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The Role of Certain Electrolytes in the Adsorption of Staphylococcal Bacteriophages

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SUMMARY: The role of electrolytes in the adsorption of the citrate-sensitive staphylococcal typing-phages and the citrate-insensitive phage K on to their propagating strains was studied in broth + Na citrate and in distilled water + CaCl_2 , MgCl_2 , and NaCl. Some phages when in the free state were partially inactivated by 1% (w/v) Na citrate. In all except two phages, K and 51, citrate inhibited adsorption in broth; adsorption occurred in distilled water. Three phages, 7, 42B and 42E, had a specific requirement of 50 μg . CaCl_2 /ml. for adsorption. With the remainder, the requirements of CaCl_2 varied from 5 to 400 μg ./ml.; similar amounts of MgCl_2 acted equally well.

With the citrate-sensitive phages adsorption in NaCl was variable and, where it occurred, recovery of viable phage from cells infected in NaCl was less than from cells infected in CaCl_2 . These phages appear to have a specific requirement of divalent cations for adsorption, suggesting that adsorption and penetration are enzymic in character.

The electrolyte requirements of the citrate-insensitive phage K were related geometrically to the valence of the cations used, 5 μg . CaCl_2 or MgCl_2 /ml. or 25 μg . NaCl/ml. giving optimum adsorption.

Early in the study of bacteriophage phenomena it was observed that citrate or oxalate in the media prevented multiplication of certain phages. On the basis of such observations Burnet (1933) proposed that bacteriophages should be divided into 'citrate-sensitive' or '-insensitive', and showed that this sub-division was of value in the classification of phages which lysed intestinal bacteria and staphylococci (Burnet & Lush, 1935).

The mode of action whereby citrate and oxalate inhibit phage multiplication has been investigated in detail for only a few phages. The action of these substances is assumed to be due to their de-ionizing effect on calcium and/or magnesium ions, but little information is available on the role which these ions play in phage synthesis (Cohen, 1949). Delbrück (1948) described a mutant of coli phage T4 which required Ca^{++} as well as tryptophan for adsorption. On the other hand, Adams (1949) showed that coli phage T5 required calcium for growth but not for adsorption. The same was apparently true for the citrate-sensitive coli phage of Elford & Andrewes (1932), coli phage T1 (Puck, 1949), and a staphylococcal phage, 51 (Rountree, 1947). At the same time, citrate may inactivate free phage, although Adams (1949) showed that, with the coli phages, inactivation in salt solutions is due to Na ions rather than to citrate.

The present work studies the point in the staphylococcal phage cycle at which citrate acts and was concerned in the first instance with its effect on

free phage and on adsorption. From this point of departure the role of Ca, Mg and Na ions in phage adsorption has been studied.

MATERIAL AND METHODS

The typing phages of Wilson & Atkinson (1945) and phage K and their respective propagating strains were used. Certain of their characteristics have been already described (Rountree, 1949). In the adsorption tests, specific adsorption of phages on to their sensitive propagating strains was studied.

All glassware was carefully cleaned in chromic-sulphuric acid mixture and well rinsed in distilled water. Distilled water (redistilled in glass) was used to make the salt solutions and, where required, to suspend bacterial cells and to dilute phages.

Phage counts were made on plates surface-inoculated with the propagating strain, each plate accommodating four separate inocula of 0.01 ml. The media used were glucose (0.15 %) veal broth and the same broth plus 1.2 % shredded agar and 0.01 % CaCl_2 .

Adsorption tests were carried out in tubes 80 mm. long $\times$ 10 mm. diam., the final volume of reactants in each tube being 1 ml.

EXPERIMENTAL

Sensitivity of free phage to sodium citrate

The phages were tested for sensitivity to 1 % (w/v) Na citrate in broth in absence of bacterial cells. The phages were diluted in broth so that the final dilution (in broth + citrate) would give an estimated count of 100–200 plaques. The results were expressed as per cent inactivation after 20 min. in a water-bath at 37° as compared with controls in broth alone. It was considered that the dilution of citrate which took place when the 0.01 ml. inoculum was spread on the agar plate was adequate to prevent any interference in adsorption of phage on to the bacterial cells.

Different batches of nutrient broth may contain variable amounts of ionizable calcium and it has been recognized (Williams Smith, 1948) that a low Ca^{++} content may render certain batches of media unsuitable for phage growth. Unfortunately no chemical method of estimating ionized calcium is available, so no very precise information can be obtained on the concentrations of citrate which inactivate free phage in broth. However, a number of estimations made with different batches of broth and the same phages showed good agreement (Table 1).

Those phages which were almost completely inactivated by citrate belong to serological group B, which has been shown (Rountree, 1949) to be markedly unstable to heat and storage. The correlation between serological type and sensitivity of free phage to citrate is not, however, absolute, since phages 29A, 31A, 42D and 44 also belong to this serological group. Since the broth in which these tests were made contained 0.5 % NaCl, and no inactivation of

Table 1. *Percentage inactivation of free phage in 1 % Na citrate broth in 20 min. at 37°*

Inactivation (%)	Phages
Nil	44, 51, K
10	42E
30-35	6, 7, 42D
45-60	3A, 3B, 29A, 31A, 42B
70-85	3C, 47, 47A, 47B, 47C
90-98	29, 31, 31B, 42C, 52, 52A

phage occurred in broth at 37° in the absence of citrate, it was concluded that the inactivating effect of the Na citrate was due to the citrate radical and not to Na ions.

Inhibition of phage adsorption in the presence of citrate

Sufficient Na citrate was added to overnight broth cultures of the appropriate propagating strain to give final concentrations of 0.1, 0.5 or 1.0 % (w/v) Na citrate. Phage was then added, and the mixtures incubated at 37° for 20 min., centrifuged (angle centrifuge), and the free phage remaining in the supernatant determined. Controls containing no bacterial cells were included in each test.

With phages K and 51 there was no inhibition of adsorption in the presence of 1 % citrate. Adsorption of phages 3A, 3B, 3C, 6, 7, 42B, 42D, 42E, 44, 47, 47A, 47B and 47C was inhibited by 0.5 % Na citrate. With the more unstable phages 31, 31B, 42C, 52 and 52A, it was difficult to evaluate the results since almost as much phage was inactivated in the broth and citrate controls as was adsorbed by the cells in absence of citrate. However, with phages 29 and 29A definite evidence of inhibition of adsorption by 0.5 % citrate was obtained. Table 2 gives the result of a typical experiment.

Table 2. *Inhibition of adsorption of phage 42 E by 1 % Na citrate*

Tube No.	Contents	Plaque count of supernatant
1	Cells+phage	11; 9*
2	Cells+phage+Na citrate 1 %	300; 322
3	Broth+phage	418; 400
4	Broth+phage+Na citrate 1 %	370; 340

* In this and subsequent tables, duplicate plaque counts are presented.

Adsorption of phage in the presence of electrolytes

Calcium chloride, magnesium chloride and sodium chloride were used in tests designed to demonstrate their effect on phage adsorption. The adsorbing cells were centrifuged from 5- or 18-hr. broth cultures and resuspended in distilled water at a concentration of approximately 10^9 cells/ml. The use of high titre phages diluted in distilled water ensured that the phage suspensions

contained negligible amounts of electrolytes derived from the medium used for preparing the phage filtrates. The salt solutions were made up in varying strengths so that addition of 0.1 ml. volumes to the adsorption tubes would result in the final concentrations required. After incubation of the mixtures in a water-bath at 37° for 20 min. the tubes were centrifuged and plaque counts made on the supernatant fluid. Some phages were unstable in simple salt solutions and in such cases it was necessary to test the centrifuged cells for the presence of viable adsorbed phage by removing the supernatant fluid, resuspending the cells in the distilled water and then plating. The minimum concentration of each salt which permitted 90 % adsorption was determined for each phage. No adsorption of phage was observed in phage + cell mixtures lacking electrolytes.

Table 3. *Adsorption of phage 42 E in presence of electrolytes*

Quantities of electrolytes shown in $\mu\text{g./ml.}$ solution.

Tube No.	Contents Cells + phage in	Plaque count of supernatant	Adsorption (%)
1	Distilled water	140; 148	0
2	CaCl ₂ 10	134; 141	0
3	20	100; 60	40
4	50	6; 3	97
5	100	4; 0	98
6	150	3; 5	97
7	200	1; 2	99
8	MgCl ₂ 200	103; 91	0
9	NaCl 100	103; 99	0
10	800	94; 82	0

Controls (phage only)		Plaque count	
		Time 0	Time 20 min.
11	Distilled water	143; 131	119; 142
12	CaCl ₂ 200	132; 112	124; 96
13	MgCl ₂	157; 152	84; 112
14	NaCl 800	138; 120	116; 124

Table 3 shows the plaque counts obtained in a typical experiment using phage 42 E. The average initial concentration of phage calculated from counts obtained from control tubes 11–14 plated at zero time was used as the base for calculating the percentage adsorption. With phage 42 E, 50 $\mu\text{g.}$ CaCl₂/ml. allowed maximum adsorption; MgCl₂ could not replace CaCl₂, and NaCl up to 800 $\mu\text{g./ml.}$ was inactive.

The results obtained with 21 phages are given in Table 4. Where no activity could be demonstrated for electrolyte in concentrations up to 1000 $\mu\text{g./ml.}$ a blank has been left in the table. Two phages, 42 C and 44 A, were so unstable in distilled water and salt solutions that no results were obtained. Phages 7, 42 B and 42 E showed a specific requirement of calcium for adsorption, 90 % adsorption occurring in 50 $\mu\text{g.}$ CaCl₂/ml. and no adsorption in MgCl₂ or NaCl. Phage 47 required 150 $\mu\text{g.}$ CaCl₂/ml. for adsorption; little adsorption

occurred with a similar concentration of MgCl_2 while with 800 μg . NaCl /ml. some adsorption took place.

Table 4. Minimum concentrations of CaCl_2 , MgCl_2 , and NaCl giving 90 % adsorption of various phages in 20 min. at 37°

Phage	Minimum concentration (μg ./ml.)		
	CaCl_2	MgCl_2	NaCl
K	5	5	25
3A, 3B, 3C	10	10	400
7, 42B, 42E	50	—	—
6, 47A	20	20	800
29, 52	400	400	—
29A, 52A	100	100	—
31, 31B	150	150	—
42D	50	50	—
44	200	200	—
47	150	—	800
47B	5	5	100
47C	50	50	800
51	50	50	1000*

— No activity demonstrated with 1000 μg ./ml.

* Partial adsorption only.

With the remainder of the typing phages, the CaCl_2 requirements varied from 5 to 400 μg ./ml., and in all 5 to 400 μg . MgCl_2 /ml. gave the same amount of adsorption. For phage K, which is citrate-insensitive, concentrations of 5 μg . CaCl_2 or MgCl_2 /ml. or 25 μg . NaCl /ml. permitted 90 % adsorption. These results indicate that the adsorption of phage K may be a simple electrolytic phenomenon which can be related to the valence of the cations used. With those citrate-sensitive phages in which adsorption in NaCl could be demonstrated, the relationship between valence and the concentration of electrolyte required for adsorption was not a geometric one. For example, phage 47B required 100 μg . NaCl /ml. compared with 5 μg . CaCl_2 or MgCl_2 /ml. With the phages 29, 29A, 31, 31B, 42D, 44, 52 and 52A no NaCl activity could be demonstrated in concentrations up to 1000 μg ./ml.; higher concentrations could not be tested because they inactivated free phage. If the relationship between valence and cation requirements were a simple geometrical one, those phages having high Ca requirements would need almost astronomical amounts of NaCl for adsorption. On the other hand, phage 42D which required 50 μg . CaCl_2 /ml. might be expected to adsorb, at least partially, on to cells in 1000 μg . NaCl /ml. However, no viable phage could be recovered from such cells.

The adsorption tests also supplied evidence that the requirements of the phages were related to the phage and not to the cell surface. Phages 42B and 47C are both propagated on staphylococcus strain 1163, but whereas phage 47C was adsorbed in 50 μg . CaCl_2 or MgCl_2 /ml., phage 42B showed a specific requirement of 50 μg . CaCl_2 /ml. Phages 31 and 44 required differing amounts of CaCl_2 or MgCl_2 for 90 % adsorption on to strain 18. Finally phage K when

adsorbed on to strain 145 required only 5 μg . CaCl_2 /ml. as compared with phage 51, which required 50 μg ./ml. for adsorption to this strain.

Estimates of the amount of viable phage recovered from sensitive cells infected with phages 3A, 3B or 3C in various concentrations of CaCl_2 , MgCl_2 or NaCl provided some interesting information. The results obtained with phage 3A are given in Table 5. With optimum amounts of CaCl_2 or MgCl_2 almost the whole amount of added phage could be recovered from the cells. With 400 μg . NaCl /ml., however, although the amount of free phage present in the supernatant indicated 95 % adsorption, only 25 % of adsorbed phage was recovered. Furthermore, there was no increasing yield of recoverable adsorbed phage with increasing concentrations of NaCl .

Table 5. *Adsorption of phage 3A in the presence of various concentrations of CaCl_2 , MgCl_2 , and NaCl , showing amounts of unadsorbed phage in the supernatants and of adsorbed phage recovered from the cells*

Quantities of electrolytes in μg ./ml. solution.

Tube No.	Contents Cells+phage in	Plaque counts	
		Free phage	Adsorbed phage
1	Distilled water	212; 250	14; 9
2	CaCl_2 5	33; 41	152; 121
3	10	5; 4	170; 130
4	MgCl_2 10	10; 11	136; 170
5	20	4; 3	158; 210
6	NaCl 100	118; 80	49; 68
7	200	37; 29	32; 43
8	400	9; 8	53; 72
9	800	18; 18	55; 75

These results resemble those obtained by Puck (1949) who found that coli phage T1 diluted in distilled water and adsorbed on to coli cells in the absence of calcium ions could not be recovered. Puck ascribed his finding to 'sensitization' of phage by dilution in distilled water or phosphate buffer, and suggested that this sensitization produces a block in some step in the virus cycle which occurs after adsorption. This step may be that of penetration of the cell as distinct from adsorption on to the cell surface. If this be so, the recovery of viable phage from staphylococcal cells when adsorption has taken place in NaCl might be due to the cells themselves providing sufficient calcium to allow 'activation' and penetration of some adsorbed phage. Since constant amounts of phage 3A were recovered from constant numbers of cells infected in increasing concentrations of NaCl , there is some evidence for this view. Cells grown in broth containing citrate should be lacking in this postulated available calcium and, if infected in NaCl , no phage should be recoverable from them. An experiment was set up in which the propagating strain for phage 3A was grown in 0.5 % citrate broth for 5 hr., the cells washed in distilled water and resuspended therein. From these cells no phage

was recovered when adsorption took place in NaCl, while in CaCl_2 all the phage adsorbed was recovered; the results of a typical experiment are given in Table 6.

Table 6. *Percentage adsorption and percentage recovery of phage 3A adsorbed on to cells grown in citrate broth and infected in CaCl_2 or various concentrations of NaCl*

Quantities of electrolytes in $\mu\text{g./ml.}$ solution.

Tube No.	Contents Cells+phage in	Adsorption (%)	Recovery (%)
1	Distilled water	0	0
2	CaCl_2 50	99	100
3	NaCl 100	60	0
4	200	80	0
5	400	90	0
6	800	100	0

DISCUSSION

Phage adsorption was originally regarded as a relatively simple process in which specific receptor structures of phage and cell surfaces were brought together by non-specific electrolytic forces. However, the observations of Anderson (1945) on the tryptophan co-factor requirements of certain coli phages and of Delbrück (1948) on a Ca^{++} -requiring mutant coli phage indicated that the electrolyte theory could no longer be regarded as sufficient and that, besides electrical forces, enzymic reactions involving either phage or cell surface or both must be postulated.

With the staphylococcal phages used here the adsorption process may be equally complex. With two exceptions, adsorption of these phages was inhibited by citrate although adequate amounts of electrolyte were present had the adsorption been a simple electrical phenomenon. With phages 7, 42B and 42E, a specific co-factor requirement for Ca^{++} was demonstrated. With the other phages Mg^{++} could be substituted for Ca^{++} . There was also evidence that each phage had a characteristic Ca^{++} requirement, rather than that the Ca^{++} requirement was related to the nature of the bacterial cell surface. The inactivation of some phages by citrate in broth suggests that citrate may combine with Ca^{++} on the surface of the phage particle and so block the sites involved in adsorption.

With phages K and 51 there was neither inactivation of free phage by citrate nor inhibition of adsorption. The requirements of phage K were directly related to the valence of the ions used. With phage 51 this could not be demonstrated experimentally since the amount of NaCl 'theoretically' required inactivated the phage in absence of the stabilizing action of nutrient broth.

The fact that certain of the phages which had low requirements of divalent cations adsorbed in NaCl does not invalidate the hypothesis that divalent

cations, particularly Ca^{++} , are of primary importance in adsorption. There was a lower recovery of viable phage from cells infected in NaCl, and furthermore there was complete loss of adsorbed phage when the cells had been previously grown in citrate broth. This suggests that under certain conditions the cell itself may supply the required Ca^{++} or Mg^{++} and NaCl may merely provide the electrical conditions necessary for apposition of cell and phage. The experimental results, therefore, suggest that Ca^{++} is required for penetration, but they do not exclude the possibility that this is also involved in an early step in the growth cycle following penetration of the cell.

The conclusion that Ca^{++} is important in phage adsorption is of interest in the light of the findings of Burnet and his associates on the importance of Ca^{++} in the reactions of influenza virus, of the receptor-destroying enzyme of the cholera vibrio with erythrocyte surfaces, and the mucoid inhibitors of haemagglutination (Edney, 1949). The adsorption phenomena involved in these reactions appear to be enzymic in nature. The present observations with staphylococcal phages suggest that, at least for some of them, adsorption and penetration may also be enzymic.

REFERENCES

- ADAMS, M. H. (1949). The calcium requirement of coli phage T5. *J. Immunol.* **62**, 505.
- ANDERSON, T. F. (1945). The role of tryptophane in the adsorption of two bacterial viruses on their host, *E. coli*. *J. cell. & comp. Phys.* **25**, 17.
- BURNET, F. M. (1933). The classification of dysentery-coli bacteriophages. III. A correlation of the serological classification with certain biochemical tests. *J. Path. Bact.* **37**, 179.
- BURNET, F. M. & LUSH, D. (1935). The staphylococcal bacteriophages. *J. Path. Bact.* **40**, 455.
- COHEN, S. S. (1949). Growth requirements of bacterial viruses. *Bact. Rev.* **13**, 1.
- DELBRÜCK, M. (1948). Biochemical mutants of bacterial viruses. *J. Bact.* **56**, 1.
- EDNEY, M. (1949). The influence of calcium ion on the reactions of the *V. cholerae* enzyme R.D.E. and influenza virus with specific mucopolysaccharides. *Aust. J. exp. Biol. med. Sci.* **27**, 252.
- ELFORD, W. J. & ANDREWES, C. H. (1932). The 'killing' of bacteria by bacteriophage. *Brit. J. exp. Path.* **13**, 13.
- PUCK, T. T. (1949). A reversible transformation of T1 bacteriophage. *J. Bact.* **57**, 647.
- ROUNTREE, P. M. (1947). Staphylococcal bacteriophages. II. Bacteriophage absorption by staphylococci. *Aust. J. exp. Biol. med. Sci.* **25**, 203.
- ROUNTREE, P. M. (1949). The serological differentiation of staphylococcal bacteriophages. *J. gen. Microbiol.* **3**, 164.
- WILLIAMS SMITH, H. (1948). Calcium-deficient media: their effect on phage action. *Nature, Lond.*, **161**, 397.
- WILSON, G. S. & ATKINSON, J. D. (1945). Typing of staphylococci by the bacteriophage method. *Lancet*, **i**, 647.

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