THE ROLE OF CALCIUM IN THE PENETRATION OF BACTERIOPHAGE T5 INTO ITS HOST[1]

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Certain peculiarities of the early phases of infection of *Escherichia coli*, strain B, with phage T5 were observed by Lanni (1954). Separation of phage desoxyribonucleic acid from phage protein and penetration of desoxyribonucleic acid into the host cell, as observed with phage T2 (Hershey and Chase, 1952), do occur under the conditions of a one-step growth experiment, but fail to take place if the cells are infected and kept in nutrient broth at high bacterial concentrations (over 2×10^9 cells per ml). Like many other phages, T5 requires calcium (or magnesium, or strontium) ions for growth (Adams, 1949; Kay, 1952). Calcium deficiency arrests phage development at an early stage following adsorption. The obvious guess, that calcium is required for the penetration of the phage desoxyribonucleic acid, was verified by experiments reported here.

MATERIALS AND METHODS

E. coli, strain B, phage T5, and the routine methods in phage experiments were employed (Adams, 1948*a*). Adsorption generally was carried out with bacteria from young cultures concentrated to a density of 2 to 8×10^9 per ml. It was over 95 per cent complete in four minutes.

The basal synthetic medium used (Kay, 1952) contained 2×10^{-2} M KH_2PO_4; 3×10^{-2} M Na_2HPO_4; 8×10^{-2} M NH_4Cl; 10^{-4} M $MgSO_4$; 0.4 per cent glucose; and 0.02 per cent gelatin. $CaCl_2$ and $MgSO_4$ were added in the desired amounts. For some experiments, traces of calcium were removed from the basal medium as follows: A solution of each salt was passed twice through a column of a cation exchange resin ("dowex 50"), which had been regenerated with a 2 M solution of the chloride of the corresponding cation (for example, KCl-regenerated resin for KH_2PO_4) and washed to a negative AgCl reaction. Gelatin and glucose solutions were treated with resin regenerated with HCl and washed to neutral reaction.

Radioactive T5 was prepared in 0.5 per cent tryptose with 0.5 per cent NaCl and 20 microcurie P^{32} per ml and purified by differential centrifugation. Thin samples of the purified preparation (8×10^{11} infectious particles per ml) gave 4×10^{-6} counts per infectious particle per minute in a flow counter. Over 98 per cent of the infectious particles and 65 per cent of the radioactivity were adsorbed by *E. coli*, strain B (and not by *E. coli*, strain B/5, resistant to T5).

Removal of protein skins of phage adsorbed upon bacteria was accomplished as by Hershey and Chase (1952), by stirring or "blending" for 2.5 minutes either with the semimicrocup of a Waring blendor (25 ml samples) or in the microcup of a Viltis homogenizer (0.8 ml samples). The samples were chilled before and again immediately after blending. The blendor cups were chilled with ice by special attachments.

RESULTS

Preliminary experiments confirmed several of the results of Adams (1949), Kay (1952), and Lanni (1954). The following points were established:

(1) There was no liberation of phage T5 by bacteria infected and kept in nutrient broth or tryptose broth at concentrations of 2×10^9 cells per ml or higher. Dilutions 1:25 or 1:200 in broth at various times allowed phage liberation to occur 40 minutes after dilution, with yields of 100 to 250 phages per cell. The results are similar to those of Adams (1949) for T5 adsorbed in absence of Ca^{++}, followed by dilution into medium with 10^{-3} M Ca^{++} at various times.

(2) Bacteria infected in synthetic medium and diluted in the same medium without added Ca^{++} or Mg^{++} showed a rapid loss of plaque forming ability and no phage liberation. Addition of 10^{-3} M $MgSO_4$ gave some protection against the loss and allowed some phage production. Addition

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TABLE 1

The effect of divalent ions on the resistance of infective centers to blendor treatment

Bacteria grown to 10^8 cells per ml in synthetic medium were concentrated 40-fold and infected with T5 (input 4.4×10^8 per ml). After 5 minutes, samples were diluted 1:100 in synthetic medium alone, or with 10^{-3} M $MgSO_4$, or with 10^{-3} M $MgSO_4$ plus 5×10^{-4} M $CaCl_2$. At various times samples were taken and diluted 1:10 in chilled medium. Aliquots of 25 ml were stirred in a Waring blendor for 2.5 minutes. All samples were plated after a further dilution 1:100 in synthetic medium at 37 C. The plaque counts are in plaques per ml.

TREATMENT	INFECTIVE CENTERS, WITHOUT STIRRING		INFECTIVE CENTERS, AFTER STIRRING		
	Count	Per cent of input	Count	Per cent of input	Per cent of unstirred controls
Synthetic medium	8.8×10^7	20	—	—	—
$MgSO_4$ for 2 minutes	2.3×10^8	50	7.6×10^6	1.7	3.4
$MgSO_4$ for 10 minutes	1.8×10^8	40	2.2×10^7	5	12
$CaCl_2$ + $MgSO_4$ for 2 minutes	3.6×10^8	80	1.5×10^7	3.4	4.2
$CaCl_2$ + $MgSO_4$ for 4 minutes	2.9×10^8	70	9.0×10^7	20	31
$CaCl_2$ + $MgSO_4$ for 7 minutes	4.1×10^8	90	2.8×10^8	64	68
$CaCl_2$ + $MgSO_4$ for 10 minutes	4.4×10^8	100	3.1×10^8	70	70

of 5×10^{-4} M $CaCl_2$ protected against the loss of plaque forming ability (see table 1) and produced a yield of about 250 particles per cell.

(3) Removal of calcium by dilution at various times allowed more and more complete production of phage, the later the dilution was made.

(4) Addition of 10^{-2} M citrate to infected bacteria causes a rapid loss of infective centers. After the infected bacteria have been exposed to calcium for two minutes or longer, however, addition of citrate fails to suppress the plaque forming ability although it still reduces the phage yield.

(5) Plating of T5 infected bacteria by the agar layer method directly from chilled samples often causes an important loss of infective centers. This was probably responsible for the loss of infected bacteria that complicated some of the experiments by Lanni (1954). We found that this loss can be avoided if the samples are warmed for several minutes at 37 C before being plated.

The role of calcium in the resistance of T5 infected bacteria to blendor treatment. Bacteria infected in synthetic medium were diluted at intervals in medium with or without added Ca^{++} (5×10^{-4} M) or Mg^{++} (10^{-3} M). After various intervals, they were diluted in chilled medium without added cations, and 25 ml samples were stirred for 2.5 minutes in a Waring blendor. This treatment removes over 95 per cent of the adsorbed phage antigens from the surface of the bacteria (Lanni, 1954). Blended and control samples were diluted in medium with added Ca and Mg and plated at intervals. The results of a typical experiment are shown in table 1. They show that the longer the treatment with Ca^{++} before blending the greater the number of infected bacteria whose phage forming ability withstands blending. Treatment with Mg^{++} has a lesser effect.

In these experiments it was found also that blending reduces and delays the phage yield considerably. This effect remains unexplained.

The role of Ca^{++} in the penetration of phage desoxyribonucleic acid. Some experiments with *E. coli*, strain B, infected with P^{32} labeled phage T5 indicated that the addition of calcium permits the penetration of phage desoxyribonucleic acid in the bacterium, as measured by the failure of the isotope to be removed by blending.

The experiment shown in table 2 includes tests with bacteria infected in synthetic medium and with bacteria infected in nutrient broth and kept at a concentration of 4×10^9 cells per ml. The labeled T5 stock contained about 30 per cent of its radioactivity in nonadsorbable form (probably inactive phage). Of the adsorbable radioactivity, blending removed 80 per cent from bacteria infected and kept concentrated in broth; over 80 per cent from bacteria infected and kept

TABLE 2

The removal of plaque forming ability and of phage phosphorus by blendor treatment

Bacteria were concentrated 40-fold by centrifugation and infected with P^{32} labeled T5 (phage input 9×10^8 per ml). The unadsorbed phage was less than 2 per cent; the unadsorbed radioactivity was about 30 per cent. After various treatments, aliquots were stirred for 2.5 minutes in a Waring blendor. Blended and unblended samples were centrifuged in the cold. Assays of plaque counts and radioactivity were made on the total samples and in the supernatants of centrifugation. The radioactivity values are in counts per ml per minute; the plaque counts in plaques per ml.

TREATMENT	CONTROL, NOT STIRRED				STIRRED SAMPLES			
	Total		Supernatant		Total		Supernatant	
	Plaque count	P^{32} activity	Plaque count	P^{32} activity	Plaque count	P^{32} activity	Plaque count	P^{32} activity
1. Bacteria grown in nutrient broth; concentrated to 3×10^9 per ml; infected in nutrient broth; after 7 minutes diluted 1:160 with iced broth	3.2×10^8	*356*	1.8×10^6	*120*	4×10^8	*320*	1.3×10^6	*277*
2. Bacteria grown in synthetic medium; concentrated to 6×10^9 per ml; infected in synthetic medium; after 7 minutes diluted 1:160 in iced medium	1.6×10^8	*290*	8.0×10^7	*89*	1×10^8	*252*	1×10^6	*270*
3. Like 2; but after 6 minutes adsorption, diluted 1:40 in medium with 5×10^{-4} M $CaCl_2$ and 10^{-3} M $MgSO_4$; after 10 minutes, diluted again 1:4 in iced medium	1.1×10^9	*355*	5.6×10^7	*103*	8×10^8	*295*	6.6×10^7	*124*

in synthetic medium; and not more than 10 per cent from bacteria exposed for 10 minutes to 5×10^{-4} M Ca^{++} plus 10^{-3} M Mg^{++}. Two points should be noted:

(1) The proportion of plaque forming bacteria was more reduced by blending than the proportion of removable phosphorus. This discrepancy (see also Lanni, 1954) indicates that injection of phage desoxyribonucleic acid, as measured by failure of phage phosphorus to be removed by blending, is not identical with acquisition of resistance to blending, as measured by the plaque forming ability of the bacteria.

(2) There was a significant loss of plaque forming ability in all the unblended samples in synthetic medium even when Ca^{++} was added after 6 minutes. Yet, there was no corresponding release of P^{32} into the supernatants. This indicates that the loss of plaque forming ability is not accompanied by a release of phage desoxyribonucleic acid into the medium such as is observed upon inactivation of free phage in citrated buffer at 45 C (Adams, 1953).

The speed of T5 injection in calcium supplemented media. Lanni (1954) observed that when bacteria in concentrated suspensions were infected with T5 and then diluted in nutrient broth, their plaque forming ability became resistant to blending at a slow rate, requiring 10 minutes or longer to reach a maximum. We explored the possibility that the slow desoxyribonucleic acid injection might be due to a calcium depletion of the cells while in concentrated suspension. Bacteria were infected at concentrations between 4×10^8 and 5×10^9 cells per ml with Ca^{++} concentrations up to 10^{-2} M. Then, the mixtures were diluted in calcium containing media and tested at intervals for resistance to blending. These experiments revealed no greater speed of injection. The plaque forming ability of bacteria never

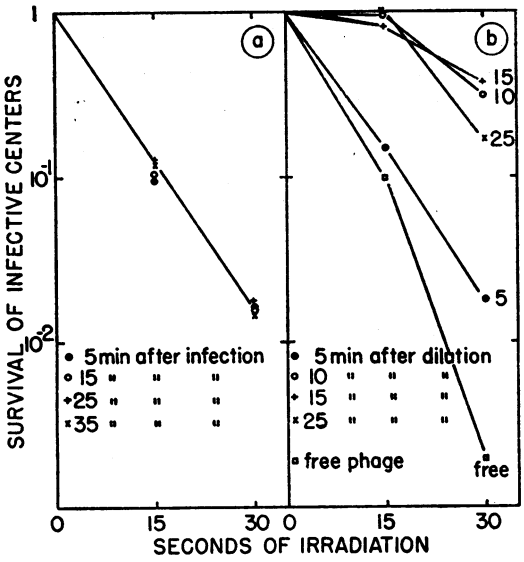

Figure 1. Ultraviolet inactivation curves for free and intracellular phage T5. (a) Bacteria infected and kept at 6×10^8 cells per ml in nutrient broth at 37 C. (b) Bacteria infected at 6×10^8 cells per ml in nutrient broth, diluted 1:100 in nutrient broth after 4 minutes, and incubated at 37 C. Samples were taken at intervals, diluted 1:200 in ice-cold salt solution, irradiated in the cold, returned to 37 C for 5 minutes, and plated for phage assay. The free phage was irradiated after dilution 1:200 in chilled salt solution. Abscissa: Duration of exposure to a 15 watt germicidal lamp at 50 cm distance. Ordinate: Ratio of plaque titers in irradiated and control samples.

did reach maximum resistance (80 to 100 per cent) until 10 to 12 minutes after infection. Thus, the slow penetration of phage T5 into *E. coli*, strain B, appears to be without evident relation to the role of Ca^{++}.

Some properties of T5 adsorbed without penetration. (1) Bacteria infected with T5 in the absence of Ca^{++} are killed (Adams, 1949). Murray and Whitfield (1953) showed that bacteria infected with T5 in the absence of Ca^{++} undergo the nuclear disintegration characteristic for T5 infected cells, without later appearance of the granular phage chromatin that appears in normal controls. We found that bacteria infected and kept concentrated in nutrient broth showed a cytological evolution similar to that observed in medium without Ca^{++}. It is possible that the irreversible loss of plaque forming ability of T5 infected bacteria, when kept in Ca^{++}-free medium, may reflect inability to carry out some essential reaction if the phage desoxyribonucleic acid is injected after disintegration of the host chromatin.

(2) Antiserum against T5 fails to suppress the plaque forming ability of bacteria infected with T5 and kept in concentrated suspensions although this ability is still removable by blending.[2]

(3) Phage T5 adsorbed to bacteria without penetration can exclude phage T2 added as early as one minute after T5. If, however, T2 is adsorbed first, subsequent addition of T5 even one minute later does not prevent T2 growth.[3] Exclusion between noninjected T5 and phages other than T2 has not been tested.

Intracellular irradiation of phage T5 with ultraviolet light. The ultraviolet sensitivity of bacteria infected with T5 was studied by infecting concentrated bacteria in broth, diluting them in broth at various times, and then, after further dilution in chilled buffer, exposing them to several doses of ultraviolet light. Platings after irradiation give the numbers of infected bacteria that retain the plaque forming ability (Luria and Latarjet, 1947; Benzer, 1952). Typical results are shown in figure 1. Immediately after phage adsorption the ultraviolet sensitivity is lower (survival higher) than for free phage T5. It remains constant as long as the infected bacteria are kept in broth at concentrations of over 10^9 cells per ml. Dilution in nutrient broth is followed, after a delay of 5 to 10 minutes, by a progressive increase in ultraviolet resistance similar to that which is observed with phage T2 immediately after infection under conditions of active metabolism.

DISCUSSION

Adsorption of phage T5 is not followed by full penetration of phage nucleic acid and initiation of phage development unless adequate amounts of calcium ions are present. Magnesium (and presumably strontium; Kay, 1952) can replace calcium, though less effectively. Nutrient broth (0.5 per cent peptone plus 0.3 per cent beef extract) and two per cent tryptose broth apparently do not contain enough calcium to allow phage penetration in the presence of 10^9 or more bacteria per ml. Preliminary tests have indicated that the addition of 10^{-3} M $CaCl_2$ to a nutrient broth suspension containing 2×10^9 cells per ml

[2] Experiments by Miss Martha Paton.
[3] Experiments by Mr. Peter Berman.

is just sufficient to allow phage penetration in a fraction of the bacteria.

Even in the presence of adequate concentrations of calcium, the penetration process is rather slow, requiring several minutes in the presence of calcium for its completion (see also Lanni, 1954). Assuming a tailwise attachment of phage T5 to bacteria and a penetration of the phage desoxyribonucleic acid through the tail, we may speculate on a role of the small diameter of the tail of phage T5 (about 10 millimicra) in rendering the penetration process a slow one.

Calcium or magnesium appears to be still needed, after the penetration stage, for a full yield of phage in synthetic medium. This may be due to an inactivation of newly produced phage when released in a medium with low content of divalent cations (Adams, 1948*b*). Kay's observations (1952) that a calcium treatment as short as one minute permits full development of phage T5*st* may reflect either the use by that author of the *st* mutant instead of the wild type T5, or other differences in experimental conditions.

Phage T5 adsorbed without penetration is not accessible to neutralization by phage antiserum. It is inactivated by exposure to low concentrations of divalent ions and, even more rapidly, in citrated media. It causes disintegration of the bacterial chromatin and can prevent another phage from multiplying. The cytological and interference effects may be due to the externally attached phage particle itself or may depend on injection of some portion of phage material not yet recognized. Some attempts to duplicate the effects of externally adsorbed phage T5 by the use of T5 ghosts produced by heating in citrated buffer (Adams, 1953) failed, presumably because of the failure of these ghosts to be adsorbed by bacteria (*see* Lark and Adams, 1953).

SUMMARY

Adsorption of coliphage T5 onto host bacteria is not followed by full penetration of phage nucleic acid and initiation of phage development unless adequate concentrations of calcium or magnesium ions are present. Even in the presence of calcium, the penetration process is slow, requiring several minutes for completion. Phage T5 adsorbed without penetration is not accessible to neutralization by antiphage serum. It is inactivated fairly rapidly in the absence of calcium or magnesium ions. It causes disintegration of bacterial chromatin and interferes with the multiplication of other phages.

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