The Effect of Host-Cell Starvation on Virus-induced Lysis by MS2 Bacteriophage

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SUMMARY

Infection of glucose, sulphur or nitrogen starved cells with MS2 virus results in the production of progeny virus but the absence of cell lysis and the failure of progeny virus release. Addition of glucose or sulphur to the correspondingly starved cells results in the normal release of virus within 40 to 60 min. Return of nitrogen to nitrogen-starved cells, however, does not result in the release of virus, even after 1½ h. In experiments with uninfected, starved cells it was found that glucose or sulphur starved cells begin dividing within 45 min after the limiting compound is returned. In contrast, nitrogen-starved cultures still have not begun to divide 1½ h after the return of nitrogen. The correlation between the time it takes for starved, infected cultures to resume lysis after the return of the limiting compound and the time similarly starved, but uninfected, cells normally begin division after addition of the limiting compound supports the hypothesis that lysis by RNA phage is related to cell division and may result at the time of cell division from failure of the cells to divide properly.

INTRODUCTION

The production of progeny RNA bacteriophage generally terminates with the release of these viruses through lysis of the host cell. Since cells which are infected with coat-protein mutants do not lyse, it has been suggested that the virus coat protein is involved in the lysis mechanism (Zinder & Lyons, 1968). There is no evidence, however, that any of the virus-coded proteins has a direct lytic action. It has recently been shown that stationary phase cultures and cultures with long generation times do not release progeny virus properly (Propst-Ricciuti, 1972; Haywood, 1974). Based on these observations, an alternate theory has been proposed in which lysis by RNA phage results from aberrant cell division, which occurs secondarily to changes in the host’s RNA and protein syntheses (Haywood, 1974). A corollary to this second hypothesis is that cells which are not dividing will not be able to lyse. Because of the lack of information about the basis of RNA phage lysis, the following study was undertaken to determine (1) whether starved cultures, where cell division is stopped and RNA and protein synthesis decreased, could produce, but not release, viruses, and (2) whether, if this were the case, the return of the limiting compound would concomitantly restore both cell growth and progeny virus release.

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Bacteria and bacteriophage. *Escherichia coli* 3000, ATCC15597 (Hfr thi- rel-) was obtained from R. L. Sinsheimer. Strain AB1621, ATCC25920 (F thi- mtl- ara- gal- lac- str) was obtained from E. A. Adelberg. Wild type MS2 virus was originally obtained from Alvin Clark. The virus was purified prior to use by the following procedure. MS2 was banded in CsCl by centrifugation for 24 h at 40000 rev/min using a type 65 rotor and a model L2-65B ultracentrifuge. The density of the virus-CsCl solution was 1.385 g/ml. CsCl fractions having high virus titres were run through a Sephadex G-75 column and collected in 0.1 M-NaCl-0.01 M-tris (pH 7.6) buffer. 

Chemicals. 2-14C-uracil, 52 to 55 Ci/mmol, was obtained from Schwartz Bioresearch, Inc. 4,5-3H-leucine 6 Ci/mmol, was obtained from New England Nuclear Corp. Lysozyme, 18600 units/mg, was obtained from Mann Research Laboratories. 

Media. GSO medium is a modification of the medium of Garwes, Sillero & Ochoa (1969) and contains per litre: 4.97 g NaCl, 7.46 g KCl, 0.047 g KH₂PO₄, 12.1 g tris base, 1.07 g NH₄Cl, 0.023 g Na₂SO₄, 0.368 g CaCl₂, 0.51 g MgCl₂, 10 mg thiamine and 5 g glucose. The pH was adjusted to 7.5 with HCl. Limiting concentrations of nitrogen, sulphur, and glucose were determined by constructing *E. coli* stationary phase OD against compound concentration curves for each of these compounds in GSO medium. The limiting concentrations selected for the following experiments fall within the linear portion of each curve and give a convenient maximum OD₄₅₀ of approx. 0.5. The limiting concentrations are nitrogen as NH₄Cl, 120 μg/ml; sulphur as Na₂SO₄, 3.2 μg/ml; glucose, 330 μg/ml. In experiments in which the limiting compound was returned to resting cells, the compound was returned to the concentration in the complete GSO medium. 

MS broth and MS agar were used as general growth and plating media for MS2 virus (Pfeiter, Davis & Sinsheimer, 1964). MS broth contains 10 g Bacto Tryptone (Difco), 8 g NaCl and 1 g yeast extract (Difco) per litre of distilled water and is supplemented with 1.0 g glucose, 0.212 g CaCl₂ and 10 mg thiamine. MS agar contains, in addition, 1.5 %(w/v) agar for plates and 1.0 %(w/v) agar for plate overlays. 

Growth conditions. Unless stated otherwise, *E. coli* was grown from a slant in the appropriate medium until the culture reached stationary phase. This culture was then diluted 1:200 or more into fresh medium. Growth was at 37 °C in a shaking water bath, and was followed by determining the optical density (450 nm) of the culture in a Bausch and Lomb Spectronic 20. Cell numbers were determined using a Petroff-Hausser counting chamber. A minimum of 250 cells/point was counted. 

Virus titre assay. To determine intracellular-plus-extracellular phage, infected bacteria were artificially lysed using a lysozyme-EDTA procedure (Haywood & Harris, 1966). Dilutions of the lysed sample were then placed on MS agar plates using *E. coli* 3000 as an indicator strain. For determination of extracellular virus, infected cells remained unlysed and were removed prior to the assay by centrifugation. 

Virus adsorption assay. Virus adsorption was measured by the technique of Newbold & Sinsheimer (1970), in which cells were exposed to virus for 10 min and the virus remaining unadsorbed was measured. The multiplicity of infection (m.o.i.) was approx. 0.2. AB1621 was used as the carrier bacterium during centrifugation. 

Incorporation of labelled precursors into RNA and protein. To measure the incorporation of 14C-uracil and 3H-leucine into acid-precipitable material, the appropriate radioisotope was added to the sample. Labelling was completed by adding 0.1 M-Na azide and unlabelled uracil (100 μg/ml) or unlabelled leucine (200 μg/ml) respectively to the sample and chilling.
it in a dry-ice ethanol bath. Duplicate 0.1 ml samples were put on to Whatman 3MM filters. Uracil labelled samples were precipitated by the addition of cold 7\% (w/v) trichloroacetic acid (TCA). The labelled filters were kept at 4°C for at least 1 h, washed twice with cold 5\% TCA, and once with cold 95\% ethanol. Leucine labelled samples were precipitated with cold 10\% (w/v) TCA for 1 h, heated in 5\% (w/v) TCA for 20 min at 80 to 90°C, and washed as above. All filters were dried, added to Liquifluor-toluene scintillation fluid and counted in a scintillation counter.

RESULTS

Effects of starvation on general cellular metabolism

Starvation conditions were determined as described in the Methods section. Cultures were starved for their carbon and energy source (glucose), sulphur source (Na2SO4) or nitrogen source (NH4Cl) by allowing cells to consume the appropriate substance, which was present in the general growth medium in limiting concentrations. Normally Escherichia coli 3000 ceases growth in complete GSO medium at an optical density at 450 nm of 1.5. Under starvation conditions for each of the above compounds, growth ceased at an optical density around 0.5. Fig. 1 shows the general shutdown of cellular RNA and protein syntheses as the cells enter the starved state. The arrow indicates the time (+15 min) after which no cell division occurred as determined by Petroff-Hausser cell count.

Virus infection of starved cells

The previous section has shown that starvation for glucose, sulphur or nitrogen results in decreased RNA and protein syntheses as well as cessation of cell division. To determine whether these conditions lead to a loss of lysis by RNA virus-infected cells, cultures of E. coli 3000 were infected with MS2 at different times in logarithmic growth and under starvation conditions. The extent of cellular lysis was monitored by optical density and the results of these experiments with limiting nitrogen, sulphur and glucose are shown in Fig. 2. These results clearly indicate that starved cells are not lysed after virus infection.

To establish that the absence of lysis in these cells was not due to failure of the cells to adsorb virus initially, virus adsorption in starved cells was measured directly. The results are shown in Table I and indicate that there is little or no difference between logarithmic phase and starved cells adsorption of virus.

Release of progeny virus by starved cells

To unequivocally demonstrate that starved cells were being infected and producing progeny virus, total virus (intracellular-plus-extracellular) was measured at different times after infection of glucose, sulphur and nitrogen starved cultures. In addition, extracellular (released) virus was measured to distinguish between the possibilities that starved cells were not lysing because (1) viruses were not being released or (2) viruses were being released via a non-lytic release mechanism, e.g. as in a ‘carrier state’ (Davern, 1964; Hoffmann-Berling & Maze, 1964; Knolle, 1964). The results of these experiments are shown in Fig. 3 (solid lines) and indicate that progeny virus is being produced by all starved cultures; however, few, if any, of these phage are being released from the host cells. Differences in total p.f.u. production among cultures starved for different compounds have previously been noted (Propst-Ricciuti & Haywood, 1974).

Release of progeny virus from ‘starved’ cells will occur for glucose or sulphur limited cells if the limiting substance is returned to the starved cells at the time of infection (Fig. 3,
Fig. 1. Shutdown of RNA and protein syntheses in starved E. coli 3000. Cells were grown in limiting glucose (a), limiting sulphur (b) and limiting nitrogen (c) media. At intervals samples were removed and labelled with either $^{14}$C-uracil (0.1 μCi/ml, 0.2 μg/ml) or $^{3}$H-leucine (2 μCi/ml, 0.06 μg/ml) as described in Methods. The amount of radioactivity incorporated into each sample during the 5 min labelling period is expressed as ct/min/10^8 cells. Arrows indicate the time of the last cell division. O——O, optical density at 450 nm. ■——■, $^{3}$H-leucine. ×——×, $^{14}$C-uracil.

dotted lines). With glucose or sulphur starved cells, having the appropriate compound returned, virus release begins within 40 to 60 min p.i. This release is presumed to be via cell lysis since it is accompanied by a decrease in the optical density of the cultures. In the case of nitrogen starved cells, however, virus release has still not begun by 85 min p.i.

**Resumption of growth by starved cells**

To determine whether a correlation exists between the time virus release occurs after the limiting compound is returned to starved, infected cells and the time starved cells would normally begin dividing after the compound was returned, cultures limited for glucose, sulphur or nitrogen had their limiting compound returned and their resumption of growth was monitored by optical density and Petroff–Hausser cell counts. The results of this
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Fig. 2. Effect of cell starvation on lysis of MS2-infected E. coli 3000. Cells were grown in limiting nitrogen (a), limiting sulphur (b) and limiting glucose (c) media. At different times in growth, as measured by optical density at 450 nm (○), cell samples were removed and infected with purified MS2, at a multiplicity of approx. 5. The optical density of both infected and uninfected cells was then followed. Numbers along the optical density curve indicate times at which samples of the uninfected culture were infected with virus. Dashed lines indicate optical density curves of infected cells.

| Table 1. Adsorption of MS2 to log phase and starved E. coli* |
|---------------------------------|----------------|----------------|----------------|
| Bacterial condition            | Input phage/ml | Unadsorbed phage/ml | Adsorption (%) |
| Log phase                      | 1.5 x 10⁵      | 4.4 x 10⁵         | 97.1%          |
| Nitrogen starved               | 2.4 x 10⁵      | 6.5 x 10⁵         | 97.3%          |
| Sulphur starved                | 2.0 x 10⁵      | 1.4 x 10⁶         | 93.0%          |
| Glucose starved                | 2.9 x 10⁵      | 1.5 x 10⁶         | 94.8%          |

* Cultures of E. coli 3000 were grown in limiting nitrogen, sulphur or glucose medium until approx. 1 h after the cultures ceased growing. The cells were then infected with MS2 virus and adsorption was measured by the method of Newbold & Sinsheimer (1970). The log phase sample was taken from a culture grown in unmodified GSO medium. Adsorption data are expressed as the percentage of input virus adsorbed at 10 min p.i. Input virus represents p.f.u./10⁶ bacteria/ml.
Fig. 3. Production and release of MS2 by starved E. coli 3000. Cells were grown in limiting glucose (a), limiting sulphur (b) and limiting nitrogen (c) media until approx. 1 h after growth ceased. Cultures were then inoculated with MS2 virus at a multiplicity of about 5. Immediately after infection the cultures were split and the appropriate limiting compound returned to half of the cells. At different times after infection samples were removed from each half of the cultures and assayed for intracellular-plus-extracellular (produced) virus and/or extracellular (released) virus. ○—O, intracellular-plus-extracellular virus produced by starved cells; •—•, extracellular virus released by starved cells; ▲—▲, extracellular virus released by cells with the limiting compound returned.

The experiment are shown in Fig. 4. With glucose and sulphur limited cells, optical density began to increase within 15 to 30 min after the appropriate compound was returned. Actual increases in cell number in these cultures, indicating the resumption of cell division, lagged behind the optical density increase but were noted by 45 min after the return of the limiting compound. With nitrogen starved cultures re-initiation of growth was extensively delayed. Optical density increases did not begin to occur until 60 min and cell division was still not apparent at 90 min after the limiting compound was returned to these cells.

DISCUSSION

It is known that infected bacteria can release RNA bacteriophage in one of several ways. Under conditions of normal infection, e.g. exponentially growing cells infected at 37 °C, host cells undergo a lytic cycle ending with virus release after cell disruption. Under less conducive conditions of infection, e.g. non-exponentially growing cells and/or infection at lower temperatures, a persistent infection or 'carrier' state may be set up and progeny viruses are released via a mechanism not involving cell lysis (Davern, 1964; Hoffmann-Berling & Maze, 1964; Knolle, 1964; Propst-Ricciuti & Haywood, 1974). The experiments described in this paper have shown that cells starved for glucose, sulphur or nitrogen can be infected with MS2 virus and will produce progeny phage, however, cell lysis does not occur.
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Fig. 4. Resumption of growth by starved E. coli 3000. Cells were grown in limiting glucose (a), limiting sulphur (b) and limiting nitrogen (c) media until approx. 1 h after growth ceased. Cultures were then split and the appropriate limiting compound returned to half of the cells. Growth in both halves of each culture was subsequently monitored by optical density at 450 nm and/or Petroff-Hausser cell counts. ○——○, optical density of culture prior to the return of the limiting compound; ●——●, optical density of culture after return of the limiting compound; □——□, cell count of culture without limiting compound returned; ×——×, cell count of culture with limiting compound returned.

In these experiments, failure of starved cells to lyse reflects the absence of virus release, and is therefore indicative of a malfunction in the lytic cycle rather than increased formation of persistent infections.

In the case of lytic infections two hypotheses have been presented to explain how viruses are released. RNA phage carry a very small genome which does not code for a lysozyme, per se, however, cells infected with certain coat protein mutants do not lyse. Thus, it has been proposed that the virus coat protein is involved in lysis of host bacteria (Zinder &
Lyons, 1968). There is no evidence that virus coat protein, or any other virus coded protein, has a direct lytic action, however. Recently a second hypothesis has been presented which suggests that lysis by RNA phage occurs through aberrant cell division which is secondary to changes in the host's RNA and protein syntheses after infection (Haywood, 1974). A corollary to this second hypothesis is that cells which are not dividing should not be able to lyse. This has previously been shown to be the case with stationary phase cells (Propst-Ricciuti, 1972; Haywood, 1974) and also, as described here, with cells starved for glucose, sulphur or nitrogen. With starved cells, in contrast to stationary phase cells, growth can be restored simply by returning the appropriate limiting compound to the cells. Using this technique it is possible to determine whether a correlation exists between the time virus release occurs after the limiting compound is returned to starved, infected cells and the time starved cells would normally begin dividing after the limiting compound was returned. The lysis-via-aberrant-division hypothesis predicts that a positive correlation should exist between return of growth and return of lysis. As indicated in the previous experiments such a correlation does exist between the time starved, infected cells begin releasing virus after the return of the limiting compound and the time similarly starved, but uninfected cells normally begin cell division after release from starvation conditions. In the case of glucose and sulphur starvation both virus release and the first cell divisions occurred about 50 min after the return of the limiting compound. In contrast, with nitrogen starved cells neither virus release nor cell division has occurred at 85 min after nitrogen was returned to the culture. Although correlations between the behaviour of infected and uninfected cells must be viewed with caution, these data nevertheless lend support to the hypothesis that lysis of infected cells by RNA phage is related to cell division and may result at the time of cell division from failure of the cells to divide properly.

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REFERENCES


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