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The Role of Divalent Cations in the Multiplication of Staphylococcal Bacteriophages

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SUMMARY: The staphylococcal typing phages require divalent cations for a stage in phage growth which is probably that of penetration. When phage is adsorbed to the cell surface of sensitive cocci and a chelating agent is added, there is a short period during which the phage particle may be inactivated and its lethal effect on the coccus prevented. Cocci attacked by phages of serological group A show penetration of the phages in distilled water + CaCl_2 , or + MgCl_2 , or to a less extent + SrCl_2 . The phages of group B are unable to penetrate in presence of SrCl_2 and in some cases MgCl_2 is only partially active as compared with CaCl_2 . Ca ions appear to be required specifically at a late stage in phage synthesis since the average yield of phage/coccus increases with increasing concentration of CaCl_2 ; the addition of a chelating agent towards the end of the latent period depresses the yield of phage.

Some bacteriophages require Ca ions either for adsorption or during phage multiplication. With coliphage T5 (Adams, 1949; Luria & Steiner, 1954), a typhoid phage (Fildes, Kay & Joklik, 1953) and lactic streptococcus phages (Potter & Nelson, 1953) Ca is not necessary for adsorption but apparently acts at an early stage in phage synthesis. Delbrück (1948) and Beumer & Beumer-Jochmans (1951) described two phages (a mutant of coliphage T4 and a dysentery phage) which apparently required Ca for adsorption. However, Fildes (1953) pointed out that in these cases the activity of alternative divalent cations was not investigated and it is therefore uncertain whether or not the Ca requirements were absolute.

Puck (1953), with coliphages of the T series, produced evidence that there were a number of stages in the process of phage invasion. He suggested that the first step of adsorption involved electrostatic forces which were supplied by cations of the medium and which permitted reversible attachment of phage to cell surface. The next step was the separation of the protein and deoxyribonucleic acid components of the phage; this is quickly followed by a process leading to cell killing, and the penetration of deoxyribonucleic acid into the cell and the initiation of phage synthesis in lytic systems. The process of penetration is probably enzymic in nature. The staphylococcal typing phages (Rountree, 1951) require divalent cations for adsorption; this process is inhibited by Na citrate. Each phage has characteristic cation requirements which are related to the phage rather than to the host cell surface. The present paper deals with the role of divalent cations in the different stages of multiplication of staphylococcal phage which follow the first step of adsorption.

METHODS

The typing phages of Wilson & Atkinson (1945) and their respective propagating strains were used. The methods of phage estimation, preparation of glassware and of salt solutions have been previously described (Rountree, 1951). In general, phage counts were made in quadruplicate. Since no chemically defined medium which regularly supported the growth of the phages was available, glucose (0.15 %, w/v) veal broth was used as basal medium which was supplemented with various cations as required. The disodium salt of ethylene diaminetetra-acetic acid (EDTAA) (supplied by Dr F. Dwyer, University of Sydney) was used as a chelating agent. Na citrate was unsuitable for this purpose since in the concentrations necessary for effective chelation it inactivated many of the phages when they were in the free state.

The agar nutrient broth medium used for plaque counting had 0.1 % (w/v) CaCl_2 added to it.

RESULTS

One-step growth curves

Knowledge of the lengths of the minimum latent period and of the one-step growth curve and average burst size were required for the various different phages. One large batch of broth was prepared which supported the growth of all the phages.

The appropriate propagating strain of staphylococcus was grown in broth with aeration in a water bath at 37° until the total count (determined turbidometrically) reached 1×10^8 cells/ml. Sufficient phage was then added to give 3–4 particles/cell. After 10 min. of adsorption the infected cells were so diluted in warmed broth that on plating 0.01 ml. samples 1–3 plaques were obtained. Incubation was continued at 37°, samples being removed for plating at 5 min. intervals. The end of the latent period was marked by an increase in the plaque count. If all cells had been infected, the end of the period of phage release was followed by a steady count. The average burst size was obtained by dividing the final phage count calculated in terms of the original volume of infected culture by the number of infected cells.

The phages showed fairly wide differences in the length of the minimum latent period; this ranged from 35 min. for phage 3B to 60 min. for phage 42B (Table 1). Most of the phages, however, gave their first release of phage between 40 and 50 min. The end of phage release ranged from 50 to 75 min. In this particular batch of broth the average yield of phage particles per cell ranged from 12 for phage 42B to 168 for 47D. Replicate estimations of the average burst size of different phages agreed well, although exact reproduction of results from day to day was not always possible. This was thought to be due chiefly to variability in cell sensitivity. Variation in average burst size also occurred with different batches of broth.

Table 1. *Times of the minimum latent period and the end of phage release of staphylococcal phages grown in broth*

Latent period (min.)	Phages	End of phage release (min.)	Phages
35-40	3 B, 44 A	50-55	47 D, 51, 52 A
40-45	3 C, 6, 7, 29, 29 A, 31 A, 31 B, 42 C, 44, 47 A, 47 D, 51, 52, 52 A	55-60	3 C, 6, 7, 29 A, 31 A, 44 A, 47 A, 52
		60-65	3 B, 29, 42 C, 42 D, 44, 47, 47 B
45-50	3 A, 31, 42 D, 42 E, 47, 47 B, 47 C	65-70	31, 31 B, 42 B, 42 E
55-60	42 B	70-75	3 A, 47 C

Effect of divalent cations on the average burst size

Williams Smith (1948) observed that the addition of CaCl_2 to broth in which staphylococcal phages could not be propagated rendered the medium adequate. This stimulatory effect of CaCl_2 might be due solely to its effect on phage adsorption but it seems probable that Ca ions were required for a later step or steps in phage synthesis. In the present investigations, one particular batch of broth which appeared to be deficient in calcium, since some phages failed to multiply in it but added CaCl_2 made it adequate for growth, was used to examine the divalent cation requirements of the phages. Experiments were so arranged that growth tubes contained two to three infected cells/0.01 ml. during the latent period and so yielded countable numbers of plaques when plated directly at the end of the rise in free phage. To a series of $6 \times \frac{1}{2}$ in. test tubes (wide tubes to ensure adequate aeration) was added 0.1 ml. of solutions of either CaCl_2 , MgCl_2 or $\text{SrCl}_2 \cdot 6\text{H}_2\text{O}$ to give final salt concentrations of 20, 50, 100 and 200 $\mu\text{g./ml.}$ Samples of young aerated broth culture (0.8 ml.) of the appropriate staphylococcus were added to each of the tubes. These were placed in a water-bath at 37° and then received 0.1 ml. of phage suspension (diluted in broth). Control tubes of cells + phage without added salt, and of the phage in broth + salts, were included in each test. Samples were plated during the latent period to determine the number of infected cells and at the end of 70 or 80 min., depending on the growth curve of the particular phage being examined. The average burst size was calculated by dividing the final phage count by the count during the latent period.

All the phages examined showed an increase of the average burst size on addition of CaCl_2 , but there were differences in the behaviour of different phages which could be correlated with their serological types. Table 2 shows the average burst size obtained for twenty different phages belonging to serological groups A and B (Rountree, 1949). The group A phages were able to establish infection in the basal broth alone and their average burst sizes were, in most cases, of a reasonably high order; however, the addition of CaCl_2 increased their burst sizes. The amounts of CaCl_2 which gave optimal phage production varied and was 20 $\mu\text{g./ml.}$ with phages 3 C, 7 and 51, 50 $\mu\text{g./ml.}$

with phage 6, and 100 $\mu\text{g.}/\text{ml.}$ with the remainder. When MgCl_2 or SrCl_2 was substituted for CaCl_2 no clear-cut evidence of increased yield was obtained. Possible exceptions were phage 6 in which an average burst size of 64 was produced in the presence of 200 $\mu\text{g.}$ $\text{MgCl}_2/\text{ml.}$ as compared with 44 in the basal broth, and phage 7 which gave average burst sizes of 16 and 21 in 100 and 200 $\mu\text{g.}$ $\text{SrCl}_2/\text{ml.}$ respectively, as compared with 7 in broth alone.

Table 2. *Effect of added divalent cations on the average burst size in broth of staphylococcal phages*

The phages in the upper half of the table (3A to 51 inclusive) belong to serological group A; those in the lower half (29 to 52A inclusive) to serological group B. Average burst size obtained by plating at 70–80 min. after infection, 0 = disappearance of phage, 1 = adsorption but no multiplication.

Phage	Composition of broth + salts mixtures												
	Broth alone	Broth + CaCl_2 $\mu\text{g. CaCl}_2/\text{ml.}$				Broth + MgCl_2 $\mu\text{g. MgCl}_2/\text{ml.}$				Broth + SrCl_2 $\mu\text{g. SrCl}_2/\text{ml.}$			
		20	50	100	200	20	50	100	200	20	50	100	200
		Average burst size											
3A	24	28	68	85	77	35	28	45	47	12	14	14	12
3B	111	116	126	152	140	93	113	80	95	108	118	100	95
3C	12	27	26	22	29	20	18	20	20	20	17	15	16
6	44	66	122	90	106	45	54	46	64	52	42	54	46
7	7	44	47	42	33	9	12	13	10	10	10	16	21
42E	3	3	12	26	60	2	3	2	2	2	2	2	3
47	20	43	56	100	68	24	22	20	12	18	21	19	17
47A	29	24	50	61	63	16	20	21	22	23	28	22	37
51	75	101	105	110	111	72	75	85	83	85	81	85	95
29	2	9	20	24	10	2	3	5	7	3	3	3	3
29A	6	41	92	91	30	6	6	15	29	4	14	14	10
31	1	18	25	26	50	3	4	7	14	3	3	5	10
31A	1	9	20	19	15	5	2	4	9	1	3	2	2
31B	10	20	45	71	102	3	3	6	12	2	4	8	9
42C	0	0	14	18	15	0	0	0	0	0	0	0	0
42D	0	0	31	52	24	0	0	0	0	0	0	0	0
44	1	33	44	85	65	0	0	0	0	0	0	0	0
44A	3	16	37	60	61	4	8	9	8	4	4	6	8
52	0	5	44	63	60	0	0	0	0	0	0	0	0
52A	12	36	57	95	91	7	16	24	41	15	11	24	26

Many of the group B phages did not grow in the basal broth. Phages 42C, 42D and 52 disappeared partly on adsorption, but chiefly during the latent period; and the addition of at least 50 $\mu\text{g.}$ $\text{CaCl}_2/\text{ml.}$ was necessary before any phage multiplication took place. With these phages and with phage 44, the addition of MgCl_2 or SrCl_2 to broth did not prevent this disappearance of phage. Other phages (e.g. 31 and 31A) established infection in broth alone but the infected cells liberated either very little phage or none until plated. All the phages gave higher average burst sizes in the presence of CaCl_2 ; 100 $\mu\text{g.}$ $\text{CaCl}_2/\text{ml.}$ was sufficient for maximal stimulation, with the exception of phages 31 and 31B which required 200 $\mu\text{g.}$ $\text{CaCl}_2/\text{ml.}$ Some phages (e.g. 29A, 31, 31A, 31B, 44A and 52A) gave slight increases in average burst size when MgCl_2

or SrCl_2 was substituted for CaCl_2 . In no case, however, did the average burst size approach that obtained with CaCl_2 . It was concluded that these other cations were at best poor substitutes for Ca ions. Since the previous work had shown that in most cases Mg ion was as effective as Ca ion in the first step of phage adsorption, it was apparent that the stimulatory effect of Ca ion was concerned with some later stage of phage growth.

Divalent cation activity early in the latent period

The phages 3A and 29, representative of groups A and B, were used for further study of the stage at which Ca ions were required, EDTAA (0.1 %, w/v) being used as a chelating agent at various times in the latent period. This concentration of the chelating agent had no effect on the infectivity of the free phages in broth at 37° during 60 min. (Table 3). The effect of the chelating

Table 3. *Stability of phages 3A and 29 in broth + 0.1 % ethylene diamino-tetra-acetic acid (EDTAA)*

Time of sampling (min.)	Phage 3A		Phage 29	
	In broth	Broth + EDTAA	In broth	Broth + EDTAA
	Phage count ($\times 10^{-3}/\text{ml.}$)			
0	19	18	145	149
60	21	18	152	141

agent on the growth of uninfected cultures of the propagating strains of staphylococci PS3A and 29 was examined. EDTAA (0.1 %, w/v) was added to 3 hr. old broth cultures aerated at 37°; turbidities were determined at intervals thereafter. Figs. 1 and 2 show that the chelating agent had no effect on the growth rate until 50–60 min. had elapsed. Since in the experiments to be described the chelating agent was not in contact with the infected cells for more than 60 min., and often only for 5–10 min., it was concluded that the effects obtained might be attributed to action on the host-phage complex rather than on the cells alone. When EDTAA was added to cells in broth before adding either phage, it completely inhibited adsorption, and all the phage added was recovered in supernatants when the cell + phage mixtures were centrifuged.

Phage 3A. A young broth culture of staphylococcus PS3A was infected with sufficient phage to give about 100–150 plaques/0.01 ml. during the latent period. At 1, 2, 4, 6 and 20 min. after infection, 1 ml. volumes were removed to tubes containing 0.1 ml. 1 % EDTAA and centrifuged (angle centrifuge). After plating samples of the supernatants to estimate unadsorbed phage, the supernatant fluids were removed, the cells resuspended in 1 ml. broth and held in an ice + water bath until plated, as a precaution against phage release before plating. Table 4 shows that, compared with the control which was centrifuged at 6 min., EDTAA added at 1–6 min. markedly decreased the number of infected cells which could be recovered. The maximal effect occurred when the

chelating agent was added at 2 min; 89 % of the adsorbed phage was then inactivated. When EDTAA was added at 20 min. there was little or no inactivation of phage.

Subsequent experiments confirmed that the period 2–3 min. after addition of the phage was the critical time at which the chelating agent had its maximal inactivating effect. Ten minutes after infection the phage+host complex

Fig. 1.

Fig. 2.

Figs. 1 and 2. The effect of EDTAA (0.1 %, w/v) added at 3 hr. on the growth in broth of propagating strains of staphylococci no. 29 and 3A.

reached a stage where the removal of divalent cations had no effect on the ability of the infected cells to register as infective centres when plated. This critical period might be called the period of vulnerability. The recovery of some infective centres when EDTAA was added at 1–4 min. indicated that already at these times a few particles had passed through this vulnerable period. The period of vulnerability for any individual particle might be of very short duration and the later in the latent period that the chelating agent was added, the greater the number of particles that would have been adsorbed and have passed this point (see Table 4). In these experiments, the chelating agent was in contact with the cells for at least 8 min. before it was removed in the

supernatant fluid on centrifuging. In order to ascertain the minimum length of time of contact necessary for inactivation of the adsorbed phage, the action of the chelating agent was halted by dilution, for 0.01 % EDTAA had no inactivating effect. The cells received ten times the previous amount of phage, EDTAA was added 2 min. later and, at intervals, 0.1 ml. samples were diluted in 0.9 ml. broth in tubes held in an ice + water bath to prevent further phage

Table 4. *Effect of time of addition of 0.1 % EDTAA on recovery of adsorbed phage from cells infected with phage 3A*

Tube	EDTAA added at (min.)	Phage added (a)	Phage in supernatant (b)	Phage adsorbed (a-b)	Phage recovered in cells	% of adsorbed phage recovered
1	Control no EDTAA	145	45	100	100	100
2	1	145	94	51	14	27
3	2	145	80	65	7	11
4	4	145	68	77	32	42
5	6	145	50	95	49	53
6	20	145	13	132	118	90

Phage/0.01 ml.; mean of 4 replicates

Table 5. *The effect of dilution into broth of phage 3A + cell mixtures to which 0.1 % ethylene diamino tetra-acetic acid (EDTAA) was added*

No EDTAA was added to tube 1; EDTAA was added to tubes 2-6 at 2 min. after the addition of phage.

Tube	Time of dilution	Time of contact with EDTAA	Phage adsorbed/ 0.01 ml.	Phage in cells	Phage inactivated
1	1 min. 55 sec.	—	82	82	0
2	2 min. 50 sec.	50 sec.	82	42	40
3	3 min.	60 sec.	82	46	36
4	3 min. 30 sec.	90 sec.	82	41	41
5	4 min.	120 sec.	82	42	40
6	*4 min. 10 sec.	> 8 min.	82	32	50

* Tube 6 diluted into broth + EDTAA

Phage/0.01 ml.; mean of 4 replicates

adsorption. The samples after chilling were centrifuged and plated as before. Table 5 shows that approximately 50 % of the phage adsorbed to the cells at 2 min. was inactivated by 50 sec. contact with the chelating agent. Tube 6, in which the mixture was diluted in broth + 0.1 % EDTAA, served as a control; it indicated that there was little if any reversal of the phage inactivation on dilution into broth. A very short period of exposure to the chelating agent was therefore sufficient for irreversible inactivation of adsorbed phage.

Infection with lytic phage normally results in death of the cell; EDTAA (0.1 %) added to cells 3 min. after infection protected at least half the cells from this lethal action. Table 6 shows the results of two experiments with different phage/cell ratios in which viable cell counts were made 10 min. after infection, the centrifuged infected cells being diluted in broth which contained phage antiserum.

Table 6. *The protection of cells infected with phage 3A by the addition of ethylene diamino tetra-acetic acid (EDTAA) 3 min. after infection*

Treatment	Cells/ml.	Phage/cell adsorbed	Viable cells/ml.	Cells killed (%)
0.1 % EDTAA	5×10^7	3	2.4×10^7	50
No EDTAA	5×10^7	3	1.5×10^6	97
0.1 % EDTAA	7×10^7	0.7	5.4×10^7	23
No EDTAA	7×10^7	0.7	3.5×10^7	50

Phage 29. In one particular batch of broth phage 29 adsorbed slowly on to its own propagating strain. Also, very little adsorbed phage could be recovered as infective centres, thus giving results resembling phage 3A-infected cells + EDTAA. The addition of CaCl_2 to the cells before adding phage resulted in more efficient cell infection. Table 7 indicates that in this broth the phage had a threshold requirement of added CaCl_2 which was between 100 and 200 $\mu\text{g./ml.}$, since in unsupplemented broth and in broth + 100 $\mu\text{g. CaCl}_2/\text{ml.}$, only 18 and 19 % respectively of the adsorbed phage was recovered.

Table 7. *Infection of staphylococcus PS 29 with phage 29 in broth and in broth + CaCl_2*

Each tube contained phage + cells and the amount of CaCl_2 indicated.

Tube	Amount of CaCl_2 ($\mu\text{g./ml.}$)	Phage adsorbed	Phage in cells	Adsorbed phage lost (%)
1	—	61	11	82
2	100	68	13	81
3	200	66	46	30
4	300	68	39	43
5	400	65	40	38

Phage/0.01 ml.; mean of 4 replicates

The substitution for staphylococcus PS 29 of another strain R 5310, resulted in more rapid adsorption of phage, but the cation requirements were the same and even in 200 $\mu\text{g. CaCl}_2/\text{ml.}$ only 42 % of the adsorbed phage was recovered. Nevertheless, when infection was established in these suspensions of cells + CaCl_2 and EDTAA added 2 and 6 min. later, inactivation of adsorbed phage could be demonstrated (Table 8).

Table 8. *Effect of time of addition of EDTAA on recovery of adsorbed phage from cells of staphylococcus R 5310 infected by phage 29*

Each tube contained phage + staphylococci and except in Tube 1 CaCl_2 , 200 $\mu\text{g./ml.}$ EDTAA (to 0.1 %) was added at the times shown.

Tube	EDTAA added	Phage adsorbed	Phage in cells	Adsorbed phage lost (%)
1	Nil	109	9	92
2	Nil	108	63	58
3	At 2 min.	106	22	79
4	At 6 min.	115	31	73
5	At 10 min.	124	54	61
6	At 20 min.	137	74	62

To summarize, shortly after adsorption, phages 3A and 29 pass through a period during which they can be inactivated by the addition of a chelating agent. This inactivation is rapid and irreversible and protects the cell from the lethal action of the phage. A reasonable hypothesis to explain these findings is that in the absence of divalent cations phage DNA cannot penetrate the cell. The divalent cations may therefore be essential to the process of penetration.

Effect of divalent cations in penetration

The experiment with a chelating agent in broth did not distinguish between a specific Ca ion effect and one due to divalent cations. The phenomenon of penetration was therefore examined by another method. When staphylococci are grown in citrated broth and resuspended in distilled water to which appropriate concentrations of cations have been added, the cocci adsorb phage which can be recovered as infective centres when the plating is done within 30 min. of infection. It can be argued that, under these conditions, phage adsorption had resulted in the first step only and that the later steps proceeded when the cells were transferred to the plating medium which contained adequate amounts of Ca ion. When, however, differences in the activity of various cations can be demonstrated under such conditions, then it may be assumed that penetration has taken place. Such differences were found. In experiments of this kind complete recovery of added phage was rare, partly because of inactivation of the free phage in distilled water and partly because of a proportion of abortive adsorptions. Nevertheless, the method can be used to compare the relative activity of equivalent amounts of cations.

Young cells grown in 0.5% (w/v) Na citrate broth were centrifuged and resuspended in distilled water. Samples (0.8 ml.) were distributed into tubes containing 0.1 ml. water or appropriate concentrations of CaCl_2 , MgCl_2 or SrCl_2 ; 0.1 ml. volumes of phage suspension (diluted in water) were then added. After 10 min. adsorption the tubes were centrifuged, the supernatant fluid, and the cells resuspended in water were plated.

Before penetration can occur the first step of adsorption must take place. This can be measured by the disappearance of free phage from the supernatant but is complicated by the fact that some phages are unstable in the salt solutions in absence of cells and it is not always possible to be certain whether their disappearance in the salt + cell suspensions is due to adsorption and inactivation or to inactivation while unadsorbed. In a previous paper (Rountree, 1951), basing evidence for lack of adsorption on the similarity between amounts of free phage in salt solutions and in salt solutions + cells, it was concluded that phages 7 and 42E had specific Ca ion requirements for adsorption. The present results, however, indicate that small amounts of phage adsorb and penetrate in the presence of Mg or Sr ion and small amounts of 42E in the presence of Mg but none in the presence of Sr ion.

The phages fell into four groups according to the relative activity of the three cations (Table 9). All the phages which showed penetration in SrCl_2 belonged to group A. On the other hand, the group B phages showed no Sr

activity and in three cases, comparatively little Mg activity. This lack of Mg or Sr activity was not due to failure of adsorption, since the free phages were relatively stable in these salt solutions in the absence of cells. Phage 42E, which is group A serologically, was aberrant in its behaviour.

Table 9. *Relative activity of Ca, Mg and Sr in penetration of staphylococcal phages*

++ = cations equally active; ± = cations partially active compared with Ca; -- = no activity.

Ca	Mg Relative activity	Sr	Phages
++	++	++	3B, 6, 7, 47
++	++	±	3A, 3C, 47A, 51
++	++	--	29, 29A, 31A, 31B, 42D, 52A
++	±	--	31, 42E, 44, 52

The results indicate that, save with phages 31, 42E, 44 and 52, no specific Ca ion effect was demonstrated for phage penetration and that even in the exceptions there was partial activity with Mg ion. The stimulatory effect of Ca ion must therefore occur at a later stage in phage growth.

The effect of calcium towards the end of the latent period

Since the addition of CaCl_2 to broth increased the average burst size with all the phages examined and in some cases converted an unsuitable broth to one which gave a good yield of phage, a chelating agent added to the infected cells after penetration has occurred should decrease the average burst size. Na citrate was found to have this effect, but owing to its inactivation of free phage, doubt existed as to the point in the growth cycle at which it acted. The effect of 0.1% EDTAA on the average burst size and growth curves of phages 3A and 29 was therefore investigated. This concentration of EDTAA (0.1%) did not completely inhibit phage production but did cause a marked decrease in average burst size. Figs. 3 and 4 show that this depressive effect occurred whether the chelating agent was added relatively early or late in the latent period. Phage 3A showed a similar degree of decrease in phage yield/cell, whether the EDTAA was added at 20, 30, 40 or 50 min. after infection. Similarly, phage 29 gave lower average burst sizes when EDTAA was added at 10 or 50 min. In the latter case, phage release was already occurring when the chelating agent was added. This depressive action of EDTAA could be partially reversed by the addition of CaCl_2 to the infected cells, provided this was done before phage release was completed (Table 10). It was concluded that in these phages the specific Ca ion requirements were concerned with a very late step in phage synthesis.

The failure of phages of group B, e.g. 42C, 42D, 44 and 52, to produce phage in the basal broth or broth + MgCl_2 was not due solely to failure of penetration. When Mg ions were adequate for penetration, successive platings of the infected cells showed their gradual failure to register as infective centres. It

would seem therefore that, in the absence of adequate amounts of Ca ion these phage + cell complexes gradually lost their ability to complete a step in phage multiplication. The present data do not, however, allow a distinction between an early and late need for Ca ion in these phages.

Fig. 8.

Fig. 8. The effect of EDTAA (0.1 %, w/v) on the one-step growth curve in broth of phage 3A. $\triangle$ — $\triangle$ = EDTAA added at 20 min.; x—x—x = EDTAA added at 50 min.

Fig. 4.

Fig. 4. The effect of EDTAA (0.1 %, w/v) on the growth curve in broth of phage 29. $\triangle$ — $\triangle$ = EDTAA added at 10 min.; x—x—x = EDTAA added at 50 min.

Table 10. *Partial annulment by CaCl_2 of the effect of EDTAA on the average burst size of phage 3A*

Tube no.	Contents of tube	Average burst size
1	Phage and cells in broth	42
2	Phage and cells in broth + 100 $\mu\text{g./ml.}$ CaCl_2 at 1 min.	70
3	Phage and cells in broth + 0.1 % EDTAA at 15 min.	10
4	Phage and cells in broth + 0.1 % EDTAA at 15 min. + CaCl_2 at 50 min.	40
5	Phage and cells in broth + 0.1 % EDTAA at 15 min. + CaCl_2 at 60 min.	20
6	Phage and cells in broth + 0.1 % EDTAA at 15 min. + CaCl_2 at 70 min.	22

DISCUSSION

From the present results and those previously reported, a general outline of the role of divalent cations in the growth of staphylococcal typing phages may be suggested. It should be understood, however, that the quantitative

details vary from phage to phage. Adsorption of the phages to their host cells requires divalent cations and can be inhibited by suitable chelating agents. Following adsorption, the phage particle passes through a short period during which it can be irreversibly inactivated by the removal of divalent cations from the medium. Although there is no direct evidence, it seems reasonable to assume that this inactivation is due to the abortive separation of phage protein and deoxyribonucleic acid at the cell surface and the inability of the deoxyribonucleic acid to penetrate the cell in the absence of divalent cations. With the possible exception of phage 42E, there is no evidence of any specific Ca ion requirements in these steps. There is, however, evidence of a correlation between antigenic structure and cation requirement since the group B phages are unable to use Sr ion for penetration.

After this 'vulnerable' period has passed, the phage material is no longer inactivated by the chelating agent EDTAA and infected cells treated with this substance from 10 min. onward in the latent period will register as infective centres when plated. There is, however, a specific calcium requirement late in the latent period. This is shown by the increase in average phage yield/cell in increasing concentrations of Ca ion and in the depression in yield in the presence of a chelating agent. Particularly with the group A phages, the amount of CaCl_2 which stimulates phage yield is much greater than that required for adsorption or penetration; for example, phage 3A requires $10\text{ }\mu\text{g. CaCl}_2/\text{ml.}$ for adsorption and $100\text{ }\mu\text{g. CaCl}_2/\text{ml.}$ for maximal stimulation. In a few cases, some stimulation of yield can be obtained with Mg or Sr but neither cation is as effective as an equivalent amount of Ca and generally no activity is found.

The results do not exclude the possibility that Ca ion is also required specifically for some process in phage synthesis which immediately follows penetration, as suggested for other phages by Fildes (1953); but they do indicate the importance of Ca ion in the final stages of phage multiplication. It is unlikely that the effect of EDTAA is a direct one on the metabolism of the host cells since in some experiments in which a decrease in phage yield was obtained the chelating agent was in contact with the cells for only 5–10 min. before they were plated. There is a possibility that the depressive effect of the chelating agent was due to its direct inactivation of completed phage particles not yet liberated from the cell. Since, however, free phage particles after liberation were not affected by the concentration of chelating agent used, this seems unlikely. It is possible that the effect of the chelating agent is an indirect one in making Ca unavailable to not yet completed phage particles or to some enzyme or enzymes concerned in phage synthesis. The stabilizing effect of Ca ions on various proteins and enzymes is a well-recognized biological phenomenon and their activity in the synthesis of the staphylococcal phages may be of a similar nature.

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