Control of Lysis of T4-infected *Escherichia coli*\(^1\)

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The lysis of *Escherichia coli* B/5 infected with T4Dr48 could be delayed by addition of 9-aminoacridine (9AA). Infected cells showed an early period of maximal response followed by a decline in sensitivity. The ultimate rate of lysis was also affected by the dye. Deoxyribonucleic acid (DNA), protein, and lysozyme synthesis began at the normal time in complexes inhibited by 9AA addition. The rates of synthesis of these macromolecules were lower in the presence of the dye, with DNA and lysozyme synthesis being more strongly affected than total protein synthesis. Penicillin-sensitive cell wall synthesis stopped at about 10 min after infection. Inhibition of oxidative metabolism by early potassium cyanide addition prevented lysis in the presence of intracellular lysozyme. The cyanide-sensitive event occurred at about 20 min in normal infections, and between 30 and 40 min in 9AA-inhibited infections. 9AA could alter both the time at which the cyanide-sensitive event occurred and the time of lysis. Addition of chloramphenicol did not prevent lysis once intracellular lysozyme was present. Lysis from without of infected cells consisted of three phases: an initial sensitivity, followed by a short period of resistance, and then a return to sensitivity in normal infections. The demonstration of the late return to sensitivity depended on the presence of intracellular lysozyme, and could be delayed by 9AA addition.

The mechanism which controls the lysis of bacteria infected with virulent bacteriophage is obscure. Lysis of infected complexes may be delayed or inhibited in several ways. Doerrmann (5) showed that cells infected with virulent bacteriophage remain intact for several hours if a secondary phage infects these cells during the latent period. Lysis inhibition by superinfection requires that the primary phage be wild type with respect to the rapid-lysing (r) genotype (5).

Lysis inhibition which is independent of the genotype of the primary phage can be demonstrated with various chemicals. Doerrmann (6) reported that cyanide (KCN) addition early in the latent period prevented lysis of T4Dr48-infected complexes, and Heagy (10), using 2,4-dinitrophenol, noted a similar effect. Susman and co-workers (18) have shown that the dye 9-aminoacridine (9AA) is capable of inhibiting both phage maturation and lysis.

An equilibrium theory of lysis has been proposed (4) which suggests that in phage-infected cells there is a dynamic equilibrium between the synthesis of bacterial cell wall and its destruction by intracellular lysozyme. This theory is based on the following observations: (i) a phage-controlled lysozyme is produced in infected cells (11, 16); (ii) addition of cyanide early in the latent period prevents lysis, whereas addition late in the latent period causes immediate lysis (6); (iii) infected complexes become resistant to lysis from without, and are therefore presumably synthesizing wall material at a rate which is sufficiently rapid to repair the damage caused by the superinfecting phage (19); and (iv) cells infected with lysozymeless mutants stop metabolizing at the time at which the cells would normally lyse (12). This theory predicts that metabolic arrest in the presence of intracellular lysozyme would precipitate immediate lysis, and that cell wall synthesis continues through infection.

In this report, the effect of 9AA on lysis has been further characterized and the delay caused by the dye has been utilized to study the lytic reaction. The predictions of the above theory of lysis and the lysis-from-without reaction have been investigated.

**MATERIALS AND METHODS**

The basic phage techniques used were those described by Adams (1).
**Bacteriophage.** T4Dr48 (5), an r1 mutant which was generously provided by R. S. Edgar, was used as the primary phage in most experiments. It was necessary to use this mutant to prevent lysis inhibition by superinfection (5). The acidine sensitivity of this phage with respect to phage production does not differ from that of wild type as reported by Susman and co-workers (18).

The amber mutant T4DamH26 which does not synthesize phage lysozyme in the restrictive host (E. coli B) was also obtained from Dr. Edgar. The double mutant amH26r48 was constructed in this laboratory.

T4D wild-type phage (7) was used as the superinfecting phage in lysis-from-without experiments.

All phage stocks were purified by differential centrifugation.

**Bacteria. Escherichia coli** strain B/5 (8) was used as the host in all experiments, and also as the indicator for the assay of T4D wild type and T4Dr48. The host cells were prepared by harvesting cells grown with aeration at 30°C to a density of 2 x 10^9 cells/mL. These were washed, resuspended in buffered saline at 4 x 10^9 cells/mL and starved by bubbling at 30°C for 1 hr. Starved bacteria were used to synchronize infection. This method of synchronizing resulted in a complete recovery of infective centers.

E. coli strain CR63 (2) was the indicator for the assay of T4DamH26r48.

**Media.** Hershey broth, pH 7.5 (15), was used in some of the experiments describing the phenomenon of lysis inhibition by 9AA. Hershey agar (15) was used for all assays.

Synthetic medium (M9S-2) was prepared as follows: Basal medium contained 1 mL of 0.2% gelatin, 0.05 mL of 2.0 μM CaCl₂, 1 mL of 1 μM MgSO₄, 20 mL of 20% glucose, 7.5 x 10⁻⁴ M L-tyrosine, and 3.0 x 10⁻⁴ M cystine. These components were added to 663 mL of distilled, deionized water and autoclaved. Immediately before use, the basal medium was supplemented with 100 mL of salts (4.76 g of KH₂PO₄, 45.45 g of Na₂HPO₄, 25 g of NaCl, 5 g of NH₄Cl, 500 mL of distilled, deionized water), 50 mL of sterile 0.01 M L-tryptophan, 50 mL of a sterile mixture of 0.01 M aspartic acid and 0.01 M glutamic acid, and 10 mL of a sterile mixture of the following L-amino acids (0.05 M): proline, serine, methionine, leucine, threonine, valine, alanine, isoleucine, lysine, arginine, histidine, phenylalanine, and glycine. The final pH of the medium was 7.4.

**Basic experimental procedures.** All experiments were carried out at 30°C. The infection was made as follows: phage in buffered saline was added to the starved bacteria to give a multiplicity of infection of about 5. Adsorption in buffer was continued at 30°C for 5 min, followed by treatment with antiphage serum for 5 min to inactivate free phage. The infected cells were then diluted 50-fold into prewarmed M9S-2 growth tubes and bubbled at 30°C. The time of dilution into complete medium was taken as zero minutes after infection. The concentration of infected cells in the growth tube was about 6 x 10⁸/mL.

The absorbance of the culture was followed by removing samples from the growth tube at various times and reading the A₄₅₀ in a Beckman DB spectrophotometer.

In all of the experiments, the time of onset of lysis was measured. This was defined as the intersection point of a line drawn through the linear portion of the A₄₅₀ drop after lysis and a horizontal line drawn through the A₄₅₀ value(s) obtained immediately prior to lysis. In using this parameter, early-lysing cells receive considerable emphasis; however, it was felt that this would give at least a minimal estimate of the lysis delay.

Lysis-from-without experiments were performed by removing 1.9 mL samples from the growth tube at various times and adding 0.1 mL of T4D wild-type phage at an input multiplicity per infected cell of between 3,000 and 5,000. This multiplicity was used to ensure rapid adsorption of sufficient phage to lyse the complexes. The samples were bubbled vigorously at 30°C for 15 min, at which time the A₄₅₀ of the sample was read. The maximal A₄₅₀ drop was observed at 15 min after addition of the lysing phage. The A₄₅₀ of the sample was corrected for the A₄₅₀ of the added phage.

**Chemicals.** 9AA, obtained from Mann Research Laboratories, New York, N.Y., was dissolved in distilled water at 1 mg/mL. This stock solution was stable for several months, and was stored in a refrigerator in the dark.

Adenine-5'-MC and DL-leucine-1-14C were obtained from Calbiochem, Los Angeles, Calif. Radioactive adenine and cold adenine were added to the growth tubes to give a final adenine concentration of 4 μg/mL and a final activity of 0.01 μCi/mL. Radioactive leucine was added to growth tubes to give a final activity of 0.1 μCi/mL and a final leucine concentration of 68.8 μg/mL.

Chloramphenicol, a gift of Parke, Davis & Co., Detroit, Mich., was dissolved with warming at 4 mg/mL in M9S-2 medium prior to use.

Penicillin G (crystalline sodium salt) was obtained from The Upjohn Co., Kalamazoo, Mich., and was dissolved at 10 mg/mL in M9S-2 medium immediately before use. Fresh solutions were used for each experiment, as penicillin is relatively unstable.

**Measurements.** Deoxyribonucleic acid (DNA) synthesis was followed by measuring the incorporation of 14C-adenine into trichloroacetic acid-precipitable, alkaline stable counts (12). Samples were taken into 0.5 N KOH (final concentration, 2.5 N) and incubated for 16 to 20 hr at 37°C. The samples were chilled, HCl and trichloroacetic acid at final concentrations of 0.3 N and 5%, respectively, were added. The precipitates were collected by filtration through 0.45-μm filters (Millipore Corp., Bedford, Mass.). Counting was carried out in a Nuclear-Chicago low background counter.

Protein synthesis was followed by measuring incorporation of 14C-leucine into trichloroacetic acid-precipitable, nonhydrolyzable counts. Samples were withdrawn into cold trichloroacetic acid (final concentration, 5%) and allowed to stand on ice for 30 min. They were then boiled for 10 min to remove label from charged transfer ribonucleic acid (RNA), and again cooled on ice for 15 min. The precipitates were collected by filtration and counted as above.
Samples for the assay of phage lysozyme were removed from the growth tubes into an ice-water bath and sonically treated on ice; the crude sonic extracts were used to assay the enzyme. The substrate was lyophilized E. coli B/S suspended at 0.4 mg/ml in 0.05 M tris(hydroxymethyl)aminomethane, pH 7.5, as described by M. Inouye, A. Tsugita, E. Terzaghi, and G. Streisinger (manuscript in preparation). The change in absorbance at 350 μM (A₃₅₀) was followed. The concentration of the extract to be assayed was adjusted to give a linear decrease in A₃₅₀ over an interval of 4 min of incubation at 26°C. An M9S-2 lysate of T4Dr48 from which the phage had been removed by centrifugation was used as a lysozyme standard, since commercial muramidase gives a slightly different standard curve. One enzyme unit is defined as that amount of enzyme which gives a linear change of 0.08 A₃₅₀ units per min at 26°C.

RESULTS

Lysis inhibition by 9AA. Susman and co-workers (18) observed that the delay in lysis caused by 9AA depends both on the concentration of dye and on the time of addition. This finding has been confirmed and extended to show (Fig. 1) that there is a period of maximal response at 0 to 10 min after infection. Addition of increasing concentrations of dye during this time (Fig. 1b) led to an increasing lysis delay which did not reach a maximum at the concentrations used. This was followed by a loss of sensitivity, with a possible second period of lowered sensitivity at 12 to 17 min (shoulder in Fig. 1a). This second delay, although small, was reproducible. Addition of increasing amounts of dye at this time (Fig. 1b) increased the delay in lysis only slightly. A maximal effect was reached at a concentration of 2 μg/ml. Addition of 9AA at 20 min or later had no effect on the timing of lysis.

The rate of lysis in the presence of 9AA was measured (Fig. 2), and it was found that the reduction in rate was maximal for addition of dye up to 14 min after infection.

Effect of 9AA on macromolecular syntheses. Since the concentration of 9AA needed to delay lysis was somewhat higher than that necessary to inhibit maturation (18), and was therefore higher than the concentration which was reported (18) to

![Fig. 1. Effect of 9-aminoacridine hydrochloride (9AA) on the timing of lysis. (a) 9AA, 3 μg/ml, was added at various times after infection with T4Dr48. The time of onset of lysis was measured for each time of addition of 9AA and corrected for the time of lysis of the control to give ΔT₉₉. These data were compiled from nine separate experiments, and the points represent average values, with the dashed lines denoting the extreme values obtained for each time of addition. (b) 9AA, 1, 2, 3, and 4 μg/ml, was added at 3 to 15 min after infection with T4Dr48. The time of onset of lysis was measured for each 9AA concentration and corrected for the time of lysis of the control, as noted for Fig. 1a. The data were compiled from seven separate experiments and were averaged as described for Fig. 1a.](http://jvi.asm.org/Downloaded.png)
have no effect on macromolecular syntheses, it was necessary to measure DNA, protein, and lysozyme production under conditions of 9AA lysis inhibition.

In all of the following experiments, 3 μg of 9AA per ml was added at the time of infection, because this gave good lysis inhibition, and was still well below the bacteriostatic concentration of the dye.

The results for DNA, total protein, and lyso-

**Fig. 2.** Rate of lysis of inhibited cells in the presence of 3 μg/ml of 9-aminoacridine (9AA) added at various times after infection. The rate of lysis was measured over the linear portion of the lysis curve. $R_{9AA}$ is the change in $A_{660}$ per minute in the presence of 9AA; $R_C$ is the change in $A_{660}$ per minute of the control for each experiment. The data were compiled from nine separate experiments, and the points represent average values, with the dashed lines indicating the extreme values obtained for each time of addition.

**Fig. 3.** Effect of 3 μg/ml of 9-aminoacridine (9AA) added at the time of infection on macromolecular syntheses. In each case, infected complexes were diluted into M9S-2 growth tubes containing the appropriate isotope with or without 3 μg/ml of 9AA. Measurement of incorporation and enzyme activity is described in Materials and Methods. (a) DNA synthesis; (b) protein synthesis; (c) lysozyme syntheses. Arrows indicate the onset of lysis.
enzyme synthesis are presented in Fig. 3. 9AA inhibited the rate of DNA synthesis, as measured by $^{14}$C-adenine incorporation. The inhibition was more pronounced early in infection, with the final amounts of DNA synthesized in both cases being approximately equal. By use of adenine of a higher specific activity, it was possible to determine that DNA synthesis began at the same time in both control and inhibited complexes.

Total protein synthesis in inhibited complexes was only slightly less rapid than in untreated complexes. There was thus an apparent differential effect of the dye on the rate of DNA synthesis.

The synthesis of phage lysozyme began at about 15 to 16 min in both untreated and inhibited complexes. 9AA did not inhibit phage lysozyme activity when mixed with an enzyme extract in vitro at concentrations of 9AA up to 6 $\mu$g/ml. Therefore, the observed lowering of the rate of lysozyme production in the presence of the dye was a real phenomenon and not simply an inhibition of enzyme activity by the dye.

Since 9AA appeared to affect synthetic processes primarily by slowing the rate of synthesis, its effect on metabolism in general was investigated by measuring the oxygen consumption of complexes treated with the dye. The rate of oxygen consumption was lowered to 80% of the control in the presence of 9AA. The duration of oxygen uptake was prolonged, with the total amount of oxygen consumed in 9AA higher than in non-treated complexes.

Lysis from without. The ability of infected cells to become resistant to lysis from without is thought to be due to rapid cell wall synthesis (19). Therefore, the synthesis of cell wall material during infection was first investigated by measuring the ability of complexes to become resistant to lysis from without. It was found, in agreement with Visconti (19), that complexes become increasingly resistant to lysis from without during the first 10 min after infection (Fig. 4). However, if the lysing phage was added 15 min after infection, a return to sensitivity is observed.

This return to sensitivity could be the result of the presence of intracellular lysozyme, and the experiment was therefore repeated with amH26r48. These complexes became resistant to lysis from without at the normal time and did not show a return to sensitivity (Fig. 4).

A possible explanation of this result would be that the infected cells had lost the ability to adsorb the superinfecting phage. This possibility was investigated by use of superinfecting phage labeled with $^{14}$C-leucine, and it was found that the complexes were capable of adsorbing at least 40 phage per cell as late as 100 min after infection.

If late lysis from without depends solely on the presence of intracellular lysozyme, 9AA-inhibited complexes should show the same pattern of susceptibility as the untreated cells. As shown in Fig. 4, 9AA-treated complexes did not become susceptible to lysis from without at late times, but remain resistant until at least 65 min. The treated complexes became resistant at the normal time, indicating that the process involved in the resistance to lysis from without is initiated at the normal time in the presence of 9AA.

Sensitivity of infected cells to penicillin, cyanide, and chloramphenicol. It has been demonstrated that phage lysozyme and penicillin are both active on the same layer of the cell wall (17, 20, 21). The penicillin-sensitivity of infected complexes was therefore investigated as an approach to the problem of cell wall synthesis after phage infection.

It was not possible to measure the sensitivity of untreated r48-infected cells to penicillin lysis, because this requires about 30 min to begin, and thus the infected cells would begin normal lysis too soon to allow one to distinguish between the two.

Cells infected with amH26r48 were sensitive to penicillin lysis up to about 10 min after infection (Fig. 5a), but became insensitive by 20 min. This indicates that murepoxide synthesis does not continue throughout infection. It was also found, by use of 90-min addition, that in the absence of

![Figure 4](http://jvi.asm.org/Downloadedfrom/fig_4.png)

**Fig. 4. Lysis from without.** T4D+ was added to Escherichia coli B/5 infected with T4Dr48 (○) or T4DamH26r48 (●) at an input multiplicity of 2,900. In one case (△), 3 $\mu$g/ml of 9- aminoacridine was added to the r48-infected cells at the time of infection, and the lysing phage had an input multiplicity of 5,000. The method of measuring lysis from without is described in Materials and Methods. $A_0$ is the absorbance of the sample read at 15 min after addition of the lysing phage, and corrected for the $A_{450}$ of the added phage. $A_C$ is the absorbance of the culture at the time at which the lysis-from-without sample was read.
oxygen uptake the complexes remained insensitive to penicillin lysis.

It is possible that the delay in lysis caused by 9AA might be due to a prolonged synthesis of mucopptide. Figure 5b shows that 9AA-inhibited complexes also became insensitive to penicillin lysis at about 10 min after infection.

The equilibrium theory of lysis predicts that addition of a metabolic poison to cells containing lysozyme will cause immediate lysis. Figure 6a demonstrates that cyanide may be added to the infected cells up to 20 min after infection without causing lysis, even though lysozyme is present at this time. Instead, addition of cyanide at this time prevented lysis. Addition of cyanide at 25 min or later allowed lysis to occur. The timing of lysis after 25 min was fairly insensitive to the cyanide. Cells lysed at the normal time even in the absence of energy metabolism once the cyanide-sensitive process had begun.
FIG. 6. Addition of cyanide to infected complexes. Samples were removed from the growth tubes at various times and KCN at a final concentration of $5 \times 10^{-3}$ M was added. The samples were incubated further at 30°C without aeration to prevent evaporation of the cyanide and recovery of the cells. (a) Addition to r48-infected cells. The data for addition at 1, 20, 25, and 30 min are presented. Addition of cyanide later than 30 min had no effect on the onset of lysis. (b) Addition to 9-aminoacridine (9AA)-inhibited complexes. The results for 1-, 30-, 40-, and 80-min addition are presented. Addition prior to 30 min prevented lysis, and addition between 40 and 80 min did not affect the timing of lysis predetermined by the 9AA.

The same experiment was performed with infected cells inhibited by 9AA (Fig. 6b). The results were analogous to those with the untreated cells, differing primarily in the time at which the cyanide-sensitive process occurred. Cells treated with 9AA became capable of lysing in the presence of cyanide at between 30 and 40 min. The time interval between the occurrence of the cyanide-sensitive commitment to lysis and lysis itself was prolonged by the 9AA.

The effect on lysis of chloramphenicol addition at various times was examined, and the results for untreated and acridine-inhibited complexes are presented in Fig. 7. Noninhibited complexes lysed if chloramphenicol was added between 10 and 20 min, and 9AA-inhibited cells lysed if
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FIG. 7. Lysis in the presence of chloramphenicol (CM). Samples were removed from the growth tubes into 50 μg/ml of CM and bubbled at 30 C. (a) Effect of CM on r48-infected complexes. The data for 10-, 20-, and 25-min addition are presented. Addition prior to 10 min prevented lysis. Addition later than 25 min had no effect on the timing of lysis. (b) Effect of CM on 9-aminoacridine (9AA)-inhibited complexes. 9AA, 3 μg/ml, was added at the time of infection. The data for 15-, 20-, and 60-min addition are presented. Addition prior to 15 min prevented lysis; addition between 20 and 60 min delayed lysis, and addition later than 60 min had no effect on the timing of lysis.

chloramphenicol was added between 15 and 20 min.

DISCUSSION

E. coli B/5 infected with T4Dr48 exhibited an early pronounced sensitivity to lysis inhibition by 9-aminoacridine. This was followed by a decrease in sensitivity, with a possible second period of lowered response to the dye. The observed change in sensitivity of the complexes cannot be explained on the basis of dye uptake, since it has been shown (14) that infected cells are capable of rapid uptake of acridine throughout infection, and that the amount of dye entering the cell is the same whether the dye is added early or late. At the concentrations used, 9AA was not capable of completely preventing lysis, but only of delaying it.
9AA reduced the rate of lysis when added earlier than 14 min after infection. The fact that the timing of the sensitivities was different suggests that the acridine-sensitive process affecting the timing of lysis was different from that affecting the rate of lysis.

Studies of macromolecular syntheses in cells inhibited by 3 μg of 9AA per ml added at the time of infection failed to show any significant differences between control and treated complexes in the time of onset of DNA, protein, and lysozyme synthesis. The rates of synthesis of DNA and lysozyme were sharply reduced in treated complexes. The decrease in rate of oxygen consumption caused by the 9AA was small in comparison with the effect of the dye on the rate of DNA and lysozyme synthesis.

The effect of 9AA on the timing of lysis cannot be explained on the basis of an inhibition of lysozyme synthesis or activity. An explanation based on the amount of intracellular lysozyme present is not tenable since inhibited complexes began to lyse when the intracellular lysozyme level was only 65% of that found in untreated complexes at the time of lysis. It has been shown by others (9), using bacteriophage λ, that there is no apparent correlation between the amount of intracellular lysozyme and the ability of a cell to lyse.

With regard to the mechanism of lysis of phage-infected cells, it has been shown that:

(i) Lysis from without is a complex phenomenon involving a rapid increase in resistance of the complexes, followed by a return to sensitivity in normal infections. In the absence of intracellular lysozyme, the complexes are incapable of undergoing a return to sensitivity even after oxygen uptake has stopped. There is, therefore, a mechanism in infected cells which, in the absence of intracellular lysozyme, preserves the integrity of the cell when it is attacked externally by phage which contain lysozyme (3). 9AA is capable of prolonging the period of resistance to lysis from without in the presence of intracellular lysozyme. The ability of an infected cell to undergo lysis from without at late times is dependent both on the presence of intracellular lysozyme and on the occurrence of some acridine-sensitive process.

(ii) Penicillin-sensitive cell wall synthesis stops at about 10 min after infection in both r48 and amH26r48-infected cells. 9AA does not prolong this period of sensitivity to penicillin. Continuing wall synthesis is, therefore, probably not responsible for the ability of infected cells to remain intact in the presence of intracellular lysozyme, nor is it responsible for the prolongation of resistance to lysis from without.

(iii) A cyanide-sensitive event occurs at 20 min after infection which commits the cells to lyse. Once the reaction leading to lysis begins, it cannot be reversed by either cyanide or chloramphenicol addition, and continues in the absence of energy metabolism. Addition of 9AA at the time of infection delays the occurrence of the cyanide-sensitive event until between 30 and 40 min. The time between this event and actual lysis is also altered by the acridine. Normally about 20 min, the interval is extended to about 50 min by the dye.

(iv) Addition of chloramphenicol to infected cells prevents lysis only if added prior to the appearance of intracellular lysozyme. One could interpret these results to mean that chloramphenicol prevents lysis only by preventing lysozyme synthesis. If this is true, it becomes apparent that only a small amount of lysozyme is necessary for lysis. Lysis occurs at the same time whether 1.3 (25-min chloramphenicol addition) or 4.8 (normal lysis) enzyme units are present intracellularly. Since the cyanide-sensitive event appears to be unaffected by chloramphenicol addition, it is apparently not based on a protein synthesized at 20 min.

A summary graph showing the time of action of 9AA, KCN, and chloramphenicol on lysis.

![Fig. 8. Time of action of various inhibitors of lysis. The curve showing synthesis of intracellular lysozyme is derived from Fig. 3c. The bars represent the times at which the various inhibitors of lysis are effective. A solid bar denotes complete inhibition of lysis by the addition of inhibitor during this period; a cross-hatched bar represents a delay.](http://jvi.asm.org/)
together with the synthesis of intracellular lysozyme, is presented in Fig. 8.

The timing of lysis is a typical "clock" phenomenon. The time at which the infected cells will lyse is determined early in the infection, and cannot be altered by addition of an inhibitor of protein synthesis nor by a respiratory inhibitor once the ingredients necessary for the lytic reaction are present. The timing of the "clock" may be altered by the addition of 9AA at various times soon after infection.

It is not possible to speculate on the nature of the cyanide-sensitive event or on the mechanism of the lysis "clock." However, it has been possible to isolate many mutants of T4 which lyse at various times, but whose phage-synthesizing machinery is normal (Couse, unpublished data). It is possible that a study of these mutants will lead to an understanding of the lytic process in cells infected with virulent phage.

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LITERATURE CITED