THE EFFECT OF DIVALENT METALS ON THE MULTIPLICATION OF COLI BACTERIOPHAGE T5st.

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Received for publication January 11, 1952.

DIVALENT metals and particularly calcium have long been recognised as essential participants in the multiplication of some strains of phage. Stassano and de Beaufort (1925) and Bordet and Renaux (1928) found that calcium or strontium was necessary for the lysis of the bacteria by shiga phages. More recently Adams (1949) with coli phage T5 and Kay and Fildes (1950) with a typhoid phage reported more detailed studies. These indicated that in the absence of calcium very little phage multiplication took place, whereas in the same culture medium containing calcium a large increase in phage titre occurred. Since no bacterial requirement for calcium was evident it appeared that calcium was involved specifically in the multiplication of the phage. The work reported in this paper concerns a survey of various metals to determine whether elements other than calcium are active, an examination of the relative activities of those elements found to be active and an attempt to determine the point in the virus multiplication cycle at which calcium acts.

The work of Adams (1949) with T5 has been confirmed and extended with the related heat-resistant variant T5st.

MATERIALS AND METHODS.

Medium.

Bact. coli B obtained from Dr. T. F. Anderson was cultivated in the following medium :

KH_2PO_4	0·2 M	100 ml.
Na_2HPO_4	0·2 M	150 ,,
NH_4Cl	4 M	20 ,,
$MgSO_4 7H_2O$	·04 M	4 ,,
Water		526 ,,
Total		800 ml.

The chemicals were "Analar" grade and the water was redistilled from glass. The medium was sterilised by autoclaving in " Pyrex " flasks capped with beakers. The pH was 7·0. Glucose was used as a source of energy and carbon. Molar solutions were sterilised separately in " Pyrex " vessels. The medium in 8 ml.

* Working with a personal grant from the Medical Research Council.

volumes was made up to 10 ml. with water after addition of glucose, bacterial inoculum and any other materials.

Gelatin (0·05 per cent) was added to the culture medium. Prior to the use of gelatin average burst sizes of 50 to 60 were the largest obtainable, whereas when gelatin was added burst sizes of 200 to 400 were regularly obtained. Commercially available gelatin contains calcium and this was removed by prolonged electrodialysis.

Culture technique.

All experiments were made in L-shaped vessels, which were rocked 54 times a minute in a water bath at 37°. A mean generation time of 40 minutes was found for *Bact. coli* growing in the above medium incubated in L tubes. The lower limb of the L had a volume of 20 ml. and usually contained 10 ml. of medium. The extent of bacterial multiplication or lysis could be determined by inserting the vessel into a nephelometer (Evans Electroselenium Ltd.) and measuring the amount of scattered light. This figure was related to viable cell count by a standard curve.

Phage.

Coli phage T5 was obtained from Brigadier J. K. S. Boyd, F.R.S. On being examined by the method of Adams (1950) it was found to contain both heat-labile and heat-stable mutants. The heat-stable mutant, T5st, was used for this work.

A stock of high titre phage was prepared as follows: Three litres of medium containing 0·0005 M calcium were inoculated with bacteria and aerated with compressed air at 37°. When the bacteria had reached 4×10^8/ml. phage was added at a ratio of 2 phages to one bacterium. Aeration was continued, and 0·1 ml. of triamylcitrate was added to control the froth when lysis occurred 50 minutes later. Aeration was continued for a further hour. The lysate was then concentrated to one-tenth of its volume by evaporation under reduced pressure. The phage is unharmed by this treatment. Much bacterial debris and precipitated material was removed by centrifugation at 2000 **g.** for 30 minutes. The opalescent supernatant was separated and dialysed against 3 changes of 5 volumes of ice-cold distilled water. The phage was again centrifuged at 2000 **g.** and the supernatant separated. This was the material used in the following experiments. It was stored at +4° saturated with thymol to prevent the growth of contaminants. The titre of the stock phage was 7×10^{11}/ml. and declined to 5×10^{11}/ml. during the course of the work reported in this paper.

The estimation of phage.

Phage counts were made by the two-layer technique of Hershey, Kalmanson and Bronfenbrenner (1943). Dilutions were made in medium containing 0·05 per cent of gelatin to prevent denaturation of the phage during manipulation.

EXPERIMENTAL.

The Effect of Divalent Metals on Lysis.

A non-toxic range of concentrations of different metals (as chlorides or sulphates) was added to the standard medium, which was then tested for ability

to support phage lysis. The bacteria were infected with a ratio of 2 phages per cell when the bacterial population was estimated to be 4×10^8/ml. Turbidities of the cultures were measured at intervals for two hours after the addition of phage.

The following metals were examined: barium, beryllium, calcium, cobalt, copper, iron (ferrous), magnesium, manganese, strontium and zinc. Concentration ranged from 1 to 100 μg./ml., or the maximum solubility in the medium if this was less than 100 μg./ml.

It was found that calcium, magnesium and strontium would support lysis while the other metals were either inhibitory to bacterial multiplication or inactive. Phage titres were determined, and it was found that only in those cultures that had lysed had the phage increased. It was also noticed that calcium caused lysis in a lower concentration than magnesium or strontium. The relative efficiencies of the three metals to support the multiplication of phage were then examined.

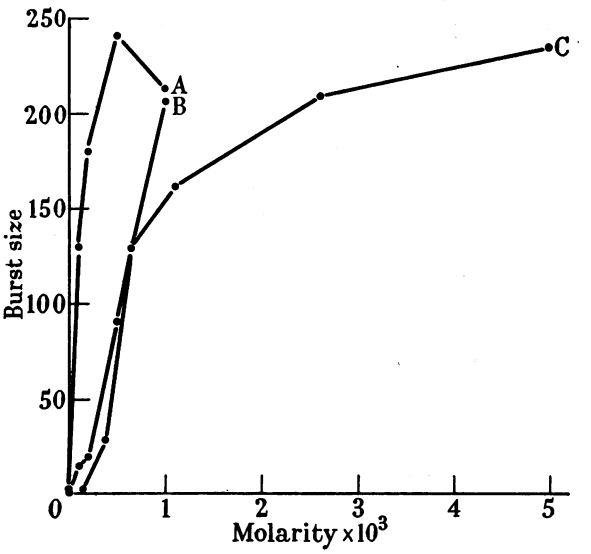

FIG. 1.—The effect of divalent metal concentration on the burst size.
A = calcium, B = strontium, C = magnesium.

The Effect of Various Concentrations of Calcium, Magnesium and Strontium on the Multiplication of Phage.

It was first determined whether these metals affected the multiplication of bacteria when added to the standard medium. No significant effect was found except with magnesium, a concentration of which if below 0·0001 M retarded growth.

The average burst size was then determined by the method of Ellis & Delbrück (1939) using bacteria grown in the standard medium supplemented with various concentrations of the three metals. The results are shown in Fig. 1, where the burst size is plotted against the molarity of the metal.

Calcium is the most effective, producing a burst size of 240 at 0·0005 M.

Strontium is the next most effective with a burst size of 205 at its maximum concentration of 0·0001 M. Magnesium, however, shows a somewhat different relationship between concentration and burst size. The curve is similar to that of strontium up to 0·0007 M, but at higher concentrations the burst size increases slowly with concentration, reaching 235 at 0·005 M. The standard medium which contains 0·00016 M magnesium gave a burst size of 2.

Having demonstrated the necessity of some divalent metal for lysis and phage production, the mode of action of the metal was investigated. Only calcium was studied as this was the most active metal.

Experiments were made to find whether bacteria infected with phage in the absence of calcium were able to produce phage if calcium was added later, and whether bacteria infected in the presence of calcium continued to produce phage when the calcium was removed from the medium before the end of the latent period.

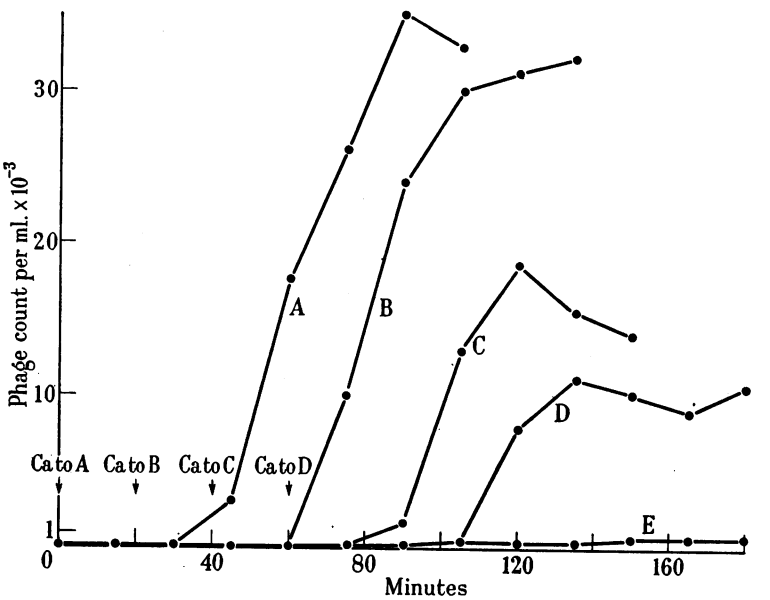

FIG. 2.—The effect of delaying the addition of calcium to phage-infected bacteria. Calcium was added at points indicated to A, B, C and D. E received no calcium.

The Addition of Calcium at Intervals to Phage-infected Bacteria.

A 10 ml. culture of actively growing bacteria containing 6×10^8/ml. was infected with phage at 6×10^8/ml. After rocking for 10 minutes to allow adsorption to proceed the bacteria were centrifuged down, the supernatant discarded, and the sediment containing the infected bacteria re-suspended in fresh medium. This suspension was diluted 1:10,000 in fresh medium and 1 ml. of this was added to each of 5 L tubes, A, B, C, D and E, containing 9 ml. of medium. These were set rocking at 37° and calcium (0·0005 M) was immediately added to tube A. Samples were taken at intervals from all the tubes. After 20 minutes calcium (0·0005 M) was added to tube B, at 40 minutes to tube C, and at 60 minutes to tube D. Tube E was retained as a calcium-free control. The

results of a typical experiment are given in Fig. 2. The following conclusions can be drawn :

1. The addition of calcium to bacteria infected with phage in its absence causes a burst to take place whether it is added early or after 60 minutes.

2. The burst size decreases the longer calcium is withheld. In tube A it was 254, in B 240, in C 190, and in D 167.

A 10-fold increase in phage titre occurred in the calcium-free culture (E) between 90 and 120 minutes. This was probably due to the magnesium in the medium.

3. There is no appreciable alteration in the time between the addition of calcium and the beginning of the burst whether calcium is added early or late.

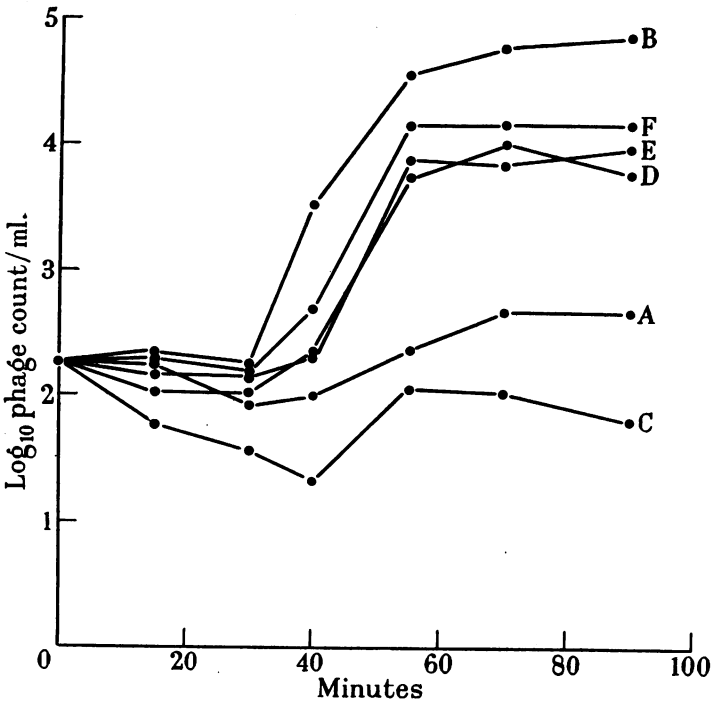

FIG. 3.—The removal of calcium with citrate. Effect on the burst size.
A = No calcium. B = Calcium all the time. C = Calcium plus citrate all the time. D = Citrate added after 1 minute, E after 5 minutes, F after 10 minutes.

The Removal of Calcium from Phage-infected Cultures.

It was technically impracticable to centrifuge and wash the bacteria infected in the presence of calcium so other ways of removing calcium had to be found. Two ways were tried. Both depended on reducing the concentration of calcium ions to a level below which they are inactive. This is about 0·00002 M. Therefore cultures could be made virtually calcium-free by dilution with calcium-free medium. The second method depended on the formation of a unionised complex between calcium and sodium citrate. If sufficient citrate was added the concentration of calcium in the medium could be reduced to below the level of activity.

It was, however, important that the citrate should not affect the multiplication of the bacteria. On being tested it was found that 0·01 M sodium citrate could be added to the medium and yet not measurably affect the bacterial growth rate. This amount was therefore used in the following experiments.

The removal of calcium with citrate.—Infected bacteria were prepared by mixing bacteria at 4×10^8/ml. with phage at 2×10^6/ml., incubating for 10 minutes, centrifuging down the cells and re-suspending in fresh medium. This suspension was diluted 1:100 in fresh medium and 1 ml. of this was added to each of 6 L tubes containing 9 ml. of medium. Five of these tubes already contained calcium 0·0005 M, while one (A) was kept as a calcium-free control. To one (C) of the 5 containing calcium 0·01 M citrate had already been added, and to 3 others (D, E

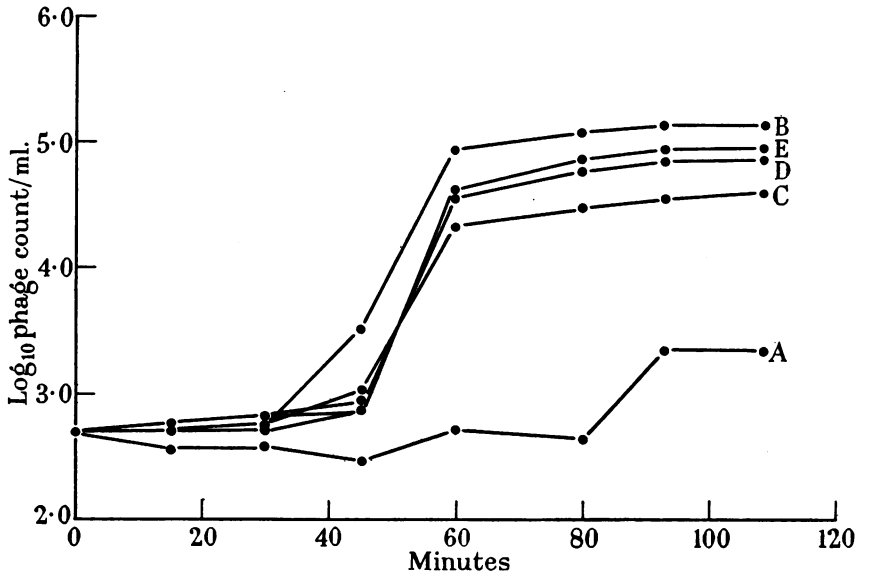

FIG. 4.—The removal of calcium by dilution. Effect on the burst size.
A = No calcium. B = Calcium all the time. C = Calcium diluted out after 1 minute, D after 5 minutes, E after 20 minutes.

and F) citrate was added 1, 5 and 10 minutes later. The remaining tube (B) was used as a calcium control with no citrate. Samples were taken at intervals for phage counts. The results are given in Fig. 3.

In the presence of 0·01 M citrate, 0·0005 M calcium is unable to cause a large burst. In fact the burst it does give (3–4) is less than that given by the standard medium (6). If, however, the infected cells are allowed contact with sufficient calcium for only one minute a burst size of 50 is given. Five minutes' contact with calcium produced about the same burst size and 10 minutes gave one of 60. The control which had calcium all the time gave a burst size of 410.

The removal of calcium by dilution.—Infected bacteria were prepared by mixing bacteria at 4×10^8/ml. with an equal number of phage particles. After incubating for 10 minutes the cells were centrifuged down and re-suspended in fresh medium. A sample of this was diluted 1 in 10^5 with medium containing 5×10^{-9} M

added calcium as a control (Tube A). (This would be the amount left in the medium after making the dilutions required in this experiment.) The re-suspended culture was then incubated with 0·0005 M calcium. After 1, 5 and 20 minutes samples were taken, diluted 1:100,000 in medium without calcium, and again incubated (Tubes C, D and E). Phage titres were determined at intervals in the diluted cultures and the undiluted one (Tube B). The results are given in Fig. 4. They are similar to those obtained by the citrate method in that as little as one minute's exposure to calcium permits a considerable burst (93) to take place. The longer the calcium is allowed to act, the greater is the burst size.

DISCUSSION.

The cells of *Bact. coli* B when infected with phage T5 can produce between two and four hundred new phage particles provided there are sufficient divalent metal ions in the medium. In the absence of added metal ions the burst size is reduced to a lower limit of between 2 and 10, which may well be due to metal impurities in the reagents. Three metals, calcium, magnesium and strontium, are active for the T5 phage though not equally so. Their different activities and the inability of other divalent ions such as manganese and iron to support phage production may be explained in terms of their ionic dimensions and electronic configurations. The ionic radii of magnesium, calcium and strontium are 0·78 Å, 0·98 Å and 1·15 Å (Glasstone, 1940). However, the ionic radii of iron, manganese, cobalt and zinc lie within the limits 0·78 Å and 1·15 Å, yet these metals are not active. The electronic configurations of magnesium, calcium and strontium are similar in that they all have two valency electrons and no partially filled inner electron sub-groups. In contrast the electronic configurations of the inactive metals are markedly different except for beryllium and barium, which have a similar type of configuration to calcium, magnesium and strontium. In the case of beryllium and barium lack of activity is presumably due to the size of the ions, beryllium (0·34 Å radius) being too small and barium (1·37 Å radius) too large to combine effectively with the appropriate parts of the bacterium or phage.

Thus for a metal to " catalyse " the production of phage, its ions must have both a particular electronic configuration and dimensions lying between certain limits.

The overall process of phage multiplication is assumed to take place in several stages. These are adsorption of the phage particle by the bacterium, multiplication of the phage within the cell and lysis of the cell, resulting in the liberation of the new particles into the culture medium.

For the following reasons it is considered that the rôle of calcium is in the intracellular multiplication of the phage. Calcium is not involved in adsorption, which proceeds equally well in its absence as in its presence. However, the burst size depends on the concentration of calcium (or magnesium or strontium) in the medium over a considerable range. This is in accordance with the view that the metals are involved in the synthesis of phage. When calcium is removed from the medium before the latent period is completed the burst size is diminished. This means that calcium must be available throughout the latent period for the maximum burst to take place, and again supports the view that the calcium is involved in phage synthesis. If calcium were involved solely in lysis and not in

phage synthesis it would be reasonable to expect that when calcium is added to cultures incubated for some time without it the burst size would be greater, phage synthesis having continued for longer without interruption by lysis ; and the period between addition of calcium and the burst shorter than if calcium had been added at the earliest moment. In fact, however, the burst size decreases, and the period between addition of phage and the burst is not significantly altered. The reduction in the burst size given by infected cultures deprived of calcium for some time may be accounted for by assuming that the phage-bacterium complex is unstable in the absence of calcium when no synthesis of phage can take place. These results and conclusions are in many ways similar to those of Fildes (personal communication) working with a typhoid-phage system which also required calcium for phage production.

SUMMARY.

Calcium, magnesium and strontium are active in causing lysis and phage production in the system *Bact. coli* B—T5st. Barium, beryllium, cobalt, copper, iron, manganese and zinc are inactive.

The relationships between ionic size, electronic configuration and biological activity are discussed.

The relative activities of calcium, magnesium and strontium have been investigated. Calcium is the most effective, causing maximal phage production at 0·0005 M.

Bacteria infected with phage without calcium can be caused to produce phage by the later addition of calcium. The burst size is reduced the later calcium is added, but the latent period after addition of calcium is not significantly altered.

Calcium may be removed almost completely from the culture medium by dilution or treatment with citrate without preventing the multiplication of phage even if the complex has had access to calcium for only one minute. The burst size is, however, reduced the shorter the exposure to calcium, and it is considered the calcium must be present in the medium throughout the major part of the latent period if the maximum burst size is to be obtained.

The author wishes to thank Sir Paul Fildes, F.R.S., the Director of the Unit, for his interest in the work and his valuable criticism of the manuscript.

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