THE EFFECT OF SODIUM CHLORIDE ON PHAGE FORMATION BY STAPHYLOCOCCI AT ELEVATED TEMPERATURES

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(Received for publication, September 1, 1948)

It has been demonstrated previously that staphylococci which have been subjected to rapid growth in an oxygenated medium at 36°C. for 2 hours and then reduced to a resting state by washing, resuspending the cells in Locke's solution, and maintaining them at 5°C. for 2 hours, possess the ability to produce an immediate six- to tenfold rise in phage activity titre when added to a phage-containing solution (1). This observation was regarded as evidence that actively growing cells contained something which on contact with active phage particles was transformed into phage.

Attempts have been made to define conditions under which cellular reproduction would progress normally while phage formation did not occur either because of the destruction of this factor or the inhibition of its synthesis. Experiments carried out several years ago showed that when a mixture of phage and growing susceptible staphylococci in ordinary nutrient broth was kept at 42.3°C. the bacteria grew without inducing the formation of phage. This was interpreted as being due to inhibition of the production of the phage-augmenting substance (2).

More recent investigations have shown that both the "activated" cells produced by the rapid growth of staphylococci under favorable conditions and the phage-augmenting property of these cells could be quickly inactivated by suspending the bacteria in distilled water and exposing them to 44°C. for a period of 15 minutes; at the same time it was observed that 1 M NaCl protected the "activated" cells against thermal destruction and preserved their ability to immediately give rise to an increase in [phage] when brought in contact with active phage (3). This observation suggested the possibility of providing an environment for the phage-bacterium reaction in which 1 M NaCl might be expected to protect the "activated" cells thereby permitting bacterial growth and phage formation to proceed at temperatures normally inhibitive.

The original purpose of the present investigations was not attained, for it was found that 1 M NaCl interferes with the sorption of phage by susceptible, actively growing bacteria, and thus prevents phage formation.

EXPERIMENTAL METHODS

The following terms are used throughout this paper: (*a*) [phage] = concentration of phage/ml. expressed as activity units; (*b*) $[phage]_0$ = initial concentration of

phage/ml. in activity units; (*c*) $[\text{phage}]_f$ = final concentration of phage/ml. in activity units; (*d*) $[\text{phage}_{\text{pl}}]_0$ = initial concentration of phage/ml. in plaque units; (*e*) $[\text{phage}_{\text{pl}}]_f$ = final concentration of phage/ml. in plaque units; (*f*) $[\text{bacteria}]_0$ = initial number of staphylococci/ml; (*g*) $[\text{bacteria}]_f$ = final number of staphylococci/ml.

These terms refer to the K strain of *Staphylococcus aureus* and to the homologous K phage.

The experimental procedures employed were as follows:

1. [Total bacteria] was determined with a Klett-Summerson photoelectric colorimeter which previously had been calibrated with bacterial suspensions of known density.

2. [Viable bacteria] was ascertained by plating proper dilutions of the suspensions in tryptose agar; counts were made after 24 hours incubation at 36°C.

3. Activity titrations for [phage] were carried out by: (*a*) removing 4.0 ml. aliquots of the experimental samples and chilling immediately in an ice water bath for 30 minutes; (*b*) adding 1.0 ml. of a phage solution containing 5.0×10^9 activity units/ml. to each of the 4.0 ml. aliquots; (*c*) chilling the bacterium-phage mixtures for 10 minutes in an ice water bath; (*d*) preparing dilutions in tryptose phosphate broth for activity assay according to Krueger's method (4).

4. Plaque counts were made by Gratia's method (5). 0.5 ml. of the experimental sample, properly diluted, was mixed with 3.5 ml. of bacterial suspension containing 16.0×10^7 bacteria/ml.; 1.0 ml. of melted agar was then added and the whole suspension was thoroughly mixed. 1.0 ml. of the latter was removed and spread out on the surface of an agar plate. All plates were read after 18 to 24 hours of incubation at 36°C.

5. [Extracellular phage] in a mixture of phage and bacteria was determined by first passing the mixture through a supercel filter (6). This procedure has been shown to remove all the staphylococci and the associated phage without reducing the concentration of phage free in solution (6). Plaque counts were then done on the filtrate.

6. The growth medium for all experiments was tryptose phosphate broth.

EXPERIMENTAL RESULTS

1. The Effect of 1 M *Sodium Chloride upon Bacterial Viability at 42°C.*— To determine the effect of 1 M NaCl upon bacterial viability at 42°C., staphylococci from an 18 hour agar culture were suspended in either plain broth or in 1 M NaCl broth and incubated at this temperature. Samples were removed at the beginning, after 1.5 hours, and again after 3.0 hours of incubation for determination of total cell counts and viable counts.

Table I summarizes the results obtained. It shows that while cellular reproduction does occur, a considerable number of the staphylococci die. Only some 55 per cent of the cells grown in plain broth are still viable after 3.0 hours of incubation at 42°C. The corresponding viable count for staphylococci suspended in 1 M NaCl is approximately 33 per cent of the total count. It is evident that 1 M NaCl does not exert any measurable protective effect on staphylococci growing at 42°C.

2. The Effect of 1 M Sodium Chloride upon Production of the Phage-Augmenting Substance at 42°C.—Suspensions containing 1.0 × 10^8 bacteria/ml. in either plain broth or in 1 M NaCl broth were prepared from an 18 hour culture of staphylococci. These cell suspensions were then "activated" at 42°C. for a period of 3.5 hours according to the procedure described in earlier reports. At the end of this incubation period total bacterial counts were made and the preparations were tested for capacity to increase the activity titre.

Table II shows that those cells which have undergone incubation at 42°C. for 3.5 hours in plain broth or in broth containing 1 M NaCl retain the capacity to

TABLE I

Effect of 1 M NaCl upon Bacterial Viability at 42°C.

Average values for 2 experiments.

Sample	Incubation	$[\text{Total bacteria}]_f \times 10^8$	$[\text{Viable bacteria}]_f \times 10^8$
	hrs.		
Staphylococci suspended in plain broth	0	1.0	1.0
	1.5	4.3	2.5
	3.0	6.5	3.6
Staphylococci suspended in 1 M NaCl broth	0	1.0	1.0
	1.5	2.9	1.5
	3.0	6.5	2.1

enhance the phage activity titre. As a matter of fact, certain other experiments in the course of these investigations demonstrated that cells suspended in either plain broth or in 1 M NaCl broth and grown at 45°C. are still capable of producing this effect.

3. The Effect of 1 M Sodium Chloride upon Bacterial Growth and Phage Formation at 42°C.—To demonstrate the effect of 1 M NaCl upon bacterial growth and phage production at a temperature of 42°C., suspensions containing 1 × 10^8 bacteria/ml. in either plain tryptose phosphate broth or in 1 M NaCl-tryptose phosphate broth were prepared from an 18 hour agar culture of *Staphylococcus aureus*. To these suspensions the homologous phage was added so as to obtain a $[\text{phage}]_0$ of 1 × 10^8 units/ml. The mixtures were shaken for a period of 3 to 4 hours in a water bath kept at a temperature of 42°C. and samples were then removed for activity assay and total bacterial counts.

Table III shows that staphylococci mixed with phage are able to grow equally well in the presence or absence of 1 M NaCl, at least as far as we are able to determine after 3 to 4 hours' incubation at this temperature. However, the presence of 1 M NaCl definitely affects the $[\text{phage}]_f$. A loss of approximately 60 per cent of the $[\text{phage}]_0$ occurs in plain broth mixtures, while those containing 1 M NaCl exhibit neither loss nor increase in [phage].

4. The Effect of 1 M Sodium Chloride on Sorption of Phage by Susceptible Bacteria.—Since a culture of staphylococci grown at 42°C. contains "activated" cells among the survivors, it might reasonably be predicted that a mixture of phage and bacteria kept at 42°C. would exhibit some production of phage. However, as noted in the previous section, the opposite appears to be true for $[phage]_f$ is actually lower than $[phage]_0$.

TABLE II

Effect of 1 M NaCl upon Capacity of Cells Grown at 42°C. to Increase Phage Activity Titre

Average values for 3 experiments. Activity titrations conducted on mixtures of cells and phage kept at 5°C.

Sample	$[Total\ bacteria]_0 \times 10^8$	$[Total\ bacteria]_f \times 10^8$	Activity titer $[Phage]_0 \times 10^8$	Activity titre $[Phage]_f \times 10^9$
Staphylococci "activated" in plain broth at 42°C.	1.0	10.0	10.0	160.0
Staphylococci "activated" in 1 M NaCl broth at 42°C.	1.0	8.8	10.0	150.0

TABLE III

Effect of 1 M NaCl upon Bacterial Growth and Phage Formation at 42°C.

Average values of 3 experiments.

Sample	Incubation	$[Total\ bacteria]_0 \times 10^8$	$[Total\ bacteria]_f \times 10^8$	Activity titre $[Phage]_0 \times 10^8$	Activity titre $[Phage]_f \times 10^8$
	hrs.				
Bacterium-phage mixture in plain broth	3–4	1.0	5.6	1.0	0.39
Bacterium-phage mixture in 1 M NaCl broth	3–4	1.0	5.2	1.0	1.0

One possible explanation of these experimental results is that the considerable number of dead cells accumulating in a staphylococcal suspension grown at 42°C. removes phage from the system through irreversible adsorption, a well recognized property of dead cells. If this is the case, then some change occurs in the presence of 1 M NaCl for the salt mixture shows neither formation nor loss of phage.

In order to get some idea of the differences in adsorptive capacity attributable to 1 M NaCl, suspensions containing 5×10^8 normal, non-activated staphylococci were prepared in either plain broth or in 1 M NaCl broth. The sus-

pensions were then mixed with phage so as to obtain an approximate initial concentration of 1×10^8 plaque units/ml. The mixtures were maintained at 36°C. and samples were removed after 0, 0.5, 1, and 2 hours for determination of [total phage] and [extracellular phage] by the plaque count method.

The results of three experiments have been averaged in Fig. 1. It is clear that the presence of 1 M NaCl entirely upsets the normal process by which phage is adsorbed to the bacterial cell. Very little, if any, phage is taken up; the cells remain uninfected and no significant amount of new phage is formed.

At first glance these results seem to contradict those recorded in section 2, for in the latter case bacteria grown at 42°C. in 1 M NaCl broth give, on the average, a 15-fold increment in phage when tested at 5°C. by the usual procedure. The explanation of this discrepancy may be derived from a separate experiment designed to evaluate the influence of temperature in testing for "activation" of cells.

For this purpose staphylococci were grown in plain broth and in broth containing 1 M NaCl for 2.5 hours at 42°C. One sample was held for 0.5 hour at 5°C.; phage was then added and the mixture was maintained for 10 minutes at 5°C. The cells were then subjected to the usual test for "activation;" in addition, plaque determinations were made on the whole suspension and on supercel filtrates. Another sample of the staphylococcal culture was kept at 42°C., phage was added, and the mixture was held an additional 10 minutes at 42°C. All the measurements mentioned above were made on this sample also. (Table IV.)

The results demonstrate that the temperature imposed in testing the cell suspension for "activation" markedly affects the results. When staphylococci are grown in 1 M NaCl broth at 42°C. for 2.5 hours and are tested at this same temperature for capacity to raise [phage], nearly 85 per cent of the phage added remains extracellular. In contrast, when the test is conducted at 5°C., only 16 per cent of the phage is left free in solution.

Much the same situation is observed in growing mixtures of bacteria and phage maintained at 42°C. When 1 M NaCl is present during the incubation period of 2.5 hours, the final plaque titre is essentially identical with the $[\text{phage}_{p1}]_0$ and 85 per cent of the phage is extracellular. In plain broth without NaCl, the titre is $< [\text{phage}_{p1}]_0$ and only 2 per cent of the phage is extracellular. The plaque determinations cited in Table V incidentally confirm the activity data of Table III.

An important point remains unanswered so far; namely, are the drops in activity titre and plaque count noted in plain broth mixtures of bacteria and phage kept at 42°C. for 2.5 hours due to loss of phage through adsorption on heat-killed staphylococci? If this proves to be the case, one would like to know also why the same or greater loss does not take place in suspensions con-

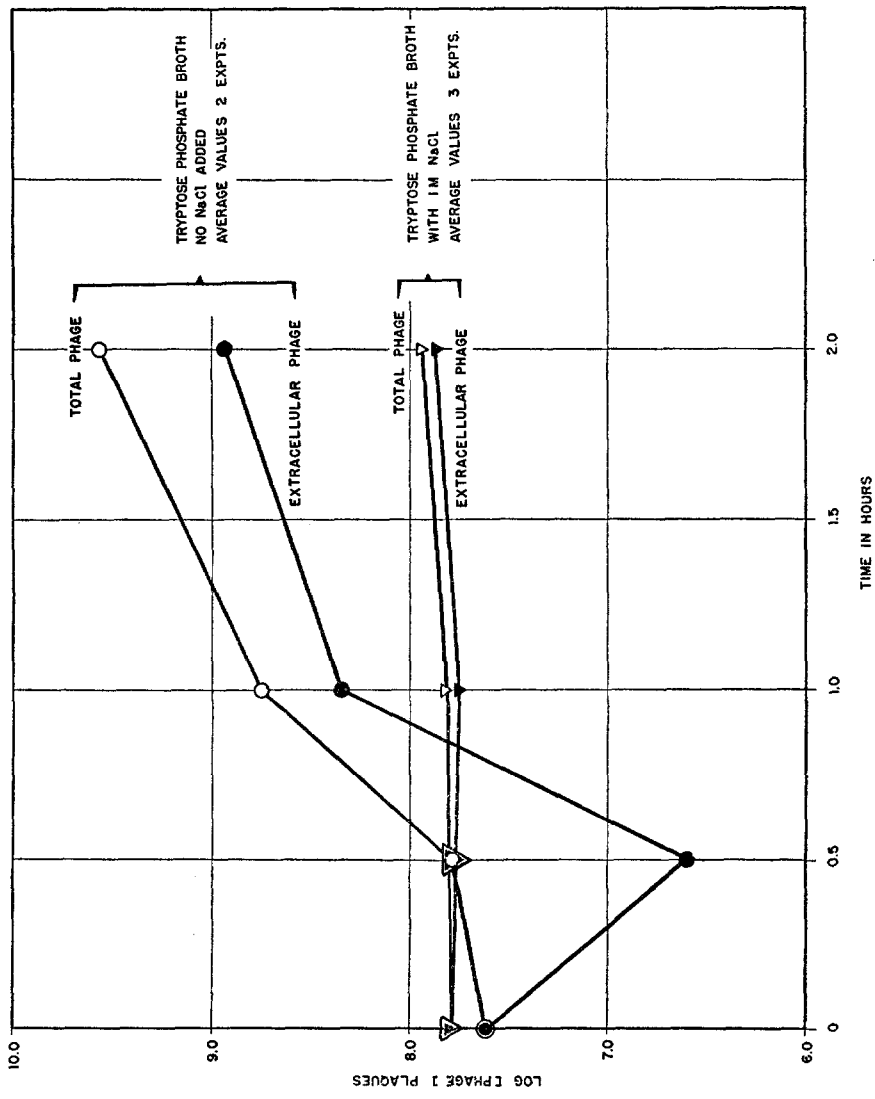

FIG. 1. Curves for [total phage] and [extracellular phage] in mixtures of staphylococci and phage suspended in tryptose phosphate broth and in 1 M NaCl-tryptose phosphate broth at 36°C. 1 M NaCl prevents sorption of phage by bacteria and the cells remain uninfected.

taining 1 M NaCl despite the fact that somewhat more dead cells accumulate here than in plain broth.

Additional experiments were performed to determine how 1 M NaCl affects the sorption of phage by heat-killed staphylococci. Staphylococci grown on nutrient agar were suspended in 1 M NaCl broth and in plain broth. Both

TABLE IV

Effect of Temperature on Results Obtained in Test for "Activation" Conducted with Staphylococci Grown at 42°C.

Sample	Treatment of sample	[Total bacteria]$_0$ $\times 10^8$	[Total bacteria]$_f$ $\times 10^8$	[Bacteria] after adding phage $\times 10^8$	Activity titre [Phage]$_0$ $\times 10^8$	Activity titre [Phage]$_f$ $\times 10^8$	Plaque titre [Phage]$_0$ $\times 10^8$	Plaque titre [phage]$_f$	
								Total $\times 10^8$	Filtrate $\times 10^8$
Staphylococci grown 2.5 hrs. at 42°C. in 1 M NaCl broth	Held 5°C. ½ hr. Phage added and kept 10 min. at 5°C.	2.5	10.0	5.0	50.0	700.0	3.3	3.28	0.52
Staphylococci grown 2.5 hrs. at 42°C. in 1 M NaCl broth	Kept at 42°C. Add phage, hold 10 min. at 42°C.	2.5	10.0	5.0	50.0	100.0	3.3	3.4	2.9

TABLE V

Effect of 1 M NaCl on the Distribution of Phage between Staphylococci and Medium at 42°C.

Sample	Plaque titre [phage$_{pl}$]$_0$ $\times 10^7$	Plaque titre [phage]$_f$	
		Total $\times 10^7$	Filtrate $\times 10^7$
Staphylococci and phage in 1 M NaCl broth at 42°C. for 2.5 hrs.	6.6	8.2	7.0
Staphylococci and phage in plain tryptose PO_4 broth at 42°C. for 2.5 hrs.	6.6	2.9	0.06

preparations were kept for 2 hours at 80°C. to kill the organisms. Equal portions of the heat-killed staphylococcal suspensions were mixed with phage and kept either at 5°C. or at 42°C. for 20 minutes. Plaque determinations were carried out on samples removed from the whole suspensions and on filtrates (supercel).

The results of this experiment (Table VI) may be evaluated by comparing the initial titres with plaque counts at the end of the test period; the drop in plaque count is a measure of the phage taken up by dead bacteria. It is evident that 1 M NaCl vastly alters the adsorptive capacity of heat-killed staphy-

lococci. In the tests conducted at 42°C. for 20 minutes, 65 per cent of the phage in the NaCl mixture is left unadsorbed, while in the absence of NaCl only some 0.3 per cent of phage remains free in solution.

When the experiment is performed at 5°C. the salt effect is somewhat less pronounced. In plain broth 3.5 per cent of the phage is unattached to cocci and when NaCl is present the extracellular fraction is 30 per cent of the total phage added.

Since the growth of staphylococci at 42°C. in tryptose phosphate broth is attended by a considerable accumulation of dead cells in the medium (Table I) and since such cells irreversibly remove homologous phage from solution, an explanation is available for the observed drop in titre (60 per cent loss in Table III and 56 per cent loss in Table V).

TABLE VI

Effect of 1 M NaCl on Sorption of Phage by Heat-Killed Staphylococci at 42°C. and 5°C.

Heat-killed staphylococci	Plaque titre $[Phage_{Pl}]_0 \times 10^8$	Medium	Temperature	Time	Plaque titre $[Phage_{Pl}]_f \times 10^8$
per ml.			°C.	*min.*	
5×10^8	4.0	1 M NaCl broth	42	20	2.6
5×10^8	4.0	1 M NaCl broth	5	20	1.2
5×10^8	4.0	Plain broth	42	20	0.011
5×10^8	4.0	Plain broth	5	20	0.14

When 1 M NaCl is present in the broth (Table I), even more cells die but they present a less efficient adsorbing surface and most of the phage remains active; *i.e.*, free in solution.

DISCUSSION

Earlier work has shown that the production of phage is inhibited in a broth mixture of staphylococci and phage maintained at approximately 42°C. although the bacteria multiply at a fairly rapid rate (2). More recently Fong (3) has experimented with the thermal destruction of "activated" cells; *i.e.*, staphylococci which have been allowed to grow rapidly in a favorable environment. Normally such organisms when reduced to a resting state at 5°C. possess the ability to produce a six- to tenfold increase in activity titre when added to phage (1). However, Fong found this characteristic to be rapidly abolished by exposure to 44°C. in distilled water along with the capacity of the young cells to survive at this temperature. He noted also that the presence of 1 M NaCl permitted the cells to resist exposure to 44°C. and to retain their phage-augmenting property.

We began the experiments described in the present paper with the idea that the protective function of 1 M NaCl conceivably could be brought to bear on

broth suspensions of staphylococci and phage incubated at 42°C. In this case, we hoped to eliminate the inhibition of phage formation reported by Krueger and Pucheu (2) and ascribed by them to repression of the cellular mechanism responsible for synthesis of phage precursor.

The current experiments were conducted in tryptose phosphate broth and revealed several pertinent facts:

1. Staphylococcal growth at 42°C. in plain broth or in broth containing 1 M NaCl proceeds at a moderate rate. It is attended by measurable rates of death, with the result that at the end of 3 hours in plain broth only 55 per cent of the total cell count represents viable cells; the corresponding figure for the salt-broth preparations is 33 per cent. These results do not appear to be due to the formation of clumps.

2. Cells grown at 42°C. in plain broth or in salt-broth are "activated;" *i.e.*, when brought to 5°C. to stop growth and tested with phage they very rapidly raise the activity titre 15- to 16-fold.

3. Mixtures of staphylococci and phage maintained at 42°C. in plain broth exhibit cellular reproduction but fail to increase either the activity titre or plaque count; in fact, the activity titre falls some 60 per cent and the plaque count about 56 per cent. When 1 M NaCl is present the final titre is precisely equal to the initial titre.

4. 1 M NaCl in broth suspensions of staphylococci and phage kept at 42°C. completely alters the distribution of phage throughout the cell population. Normally, phage is taken up very rapidly by the cells but virtually all of it remains extracellular when this concentration of salt is in the medium.

5. A similar result is observed when dead staphylococci are tested for adsorbing capacity. Phage is readily removed from solution by dead cocci and the rate increases with temperature. With 1 M NaCl in the broth, relatively little phage is adsorbed at 5°C. and even less at 42°C.

6. The temperature at which the test for "activation" of organisms grown at 42°C. in NaCl-broth is conducted greatly influences the activity titre obtained. At 42°C. much of the phage remains free in solution; this is reflected in an insignificant rise in phage titre. At 5°C. under the conditions of the test, most of the phage is picked up by the cells with the result that a large increment in activity titre follows.

These experimental data furnish a basis for certain generalizations with reference to the effect of an elevated temperature (42°C.) and increased electrolyte concentration upon the relations obtaining between phage and host cells. In the first place, the increased temperature seems to favor "activation" of staphylococci. The immediate rise in activity titre when phage is added to cells grown at 42°C. and subsequently held at 5°C. to inhibit reproduction, is 15- to 16-fold as contrasted with the 5- to 10-fold increment commonly observed when the growth temperature is 36°C. The addition of 1 M NaCl to the growth medium has no appreciable influence on this phenomenon.

Despite this evidence that staphylococci growing and metabolizing over a period of time at 42°C. efficiently synthesize the substrate required for phage formation, a mixture of phage and cocci maintained at 42°C. not only fails to develop an increase in [phage] but actually registers a considerable loss. However, if 1 M NaCl is present in the suspension the loss is prevented and $[\text{phage}]_0 = [\text{phage}]_f$. At least two factors appear to participate in this seemingly anomalous result:

(*a*) If living cells infected at 42°C. behave as they are known to do at 36°C., 20 to 30 phage particles are produced in each bacterium which then undergoes lysis releasing the newly formed phage to infect other organisms. However, the rate of bacterial death at this temperature is considerable and a sufficient number of dead cells accumulates in the medium to provide an adsorbing surface for the uptake of phage. Phage removed from solution in this fashion is irretrievably lost and cannot later participate in the phage-forming reaction which normally ensues when living "activated" cocci and phage are brought into contact. This, then, might be cited as the reason for the observed drop in $[\text{phage}]_f$ in the mixture of phage and bacteria kept at 42°C.

It is possible, also, that the same end result may be attained through the operation of another mechanism. If the bacterium-phage complex were quite thermolabile, [phage] would be expected to fall off in similar fashion even without the added factor of loss by adsorption on dead cells. Experiments which will be reported in a separate paper indicate that, in fact, such thermal sensitivity on the part of phage attached to susceptible organisms may exist. The data presented here afford no proof that it is an important consideration in the experiments discussed but in certain respects it provides a more logical explanation of the experimental observations than does loss of phage through sorption on dead bacteria. For this mechanism to function there is no necessity to postulate the liberation of newly formed phage under the conditions obtaining in these experiments; even if lysis did not occur, thermal inactivation of phage-cell complexes would produce the observed drop in titre.

If removal of phage through adsorption on dead cells is invoked in the case of the growing mixture, it should operate equally well in the course of testing cocci "activated" by growth at 42°C. in the absence of phage. Viable and total bacterial counts have shown that 45 per cent of the cells present after 3.0 hours are dead. Yet this relatively large fraction somehow fails to compete successfully with the adsorbing surface presented by living" activated" organisms. As a consequence, infection of the "activated" cells occurs and the activity titre exhibits a 15- to 16-fold rise.

It is true that the efficiency of adsorption of phage by dead staphylococci is greater at 42°C. than at 5°C. so that one might be tempted to introduce this difference as a factor in accounting for a positive "activation" test at 5°C. and a negative one at 42°C. However, the degree of difference is minor in com-

parison with the large quantity of phage which could be adsorbed during the periods of exposure of test suspensions to phage.

(*b*) 1 M NaCl has a pronounced effect on the uptake of phage by living and dead cells. When staphylococci are grown in salt-broth at 42°C. and subsequently are tested for "activation" at 5°C., they adsorb 85 per cent of the phage added and produce a 14-fold increase in activity titre. However, if the test is performed at 42°C. (actually not a satisfactory temperature since it permits continued cellular reproduction) little phage is adsorbed and the increment in activity titre is negligible.

CONCLUSIONS

In experiments with the K strain of *Staphylococcus aureus* and the K race of bacteriophage suspended in tryptose phosphate broth and maintained at 42°C. it was found that the presence of 1 M NaCl produced certain drastic changes in the relationship between the host cells and the infecting virus:

1. Staphylococci grown at 42°C. in plain broth or in NaCl-broth are "activated," *i.e.* when growth is stopped by lowering the temperature to 5°C. and phage is added, the activity titre immediately displays a rise of 15- to 16-fold.

2. 1 M NaCl tends to prevent the sorption of phage by cocci and this effect is more pronounced at 42°C. than at 5°C. When the activation test is conducted at 5°C. (the usual temperature) most of the phage is picked up by the cells and the described increase in activity titre follows. If the test takes place at 42°C. there is little sorption and correspondingly little rise in phage titre.

3. Mixtures of staphylococci and phage incubated at 42°C. in NaCl-broth fail to produce phage; the final plaque and activity titres are identical with the initial titres. Here, also, the influence of 1 M NaCl in preventing contact of phage with cocci appears to account for the results.

4. Similar mixtures held at 42°C. in plain broth exhibit a drop of about 60 per cent in activity and plaque titres. The loss of phage may be due to adsorption on dead cells accumulating in the suspension or to the thermolability of the bacterium-phage complex, or to both.

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