Relationship of Sodium Chloride Tolerance and Serological Group of Staphylococcal Phages

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SUMMARY

Twenty-two staphylococcal bacteriophages representing serological groups A, B, F and L were examined for activity after incubation of free phage in 10 % (w/v) NaCl Trypticase soy broth for 24 hr. at 30°. The mean decrease in plaque-forming units for ten phages of serological group A was 13 %; in no instance was inactivation of any group A phage greater than 33 %. All but one of nine group B phages were 88 % or more inactive after incubation in the 10 % NaCl broth; the single exception was phage 83 with slightly more than 40 % inactivation in both 0.5 % or 10 % NaCl broth. Serological group F phage 42D resembled phages of group A, whereas phage 77 of group F and group L phage 187 were intermediate between group A and B phages with respect to NaCl stability.

INTRODUCTION

The effect of the ionic environment on bacteriophages has been the subject of many investigations. Relatively few of the studies reported, however, have been concerned with the stability of free phages in the presence of high concentrations of salt. Bronfenbrenner (1925) observed that concentrations of NaCl greater than 1 % inactivated 99 % of phage, although the particular phage tested and time required for inactivation were not specified in his report. Adams (1949) examined the inactivating effects of temperature on *coli* phages, particularly T5, in concentrations of NaCl ranging from 0.01 to 2 N. He found that inactivation of phage T5 was almost complete after 1 hr. at 37° in solutions containing less than 0.2 N-NaCl, whereas at concentrations of 0.4 N and 0.8 N there was no significant decrease in plaque count. Increase in sodium ion concentration resulted in increased stability of phages to the inactivating effects of temperature (about 50° to 70°); phage T5 was equally stable in 2 N-NaCl solution and in nutrient broth. In studies on the effect of electrolytes on the adsorption of staphylococcal phages, Rountree (1951) noted that, in the free state, some phages were partially inactivated by 1 % sodium citrate. Experiments to determine the quantity and kind of electrolytes giving maximum adsorption included 20 min. incubation of free phages, as well as phage + bacteria mixtures, in aqueous solutions which contained a high percentage of NaCl. Some staphylococcal phages were unstable in such simple salt solutions although adsorption occurred in certain instances.

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The present work was begun when it was observed that staphylococcal phage 81 lysed its propagating staphylococcus on agar medium containing concentrations of NaCl up to 10 %, whereas some other staphylococcal phages were inactive at NaCl concentrations greater than 3 %.

METHODS

Phages and staphylococci. Phages were selected on the basis of preliminary observations and as examples of each of the staphylococcal typing groups. Arranged according to serological group the phages used were as follows

Serological group	Phages
A	3A, 3B, 3C, 7, 42B, 42E, 47, 47C, 75, 81
B	29, 52, 52A, 79, 80, 55, 71, 44A, 83
F	42D, 77
L	187

Staphylococci used were the phage propagating strains originally obtained, together with the phages, through the courtesy of Dr J. E. Blair.

Preparation of phage suspensions. Corresponding dilutions of each phage, designed to contain from 100 to 300 plaque-forming units/0.1 ml., were made in 10 ml. amounts of (1) commercially prepared (Baltimore Biological Laboratories; BBL) Trypticase soy broth of 0.5 % (w/v) NaCl and (2) the same broth to which sufficient NaCl was added to make a final concentration of 10 % (w/v). At 10 % NaCl concentration the pH value of the broth was decreased from 7.2 to 6.8. No adjustment of pH value was made since, in previous experiments, variation of pH value within this range had no effect on the lytic activity of ten representative phages tested in media containing 0.5, 3, 7.5 and 10 % (w/v) NaCl.

Plaque counts. Within 5–10 min. after dilution samples (0.3 ml.) of each phage broth were withdrawn and dispensed in 0.1 ml. amounts to each of three 13 × 100 mm. tubes which contained 0.1 ml. of a 6-hr., 37°, Trypticase soy broth culture of the appropriate staphylococcus. Immediately thereafter 3 ml. melted cooled soft nutrient agar (Trypticase soy broth + 0.6 %, w/v, agar) was added to each tube; the contents were mixed and layered over the surface of regular Trypticase soy agar (BBL) in a standard Petri plate. When the soft agar layer was firm, the plates were inverted and incubated at 30°. All plaque counts except those of phages 71 and 79 were made after 24 hr. incubation. Plaques of phages 71 and 79 were counted after 18 hr. because further incubation resulted in overgrowth by the staphylococci. After withdrawal of the initial (0) sample, each phage-broth was incubated at 30°. At the end of 24 hr. incubation, plaque counts of both the 0.5 % and 10 % NaCl broth suspensions were made according to the method used for determining the initial counts. In a series of similar experiments plaque counts were made at intervals throughout the 24 hr. period.

RESULTS

The results of plaque counts before and after 24 hr. incubation at 30° in 0.5 % and 10 % NaCl broth indicated that phages of serological group A were more stable in the presence of 10 % NaCl than were the group B phages. Of the ten group A

phages studied only phage 47C showed as much as 33 % decrease in plaque-forming units, whereas the others of this group retained 72 % or more of their activity after exposure to the higher concentration of NaCl (Fig. 1). The mean survival of the ten group A phages, after 24 hr. in 10 % NaCl broth, was 87 %. Conversely, eight of the nine group B phages tested showed 88 % or more inactivation under the same conditions (Fig. 2). The one exception, phage 83, though less sensitive to

Fig. 1. Activity of serological group A staphylococcal phages after incubation of free phage in 0.5 % NaCl or 10 % NaCl in Trypticase soy broth at 30°. Percentages based on plaque counts of initial phage suspension (0) and after 24 hr. (1). ■, 0.5 % NaCl broth; ▨, 10 % NaCl broth.

NaCl than the rest of the group B phages, was inactivated to about the same degree in 0.5 % NaCl broth as in 10 % NaCl broth, 41 and 44 % decrease, respectively. (Results with phage 83 do not appear in Fig. 2 because its serological group became known to us, through personal communication from Miss Miriam Carr, after completion of the figure.) The NaCl sensitivity of the group B phages was also shown by a decrease in the number of plaque-forming units within 5–10 min. after initial contact of most of these phages with 10 % NaCl broth. Such an immediate effect was not produced by exposure of group A phages to the same 10 % NaCl broth. Results with group F phages (42D and 77) and group L phage 187 indicated that they were more NaCl tolerant than the group B phages, but only phage 42D resembled a typical group A phage in this respect (Fig. 2).

Comparison of the mean percentage decrease in plaque counts after holding the group A and group B phages in 0.5 % NaCl broth and in 10 % NaCl broth for 24 hr. is presented in Table 1; the extremes in variation of the stability of individual phages within each group are also shown. It will be noted that survival of active group A phages in 0.5 % NaCl broth and in 10 % NaCl broth are remarkably similar. Most of the group B phages, as well as those of group A, appeared to be

quite stable in 0.5 % NaCl broth, although plaque count decrease in the case of phage 55 after 24 hr. incubation in 0.5 % NaCl broth was 59 %, as compared to 88 % in 10 % NaCl broth. The instability of phage 55 in ordinary Trypticase soy broth was exceeded only by that of group L phage 187, which proved to be 67 % inactive after 24 hr. in 0.5 % NaCl broth and 72 % inactive after the same time in 10 % NaCl broth.

In other experiments, plaque counts of samples taken at intervals throughout the incubation period revealed that, when the activity of a phage was decreased after

Fig. 2. Activity of serological group B, F and L staphylococcal phages after incubation of free phage in 0.5 % NaCl or 10 % NaCl in Trypticase soy broth at 30°. Percentages based on plaque counts of initial phage suspension (0) and after 24 hr. (1). , 0.5 % NaCl broth; , 10 % NaCl broth.

Table 1. *Percentage inactivation of staphylococcal phages after 24 hr. incubation in 0.5 or 10 % NaCl Trypticase soy broth at 30°*

Serological group	% NaCl in broth	Group mean	Most stable phage	Least stable phage
			After 24 hr., 30°	
			% decrease in plaque count	
A (10 phages)	0.5	11	0	24
	10	13	0	33
B (9 phages)	0.5	17	0	59
	10	88	44	99
F (2 phages)	0.5	—	4	39
	10	—	8	58
L (1 phage)	0.5	—	67*	—
	10	—	72*	—

* Only one phage (187) studied.

24 hr. incubation in the presence of either 0.5 % or 10 % NaCl, a continuous decline in the quantity of active phage occurred throughout this period. Some typical results of plaque counts made during the first half of the period are given in Table 2. In the case of phage 29, for example, diminution in number of plaque-forming units after incubation in 10 % NaCl broth for 4 hr. was 24 %, after 12 hr. it was 70 % and over 90 % inactivated after 24 hr.

Table 2. *Plaque counts of typical staphylococcal serological group A phages (42B and 81) and group B phages (29 and 70) after initial dilution and incubation at 30° in 0.5 % or 10 % NaCl Trypticase soy broth*

Time (hr.)	Staphylococcal phages							
	Phage 42B		Phage 81		Phage 29		Phage 80	
	0.5 %	10 %	0.5 %	10 %	0.5 %	10 %	0.5 %	10 %
	NaCl	NaCl	NaCl	NaCl	NaCl	NaCl	NaCl	NaCl
	broth	broth	broth	broth	broth	broth	broth	broth
	Number of plaque-forming units							
0	250	247	137	137	188	178	203	179
4	247	260	128	141	171	135	142	43
10-12*	217	223	120	112	168	53	103	16

* All 12 hr. counts except those of phage 81 which were made after 10 hr.

DISCUSSION

In addition to distinctive antigenicity, serological groups of staphylococcal phages are characterized by other properties including host range, plaque size, ability to form lysogenic systems and stability (Rippon, 1956). Rountree (1949) pointed out the relative instability of serological group B phages, particularly their marked sensitivity to heat and storage at 4°, as compared with that of the group A and F phages. In the first serological investigation of staphylococcal phages Burnet & Lush (1935) observed that these phages could be divided into two categories, citrate-sensitive and citrate-insensitive. Rountree (1951) reported that most staphylococcal phages, when in the free state, were inactivated to some degree by 1 % (w/v) Na citrate and those most completely inactivated belonged to serological group B. In the same study, which was concerned chiefly with the effect of electrolytes on adsorption, evidence was presented that each phage had a characteristic calcium requirement. In all but two of the phages examined Na citrate inhibited phage adsorption and this effect was attributed to the action of the citrate radical which, it was suggested, bound the calcium on the surface of the phage and thus blocked the adsorption site. The absolute calcium requirement for propagation of serological group B phages was reported by Blair & Williams (1961); phage 47C (group A) and phage 187 (group L) were the only other staphylococcal phages in this category.

In the present work the staphylococcal phages which were least stable when incubated without staphylococci in 10 % NaCl Trypticase soy broth were also those of serological group B. The marked diminution in activity of these phages might be due to the effect of the NaCl on free phage before contact with the staphylococci

or to the failure of adsorption because of the NaCl introduced with phage to the phage + bacteria mixture. If inhibition of adsorption as proposed by the latter explanation were solely responsible, equal diminutions in plaque counts should occur in the initial (0) sample and the 24 hr. sample which were both taken from 10 % NaCl broth. Mixing of 0.1 ml. of this broth with an equal amount of the broth culture of the propagating strain of staphylococcus, followed by immediate addition of 3 ml. soft agar, quickly diluted the concentration of NaCl to about 0.8 %. The quantity of NaCl was further decreased on plating the soft agar. Rountree (1951) found that with the citrate-sensitive phages adsorption in NaCl was variable and, when it occurred, recovery of active phage from the bacteria infected in NaCl was less than from bacteria infected in CaCl_2 . These conclusions were based on experiments in which phages and bacteria were held 20 min. in aqueous solutions of NaCl at lower concentrations than those in the phage + bacteria mixtures tested here. In our experience the most unstable phages of group B lyse susceptible staphylococci when 1 % NaCl Trypticase soy agar is used in the routine typing procedure.

If inactivation of free phage occurs in the NaCl broth before mixing with the staphylococci, one would expect a continuing decrease in the number of plaque-forming units with increase in time. This was observed to occur. In the case of the group B phages there was an immediate decrease, followed by progressive decrease in plaque count until, at the end of 24 hr., 12 % or less of the original activity remained. Group A phages showed little, if any decrease in activity on initial contact with 10 % NaCl broth; when the activity was decreased after 24 hr. plaque counts made at 4–8 hr. intervals became successively lower. The inactivation of staphylococcal phages observed here appears to be an effect of NaCl on free phage.

The NaCl instability of serological group B staphylococcal phages observed in this work parallels their requirement for calcium. Two phages other than those of serological group B, which have an absolute calcium requirement are 47C (group A) and 187 (group L) (Blair & Williams, 1961). Phage 47C proved to be the most NaCl sensitive of the group A phages tested, but it was much more NaCl stable than any group B phage. The salt tolerance of phage 187 and of the exceptional group B phage 83 was intermediate between that of the average group A and group B phages, but they were almost as unstable in 0.5 % NaCl broth as in 10 % NaCl broth. In general, inactivation in the higher concentration of NaCl was independent of stability in regular broth. Most phages retained their activity in 0.5 % NaCl broth, although there were individual differences within each group. No phage was more stable in 10 % than in 0.5 % NaCl broth. NaCl tolerance appears to be a characteristic of certain staphylococcal phages, particularly those of serological group A, and this property may be useful in presumptive identification of serological group or in other classification schemes.

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REFERENCES

- ADAMS, M. H. (1949). The stability of bacterial viruses in solution of salts. *J. gen. Physiol.* **32**, 579.
- BLAIR, J. E. & WILLIAMS, R. E. O. (1961). Phage typing of staphylococci. *Bull. Wld Hlth Org.* **24**, 771.
- BRONFENBRENNER, J. (1925). Effect of electrolytes on the rate of inactivation of bacteriophage during precipitation. *Proc. Soc. exp. Biol., N.Y.* **23**, 187.
- BURNET, F. M. & LUSH, D. (1935). The staphylococcal bacteriophages. *J. Path. Bact.* **40**, 455.
- RIPPON, J. E. (1956). The classification of bacteriophages lysing staphylococci. *J. Hyg., Camb.* **54**, 213.
- ROUNTREE, P. M. (1949). The serological differentiation of staphylococcal bacteriophages. *J. gen. Microbiol.* **3**, 164.
- ROUNTREE, P. M. (1951). The role of certain electrolytes in the adsorption of staphylococcal bacteriophages. *J. gen. Microbiol.* **5**, 673.