Enhancement of Sindbis Virus Infectivity by Reduced Salt Concentration[1] (35259)

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(Introduced by E. E. Baker)

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The relatively few studies which have examined the effect of salt concentration on the ability of viruses to infect animal cells support the generalization that, within the physiologic range, increasing the salt concentration of the infecting medium enhances infectivity to a slight or moderate degree (1, 2). At least in the case of lipid-containing viruses, this has been attributed to the ability of salt to mask the negative charges on the surface of both virions and cells, and thus prevent electrostatic repulsions from interfering with viral attachment (2).

Recently, we have observed a striking exception to this generalization in the case of Sindbis virus and certain of its mutants. The preliminary observations which led us to study this phenomenon involved a class of temperature-sensitive mutants of Sindbis virus which Burge and Pfefferkorn (3), who isolated and characterized them, have termed "RNA+". At 28°, these mutants replicate normally. At 40°, they remain capable of synthesizing viral RNA, but are defective in a later function. When we infected monolayers of chick embryo fibroblasts with one such mutant (TS-2) at an input multiplicity we thought to be 1 plaque-forming unit (PFU)/cell, we observed extensive cytopathogenic effects (CPE), typical of Sindbis virus, following incubation at 40°. In cultures infected and incubated in medium of normal NaCl concentration, this CPE appeared slowly, and was often inapparent until 36 to 48 hr after infection. In contrast, cultures infected and incubated in medium containing 66% of the normal concentration of NaCl manifested striking CPE within 18 to 22 hr following infection. This accelerating effect was not an osmotic one, as substitution of sucrose for the missing salt failed to diminish the CPE. Enhancement of late viral functions did not appear to be involved, as the cultures remained incapable of yielding significant quantities of progeny virus at 40°; furthermore, when cultures were infected in medium of one salt concentration, washed, and then incubated in medium of the other salt concentration, the appearance of early CPE depended on the infecting medium and not on the medium used subsequently for incubation. Finally, the early appearance of CPE could be reproduced even in the presence of normal salt concentration by raising the input multiplicity to 10 PFU/cell. We therefore turned our attention to the effect of NaCl concentration on the efficiency of plaquing (EOP) of Sindbis virus and a series of temperature-sensitive Sindbis mutants.

Materials and Methods. Viruses. Wild-type Sindbis virus was obtained from Dr. John Enders, and the "HR" strain, which is wild-type with respect to temperature-sensitive mutations, from Dr. Elmer Pfefferkorn. Temperature-sensitive mutants TS-2, TS-10, and TS-20, which are "RNA+" mutants, and TS-4, which is "RNA—"; *i.e.*, incapable of synthesizing RNA at 40°, were also gifts from Dr. Pfefferkorn. The titers of our working stocks ranged from about 1 to 5 $\times$ 10^9 PFU/ml for chick fibroblasts in the case of our wild-type and the HR strains, and were about 1 log lower in the case of the tempera-

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ture-sensitive mutants.

Infection of cells. Virus was diluted either in cell culture medium (Eagle's minimal essential medium containing 0.2% $NaHCO_3$; 2% inactivated fetal calf serum; penicillin, 100 units/ml; streptomycin, 100 μg/ml; and amphotericin, 5 μg/ml), or in Hanks' salt solution (HSS), containing 0.03% $NaHCO_3$ and 2% inactivated fetal calf serum. In each case, variation in salt concentration was achieved by using stock solutions of Hanks' salts differing in NaCl concentration, but otherwise identical. The final concentration of NaCl in our standard minimal essential medium with Hanks' salts (including the sodium and chloride ions added separately as HCl and as components of other salts) is normally 0.15 *M*. We therefore have expressed NaCl concentrations in the text as percentages of this normal value.

Confluent monolayers of fibroblasts prepared from 9-day-old chick embryos and grown in 60-mm plastic petri dishes were infected with 0.1 ml of the appropriate dilution of virus. After a 1-hr adsorption period in a 36°, CO_2-flushed incubator, maintained at sufficient humidity to prevent any significant drying of the inoculum, the cultures were washed with HSS, and overlaid with culture medium of normal salt concentration, containing 1.25% agar. Following incubation in a humidified, 5% CO_2 atmosphere at 36° for 2 days (for wild-type virus), or at 28° for 4 days (for temperature-sensitive mutants), the cultures were stained with 0.04% neutral red for counting of plaques.

Results. Effect of NaCl concentration on the plaquing efficiency of TS-2. Figure 1 demonstrates that reducing the NaCl concentration of the infecting medium by as little as 18% yields a marked enhancement in infectivity. This degree of enhancement is maintained when the NaCl concentration drops to 60% of normal. Further reductions are associated with an EOP which is somewhat lower, but still greater than that achieved at normal salt concentration. Conversely, a lower than normal EOP results when the NaCl concentration of the infecting medium is 26% above normal.

Effect of NaCl concentration on other temperature-sensitive Sindbis mutants and on wild-type virus. Since TS-2 is not known to suffer from any defect in infectivity, we expected that its response to salt should be mimicked by the wild-type. This expectation proved false. As Fig. 2 demonstrates, both our own wild-type strain, and the HR strain from which Burge and Pfefferkorn derived the temperature-sensitive mutants under study, exhibit an increased plaquing efficiency at reduced NaCl concentration, but this increase—2- to 3-fold—is well below the 10- to 11-fold increase observed with the three RNA+ mutants, TS-2, TS-10, and TS-20. The response of TS-4, an RNA− mutant, although probably greater than those of the wild-types, is closer to them than it is to the three RNA+ mutants.

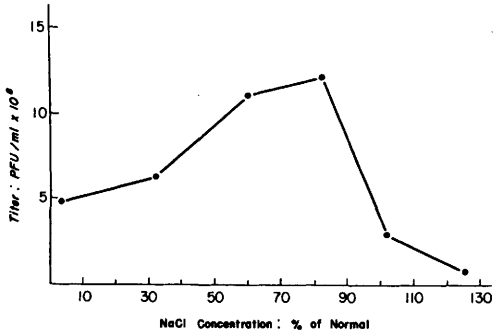

FIG. 1. Effect of NaCl concentration on plaquing efficiency of TS-2. Quadruplicate cultures were infected with virus diluted in HSS containing 2% inactivated fetal calf serum, 0.03% $NaHCO_3$, and the indicated percentage of the normal concentration of NaCl. After a 1-hr adsorption period, the cultures were washed, overlaid with agar-containing medium of normal salt concentration, and incubated for 4 days at 28° to allow plaques to develop.

The greater responsiveness of TS-2, TS-10, TS-20, and possibly TS-4 to salt concentration is not attributable to their subsequent incubation at 28° for 4 days; wild-type cultures similarly treated have proved no more responsive to reduced salt concentration than when incubated at 36° for 2 days.

Nature of the salt effect; direct interaction between virus and diluting medium. We considered the possibility that a reduction in NaCl concentration enhanced infectivity either by retarding the thermal or chemical inactivation of virus, or by preventing neutralization of virus by inhibitors in the medi-

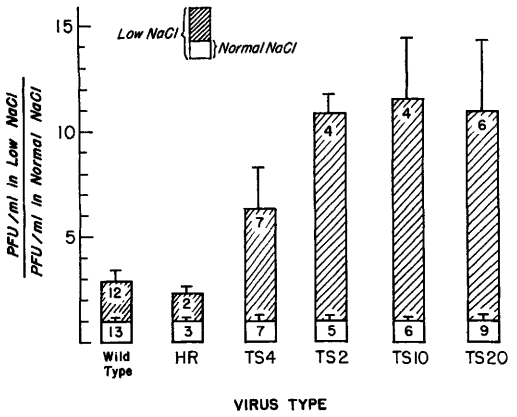

FIG. 2. Comparison of the effects of reduced NaCl concentration on the plaquing efficiency of wild-type Sindbis virus and temperature-sensitive mutants. The HR strain is wild-type with respect to temperature-sensitive mutations. TS-4 is an RNA— mutant; TS-2, TS-10, and TS-20 are RNA+ mutants. For each strain or mutant, the height of the bar represents the ratio of the titer obtained with medium of reduced NaCl concentration (66% of normal) to that obtained with normal medium. The latter titer is represented by the lower segment of the bar, and is arbitrarily assigned a value of unity. Vertical lines indicate standard errors, and numbers within the bars, the number of cultures used for each group. Conditions of infection were similar to those in Fig. 1, except that virus was diluted in complete cell culture medium, rather than in HSS. In contrast to HSS, the complete medium was somewhat hypertonic at this NaCl concentration, since considerably more $NaHCO_3$ was added during its preparation (24 mmoles/liter vs 4 mmoles/liter for HSS) to insure adequately prolonged buffering capacity for cell cultures. This greater tonicity may be responsble for the greater contrast between the effect of "normal" and low-NaCl solutions on the plaquing efficiency of TS-2 above compared with Fig. 1.

um. To test this, we incubated suitably diluted suspensions of TS-2 for 1 hr at 37° in cell culture medium of normal or reduced (66%) NaCl concentration, then diluted each a further 100-fold in the low-NaCl medium for plating onto chick cell monolayers. Virus pre-incubated in normal medium yielded a final titer of 5.1×10^8 PFU/ml (mean of 3 replicates). Virus pre-incubated in low-NaCl yielded an almost identical titer of 4.6×10^8 (mean of 4 replicates). We concluded, therefore, that the effect of salt was exerted not on the virus particle alone but on the interaction between virion and host cell.

We then examined the additional possibility that the fetal calf serum or other nutrients in the diluting medium interfered with this interaction, but to a different degree, depending on salt concentration. We diluted wild-type virus in HSS of either normal or reduced (66%) salt concentration, containing 0.1% egg white as the only protein (complete absence of protein yielded low and erratic titers). The use of such serum-deficient medium neither increased the titers obtained, nor abolished the enhancing effect of reducing the salt concentration (Table I).

Effect of salt concentration on virus adsorption. To determine whether salt concentration influenced the ability of virions to attach to host cells, we infected five replicate chick embryo fibroblast monolayers grown in 100-mm plastic petri dishes with 0.3 ml of inocula containing 5700 PFU of TS-2, diluted either in normal medium or in medium of reduced (66%) NaCl concentration. After incubation of the cultures for 1 hr to permit attachment, 0.1-ml aliquots of the inocula were removed, and diluted 100-fold into normal medium. Plaque assay of these latter dilutions to determine the titer of residual virus demonstrated that in each case 22% of the initial titer of virus remained after 1 hr. Thus, an identical 78% of the virions had been adsorbed regardless of the NaCl concentration of the infecting inoculum. These results indicate that the relative inability of the virus to infect chick cells at normal salt concentration is not due to a failure to attach, but presumably reflects an obstacle later in the process of infection.

To delineate further the interval during which salt concentration retained its ability to influence plaquing efficiency, we infected cultures with TS-2 for 1 hr in normal medium, washed the cultures twice with HSS, reincubated them for a second hour at 40° in 3 ml of either normal or low (66%) NaCl medium, rewashed them, and then added the agar overlay. The monolayers tolerated this procedure poorly, perhaps because of excessive washing, and erratic results were obtained. Nevertheless, the lack of any discernible enhancement of EOP by reduced NaCl

TABLE I. Effect of Protein and Nutrient Components of the Infecting Medium on the Ability of Reduced NaCl Concentration to Enhance Plaquing Efficiency.[a]

NaCl conc of medium	Titer (PFU/ml)	
	Virus diluted in complete medium with 2% inactivated fetal calf serum[b]	Virus diluted in HSS with 0.1% egg white[c]
(A) 100%	1.0×10^9	8.5×10^8
(B) 66%	3.1×10^9	2.0×10^9
Degree of enhancement (ratio of B:A)	3.1	2.3

[a] Chick embryo fibroblast monolayers were infected with wild-type Sindbis virus as described in Materials and Methods.

[b] Mean of 11 cultures in normal medium, and 10 in low-NaCl medium.

[c] Mean of 4 cultures in each type of medium.

concentration (Table II) suggests that few, if any, virions remain in the salt-sensitive phase of the infecting process more than 1 hr after adsorption.

Discussion. The striking enhancement of plaquing efficiency documented above is noteworthy for several reasons. First, it challenges the generalization, suggested by studies on fowl-plague virus (4), Newcastle disease virus (5), poliovirus (1, 2), and Venezuelan equine encephalitis virus (6) (like Sindbis, a Group A arbovirus), that at NaCl concentrations between 0 and 0.15 *M*, virus adsorption and/or infectivity increases as salt concentration increases. Second, its occurrence in the physiologic range of salt concentration suggests the possibility that in some cases, the virulence of naturally occurring viral infections of man or animals may be modified by apparently minor changes in the composition of body fluids. Third, the magnitude of the effect (*e.g.*, a 10- to 11-fold increase in plaquing efficiency resulting from a 34% reduction in NaCl concentration) suggests that the salt-sensitive step in infection may be a highly "cooperative" event; *i.e.*, one involving the participation of large numbers of similar or identical molecules, or large numbers of intramolecular bonds. Finally, the considerable difference in the response to salt between wild-type strains of Sindbis virus on one hand, and three different RNA+ mutants on the other, suggests that salt-responsiveness may be profoundly influenced

TABLE II. Effect of Reduced NaCl Concentration During the Second Hour of Infection on the Plaquing Efficiency of TS-2.[a]

Treatment subsequent to 1 hr adsorption period in normal medium	Titer (PFU/ml)
Agar overlay	1.90×10^{8}[b]
Incubation in normal medium for 1 hr followed by agar overlay	1.80×10^{8}[c]
Incubation in medium containing 66% of normal NaCl concentration for 1 hr followed by agar overlay	0.70×10^{8}[d]

[a] Cultures were infected with virus in normal medium. After a 1-hr adsorption period, the cultures were washed twice with HSS to remove unadsorbed virus, and reincubated for a second hour in cell culture medium of the indicated NaCl concentration. They were then rewashed, overlaid with agar-containing medium, and incubated at 28°.

[b] Mean of 3 cultures.

[c] Mean of 5 cultures.

[d] Mean of 4 cultures.

by small modifications in the envelope or capsid proteins of the virus. This interpretation is clouded by several uncertainties—for example, the common salt-responsiveness of the three RNA+ mutants is not easily attributable to any mutation which they share in common, since the only recognized mutations they bear—those responsible for temperature-sensitivity—are known to be different (7). In fact, the temperature-sensitive defect in TS-20 cannot, at present, even be assigned to a structural protein. Furthermore, the temperature-sensitive defect in the RNA— mutant TS-4 is known not to be in a capsid or envelope protein (3), yet this mutant also appears somewhat more salt-sensitive than the wild-type. How often temperature-sensitivity and salt-sensitivity represent the same mutation, and how often salt-sensitivity represents an additional, unrecognized mutation is thus unclear. Some evidence favoring the latter is provided by preliminary experiments which indicate that even when infection is carried out at 22°, TS-2 remains more responsive to salt concentration than wild-type virus, although the difference between the two strains is less at this temperature than at 36°. Certainly, each of the temperature-sensitive mutants, derived as it is from the action of potent mutagens, may harbor multiple mutations. Conceivably, any of a variety of such deleterious changes in protein structure might increase salt-sensitivity, perhaps by retarding the progress of the virion through the various stages of infection, thus increasing the time spent in that particular stage which is salt-sensitive.

Which stage is salt-sensitive is a question unresolved by the present data. Our results appear to eliminate the initial attachment of virus to cell, but do not distinguish between the subsequent, "irreversible" phase of attachment (2), penetration, and uncoating. Although a definitive distinction may require studies with radioactively labeled virus, and electron microscopy, some relevant clues may be discernible in the recent demonstration by Waite and Pfefferkorn that at low salt concentrations, cells infected with Sindbis virus release markedly reduced quantities of virus into the medium, although intracellular synthesis of viral components is unimpaired (8). Since raising the salt concentration promotes the rapid release of virus from such cells, or even from sonic extracts of infected cells, they conclude that either a terminal stage of virus assembly, or the release process itself is inhibited at low-salt concentrations. The relevance of these observations to our own data is uncertain, but conceivably, reduced salt concentrations, by unmasking surface charges on capsid proteins, might promote mutual repulsion among such protein molecules, inhibiting virus assembly on one hand, and facilitating virus uncoating on the other. Alternatively, reduced salt concentrations might increase the affinity of virions for an internal component of the host cell plasma membrane, thus facilitating penetration, but impeding release. The likelihood that the salt-sensitive step is a cooperative process is consistent with either possibility.

Also unresolved is the extent to which changes in infectivity reflect the influence of ionic strength in general, as opposed to specific effects of sodium and/or chloride ions. This problem probably cannot be confronted directly by substitution of other salts for NaCl, since such substitution, on the scale required, would be toxic to cells. However, if specific ion effects are involved, other ions, particularly multivalent ones, may well be considerably more active at a given ionic strength than sodium or chloride, either in the same or in the opposite direction. Experiments are currently in progress to determine whether small changes in the concentration of such ions magnify or inhibit the effect of NaCl on plaquing efficiency.

Summary. The efficiency of plaquing (EOP) on chick embryo fibroblast monolayers of three temperature-sensitive mutants of Sindbis virus defective in late functions was enhanced 10- to 11-fold when the inoculum was applied in medium containing 66% of the normal concentration of sodium chloride. Studies with one of these mutants revealed similar enhancement over a broad range of reduced salt concentrations. The EOP of wild-type virus and of a temperature-sensitive mutant defective in RNA synthesis were also enhanced, but to a lesser degree. No increase in EOP occurred if the exposure of virus to reduced salt concentration ended

before the virus suspension was applied to the monolayers; thus, a virus–cell interaction appeared to be involved. The salt-sensitive step occurred within the first hr of infection, but subsequent to virus adsorption.

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