# THE EFFECT OF DIVALENT METALS ON THE STABILITY OF A TYPHOID BACTERIOPHAGE AND ITS REACTION WITH ITS RECEPTOR

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Received for publication August 7, 1956

DIVALENT metals are implicated in the multiplication of many bacteriophages and appear to be concerned in most of the recognised stages of the growth cycle (Fildes, Kay and Joklik, 1953). Phage 1 of our collection, active on several strains of *Salmonella typhi*, requires calcium and fails to cause lysis unless it is added to the medium (Fildes, 1954). In the course of experiments on the reaction of this phage with its isolated receptor (Kay, 1955) it was noted that magnesium or calcium were needed to maintain the activity of the phage when suspended in phosphate buffer. Failure to add magnesium or calcium resulted in the complete loss of the phage within a few minutes at 37°. In the present paper the mechanism of this inactivation is examined and its relation to the reaction between the phage and its receptor is discussed.

## MATERIALS AND METHODS

### *Bacteria*

*Salm. typhi* R4 was used to assay the phage. *Salm. typhi* O 901 R was used to prepare the phage receptor. Both these organisms were described in a previous paper (Kay, 1955).

### *Bacteriophage*

Phage 1 was grown and purified by differential centrifugation as described previously (Kay, 1955). It was usually suspended in phosphate buffer pH 7·6 (0·067 M) containing magnesium (0·002 M) and electro-dialysed gelatin (0·02 per cent) but when wanted in collidine buffer it was centrifuged again, washed in collidine buffer and re-suspended in the same diluent. Radioactive phage was made by growing the phage in medium containing $^{32}P$ (2 μc./ml.) as orthophosphate. Phage was assayed by the two-layer method using R4 as the background organism.

$^{32}P$ was assayed in 5 ml. samples which were diluted to 10 ml. with water and counted in a 10-ml. liquid counter tube.

### *Collidine buffer*

This consisted of collidine, re-distilled and kept under nitrogen, 0·05 M solution in water adjusted to pH 7·4 with acetic acid (Analar grade). I am indebted to Dr. G. G. F. Newton of this department for the details of this buffer. It has two properties which were of use in the present work. It is completely volatile and is therefore useful in electron microscopy and unlike phosphate buffer it does not form unionised complexes with calcium and magnesium.

### *Electron microscopy*

Phage suspensions in collidine buffer were suitably diluted in the same buffer, placed on thin carbon films and allowed to dry in air. Aqueous suspensions of the phage could not be used as this procedure appeared to damage the particles by separation of the " head " and

" tail " parts. Nor could non-volatile buffers be used since it proved impossible to dialyse the salts away from the films without causing an apparent rupture of the phage " heads ". The dried specimens were shadowed with a gold-palladium alloy.

*Phage receptor*

This was isolated from *Salm. typhi* O 901 R by the hot water extraction procedure and purified as described previously (Kay, 1955). It was used as a suspension in water and diluted appropriately in water before use.

## EXPERIMENTAL

### *Inactivation of Phage* 1 *and its Prevention by Various Cations*

Tubes containing phosphate buffer pH 7·6 (0·033 M) and Ca-free gelatin (0·01 per cent) supplemented with a range of different cations at 0·0001 M (as chlorides or sulphates) were inoculated with phage 1 at $8 \times 10^3$/ml. and kept at 37°. After 40 min. each tube was assayed for phage remaining active. It was found that all phage had disappeared from the tubes containing buffer alone, beryllium, manganese, zinc, copper and ferric iron. All the phage had survived in the calcium tube, 70 per cent in strontium, 36 per cent in barium and 16 per cent in magnesium. Later experiments with magnesium showed that concentrations of 0·002 M or more completely protected the phage for 1 hr. at 37°. Attempts to recover the lost phage by addition of calcium were unsuccessful. It was at first thought that the phage had been lost by adsorption to the walls of the vessel but this was shown to be unlikely when incubation of the phage in paraffin wax-coated tubes and cellulose nitrate tubes also caused inactivation unless calcium was present. The possibility that the phage particles were suffering physical damage was next examined. The release of nucleic acid from inactivated phage was looked for and morphological changes in the phage particles were examined in the electron microscope.

### *Release of Nucleic Acid*

Phage 1 ($3 \times 10^{11}$/ml.) labelled with $^{32}P$ was diluted 1/20 with phosphate buffer (0·067 M) to reduce the magnesium concentration to 0·0001 M. It was then added to each of two tubes containing buffer (final concentration 0·033 M) one of which also contained calcium (0·0001 M). The residual magnesium, 0·00001 M was too small to prevent inactivation of the phage. The initial phage titre, the total $^{32}P$ and the unsedimentable $^{32}P$ after centrifuging at 20,000 r.p.m. were all determined on samples from the calcium tube. After keeping the tubes at 37° for 1 hr. the phage titres and unsedimentable $^{32}P$ were determined in both. The phage titres obtained and radioactivity measurements after correction for background are given in Table I.

TABLE I.—*Inactivation of Phage* 1 *and Release of* $^{32}P$ *after* 1 *hr. at* 37° *in Phosphate Buffer*

| | Phage titre/ml. or $^{32}P$ counts/min./5 ml. Sample tested. | |
|---|---|---|
| | Calcium 0·0001 M. | No calcium. |
| Initial phage | $1·5 \times 10^9$ | — |
| Final phage | $1·3 \times 10^9$ | $9 \times 10^4$ |
| Total $^{32}P$ | 244 | — |
| Initial unsedimentable $^{32}P$ | 71 | — |
| Final unsedimentable $^{32}P$ | 58 | 163 |

The figures show that inactivation of the phage (99·99 per cent) is accompanied by an increase in the amount of $^{32}P$ which is unsedimentable at 20,000 r.p.m. and therefore no longer part of the phage particles. After allowing for the initially unsedimentable $^{32}P$ (71 counts/min.) this amounts to 53 per cent of the $^{32}P$ which was initially sedimentable and which was part of the phage particles. The phage in calcium is not inactivated and does not show any release of $^{32}P$. In another experiment 59 per cent of the $^{32}P$ became unsedimentable after inactivation of the phage. The U.V. absorption spectra of the sedimentable and unsedimentable fractions of the phage were examined and it was found that the latter had a marked absorption maximum at 260 m$\mu$ whereas the former showed none. The properties of the unsedimentable fraction were not examined further but from its phosphorus content and its absorption at 260 m$\mu$ it was assumed to be phage nucleic acid or its degradation products. It is considered therefore that phage 1 is inactivated in phosphate buffer by the loss of part at least of its nucleic acid.

*Electron Microscopy of Phage* 1 *and its Inactivation Products*

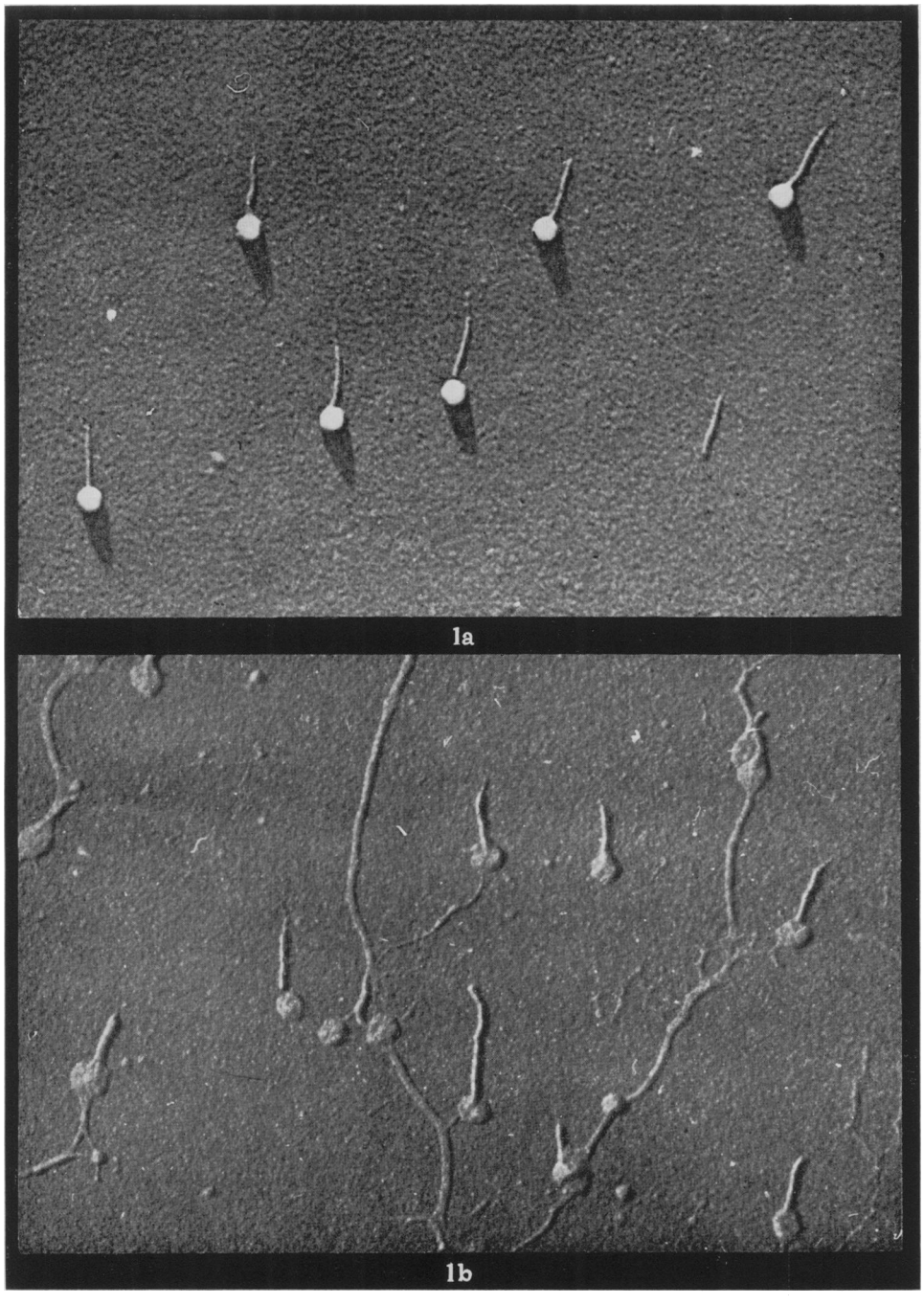
Phage 1 was examined in the electron microscope to determine its morphology in the active state and to observe any change that might occur during inactivation. The best pictures were obtained with the phage suspended in collidine buffer which evaporates from the films used in the microscope without leaving any solid matter. Fig. 1a shows the appearance of phage 1 particles. The " heads " are spherical, 80 m$\mu$ diameter, and the tails are 220 m$\mu$ long.

Attempts to inactivate the phage in collidine buffer at 37° were unsuccessful as will be described later, and it proved necessary to treat the phage with one of the calcium complexing agents, citrate, phosphate or " versene " (ethylenediamine tetra-acetic acid). Versene was chosen as the most suitable agent to inactivate the phage since it was the most active and would leave the least residue on the films. The addition of versene (sodium salt pH 7·6) at 0·0004 M to phage 1 in collidine buffer results in extremely rapid inactivation even at room temperature. The loss in activity is accompanied by the disappearance of the bluish turbidity of the phage suspension. The inactivated particles have an entirely different appearance from the active ones as is shown in Fig. 1b. The " heads " are now flattened or deflated as evidenced by the lack of shadow. Long filaments are seen in every field examined. Phage particles inactivated in phosphate buffer show a similar appearance to those inactivated by versene but the photographs were of poorer quality due to the phosphate on the film. Attempts to wash the inactivated phage free of phosphate reduced the particles to unrecognisable fragments. It is concluded that inactivation of the phage is accompanied by damage to its structure involving the escape of its nucleic acid and the consequent deflation of the head. Although the fine filaments seen in Fig. 1b could be nucleic acid which had been expressed from the tails of the phage particles the micrographs are not clear enough to be certain whether the nucleic acid did come out through the tail or through the damaged head membranes.

EXPLANATION OF PLATE.

FIG. 1a.—Phage 1 prepared in collidine buffer. Air-dried on carbon film and shadowed with gold-palladium. × 62,000.

FIG. 1b.—Phage 1 in collidine buffer inactivated by versene (0·0004 M). Air-dried on carbon film and shadowed with gold-palladium. × 62,000.



1a

1b

*Inactivation of the Phage by Complexing Agents*

The unexpected stability of phage 1 in collidine buffer compared with its instability in phosphate buffer suggested that the agent responsible for the breakdown of the phage was the phosphate. This is shown to be so by the results given in Table II which also includes the effect of citrate and versene on phage 1 in collidine buffer.

The phage ($1{\cdot}8 \times 10^4$/ml.) was kept at 37° in collidine buffer together with the additions mentioned in the Table in glass-stoppered tubes to prevent the evaporation of the collidine acetate which would occur through the normal cotton wool plug. Samples were taken at intervals and assayed for phage after dilution 1/10 in 2 per cent peptone water.

TABLE II.—*Inactivation of Phage* 1 *in Collidine Buffer by Complexing Agents*

| Additions to buffer. | Percentage phage active. 2 min. | 10 min. | 20 min. |
|---|---|---|---|
| Buffer alone | 100 | 100 | 100 |
| Versene 0·0004 M | 0 | 0 | 0 |
| Citrate 0·0004 M | 77 | 62 | 51 |
| Phosphate pH 7·4, 0·025 M | 0 | 0 | 0 |
| ,, 0·025 M + Mg 0·002 M | 100 | 100 | 100 |

Phage 1 is completely inactivated by phosphate at 0·025 M and by versene at 0·0004 M. Citrate at 0·0004 M causes a slower inactivation. Magnesium at 0·002 M is seen to prevent completely the inactivation caused by the phosphate.

*Inactivation of Phage* 1 *by Receptor*

Previous experiments to determine whether phage 1 could react with its receptor isolated from the host bacteria in the absence of calcium were always vitiated by the instability of the phage in the phosphate buffer used. However, with the discovery that phage 1 is stable in collidine buffer this experiment became feasible. Five stoppered tubes were set up containing collidine buffer (plus 0·01 per cent calcium-free gelatin) alone, buffer and calcium (0·005 M), buffer and receptor, and buffer, receptor and two concentrations of calcium (0·005 M and 0·0005 M). Each was inoculated with phage in collidine buffer ($1{\cdot}1 \times 10^4$/ml.) and kept at 37°. Phage was assayed at intervals after dilution 1/10 in 2 per cent peptone water. The results are shown in Fig. 2. This shows that phage 1 is stable in collidine buffer and that it is inactivated by receptor when calcium is absent. An unexpected result is that calcium (0·0005 M) reduces the rate of inactivation and at 0·005 M almost inhibits it. It appears therefore that phage 1 can react with its receptor in the absence of calcium and is thereby inactivated. If, as suggested previously (Kay, 1955), inactivation by isolated receptor corresponds to the infective stage in the phage multiplication cycle in whole bacteria then phage 1 could probably adsorb to and infect its host in the absence of calcium. The actual experiment with growing bacteria has not been done yet because the phosphate normally present in the media used is sufficient to inactivate the phage. The calcium requirement of this phage is probably confined to the function of protecting the phage against the phosphate in the medium.

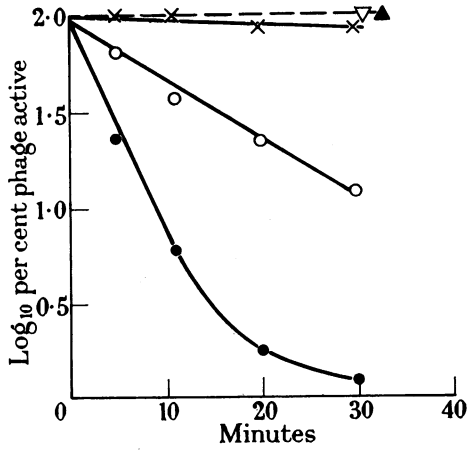



FIG 2.—Inactivation of phage 1 by receptor in collidine buffer at 37°. Effect of calcium.

●——● Receptor alone.
⊙——⊙ Receptor 0·0005 M-Ca.
×——× Receptor 0·005 M-Ca.
- - - -▲ Phage alone 0·005 M-Ca.
- - - -▽ Phage alone. No Ca.

## DISCUSSION

It has been shown previously that phage 1 is caused to release its nucleic acid when it reacts with its isolated receptor (Kay, 1955) and it is now shown to release its nucleic acid also when treated with the calcium and magnesium complex-forming agents: phosphate, citrate and versene. It has been shown that the inactivation of the phage by either phosphate or receptor is prevented by magnesium or calcium. This effect could be accounted for by assuming the phage to be a calcium (or magnesium) complex and the bacterial receptor to have the property like the phosphate, citrate and versene of forming poorly ionised complexes with the calcium. The action of the complexing agent in each case is to remove the calcium from the phage and leave it in an unstable state which readily releases its nucleic acid. The addition of calcium ions to the medium would affect the equilibrium between the stable and unstable forms of the phage in favour of the stable form and so reduce its likelihood of being deprived of calcium by the receptor or the phosphate and consequent inactivation. Adams (1949) working with coli phages noted that the inactivation which took place in the presence of certain concentrations of sodium ion could be prevented by magnesium and proposed a stable magnesium-phage complex in equilibrium with an unstable magnesium-less phage to explain this. However, he did not take into account the effect of the small amount of phosphate present in all his experimental media. This may have been the major cause of phage inactivation as in the present case of the typhoid phage.

The rôle of calcium in the multiplication of phage 1 is now seen to be an indirect one since it is probably confined to preserving the phage against inactivation by one of the normal constituents of the medium, phosphate. In order to prove that calcium is not needed in the multiplication of the phage it would be necessary to grow the host bacteria in a medium containing insufficient phosphate to inactivate the phage. This introduces difficulties in maintaining the pH of

the medium constant since phosphate buffer at 0·05 M is necessary in a culture of R4 growing in a synthetic medium containing glucose. Attempts are being made to overcome this by constantly adjusting the pH in a medium containing minimal phosphate and by using buffer systems other than phosphate.

SUMMARY

A typhoid phage is inactivated by phosphate, citrate and versene (ethylene-diamine tetra-acetic acid) which have the common property of complexing divalent metals. Calcium, magnesium and to a lesser extent strontium and barium protect the phage against inactivation. Inactivation is accompanied by the loss of nucleic acid from the phage and the " deflation " of its " head " as seen in the electron microscope. The isolated phage receptor inactivates the phage in the absence of calcium when examined in collidine buffer in which the phage is stable, but calcium tends to prevent the action of the receptor. It is suggested that the phage is itself a complex of calcium (or magnesium) ions and that the complex formation of these cations with the bacterial receptor causes the phage to become unstable and is the mechanism by which the phage is caused to release its nucleic acid into the host cell.

The author wishes to thank Sir Paul Fildes, F.R.S., Director of the Unit, for his interest and encouragement during the course of the work, and Miss J. Sampson for the electron micrographs.

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