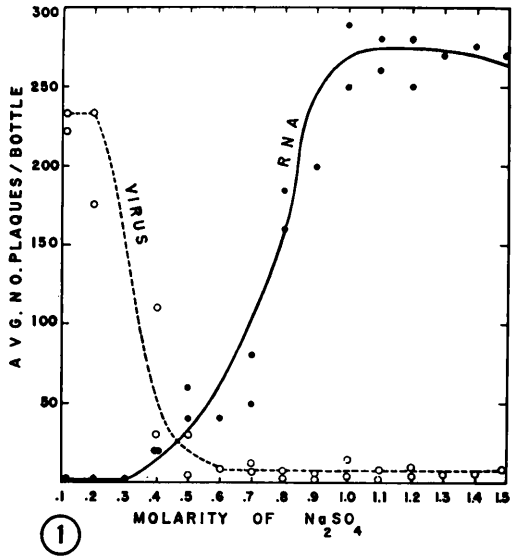
Diluent	Log PFU/0.2 ml Virus	Diff.*	RNA	Diff.*
.14M NaCl	7.4	− .3	<2.0	>1.3
1.0 M "	7.1		3.3	
.1 M Na_2SO_4	7.0	−1.5	<2.0	>2.4
1.0 M "	5.5		4.4	
.1 M $MgSO_4$	6.6	− .6	<2.0	>1.0
1.5 M "	6.0		3.0	
.1 M $MgCl_2$	7.0	−1.9	<2.0	> .3
1.0 M "	5.1		2.3	
.1 M KCl	5.3	+1.0	<2.0	>1.3
1.0 M "	6.3		3.3	
.1 M $CaCl_2$	6.7	−1.7	<2.0	0
1.0 M "	5.0		2.0	

* Difference = PFU obtained by hypertonic salt diluent / PFU obtained by isotonic salt diluent

fered saline (PBS) at pH 7.4 prior to seeding 0.2 ml of virus or RNA to each bottle. Following incubation at room temperature for the appropriate time, the infected cultures were washed thrice with PBS and were overlaid with 3.0% agar mixed with Hanks' solution containing 0.4% lactalbumin hydrolysate, 0.3% tryptose, 0.3% bovine serum albumin V fraction, neutral red, $NaHCO_3$, and antibiotics. Plaques were counted after 3 or 4 days' incubation at 36°C.

Results. Selection of most suitable diluent for yielding the maximum plaques formed by RNA. RNA and virus were 10-fold diluted with various kinds of salt solution indicated in Table I, and 0.2 ml of diluted materials was seeded per chick embryo cell monolayer for titration of plaque forming units (PFU). As shown in Table I, PFU of virus or RNA was affected by the kind of salt and by its molarity as is well known(5-10). It was demonstrated that, among the solutions tested, Na_2SO_4 had the following properties: 1) maximum plaques were produced when RNA was diluted with 1.0 M Na_2SO_4, rather than 1.0 M NaCl and 2) there was a great difference between the PFU by virus diluted with 0.1 M and that with 1.0 M Na_2SO_4.

The following experiment gives more detail and precise results. Appropriate concentration of virus and RNA were diluted 1:10 with different molarities of Na_2SO_4 indicated in Fig. 1, and 0.2 ml of diluted materials was inoculated in the chick embryo cell monolayers previously washed with PBS. After 10 to 15 minutes, the monolayers were washed with PBS, then were overlaid with agar. There were distinct differences between the effects of Na_2SO_4 on the virus and those on RNA (Fig. 1). It is seen that increasing the molarity of Na_2SO_4 as a diluent



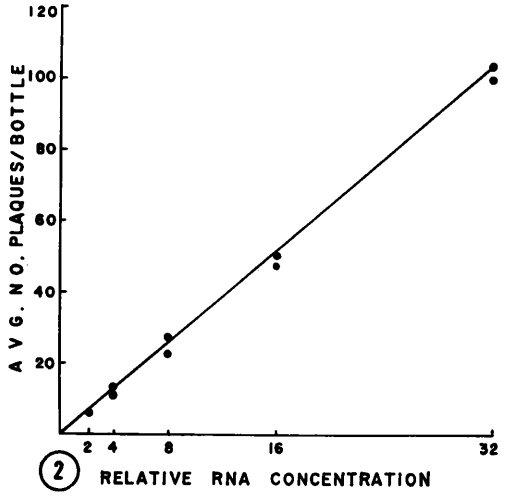


FIG. 1. Relationship of number of plaques produced by JBE virus and its RNA diluted with different molarities of Na_2SO_4.

FIG. 2. Correlation of degree of infectivity of JBE RNA to dilution factor on chick embryo cell monolayers in 1.0 M Na_2SO_4 solution.

TABLE II. Adsorption Time of RNA to Chick Embryo Cell Monolayers.

Exposure time of RNA* (min)	Diluent of RNA	
	1.0M Na_2SO_4	1.0M NaCl
1	105†	11
5	247	53
10	261	235
15	300	300
20	300	226
30	200	220
45	150	151

* At indicated exposure time, the cells were washed 3 times with 1 ml of PBS.
† Avg No. of plaques per bottle (Inoculum: 0.2 ml).

for the *virus* decreased the number of plaques formed, whereas increasing the molarity of Na_2SO_4 as a diluent for RNA increased the number of plaques produced.

Adsorption time of RNA to cells. Infectious RNA was appropriately diluted with 1.0 M Na_2SO_4 or 1.0 M NaCl. RNA thus diluted was inoculated to the monolayers previously washed with PBS, and the monolayers were washed at indicated exposure time shown in Table II, and were overlaid. RNA diluted with Na_2SO_4 adsorbed to the monolayers more rapidly than those with NaCl; the former RNA requires one minute to initiate one-third maximal infection.

Relationship between plaque counts and relative RNA concentration. A strict linearity between plaque count on cell monolayers and relative RNA concentration was observed when 0.2 ml amounts of material, serially diluted in 2-fold steps with 1.0 M Na_2SO_4, was tested (Fig. 2).

Discussion. It is well known that the plaques could be produced by RNA only when the RNA was diluted with high molarity salts or when the cells were treated with high molarity salts prior to seeding the RNA. In most cases, however, the plaques were also produced by the virus from which RNA was derived under similar conditions which were efficient for the RNA infection, although the number of plaques thus produced was sometimes fewer than those formed under optimal conditions for the intact virus.

It is indicated here that it would be possible to distinguish the plaques produced by the RNA from those by the virus, when Na_2SO_4 is used as the diluent in appropriate concentrations for the desired purpose. Also, optimal ranges of molarity of this reagent, as a diluent of RNA, for yielding the maximum plaques in chick embryo cell monolayers are more extensive, *i.e.*, 1.0 to 1.5 M compared with that of NaCl, 1.0 M, previously determined(1). Moreover, the former is less toxic than the latter to chick embryo cell monolayers. Hence, exposure time of the monolayers to materials diluted with Na_2SO_4 could be more prolonged than in the case of NaCl for any experimental design. Furthermore, RNA diluted with Na_2SO_4 adsorbed to the cells more rapidly than those with NaCl. Therefore, Na_2SO_4 might be recommended as a diluent, presumably as a washing solution for the cells, for the studies on infectious RNA using the chick embryo cell monolayers.

Summary. It was demonstrated that Na_2SO_4 is an efficient salt for a diluent of JBE RNA, presumably for a washing solution of the cells prior to seeding infectious RNA, because this is less toxic than NaCl for the chick embryo cells, and increasing the molarity of Na_2SO_4 as a diluent for RNA increased the number of plaques produced, whereas increasing the molarity for the virus decreased the number of plaques formed.

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