THE CALCIUM REQUIREMENT OF A TYPHOID BACTERIOPHAGE.

D. KAY* AND PAUL FILDES.

From the British Empire Cancer Campaign Unit, School of Pathology, Oxford.

Received for publication March 25, 1950.

IN the course of work with a strain of *Bact. typhosum* and its phage it was found that normal lysis and free phage production took place in peptone media, but that the results on a simple ammonia medium were entirely unpredictable. Lysis detectable by the eye or even by a Hilger "Spekker" absorptiometer seldom occurred and production of free phage was usually poor. This was all the more curious because the phage plated on the synthetic medium *plus* agar produced plaques almost as well as on peptone agar.

If in the course of growth in the ammonia medium traces of peptone were added, visible lysis set in rapidly and therefore peptone was fractionated to find the active factor in it. The first step was a 66 per cent alcohol precipitation. One fraction contained inorganic material which was active in traces. This contained Ca, Fe and Cu among other elements and, these being tested, Ca was found to be the factor responsible. Calcium chloride was then used in the ammonia cultures and always produced lysis within an hour or two of its addition at any point in the log phase of growth.

This paper is a study of this matter.

PREVIOUS WORK.

Calcium has been implicated, directly or indirectly, as a factor in phage growth for a number of years.

It was found by Stassano and de Beaufort (1925) that citrate inhibited phage growth. This citrate effect was confirmed by Andrewes and Elford (1932). In neither of these papers, however, were experiments carried out directly on the effect of calcium.

Bordet and Renaux (1928), working with Shiga phage, found that if broth was "highly alkalinized" in order to remove the phosphate precipitate and then used for the growth of phage, no lysis took place. But lysis followed the addition of one drop of 1 per cent $CaCl_2$ to 5 ml. culture if this was added before the culture had progressed too far. Further, normal broth could be made deficient for phage growth by adding 1 : 1000 oxalate. Other phages tested were not inhibited by oxalate, but, in view of the fact that oxalate does not remove all calcium from solution, they thought it possible that all phages might require calcium in varying concentrations.

Wahl (1946) and Wahl and Blum-Emerique (1949) have described two phages which required calcium, but this was only effective in a narrow zone of concentrations.

* Medical Research Council Research Student.

Delbruck (1948) found calcium to be a necessary component in the adequate adsorption of phage T_4 on Coli B, in the sense that the particles adsorbed were increased in number five times by addition of 4 μg./ml. $CaCl_2$.

Puck (1949) has shown that a Coli phage can be " sensitized " by dilution in distilled water or dilute buffer, but not if calcium is present. These sensitized phages are inactivated when adsorbed by bacteria, and form no plaques when plated.

Adams working with the T5 phage and Coli B also found that lysis and phage production did not occur in an ammonia medium in the absence of added calcium.

TECHNICAL DETAILS.

Host bacterium.—All work has been carried out on a strain of *Bact. typhosum* selected from No. 3390 N.C.T.C.

Typhoid phage.—This was derived from a phage obtained from Brigadier J. S. K. Boyd (Wellcome Laboratories) active on No. 3390 N.C.T.C. The actual phage preparation used in this work were not lysates in the ordinary sense, because lysis did not take place. They were supernatants of cultures of bacteria and phage made in the culture medium described below. They were heated at 58° for forty minutes to destroy phage-resistant strains.

The initial material was obtained by plaque selection from platings on single-colony isolations of the bacterium. The plaques produced are small.

Culture medium.—All work has been carried out on the following medium.

KH_2PO_4	9·0 g.
Ammonium chloride	·5 g.
Ammonium sulphate	·5 g.
Anhydrous magnesium sulphate	·01 g.
N/1 NaOH	ca. 63 ml.
Double distilled water	to 500 ml.

This was autoclaved in 100 c.c. lots at pH 7·6 in eight-ounce " medical flat " bottles. For use this mixture was diluted with water 1/2 and 8 ml. 11·25 per cent glucose (autoclaved in water) added per 200 ml.

A solid medium was made by diluting with 3 per cent Difco agar in place of water.

Culture technique.—Erlenmeyer flasks were used for large quantities, the amount of culture being 1/10 of the flask volume. The flasks were shaken in the hot room.

For smaller, 10 to 15 ml., experimental volumes the T-shaped test tube of Monod was used. This was rocked mechanically in a water bath.

The vessel used by Dr. J. Monod (Institut Pasteur) is an inverted T-shaped apparatus with the opening at the top and the culture in the horizontal arms to facilitate aeration when the tube is rocked in the water bath. It is square in section and optically worked, so that the opacity of the culture can be measured directly in the absorptiometer. Dr. W. E. van Heyningen (School of Pathology, Oxford) has modified the vessel so that it is made of Pyrex glass, circular in section, and this modification has been used in the present work. We are indebted to Dr. Monod and Dr. van Heyningen for this unpublished information.

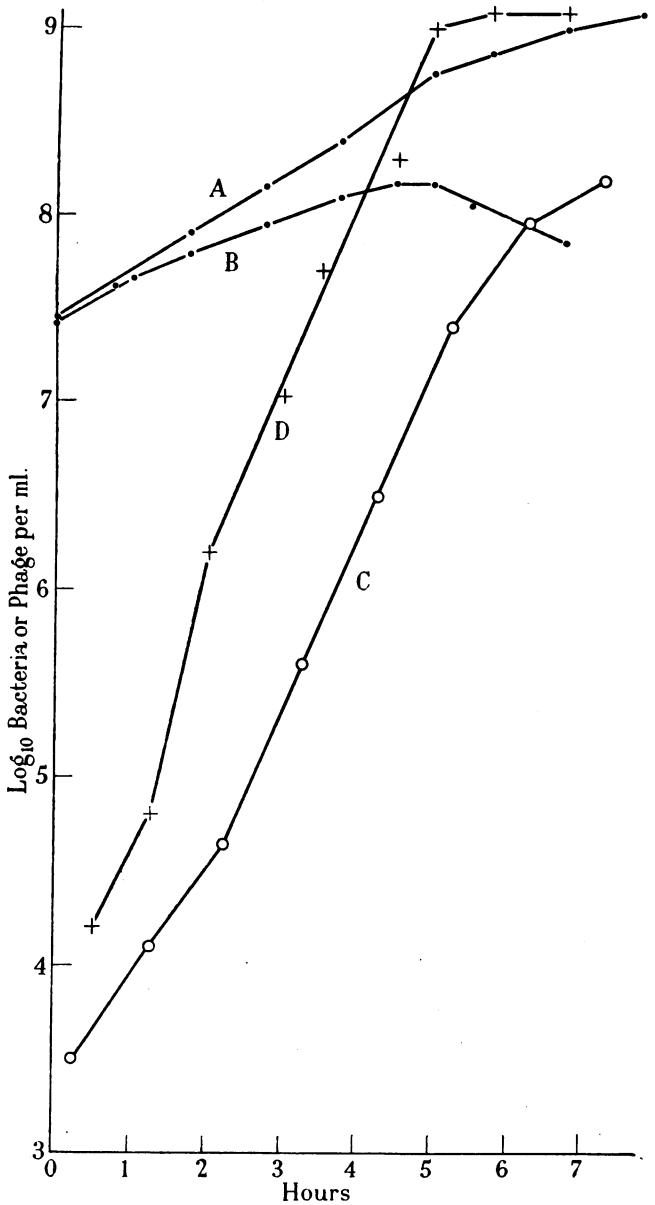

FIG. 1.—Effect of calcium on lysis and phage production. A = Bacterial growth without added calcium, but with added phage; C = phage production in the same whole culture; B = bacterial growth with added calcium (M/3500) and phage showing lysis; D = phage production in the same whole culture.

Estimations of bacteria.—All bacterial estimations were made by opacity, using Hilger's " Spekker " absorptiometer, so modified that the Monod's tubes could be used in place of cells. The reading from the calibrated tube could then be referred to bacteria per ml. by means of standard curves. In an uncomplicated growth experiment this method gave results which closely agreed with bacterial counts, but, of course, during phage lysis, for instance, it did not follow that the opacity reading corresponded to viable bacteria per ml.

Estimation of phage.—Dilutions of phage were always made in the culture medium. Aliquots were plated by the two-layer method, using an agar (2 per cent) containing 1 per cent Evans' peptone and M/40 glucose for the bottom layer. The top layer was prepared by mixing 0·5 c.c. phage dilution with 0·5 c.c. bacterial suspension (ca. 5×10^8/ml.) and after one minute adding 2 c.c. 1·5 per cent glucose-peptone agar. The whole was then poured onto the bottom layer.

The dilutions of phage, made in the culture medium, were arranged to give about five hundred plaques per plate and two plates were used for each dilution.

EXPERIMENTAL.

Effect of Calcium on Lysis and Phage Production.

Fig. 1 shows (Curve A) that the generation time of the bacterium in the medium containing phage but no added calcium was 72 minutes, which does not differ from a culture without phage. The growth rate of the culture (Curve B) with both phage and calcium added soon became reduced and at 4½ hours lysis became visible. The production of phage in the whole culture was more rapid with calcium and a higher yield was obtained. At 5 to 6 hours phage production fell off in the culture without calcium because the log phase of bacterial growth had come to an end.

It is clear that visible lysis can only take place when most of the bacteria in a culture are infected, i.e. when the phage titre approaches or exceeds the bacterial count. When calcium is not present the phage titre (Curve C) has only attained about 1/10 of the bacterial count (Curve A) before the culture ceases, and this is sufficient explanation for the absence of visible lysis.

The Effect of Substances which React with Calcium.

Fig. 1 indicates that calcium added to a culture has a marked stimulatory action on lysis and phage production and suggests the possibility that phage produced in the absence of added calcium is due to calcium as an impurity in the medium. Experiments were therefore carried out with substances which might be expected to reduce the number of available calcium ions.

Phosphate.

Wahl and Blum-Emerique (1949) have shown that a certain Coli phage, S13, would not multiply on their ordinary synthetic medium, which contained M/10 buffer even when calcium was present, but they found multiplication when the buffer concentration was greatly reduced. For instance, in the presence of a " très faible " concentration of $CaCl_2$ and M/215 buffer (0·442 g. PO_4/litre) the initial titre of phage was 1×10^5 and the final titre $3{\cdot}5 \times 10^5$/ml. With M/270 buffer, the final titre was 2×10^7; with M/540, $1{\cdot}5 \times 10^8$, and with M/1610, $4{\cdot}5 \times 10^8$.

We have tested the effect of phosphate concentration in the medium without and with added calcium. It was not practicable to reduce the phosphate greatly because then complication arose from changes in pH. Our ordinary medium contained M/15 phosphate and this was tested against the same medium containing, however, M/30 phosphate. In both media the bacteria grew at the same rate. When the cultures had reached 1×10^8/ml., phage was added to an aliquot of each to make 1×10^5 phage particles/ml. As usual no lysis was observed in the M/15 buffer tube during the period of growth, but in the M/30 buffer tube

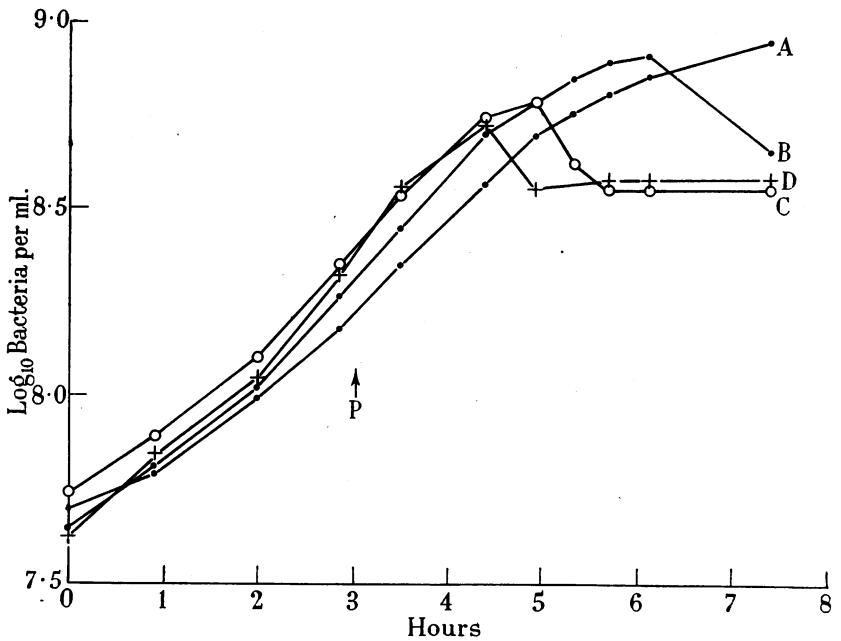

FIG. 2.—The effect of phosphate concentration on lysis. A = M/10 phosphate; B = M/15 phosphate; C = M/30 phosphate; D = M/75 phosphate. Phage added at P.

lysis started three hours after addition of phage. At 4½ hours after addition of phage, the partially lysed tube, when plated, gave $1{\cdot}1 \times 10^9$ plaques/ml. in the whole culture and $7{\cdot}6 \times 10^8$ in the supernatant. On the other hand, the M/15 buffer tube gave at the same time $5{\cdot}8 \times 10^8$/ml. in the whole culture and $2{\cdot}9 \times 10^8$ in the supernatant. Thus reduction in phosphate caused a two-fold increase in phage production and a larger proportion free in the culture.

The effect of phosphate was also tested quantitatively in the presence of added calcium (M/4000).

Fig. 2 shows that lysis did not occur with M/10 phosphate (Curve A), even though calcium was added but that on reduction of phosphate lysis set in progressively sooner.

The results of these experiments accord with the view that available calcium ions can be reduced by increasing the phosphate concentration and so reducing phage production and lysis.

Oxalate.

The action of oxalate on phage production was studied in two ways.

(*a*) The effect on lysis induced by added calcium.

(*b*) The effect on phage production in the absence of added calcium.

As a preliminary it was found that concentrations of oxalate as high as M/100 had no effect on the bacterial growth curve, except in so far as the higher concentrations caused precipitates in the presence of added calcium. These caused the opacity curve to stand at a higher level, but did not interfere with the slope of the curve.

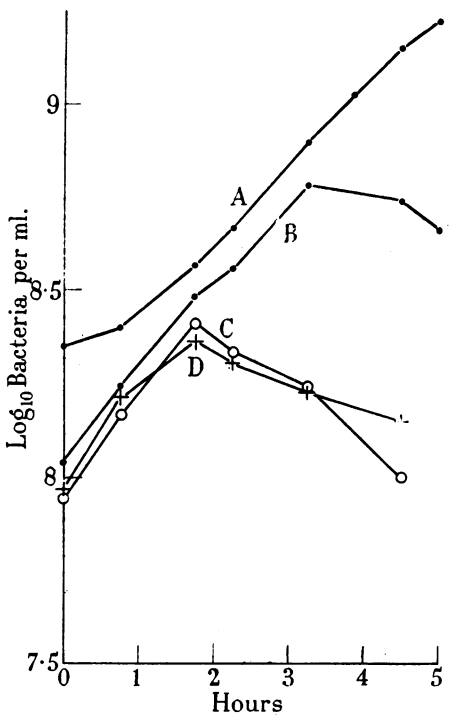

FIG. 3.—The effect of oxalate on phage lysis induced by calcium (M/3500). A = Bacterial growth (plus oxalate precipitate) with M/100 oxalate; B = bacterial growth with M/200 oxalate; C = bacterial growth with M/400 oxalate; D = bacterial growth control without oxalate.

Four tubes were set up (Fig. 3) containing 10^8 bacteria, 2×10^4 phage/ml., and M/3500 calcium. One tube (D) contained no oxalate and the others, three concentrations of oxalate. It is seen that M/400 oxalate had little effect, but that M/200 caused a marked delay in lysis, while M/100 did not lyse. The effect of oxalate is therefore to delay the lysis induced by calcium, even though no precipitate is formed. In the high concentrations visible lysis is abolished.

The effect of oxalate on phage production was shown by diluting phage in the medium containing no added calcium, but different concentrations of oxalate, namely, M/420, M/240 and M/120. A control was set up without oxalate. The cultures were then incubated, the bacterial growth recorded and samples plated

for phage concentration from time to time. The bacterial growth curves with or without oxalate were practically identical, starting at 1×10^8. The initial phage concentration was approximately 1×10^4. Without oxalate there was the normal continual increase similar to that shown in Fig. 1c. With M/240 oxalate there was a marked delay before increase started ; with M/420 there was even a slight fall in the phage content followed by an increase. The rate of increase did not much differ from that with M/420, nor even from the control. With, however, M/120 oxalate there was a heavy fall in concentration of phage. This also was followed by an increase, but this was much slower.

Fig. 4 shows the effect of another experiment with M/100 oxalate in detail. The initial titre of phage was $1{\cdot}5 \times 10^4$/ml. In two hours this fell to 1/10 and

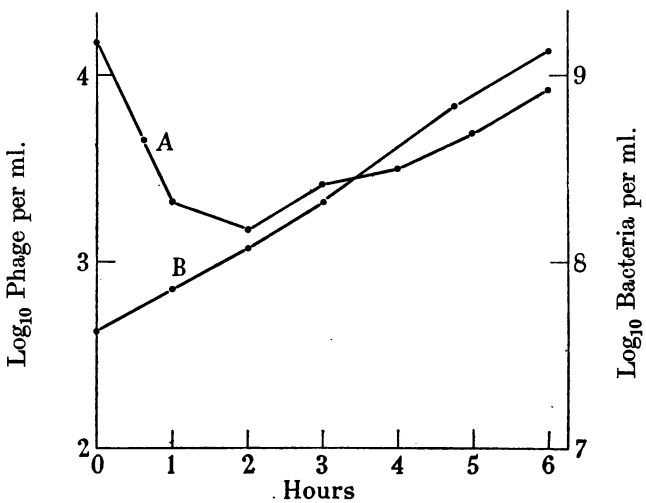

FIG. 4.—The effect of oxalate (M/100) on phage production without added calcium. At the start there were $4{\cdot}3 \times 10^7$ bacteria and $1{\cdot}5 \times 10^4$ phage. A = Phage production ; B = bacterial growth.

then an increase set in at a rate which is some five times slower than without oxalate.

These experiments show that phage is still produced in the presence of oxalate, though more slowly especially in the higher concentrations. Phage lysis induced by calcium is also delayed by higher concentrations of oxalate and eventually abolished. Addition of oxalate thus produces the same effect as reduction in calcium, namely, a fall in phage production and abolition of lysis.

Attention is called to the initial fall in phage titre shown in Fig. 4. It is clear that in the presence of bacteria and M/100 oxalate 90 per cent of the phage particles are rapidly inactivated. This matter will be referred to later.

THE PRESENCE OF CALCIUM IN CULTURE MEDIA.

The experiments described suggest that phage production by the system in use is absolutely dependent upon the presence of free calcium ions. Failure to

show a conclusive result seemed likely to be due to undisclosed calcium ions in the culture medium.

The following experiments were therefore carried out:

The medium freshly prepared from " Analytical Reagent " chemicals and double distilled water did not give a reaction for calcium by the oxalate test either at its normal strength or after a ten-fold concentration by evaporation. After autoclaving in eight-ounce " medical flat " bottles no reaction was given, but a ten-fold concentration showed calcium to be present in some samples. In view of this, a bottle of medium was autoclaved for twelve hours at 15 lb./sq. in.

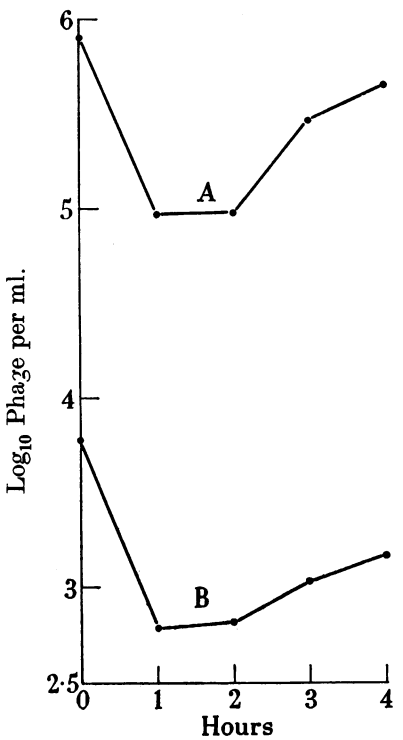

FIG. 5.—Production of phage in " calcium-free " medium. A = Phage production from an initial concentration of $7{\cdot}9 \times 10^6$ phage/ml.; B = phage production from an initial concentration of $5{\cdot}8 \times 10^3$ phage/ml.

and tested after concentration. A strong positive reaction was given. Calcium was also found in the cotton-wool used to plug the bottles. It appeared that calcium could be introduced into the medium from cotton-wool, fibres of which fall into the medium, and from the bottles during autoclaving. The medium was therefore prepared and sterilized in " Pyrex " flasks fitted with beakers instead of plugs. Further, the N/1 soda used for neutralizing was kept in paraffined bottles. After 24 hours' autoclaving no calcium was detectable in the medium thus prepared.

The method used by Bordet and Renaux (1928) to decalcify a culture was also tested.

A batch of the ammonia medium made in soft glass was divided into two. One part was kept as a normal control and the other was adjusted red to phenolphthalein and warmed to about 90°. A small precipitate was filtered off, the pH readjusted to 7·6 and the medium finished. These two batches of medium were then used to test inactivation of phage. Phage diluted in each medium

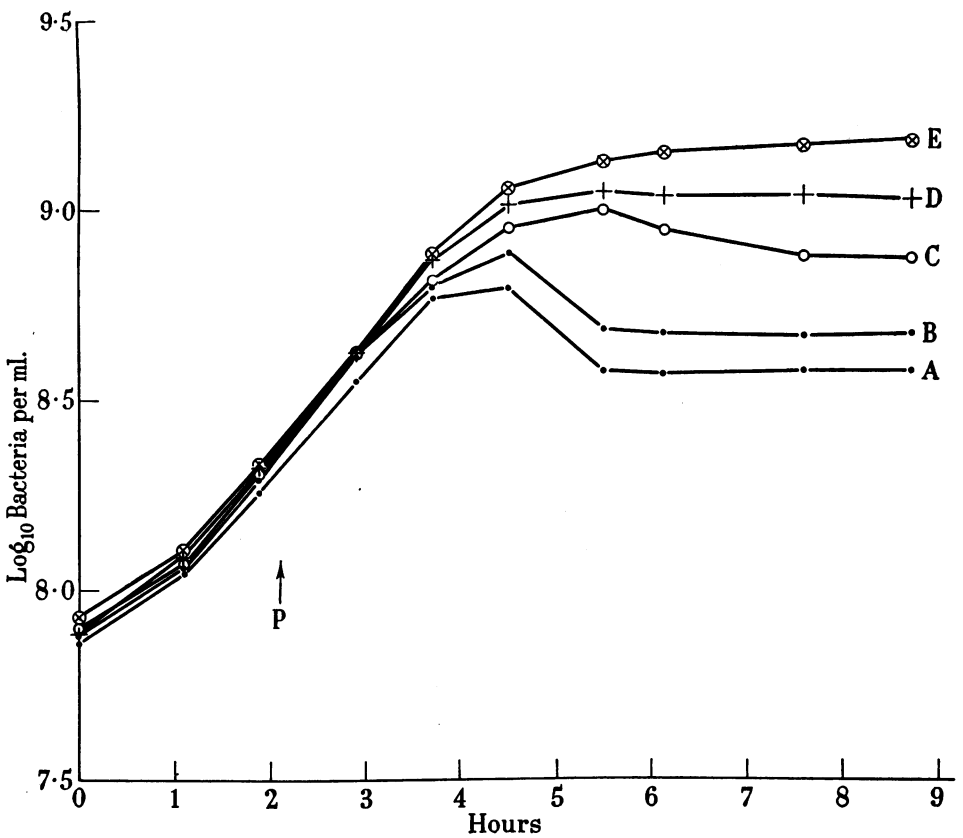

FIG. 6.—The effect of calcium concentration on lysis. A = Calcium M/2100; B = calcium M/4200; C = calcium M/10,500; D = calcium M/21,000; E = no calcium. Phage added at point P.

was mixed with bacteria grown in each medium and after one and two hours plated for active phage in the whole culture.

In the medium made with soft glass, the plaques rose as usual from $1{\cdot}7 \times 10^6$ to $3{\cdot}3 \times 10^6$ in one hour, and to $1{\cdot}7 \times 10^7$ in two hours. In the decalcified medium the plaques fell from $1{\cdot}6 \times 10^6$ to $4{\cdot}7 \times 10^5$ in one hour and then rose to $4{\cdot}6 \times 10^6$.

Thus a process which is known to remove calcium, transforms an adequate medium to the same condition as one which contains oxalate.

Experiments on "Calcium-free" Media.

Experiments were now conducted in which all glassware used was "Pyrex," and cotton-wool was eliminated as far as possible. Otherwise the materials and conditions were precisely the same.

This alteration had no effect on the rate of growth of the bacterium in use.

Fig. 5 shows the rate of phage production in the whole culture under these conditions. It is seen that the curves resemble closely that obtained with oxalate in the normal medium (Fig. 4). There was an initial heavy fall in phage content, and a slow and small increase in phage later. This small increase may be compared with the increase shown in Fig. 1, Curve C. The only difference between the experiments was the avoidance of known sources of calcium in Fig. 5.

Nevertheless, the fact that phage growth took place at all indicates, in our view, that calcium ions were still present. This, however, is inevitable under present conditions because our phage itself, although produced without added calcium, was made in part in soft glassware and can only be produced in this way, or by adding calcium. In fact the curves in Fig. 5 indicate that the concentration of phage lysate affects the result. In Curve A the rate of phage growth is faster and the number of calcium ions added with the phage is one hundred and thirty-six times greater than in Curve B.

The concentration of calcium necessary to produce lysis was tested in the medium prepared in hard glass. Fig. 6 indicates that the effect of calcium is quantitative. It is most marked at M/2100, which is the highest concentration which can be used without producing a precipitate, and it is least marked in the lowest concentration. There is no evidence of an optimum concentration, above or below which the effect is less, as recorded by Wahl and Blum-Emerique (1949).

Inactivation of Phage by Bacteria in the Absence of Calcium.

Attention has been called to the inactivation of phage by bacteria in the presence of oxalate (Fig. 4) after removal of calcium phosphate or in the absence of calcium (Fig. 5).

Puck (1949) has shown that T 1 phage diluted with distilled water or with dilute buffer becomes "sensitized" in the sense that it is inactivated when brought into contact with *E. coli* B and forms no plaques when plated. This sensitization is prevented by the presence in the diluting fluid of $CaCl_2$ M/5000 or M/10,000, but not by M/100,000. The phenomenon noted by us, thus resembles closely that found by Puck.

Our experiments suggest that if, under the conditions of the test, phage is exposed to bacteria in the absence of calcium it is permanently inactivated, while if it is exposed in the presence of calcium it is not. This being so, it is clear that the phage associated with bacteria in a "calcium-free" medium and relying only on traces of calcium carried over with the lysate, cannot multiply effectively. A large fraction of each burst will be inactivated in the absence of adequate calcium and the overall increase of active phage will be little or none.

CONCLUSIONS.

A particular typhoid phage is permanently inactivated on exposure to bacteria in the absence of calcium.

Thus it requires calcium for continued propagation under the conditions imposed.

Practically all the phage estimations in this work have been carried out by Miss Audrey Hearle. We have pleasure in thanking her for this and other assistance.

REFERENCES.

ADAMS, M. H.—(1949) *J. Immunol.*, **62**, 505.
ANDREWES, C. H., AND ELFORD, W. J.—(1932) *Brit. J. exp. Path.*, **13**, 13.
BORDET, J., AND RENAUX, E.—(1928) *Ann. Inst. Pasteur*, **42**, 1283.
DELBRUCK, M.—(1948) *J. Bact.*, **56**, 1.
PUCK, T. T.—(1949) *Ibid.*, **57**, 647.
STASSANO, H., AND DE BEAUFORT, A. C.—(1925) *C. R. Soc. Biol., Paris*, **93**, 1380.
WAHL, R.—(1946) *Ann. Inst. Pasteur*, **72**, 73.
Idem AND BLUM-EMERIQUE, L.—(1949) *Ibid.*, **77**, 137.