THE LYSIS MECHANISM OF PHAGE T4

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"TITHONUS"

"The woods decay, the woods decay and fall,
The vapours weep their burthen to the ground,
Man comes and tills the field and lies beneath,
And after many a summer dies the swan.
Me only cruel immortality
Consumes: I wither slowly in thine arms,
Here at the quiet limit of the world,
A white-haired shadow roaming like a dream
The ever silent spaces of the East,
Far-folded mists, and gleaming halls of morn.

"Alas! for this gray shadow, once a man -
So glorious in his beauty and thy choice,
Who madest him thy chosen, that he seem'd
To his great heart none other than a God!
I ask'd thee, 'Give me immortality.'
Then didst thou grant mine asking with a smile,
Like wealthy men who care not how they give;
But thy strong Hours indignant work'd their wills,

And beat me down and marr'd and wasted me,
And tho' they could not end me, left me maim'd
To dwell in presence of immortal youth,
Immortal age beside immortal youth,
And all I was, in ashes. Can thy Love,
Thy beauty, make amends, tho' even now,
Close over us, the silver star, thy guide,
Shines in those tremulous eyes that fill with tears
To hear me? Let me go; take back thy gift;
Why should a man desire in any way
To vary from the kindly race of men,
Or pass beyond the goal of ordinance
Where all should pause, as is most meet for all?

"A soft air fans the cloud apart; there comes
A glimpse of that dark world where I was born.
Once more the old mysterious glimmer steals
From thy pure brows, and from thy shoulders pure,
And bosom beating with a heart renew'd.
Thy cheek begins to redden thro' the gloom,
Thy sweet eyes brighten slowly close to mine,
Ere yet they blind the stars, and the wild team

Which love thee, yearning for thy yoke, arise,
And shake the darkness from their loosen'd manes,
And beat the twilight into flakes of fire.

"Lo! ever thus thou growest beautiful
In silence, then before thine answer given
Departest, and thy tears are on my cheek.

"Why wilt thou ever scare me with thy tears,
And make me tremble lest a saying learnt
In days far-off, on that dark earth, be true?
'The gods themselves cannot recall their gifts.'

"Ay me! ay me! with what another heart
In days far-off, and with what other eyes
I used to watch - if I be he that watched -
The lucid outline forming round thee; saw
The dim curls kindle into sunny rings;
Changed with thy mystic change, and felt my blood
Glow with the glow that slowly crimson'd all
Thy presence and thy portals, while I lay,
Mouth, forehead, eyelids, growing dewy-warm
With kisses balmier than half-opening buds

Of April, and could hear the lips that kiss'd
Whispering I knew not what of wild and sweet,
Like that strange song I heard Apollo sing,
While Ilion like a mist rose into towers.

"Yet hold me not forever in thine East:
How can my nature longer mix with thine?
Coldly thy rosy shadows bathe me, cold
Are all thy lights, and cold my wrinkled feet
Upon thy glimmering thresholds, when the steam
Floats up from those dim fields about the homes
Of happy men that have the power to die,
And grassy barrows of the happier dead.
Release me, and restore me to the ground;
Thou seest all things, thou wilt see my grave:
Thou wilt renew thy beauty morn by morn;
I earth in earth forget these empty courts,
And thee returning on thy silver wheels."

ALFRED TENNYSON

ABSTRACT

Under normal conditions, phage T4-infected bacteria lyse at a characteristic time after infection, each infected cell releasing a few hundred progeny phage. To investigate the mechanism of this lysis, a selection technique was used to isolate lysis-defective mutants of phage T4. A new class of lysis-defective mutants was found which define a new T4 gene called t. During infection, t-defective mutants synthesize both phage and phage lysozyme, yet fail to either cease metabolism or lyse at the usual time. Thus, T4-induced lysis entails a sequence of at least two events: function of t gene product, resulting in the cessation of host metabolism and allowing e gene product, phage lysozyme, to degrade the host cell wall and release progeny phage.

Although the mechanism of T4 t gene product remains to be determined, some alternatives were ruled out. Neither a metabolic poison nor exogenous lysozyme will phenotypically revert the t gene defect. These results show that the normal function of gene t involves something besides cessation of host metabolism and disruption of the host cell wall. To test whether t gene product acts by degrading host membrane phospholipid, hydrolysis of host phospholipid was measured during T4 infection. It was found that lysis, although normally accompanied by phospholipid hydrolysis,

can occur in the absence of this reaction. Thus the phospholipid hydrolysis observed is a by-product of the t gene event rather that a requirement for lysis. The role of gene t in the lysis mechanism of phage T4 is discussed in light of these results.

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INTRODUCTION

"The first act of bacteriophagy consists in the fixation of the bacteriophage corpuscle toward the bacteria, then in the fixation of the corpuscle to the latter. When, as a result of its faculty of multiplication, the bacteriophage corpuscle which has penetrated into the bacterium forms a colony of a number of elements, the bacterium ruptures suddenly, liberating into the medium young corpuscles which are then ready to continue the action." (d'Herelle, 1926)

During the last fifty years the "faculty of multiplication of bacteriophage corpuscles" has served as a cornerstone for research in molecular biology (Adams, 1959; Stent, 1963; Cairns et al., 1966). The mechanisms of "fixation and penetration" and of "rupture and liberation," nevertheless, remain a mystery. I chose to study the rupture of phage T4-infected bacteria and the liberation of progeny phage, the terminal event in the phage life cycle.

This thesis presents the results of my investigations and is divided into three sections. The introduction reviews our current knowledge on the termination of the life cycle of virulent phage in Escherichia coli. The results reports my observations on the lysis mechanism of phage T4 and is comprised of three manuscripts which have been prepared for publication. The conclusion analyzes

the mechanism of lysis of phage T4.

Lysis, the Terminal Event in the Phage Life Cycle

D'Herelle's conception of the life cycle of virulent coliphage was verified by Ellis and Delbruck in 1939. They found that in an infected bacterial culture the phage titer remained constant for 40 min, and then increased 100-fold within a few min. This cycle was repeated usually two or three times until the culture lost its turbidity. Furthermore, like the timing of bacterial divisions, the timing of these bursts of phage varied with temperature. These observations suggest that in an infected bacterial culture, phage growth occurs in bursts at temperature-dependent intervals until all the bacteria are lysed. When phage and bacteria were mixed, incubated for various times, and then centrifuged at a speed which fractionated bacteria from phage, the phage titer in the supernatants decreased with first order kinetics suggesting that phage rapidly adsorb onto bacteria. Furthermore free phage and infected bacteria have approximately the same probability of forming a plaque since, under certain conditions, the disappearance of free phage in the supernatants was quantitatively accompanied by the appearance of infected bacteria in the pellets. In an infected culture the phage titer in both the culture and the culture supernatants began

to increase at the same time and rose to the same final value. Supposedly, these free phage could reinitiate a further growth cycle resulting in a further 100-fold rise in phage titer in 40 min. However, when an infected culture was diluted by a large factor soon after infection, reducing the rate of phage adsorption onto bacteria, only one burst of phage was observed. Ellis and Delbruck (1939) concluded that by diluting a culture soon after infection, one cycle of phage growth in a bacterial population could be measured. Furthermore, experiments showed that the growth features of a population of phage-infected bacteria correspond to those of an individual infected cell. Growth of phage in individual bacteria was measured by diluting the infected culture before the burst by a factor sufficient to insure that on the average there were fewer than one infected cell per aliquot. After incubation the phage titers in a number of different aliquots were then measured. Bursts from individual infected bacteria so measured varied in size from one to a few hundred; moreover, the same variation was found when early bursts were measured or when all bursts were measured late. Thus the phage life cycle has three distinct features: 1) rapid phage adsorption onto bacteria; 2) phage growth, during the latent period, in a space constrained by the cell volume, climaxed in 3) release of a burst of approximately a hundred progeny phage per infected cell.

Moreover, phage liberation occurred in single bursts and all the evidence suggested that bursts of phage liberation occur only if and when a cell lyses.

The results of microscopic observations and of one step growth experiments led Delbruck (1940) to conclude that phage can lyse bacteria in two distinct ways depending on the number of phage which infect a cell. In lysis from within, caused by the infection of a bacterium with a low number of phage, phage growth occurs for some minimum time and stops when the cell contents are liberated into the medium without deformation of the infected cell wall. In lysis from without, caused by adsorption of a high number of phage, the cell contents are liberated soon after infection by distention and destruction of the cell wall. The adsorbed phage are not recovered after lysis, nor does phage growth occur. The discovery of lysis from without clarified paradoxical observations in the literature although its mechanism remains obscure.

Lysin, a Phage-Induced Factor which Promotes Bacterial Lysis

The mechanism of phage-induced lysis appeared to be solved by the discovery of lysin, a phage-induced factor which promotes cell lysis by digestion of the cell envelope. Lysin was discovered

by Sertic (1929a) who observed that plaques of coliphage Fcz had not only a central zone containing phage, but also a phage-free peripheral zone, suggesting the existence of a diffusable substance, lysin, which lysed bacteria. Filtered glycerine extracts of peripheral zones, containing neither cells nor phage, lysed chloroform-killed bacteria. The lysing activity of the extract was proportional to extract concentration but was not transmissible. These observations showed that phage plaques contain two anti-bacterial factors: phage particles which reproduce and smaller lysin particles which do not.

Sertic (1929b) showed that lysin activity was not found in filtrates of either growing or artificially lysed bacterial cultures suggesting that lysin is induced specifically by phage infection. Antisera was prepared against two filtrates: 1) an extract of peripheral zones, containing lysin activity, and 2) filtered growing bacterial cultures, lacking lysin activity. While the antiperipheral zone serum contained 10 times the anti-phage activity (end point assay) as did the anti-bacterial serum, when measured against extracts containing lysin activity (end point assay), the anti-peripheral zone serum contained 10^3 times more activity required to block lysin activity than did the anti-bacterial serum. These antiserum studies suggest that

antibody could be prepared against a factor, lysin, which promotes cell lysis and that phage infection greatly increases the amount of lysin found in a bacterial culture.

Sertic and Boulgakov (1931) showed that while the activity of phage Fcz lysin was destroyed by heating for 60 min at 78° but not at 76°, heating for 60 min at 88° but not at 86° destroyed the activity of lysin of another phage, phage #49. Since the unheated extracts contained the same lysin activity and since the same host was used to prepare the two lysin extracts, the difference in the heat sensitivity of the two lysin activities must be phage specific. Thus, while the heat sensitivity studies do not prove that phage direct the synthesis of lysin, they rule out the possibility that the same lysin is induced during infection by all phage.

The Identification of Lysin as Phage Lysozyme

Koch and Jordan (1958) isolated a phage-free factor from lysates of phage T2-infected cultures which promoted the digestion of purified *E. coli* cell envelopes. As the activity was pH dependent, heat sensitive, and non-sedimentable, the authors conclude that the factor was an enzymatically active protein. Koch and Dreyer (1958) showed that the T2-induced lysin

enzymatically degraded cell wall polysaccharide. Since the T2 lysin is functionally identical to egg white lysozyme, it was called phage lysozyme.

The Phage Genome Codes for Phage Lysozyme

To investigate the role of the phage in its induction of phage lysozyme, Streisinger et al. (1961) isolated lysozyme-defective mutants of phage T4. Streisinger et al. (1961) observed that exposure of T4 plaques to chloroform vapor resulted in plaques surrounded by large halos, presumably due to the action of phage lysozyme, and isolated presumptive lysozyme-defective mutant phage from plaques which lacked halos after chloroform treatment. While heat treatment at 45° for 20 min did not affect the lysozyme activity in wild-type T4 lysates, it resulted in a 100-fold decrease in lysozyme activity in mutant phage lysates suggesting that mutant lysozyme differed structurally from wild-type lysozyme. The lysozyme activity of mixtures of wild-type with mutant lysates had the heat stability of wild-type lysates suggesting that the heat sensitivity of mutant lysozymes was a property of the lysozyme molecule itself. Furthermore, the lysozymes of wild-type and mutant lysates were not separated by column chromatography. The authors concluded that these mutant phage produced lysozyme molecules that were structurally different from those produced by wild-type phage.

The results of genetic crosses showed that this mutant phenotype was due to any one of a number of single mutations at separate but closely-linked sites on the phage genome. Complementation tests, performed by measuring the heat sensitivity of the lysozyme activity in lysates of mixedly-infected cells, showed that a lysate of cells mixedly-infected with two single mutant phage retained the mutant phenotype yet the lysozyme activity in a lysate of cells infected with both wild-type and mutant phage was only slightly inactivated by heat treatment. Therefore, the mutations affecting phage T4 lysozyme belong to the same gene, called gene e (for endolysin).

Streisinger et al. (1966) examined the lysozymes synthesized by bacteria infected with pseudowild strains of phage T4 carrying certain pairs of frameshift mutations in gene e and found that a short sequence of amino acids is changed in the lysozyme produced by each of these strains. Therefore phage lysozyme is the product of T4 gene e.

Cessation of Host Metabolism at the Usual Lysis Time

Mukai et al. (1967) studied the physiology of cultures infected with lysozyme-mutant phage. As detected by a sharp decrease in culture turbidity, a culture infected with rII mutant phage and grown at 37° lysed at 25 min, yet one infected

with e:rII double mutant phage retained its turbidity for at least 60 min suggesting that functional e gene product, phage lysozyme, is required for lysis. At various times after infection aliquots of cultures infected with either e^+ or e mutant phage were plated for infective centers at 30° (at which temperature the e mutant phage formed plaques). While the control culture lysed by 35 min, as detected by a 200-fold increase in infective centers, the concentration of infective centers in the e mutant-infected culture failed to increase but began to decrease after 20 min suggesting that cells infected with e mutant phage failed to lyse. Furthermore, the e mutant-infected cells lost their ability to form plaques upon transfer to the lower temperature around the normal lysis time. Artificial lysis of e mutant infected cells at either 26 min or 100 min yielded a burst of 200 phage per infected cell showing that progeny phage were produced. Moreover, the lack of increase in burst size from 26 min to 100 min suggested that by 26 min e mutant-infected cells had lost their ability to synthesize not only phage lysozyme but other phage components as well. This idea was supported by the observation that in an e mutant-infected culture, oxygen uptake stopped at 25 min. The authors concluded that metabolism of T4-infected cells stops at 25 min and that this cessation of metabolism is independent of the presence of phage lysozyme. Since lysis of wild-type T4-infected cells occurs at the same

time as does cessation of metabolism in lysozyme-defective infected cultures, Mukai et al. (1967) proposed that infected cell lysis is induced by cessation of host metabolism at the usual lysis time.

The Phage Genome Codes for More than One Factor Which Affects Lysis

If cessation of host metabolism induces lysis and is a phage-induced function then it would be expected that mutant phage could be obtained which were defective in both this function and lysis as well. In addition to lysozyme-defective mutants, Streisinger et al. (1961) found lysis-defective mutant phage (on the basis of "haloless" plaques after chloroform treatment) which produced lysozyme. Genetic evidence showed that the mutations responsible for the phenotype of these mutants map at sites not closely linked to gene e suggesting that in addition to phage lysozyme, T4 codes for at least one other product required for lysis.

The star mutants of T-even phage might be defective in this second lysis function. The star mutant phenotype, first observed by Hershey (1946) with phage T6, affects plaque morphology. Star plaques have a central zone which looks like a usual plaque; however, surrounding the plaque are areas of lysis which give the plaque a sectored, "starlike" appearance. Upon replating, the same sectored plaques are found together with r plaques in approximately equal numbers. The r plaques when replated give

only r plaques. Hershey (1946) explained these observations by the proposal that star mutant phage have an increased frequency of mutation.

In the hope of studying mutations which affect the mutation rate, Symonds (1958) and McFall and Stent (1958) studied the genetics of the star phenotype in phage T2. They showed that star mutant phage differ from wild type phage by a single mutation at a site, s, closely linked to the rI site and that r mutants found in star plaques contain not only an r mutation but also the original s mutation. These observations were explained on the idea that 1) s mutant phage grow slower than either wild-type or r mutant phage; 2) r mutations suppress the s phenotype; and 3) the high proportion of r mutants in star plaques is due to selection of r mutants during growth of s phage. On this notion an s mutation results in a decreased rate of phage growth and the r mutants in star plaques arise by selective advantage rather than as a result of an increased mutation rate due to the s mutation.

Symonds (1958) ruled out the possibility that s phage had either a relative delay in or a decreased rate of intracellular phage synthesis. Yet while the rise of either s or s:r infective centers began between 20 and 30 min, the increase in s infective

centers continued for at least 2 hr. This observation suggested that in contrast to cultures infected with s:r double mutant phage, which lyse more or less synchronously at 30 min, lysis is prolonged in cultures infected with s mutant phage. The net effect of lysis delay due to the s mutation is that during multiplication, r phage go through more growth cycles than do s phage and as a result, accumulate more rapidly.

Manuscript I in the results section of this thesis describes the isolation of a new class of lysis-defective mutants of phage T4 which define a new T4 gene, gene t. As expected, during infection t-defective mutants synthesize both phage and phage lysozyme, yet fail to either cease metabolism or lyse suggesting that functional t gene product is required for the cessation of metabolism and consequently lysis at the usual time. Surprisingly the sites of the s and t mutations on the phage genome are quite distant from each other suggesting that T4 codes for at least two factors required for the cessation of host metabolism at lysis.

The Disruption of the Host Cytoplasmic Membrane at the Usual Lysis Time

The mechanism of cessation of infected cell metabolism at lysis may involve the disruption of the host cytoplasmic membrane. Examining thin section of cells infected with e:rII mutant phage, Sechaud et al. (1967) found that at 60 min the rodlike cells were

full of progeny phage surrounded by an intact cell wall suggesting that a complete phage growth cycle had occurred yet the cells failed to lyse as lysozyme was not synthesized. Nevertheless, the cell interior did not appear electron dense and the cytoplasmic membrane was not readily visible, suggesting that the membrane had lost its integrity and allowed the cytoplasm to leak into the surrounding medium. Furthermore, in e:rII infected cultures prelabeled with P^{32}, the rate of release of P^{32} into culture filtrates markedly increased between 20 and 30 min. Independently Bode (1967) using the luciferin-luciferase assay showed that in e mutant infected cultures, the rate of release of ATP into culture filtrates markedly increased at 30 min. These observations suggest that phage infection induces the disruption of the host cytoplasmic membrane at lysis and that this accounts for the cessation of host metabolism.

Chemicals which Induce Premature Lysis

A variety of treatments induce premature lysis of phage-infected cultures. Doermann (1952) studied the effect of cyanide on T4 r^{48} mutant-infected cultures. Addition of cyanide during the first half of the latent period prevented any later rise in infective centers and any later decrease in culture turbidity

suggesting that lysis was prevented under these conditions. Nevertheless, during the last half of the latent period, addition of cyanide resulted in an increase in infective centers and an immediate decrease in culture turbidity suggesting that cyanide induced premature lysis and liberation of progeny phage. Since cyanide blocks respiration, artificial inhibition of host metabolism during the last half of the latent period induces premature lysis.

Brown (1956) made a systematic study of the ability of various chemicals to induce premature lysis of T6-infected cultures. He failed to find an agent which induced premature lysis when added during the first half of the latent period. During the last half of the latent period, immediate lysis was induced by chloroform or cetylpyridinium chloride, a cationic surfactant; delayed lysis was induced by phenol, ethyl or amyl acetate, xylene, toluene, and cyanide (ordered with respect to increasingly delay).

Manuscript II shows that since artificial inhibition of host metabolism induces infected cell lysis only if the t gene is functional, the function of the t gene product is not solely to block host metabolism. This result shows that cessation of host metabolism is not the primary cause of infected cell lysis and leaves open the possibility that the t gene product may be involved in the disruption of the host cytoplasmic membrane.

Hydrolysis of Host Membrane Phospholipid During Infection

The idea that phage infection involves the disruption of the host cytoplasmic membrane together with the observation that phospholipid accounts for 30% of the mass of the E. coli membrane (Kaback and Stadtman, 1966) led Cronan and Wulff (1969) to measure phospholipid hydrolysis during T4 phage infection. They showed that the free fatty acid (FFA) produced during infection arises from hydrolysis of host membrane phospholipid, and that FFA production therefore reflects phospholipid hydrolysis. During infection at 37°, production of FFA began at 10 min and increased linearly such that at 25 min, the lysis time of the culture, the level of FFA was 50-fold greater than that found in uninfected cells. Since addition of puromycin (but not of chloramphenicol) at 0 min inhibited production of FFA, Cronan and Wulff (1969) proposed that hydrolysis of host membrane phospholipid, and consequently FFA production, is a phage-induced function which may be involved in the phage lysis mechanism.

Manuscript III investigates in general the role of T4 infection and in particular that of the t gene product in host phospholipid hydrolysis. The results show that T4 infection does not necessarily alter the rate of phospholipid hydrolysis detected in uninfected cells and that uninfected cells contain a latent phospholipid hydrolyzing activity which becomes activated upon

lysis of the cells by any of a number of treatments, including T4 infection. No evidence was obtained for the idea that host phospholipid hydrolysis plays any role specific to the phage life cycle.

Lysis Can Be Naturally Delayed

Any model of phage-induced host lysis must account for "lysis inhibition." The phenomenon of lysis inhibition (Doermann, 1948) is observed as the difference in the clearing times of cultures infected with either r^+ or r mutant phage. As measured in a one step growth experiment, the latent period for either wild-type of r mutant T-even phage grown at 37° is 25 min. Yet when infected with wild-type phage, a visably turbid culture will not become clear for several hours. When infected with r mutant phage, the culture becomes clear at 25 min. The notion of lysis inhibition may also account for the difference in plaque morphologies of wild-type and r mutant phage (Hershey, 1946). While wild-type phage form small plaques with turbid halos, r mutant phage form large plaques with clear halos. Doermann (1948) explained these observations on the idea that a diffusible factor inhibits the lysis of infected bacteria. Normal lysis is seen only in dilute cultures, in which the concentration of factor is too low to inhibit lysis, or in cultures infected with r mutant phage, which have lost the ability to be lysis inhibited. Doermann (1948)

isolated an activity from lysates of wild-type infected cultures which could inhibit the timing of phage bursts in dilute culture of wild-type but not of r mutant infected cells. Since activity was found in preparations of purified phage, and had the same sedimentation rate, sensitivity to anti-phage serum, and host-range as phage, Doermann concluded that lysis inhibition is caused by the secondary adsorption of phage, arising experimentally by addition of phage during infection, or naturally by the progeny of the infected cells which are the first to lyse. Arguments based on the kinetics of lysis inhibition showed that one, or at most a few, phage are required to cause lysis inhibition and that the primary and secondary infections must be separated by a few minutes.

Though Doermann (1948) inferred that the genome of the superinfecting phage plays some role in the induction of lysis inhibition, both T2 phage inactivated with either X-rays or ultraviolet light (Watson, 1950) and T4r phage (Stent and Maaloe, 1953) induce lysis inhibition. Since the X-ray treated T2 phage had lost 98% of its cell killing titer, the ability to adsorb to bacteria may be the sole requirement for superinfecting phage in their induction of lysis inhibition.

Since uptake of oxygen (Mukai et al., 1967), synthesis of both phage (Doermann, 1948) and phage lysozyme (Bode, 1967), and the

low rate of ATP leakage into the medium (Bode, 1967) continue past the usual lysis time in superinfected cultures, superinfection not only delays lysis but also allows host metabolism to continue past the usual lysis time. Thus superinfection appears to prevent the cessation of host metabolism at the usual time which in turn causes lysis to be inhibited.

Manuscript I shows that t mutant-infected cells continue to synthesize both phage and phage lysozyme past the usual lysis time. Therefore t mutant-infected cells have the same phenotype as superinfected cells and thus appear to be "auto-lysis inhibited."

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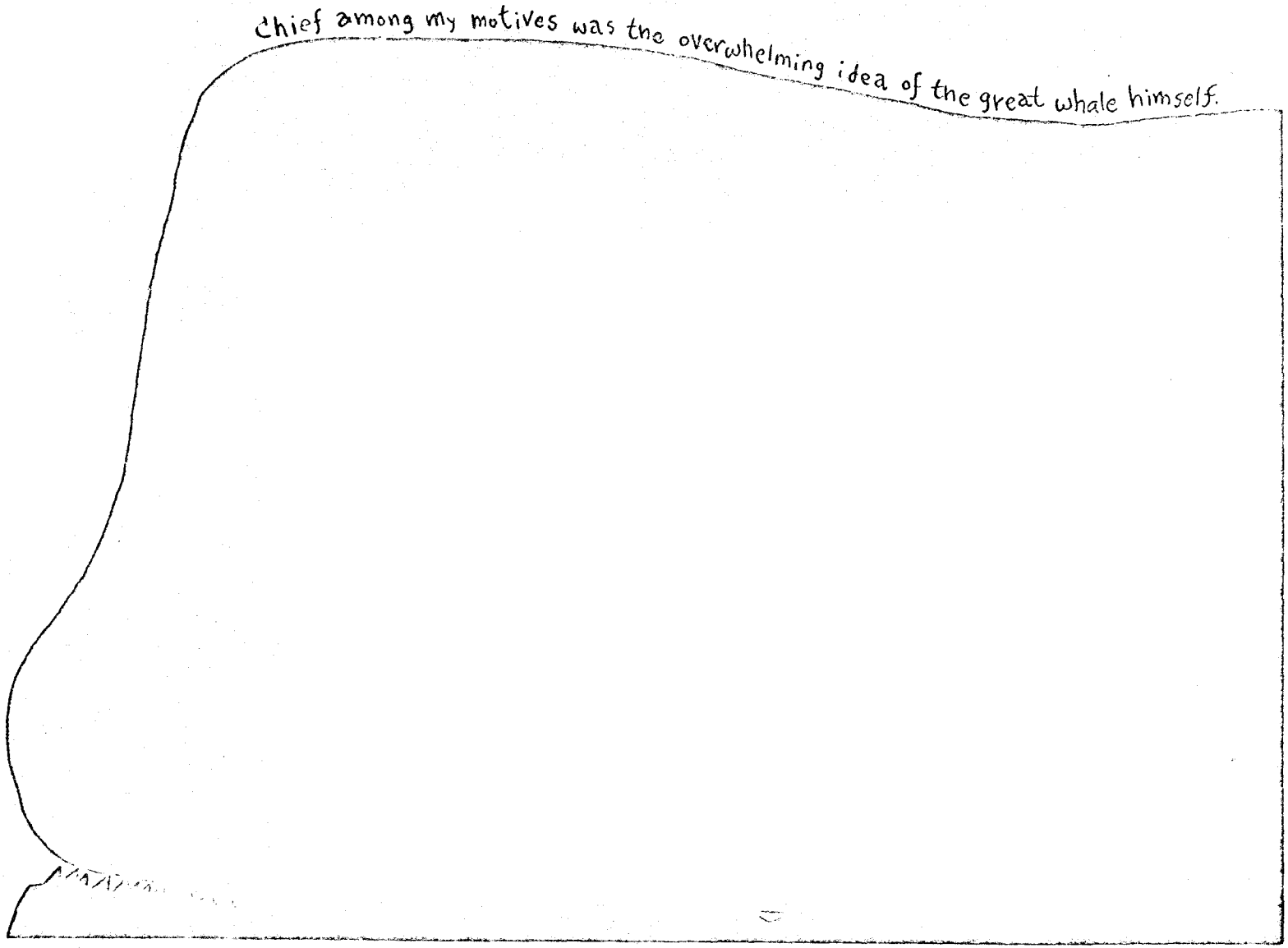
Chief among my motives was the overwhelming idea of the great whale himself.

The Lysis Mechanism of Phage T4:

Mutants Affecting Lysis

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SUMMARY

A selection technique is used to isolate a new class of lysis-defective mutants of phage T4, the T4 amber t mutants. Under normal conditions, T4-infected bacteria lyse at a characteristic time after infection, each infected cell releasing a few hundred progeny phage. However, Su^- bacteria infected with amber t mutant phage fail to lyse and continue to manufacture phage beyond the normal latent period even though phage lysozyme is present. Genetic crosses and complementation tests show that t mutant phage define a new T4 gene, gene t. Functional t gene product is required for the cessation of phage-infected cell metabolism at the characteristic lysis time. The T4 lysis mechanism is shown to be a sequential process of at least two steps: function of phage t gene product followed by function of phage e gene product. The e gene product, phage lysozyme, is known to degrade the host cell wall. It is proposed that the t gene product may be involved in degradation of the host cytoplasmic membrane.

INTRODUCTION

Thirty years ago Ellis and Delbrück (1939) showed that E. coli bacteria infected with bacteriophage burst and release their accumulated progeny virus at a characteristic time after infection. However the mechanism of this lysis and its temporal control are still poorly understood. The T4-induced enzyme, phage lysozyme, is responsible for the degradation of the bacterial cell wall (Streisinger et al., 1961). Although cells infected with mutants unable to induce the synthesis of lysozyme fail to lyse, the cells nevertheless cease metabolism at the characteristic time (Mukai et al., 1967). These facts suggest that T4-infected cell lysis may be at least a two-step process: the phage genome directing the synthesis of two lysis factors--one which degrades the host cytoplasmic membrane resulting in the cessation of infected cell metabolism and the other, phage lysozyme, which then degrades the cell wall releasing the progeny phage. If this picture is correct, it should be possible to obtain phage mutants unable to synthesize the hypothesized cell membrane lysis factor. It would be expected that cells infected with such mutants would continue to manufacture phage beyond the characteristic latent period time even though phage lysozyme is present. This paper describes the isolation and characterization of mutants of phage T4 with this expected lysis-defective phenotype.

MATERIALS AND METHODS

Phage and bacterial strains. Phage and bacterial strains were from the collection of R. S. Edgar. Phage amber mutants are defined by their ability to form plaques on the permissive host, Escherichia coli CR63, but not on the restrictive host, E. coli S/6. The E. coli K12 amber suppressor host strains employed were S26(λ) (Su^-), S26(λ) RIE (Su-I), S26(λ) RID (Su-II) originally obtained from A. Garen and K110(λ) (Su-III) obtained from R. Haselkorn. All experiments with phage and bacteria were performed at 30° unless otherwise indicated.

Preparation of phage and bacteria. Phage stocks were made by the confluent layer technique (Adams, 1959). Plating bacteria were prepared as in Edgar and Lielausis (1964). Host bacteria were similarly prepared except that the initial dilution was by a factor of 500 instead of 100.

Media and reagents. H broth was used as growth medium and diluting fluid for both phage and bacteria. EHA bottom agar and EHA top agar were used as media for plates. In the preparation of 2-aminopurine-treated phage, T broth instead of H broth was used. The recipes for these media are given by Steinberg and Edgar (1962). Chloroform was Mallinckrodt analytical grade. Dow-Corning antifoam A was frequently used as an antifoaming agent. Mutagens employed, 2-aminopurine (2-AP) and 5-bromodeoxyuridine (5-BUdR), were obtained

from California Corporation for Biochemical Research, and Cyclochemical Corporation, respectively. Egg white lysozyme (grade I, 3X crystallized) was obtained from Sigma Chemical Corporation. Anti-T4 antiserum was a gift from J. E. Flatgaard.

Treatment of phage with mutagen. 2-AP-treated phage: Log phase CR63 bacteria grown in T broth and concentrated to 10^9/ml were incubated for 10 min with T4D wild-type phage at 5 x 10^9/ml. The reaction mixture was then diluted 10-fold into T broth containing 2-AP at 500 μg/ml, aerated for 60 min and then lysed with chloroform. A dilution of this lysate was used as a source of mutants.

5-BUdR-treated phage: The same procedure was used for the preparation of 5-BUdR phage as was used for 2-AP phage, except that H broth rather than T broth was used for bacterial growth medium. 5-BUdR was used at a concentration of 5 μg/ml. Control experiments showed that mutagen treatment at these concentrations increased the frequency of r mutants in the progeny from 0.2 to 0.7%. This level of mutagen treatment was chosen to minimize the frequency of multiple mutations.

Mutant selection. S/6 bacteria at 4 x 10^8/ml were infected with an equal volume of mutagen-treated phage at 4 x 10^7/ml. The reaction mixture was incubated 5 min at 39.5°C then diluted by 10^6 into H broth at 39.5°C. At 36 min, the culture was filtered through

a Millipore filter apparatus (0.45 μ pore size). The filter was washed with H broth at 39.5°C by filtration and resuspended in H broth saturated with chloroform. Free virus in this suspension represent the progeny of the selection. Control experiments showed that this filtration procedure quantitatively separated bacteria from phage.

RESULTS

Mutant Selection

A selection procedure was designed to select for conditional lethal mutant phage, either temperature sensitive or amber, which would synthesize phage lysozyme but would not lyse their host bacterium during the selection growth cycle. Restrictive bacteria were infected with mutagen-treated phage and grown at the restrictive temperature. At a time after lysis normally occurs at this temperature, the culture was filtered to separate intact bacteria from free phage and the filter was washed with chloroform-saturated broth which should lyse only those infected bacteria which contain phage lysozyme. A phage stock grown under permissive conditions (in CR63 at 30°C) was made from this lysate for use as parental phage for a further cycle of selection.

After four cycles of selection, as described above for both 2-AP-and 5-BUdR-treated phage, the lysates were plated with CR63. Five isolated plaques from the selection with 2-AP-treated phage

and five from that with the 5-BUdR-treated phage were transferred onto a pair of plates, one seeded with S/6 and the other with CR63. In both cases one of the five phage which plated on CR63 failed to plate on S/6. These tentative lysis-defective amber mutants were designated amA3 (2-AP) and amB5 (5-BUdR).

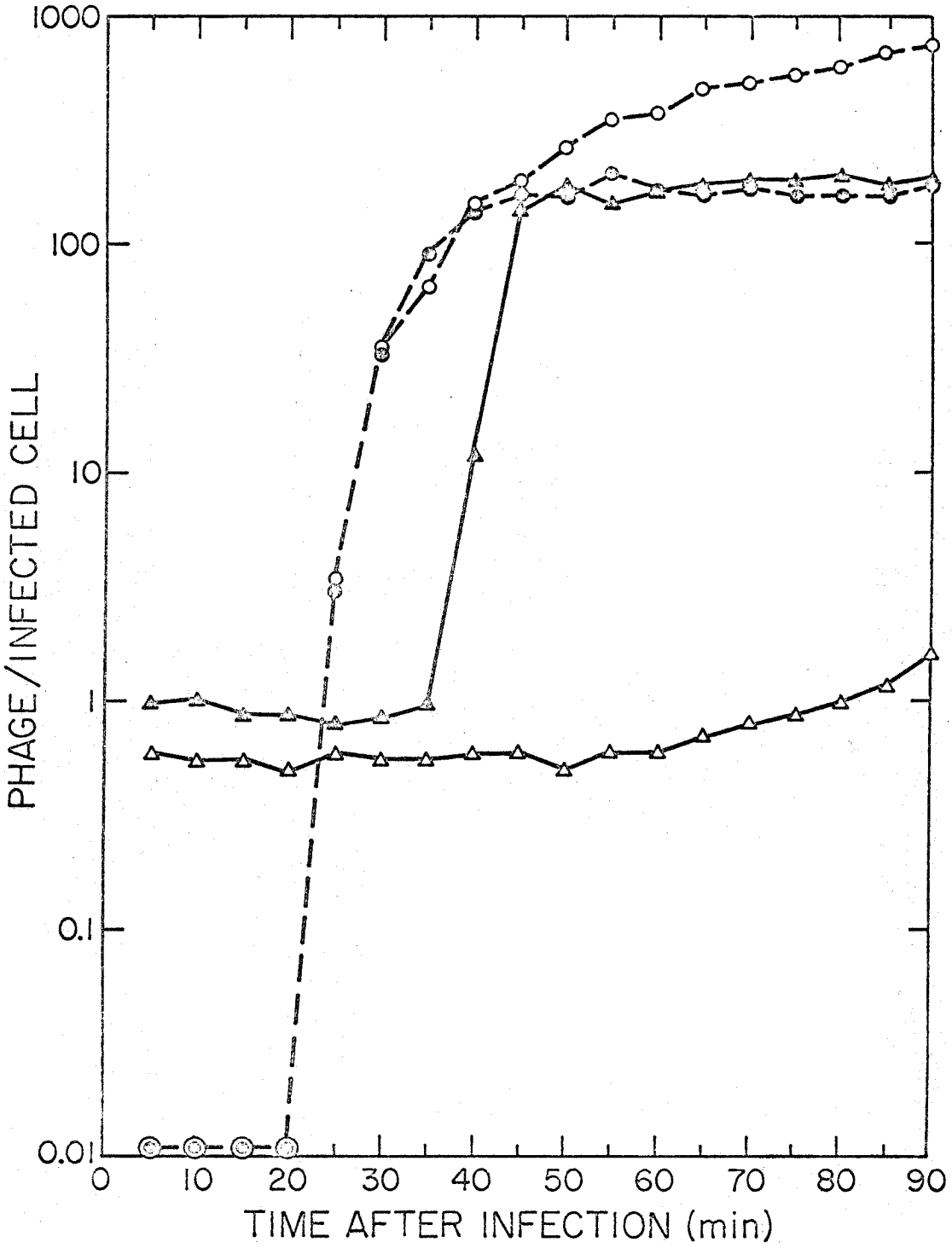
In the hope of eliminating possible multiple mutations in amA3 and amB5, each was backcrossed to wild-type phage at a multiplicity of infection of 1.5 mutant phage and 13.5 wild-type phage. One clone of mutant genotype was reisolated from the progeny of each cross.

In the permissive host, CR63, amA3 and amB5 give plaques which are indistinguishable in morphology from wild-type plaques. In the restrictive host, S/6, amA3 and amB5 plate with efficiencies relative to CR63 of about 10^{-5} at 18°, 10^{-2} at 30°, and 0.5 at 42°. The resulting plaques appear either sectored or r-like and are readily distinguishable from wild-type plaques.

Are amA3 and amB5 Lysis-Defective Mutants?

One-step growth experiments were performed with amA3, amB5 and am^+ (wild type) phage under restrictive (S/6 bacteria) and permissive (CR63 bacteria) conditions at 30°C as shown in Fig. 1. The data are not shown for either amB5-infected S/6, as it behaved like amA3-infected S/6, or for am^+- or am-infected CR63, as they behaved like am^+-infected S/6. These results show that all the phage growth cycles in CR63 and and am^+ growth cycle in S/6 are identical.

Fig. 1 One step growth curves of phage-infected cells. S/6 bacteria $4 \cdot 10^8$/ml. in 4 mM KCN were infected with an equal volume of phage at $4 \cdot 10^7$/ml. The reaction mixture was incubated for 10 min then diluted by at least $4 \cdot 10^4$-fold into growth tubes (time t = 0). Platings from appropriate tubes before (▲,Δ) and after (●,○) treatment with $CHCl_3$ represent infective centers and intracellular phage, respectively. ▲ and ●) $\underline{am}^+$; Δ and ○) $\underline{am}$tA3.

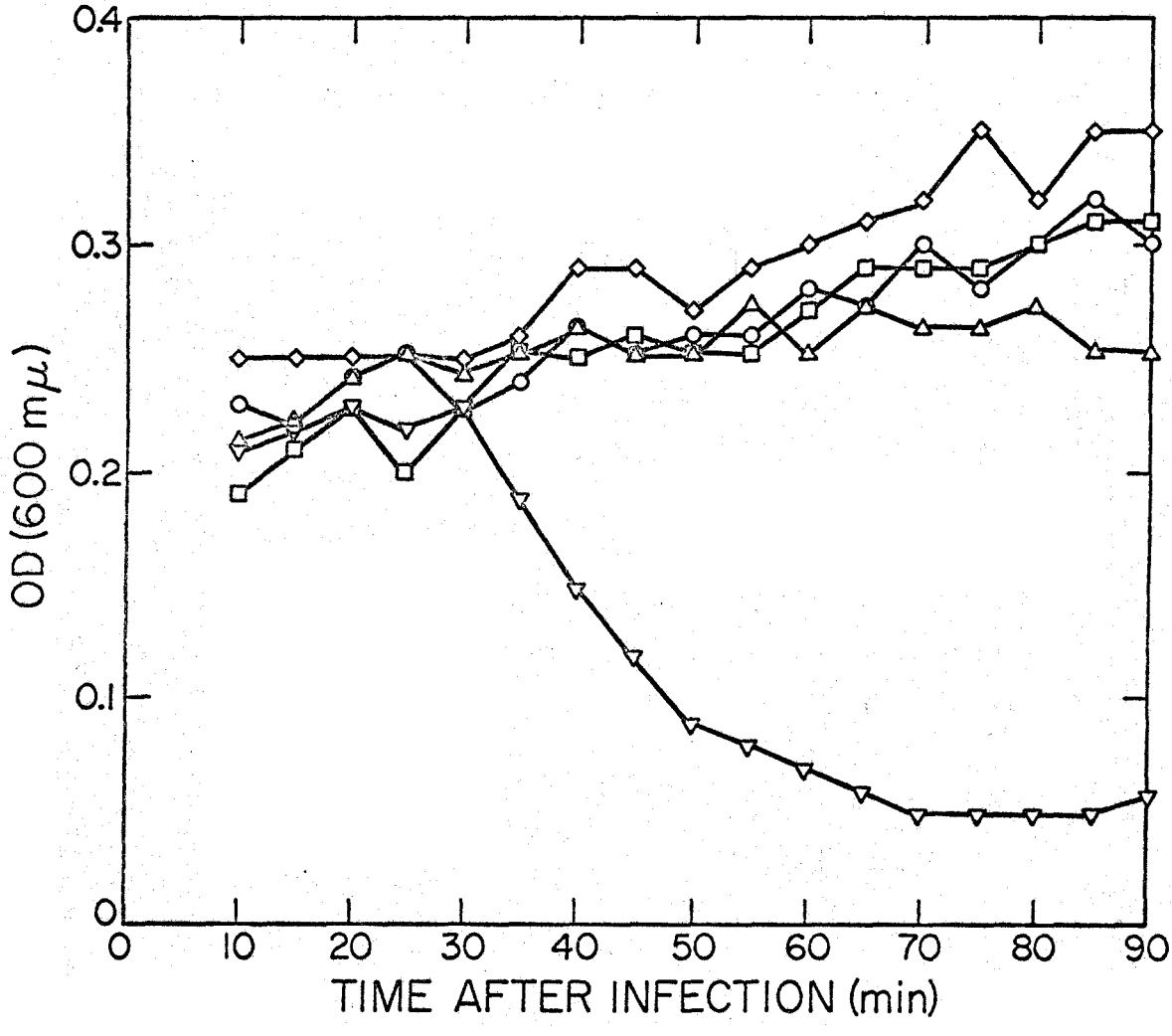

1000
100
10
1
0.1
0.01
PHAGE/INFECTED CELL
0
10
20
30
40
50
60
70
80
90
TIME AFTER INFECTION (min)

I define the lysis time of a phage-infected culture as the time at which half of the progeny phage have been spontaneously released. In these four cases the phage-infected cells lyse at 42 minutes after infection, each infected cell releasing an average burst of 150 progeny virus into the medium. However, for S/6 cells infected with either amA3 or amB5, by 90 minutes only an average of 1 phage per infected cell has been spontaneously released. Nevertheless the infected cells readily release phage after treatment with chloroform. Furthermore, in both cases synthesis of viable intracellular phage (as determined by chloroform treatment) continues past the characteristic lysis time and by 90 minutes every infected cell contains an average of 750 viable progeny phage.

To determine whether the low spontaneous burst size of S/6 bacteria infected with amA3 phage was due to a low yield from each infected cell or to a normal yield from a fraction of the infected cells, a single burst experiment was performed as described by Emrich (1968). This experiment showed that by 180 min in cultures of amA3-infected S/6 bacteria, only a few percent of the infected cells had lysed, each releasing a large burst of progeny phage.

Infected cell lysis can also be measured optically by the decrease in turbidity of concentrated infected cell cultures. As shown in Fig. 2, the turbidity of amA3- or amB5-infected S/6 cultures increases for at least 90 min after infection. Therefore neither of these infected cultures has lysed by 90 min. The

Fig. 2 Turbidity of infected cell cultures. S/6 bacteria at $2 \cdot 10^9$/ml. were infected with an equal volume of phage at $6 \cdot 10^9$/ml. After 10 min of incubation, the reaction mixture was diluted 10-fold into a growth tube and aeration was begun (time t = 0). Optical density was measured at 600 mμ with a Bausch & Lomb Spec-20 spectrophotometer. ▽) amB25; △) am^+; 0) amtB5; □) amB25:amtA3; ◇) amtA3.


0.4
0.3
0.2
0.1
0
OD (600 mμ)
0
10
20
30
40
50
60
70
80
90
TIME AFTER INFECTION (min)

turbidity of the $\underline{am}^+$-infected S/6 culture increases for 75 min then begins to decrease. Presumably the $\underline{am}^+$-infected culture has become lysis-inhibited due to superinfection (Doermann, 1948). To exclude possible superinfection by viable progeny phage, turbidity measurements were made with S/6 infected with amB25 (gene 34) and with a double mutant amB25:amA3. Under restrictive conditions these strains produce non-infective, tail-fiberless particles which cannot induce superinfection. The amB25-infected S/6 culture lysed at 45 min, yet lysis was not observed in the amB25:amA3-infected S/6 culture for at least 90 min.

These experiments reveal two aberrant features of growth of amA3 and amB5 under restrictive conditions: the absence of lysis at the characteristic time and the continuation of intracellular phage synthesis beyond the characteristic lysis time. This lysis-defective phenotype I call the t phenotype (for Tithonus).

The Mutations Responsible for the Lysis-Defective Phenotype of t Mutants

To eliminate the possibility that the t phenotype of amtA3 and amtB5 was due to strain differences in the bacteria other than the presence or absence of amber nonsense suppressors, one-step growth experiments were performed with K12 suppressor host strains infected with amtA3, amtB5, and $\underline{am}^+$ phage. All the phage growth

cycles in Su-I, Su-II, and Su-III bacteria and the $\underline{am}^+$ growth cycle in Su^- bacteria were identical. However amtA3- and amtB5-infected Su^- bacteria exhibited the lysis defective, t mutant phenotype (i.e., delayed spontaneous phage release and continued intracellular phage synthesis). Therefore, the mutations in amtA3 and amtB5 responsible for the t phenotype are nonsense mutations suppressible by host strains carrying amber suppressors.

Data from genetic crosses between amtA3 and amtB5 and of these mutants to other T4 amber mutants, are presented in Fig. 3. These results show that the amber mutations in amtA3 and amtB5 are at separate closely-linked sites. Furthermore, both sites lie between sites defined by amber mutations in genes 38 and 52. Cells infected with phage mutants in genes 38 and 52 are defective in phage tail fiber synthesis and phage DNA synthesis, respectively (Edgar and Wood, 1966).

Lysis Phenotype of Mixedly Infected Cells

One-step growth experiments were performed with S/6 bacteria mixedly infected with t^+ and t mutant phage as shown in Fig. 4. $\underline{am}^+$-infected cells lysed at 45 min whereas those infected with amtA3 phage did not lyse. Bacteria mixedly infected with equal amounts of $\underline{am}^+$ and amtA3 phage lysed at 54 min. The lysis properties of bacteria mixedly infected with amtA3 and amtB5

Fig. 3 Mapping data for mutants in genes 38, t, and 52. Genetic crosses were performed in CR63 bacteria (moi = 10) as described in Edgar and Lielausis (1964). Recombination frequencies are expressed as 200 X the ratio of progeny which plate with S/6 bacteria relative to that with CR63 bacteria.

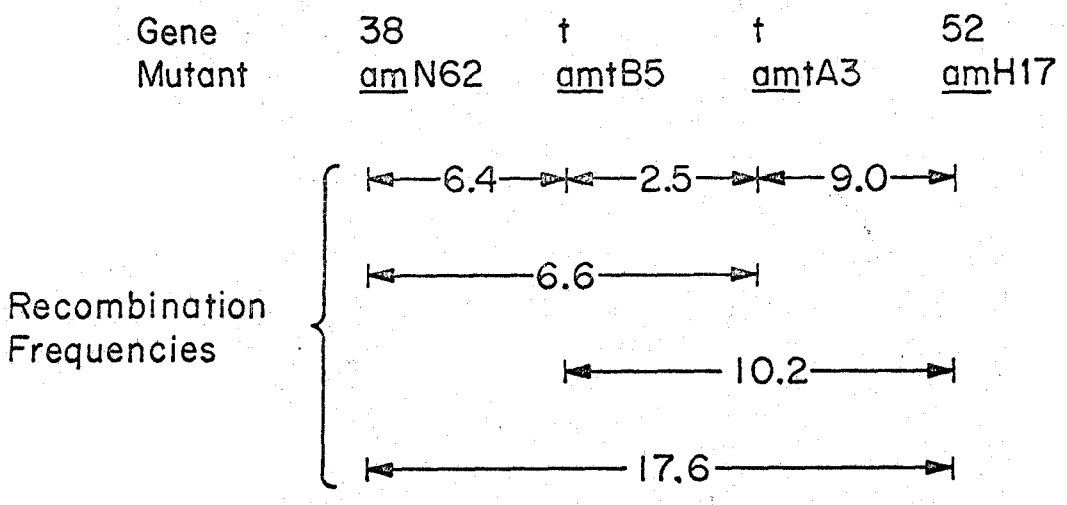
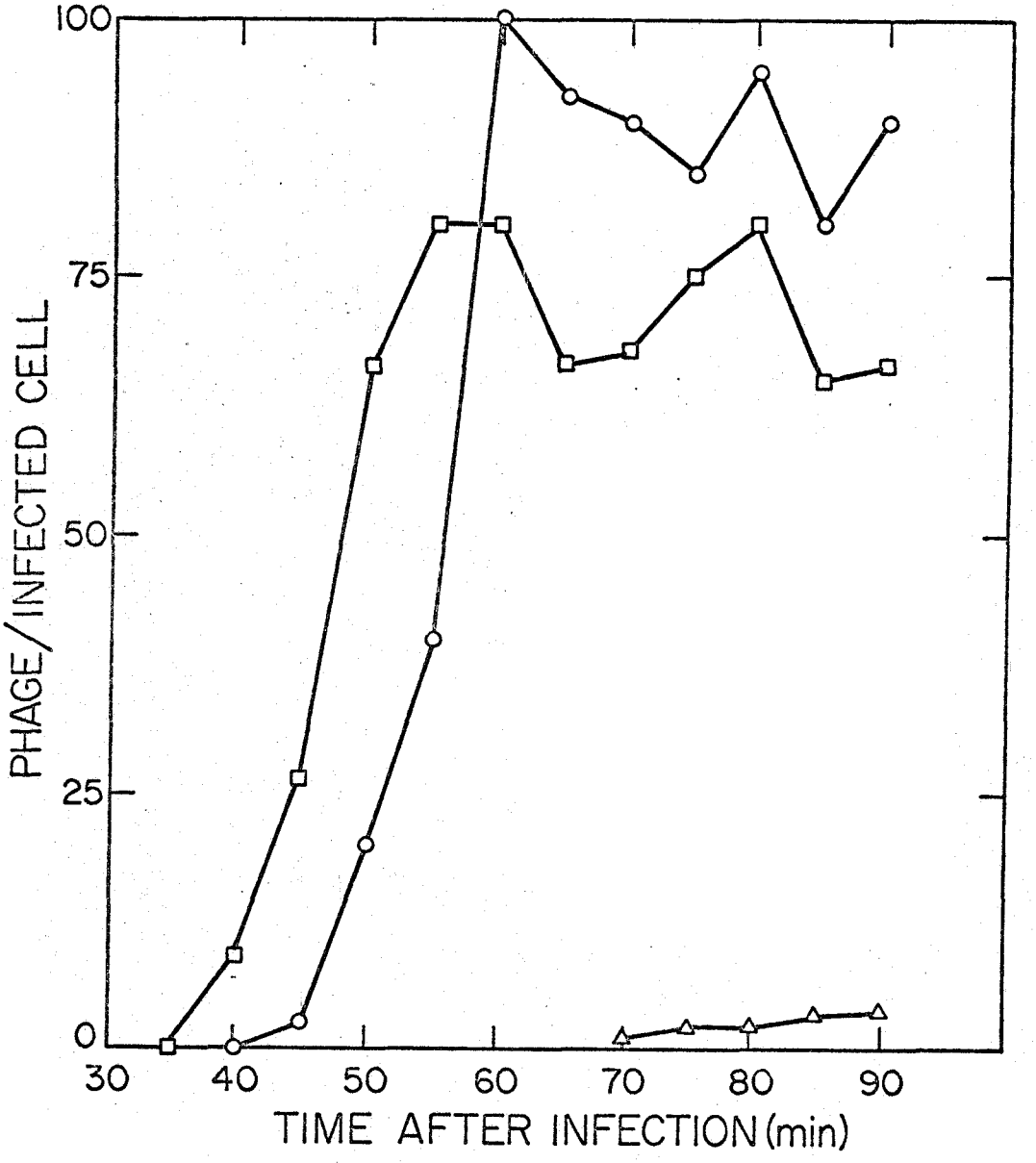

Gene
Mutant
38
amN62
t
amtB5
t
amtA3
52
amH17
Recombination
Frequencies
6.4
2.5
9.0
6.6
10.2
17.6

Fig. 4 One step growth curves of mixedly-infected cells. S/6 bacteria were infected as described for genetic cross procedure. Anti-T4 serum (K = 2/min final concentration) was added to the adsorption tube for the last 5 min of adsorption. The reaction mixture was diluted by $5 \cdot 10^6$ into a growth tube (time = 0). Samples from the growth tube were plated for infective centers. □) $\underline{am}^+$ alone; 0) $\underline{am}^+$ and $\underline{amt}$A3; Δ) $\underline{amt}$A3 alone; $\underline{amt}$A3 and $\underline{amt}$B5.


100
75
50
25
0
PHAGE/INFECTED CELL
30
40
50
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70
80
90
TIME AFTER INFECTION (min)

were indistinguishable from those infected with amtA3 alone. These results show that the t phenotype is due to the absence of a product that is normally present in t^+ phage-infected cells, since in mixed infection t^+ is dominant to t in permitting relatively normal lysis to occur. The lack of complementation between amtA3 and amtB5 indicates that both mutants are defective in the same gene. Therefore the t mutants define a new gene, gene t, which lies between genes 38 and 52 on the T4 genetic map.

The observation that am^+-infected cells lyse at 45 min while cells mixedly infected with t and t^+ phage lyse at 54 min suggests that perhaps the lysis time of phage-infected cells is determined by the fraction of t^+ genes present in the infected cell. To test this idea, S/6 bacteria were mixedly infected at a total multiplicity of 10 phage/cell but at various t^+/t input ratios. One-step growth experiments were performed with cultures infected with eight different t^+/t phage input ratios. As shown in Table 1, the lysis times of these gene dosage experiments show that Su^- cells infected with various ratios of amtA3 and t^+ phage lyse at a time proportional to the fraction of t genes in the infected cell.

Lysozyme Activity in amtA3-Infected S/6 Bacteria

amtA3-infected S/6 bacteria lyse upon the addition of chloroform. This observation suggests that these infected cells contain lysozyme. To show that this is the case, extracts of infected cells were

TABLE 1

Lysis Times of Mixedly Infected Cells at Various t^+/t Phage Input Ratios

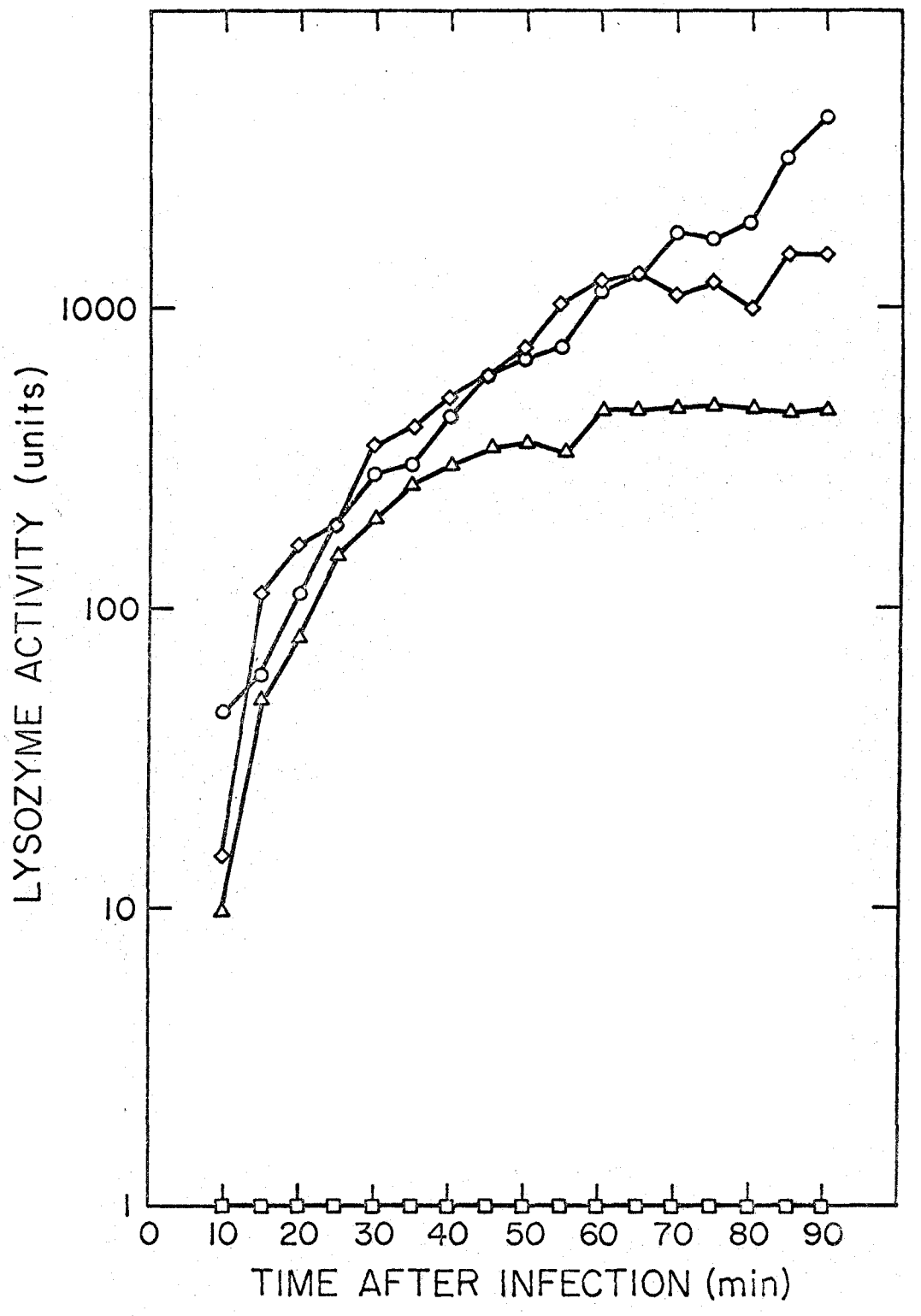
Multiplicity of t^+ phage	10	7.5	6.7	6.0	5.0	4.0	3.3	2.5
Multiplicity of amtA3 phage	0	2.5	3.3	4.0	5.0	6.0	6.7	7.5
t^+/t input ratio	1:0	3:1	2:1	3:2	1:1	2:3	1:2	1:3
Lysis time (min)	37.5	41.5	43.5	44.5	46.5	49.5	51	53

assayed for lysozyme activity as shown in Fig. 5. Lysozyme activity was undetectable (<1 unit/ml) in the amH26 (lysozyme defective)-infected cell extracts. Lysozyme activity in amB25-infected cells was found at 10 min after infection and increased at the rate of 10 units/ml/min until 60 min when it reached a maximum of 450 units/ml. In am^+-infected cells, lysozyme activity was found at 10 min and increased at the rate of 10 units/ml/min. By 90 min the lysozyme activity was 1500 units/ml. Finally, in amB25:amtA3-infected cells, lysozyme activity was found at 10 min and increased at the rate of 15 units/ml/minute, but later the rate increased. By 90 min the lysozyme activity in amB25: amtA3-infected cells was 4500 units/ml, 10 times greater than that in amB25-infected cells. t mutant-infected cells fail to lyse, but not for lack of lysozyme.

DISCUSSION

These experiments describe a new class of lysis-defective mutants of phage T4--the amber t mutants. Under normal conditions, T4-infected cells lyse at a characteristic time after infection, each infected cell releasing a few hundred progeny phage. On the other hand, Su^- cells infected with amber t mutant phage fail to lyse and continue to manufacture phage even though phage lysozyme is made. At times much later than the normal lysis time, t mutant-infected cells contain an average of five times both the progeny

Fig. 5 Lysozyme activity in extracts of infected cells. S/6 bacteria were infected as described in the legend to Fig. 2. Samples were plunged into a dry ice/acetone bath and stored at -30°. Before assaying for lysozyme activity, the samples were incubated at 30° for 5 min then stored at 0°. Lysozyme activity was measured by assay 2 of Tsugita et al. (1968) modified by incubation for 30 min at 30°. A standard curve for egg white lysozyme was constructed by plotting various concentrations of egg white lysozyme against absorbance. The relative activity of phage lysozyme sample was determined by referring the absorbance to this standard curve. Absorbance was measured at 350 mμ with a Zeiss PMQ-II spectrophotometer. One unit of phage lysozyme activity is defined as the lysozyme activity of 1 mμg of egg white lysozyme. □) amH26; Δ) amB25; ◇) am^+; O) amB25:amtA3.


LYSOZYME ACTIVITY (units)
1000
100
10
1
0
10
20
30
40
50
60
70
80
90
TIME AFTER INFECTION (min)

phage and the lysozyme activity produced during wild-type infection. This lysis-defective phenotype is shown by the amber t mutants in Su^- but not in Su^+-infected cells. Genetic crosses indicate that t mutant phage contain amber mutations at closely linked sites which lie between genes 38 and 52 on the T4 genetic map. Complementation tests with mixedly infected Su^- cells show that the defective phenotype of t mutant phage-infected cells results from the absence of a product found in wild-type infected cells. These data show that the t mutant phage define a new gene in phage T4, gene t.

I conclude that functional t gene product is required for cessation of metabolism in phage-infected cells at the characteristic lysis time. Without such a metabolic cessation, the e gene product, phage lysozyme, cannot function to degrade the host cell wall. Nevertheless Mukai et al. (1967) have shown that in the absence of host cell wall degradation, infected cell metabolism stops at the characteristic lysis time. Therefore the lytic mechanism operating in wild-type T4 phage-infected cells is a sequential process of at least two steps: function of t gene product followed by function of e gene product.

The T4 lysis mechanism appears analogous to that of phage λ (Harris et al., 1967). The function of T4 gene t or that of λ gene S is required for the cessation of phage-infected cell metabolism at the characteristic lysis time. And the function of

T4 gene e or that of λ gene R is required for the degradation of the host cell wall. Nevertheless this analogy does not entirely extend to the lytic mechanism employed by the smaller DNA coliphage, ØX174 (Hutchison and Sinsheimer, 1966) and S13 (Baker and Tessman, 1967). Although these phage have a gene (cistron I and cistron V, respectively) whose function seems analogous to that of T4 gene t, no phage-induced lysozyme activity can be found in ØX174-infected cell extracts (Fujimura and Kaesberg, 1962; Eigner et al., 1963; Markert and Zillig, 1965; Hutchison, personal communication).

The mechanism of action of functional t gene product remains to be determined. The t gene dosage experiment suggests that the time of lysis depends on the fraction of functional t genes in the infected cell. Sechaud et al. (1967) found the cessation of metabolism in T4-infected cells correlated in time with the degradation of the cytoplasmic membrane and the breakdown of the permeability barrier.

These data are consistent with the hypothesis that t gene product has antimembrane activity. When a certain amount of t gene product has been synthesized, it degrades the cytoplasmic membrane. Metabolism ceases. Lysozyme, compartmentalized in the cytoplasm, gains access to its substrate, the host cell wall. The cell wall is degraded. Progeny phage are released. Some alternative hypotheses are the following:

1) t gene product blocks metabolism causing secondarily the

degradation of the cytoplasmic membrane.

2) t gene product modifies the cytoplasmic membrane so that it is sensitive to a membrane degrading factor.

3) t gene product modifies the cell wall so that it is sensitive to phage lysozyme.

4) t gene product activates phage lysozyme.

Work is in progress to illuminate the mechanism of action of t gene product.

ACKNOWLEDGMENTS

I have profited from and enjoyed the constructive criticism and moral support from R. S. Edgar as well as from W. B. Wood, R. L. Russell, J. A. King, J. S. Parkinson, S. K. Beckendorf and the late J. J. Weigle.

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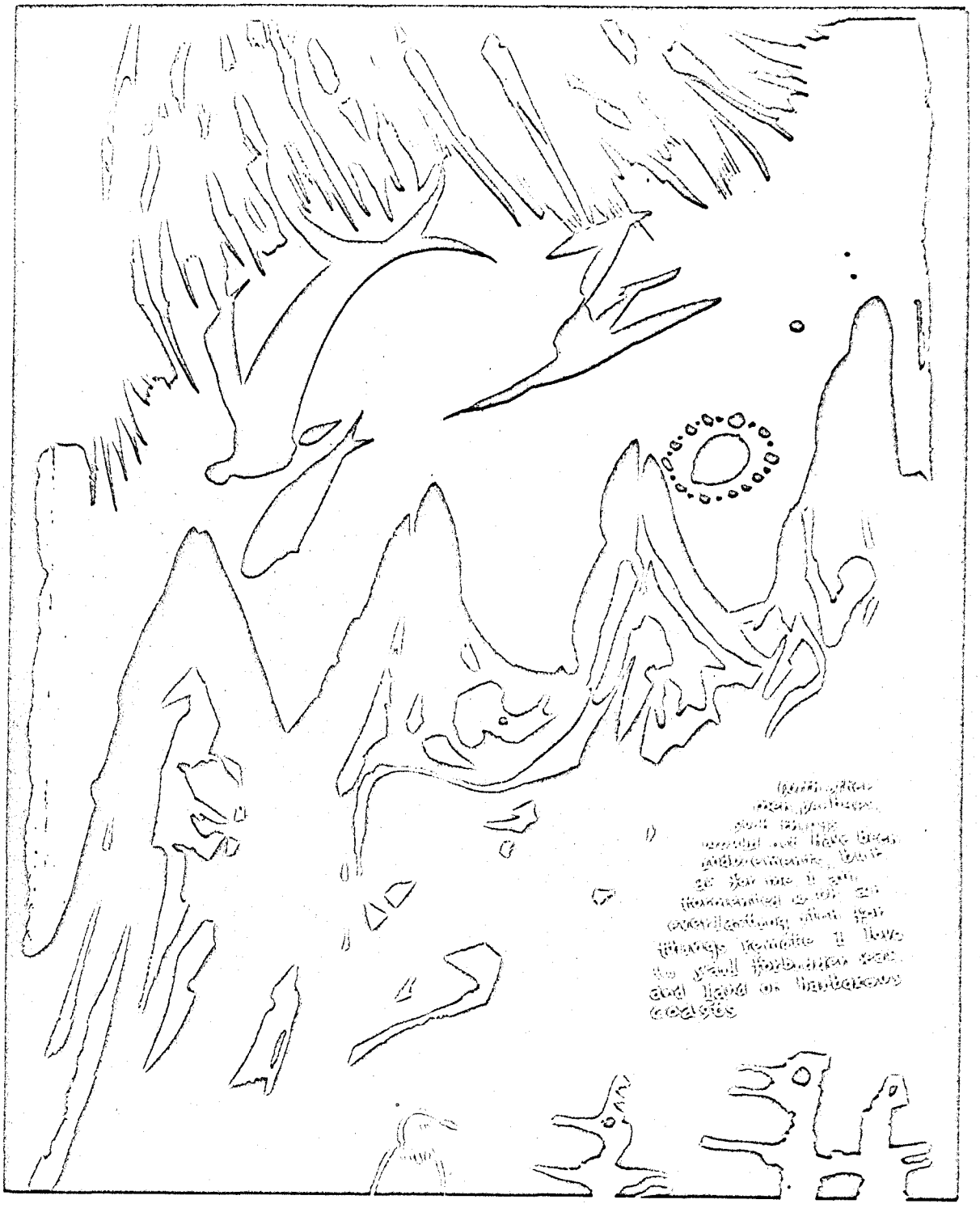

Physiological Studies on the *t* Gene Defect in T4-Infected *E. coli*

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SUMMARY

T4-infected bacteria lyse at a characteristic time after infection, each infected cell releasing a few hundred progeny phage. *Su*$^-$ bacteria infected with phage carrying an amber mutation in gene *t* synthesize both phage and phage lysozyme, yet fail to either cease metabolism or lyse at the usual time (Josslin, 1970). The experiments reported in this paper show that (1) addition of egg white lysozyme allows a normal lysis of cells infected with lysozyme-defective mutant phage but does not significantly affect the lysis phenotype of cells infected with *t* mutant phage; (2) anaerobiosis or addition of cyanide induces infected cell lysis only if the *t* gene is functional; (3) mutations in the *rII* genes partially suppress the lysis-defective, *t* mutant phenotype; (4) revertants of *t* mutant phage are often double mutants carrying the original *t* mutation as well as a mutation in either of the two *rII* genes or, less frequently, at a site not closely linked to *rII*.

The first two observations show that the *t* gene defect cannot be overcome simply by artificially disrupting the cell wall or inhibiting metabolism. They are consistent with the hypothesis that the *t* gene product induces the disruption of the cell membrane, which causes cessation of host metabolism and allows the *e* gene product to initiate digestion of the cell wall.

INTRODUCTION

Under normal conditions, T4-infected bacteria lyse at a characteristic time after infection, each infected cell releasing a few hundred progeny phage. The mechanism of this lysis is not understood. Mukai *et al.* (1967) found that cells infected with lysozyme-defective mutant phage failed to lyse, yet their respiration stops at the usual lysis time. This observation suggests that the phage genome directs the synthesis of two lysis factors -- one which degrades the host cytoplasmic membrane, resulting in the cessation of infected cell metabolism and allowing the other, phage lysozyme, to degrade the cell wall and release progeny phage.

The existence of a second lysis factor was supported by the isolation of a novel class of lysis-defective mutants of phage T4, the amber *t* mutants (Josslin, 1970). *Su*$^-$ bacteria infected with amber *t* mutant phage synthesize both phage and phage lysozyme, yet fail to either cease metabolism or lyse at the usual time.

While functional *t* gene product is required for cessation of metabolism at the normal lysis time, its mechanism of action is not understood. To investigate its mechanism, experiments were designed to study the physiology and phenotypic reversion of the *t* defect in the hope that conditions which promote reversion might share a common mechanism with *t* gene product. It is concluded that the *t* gene product may be involved in the disruption of the host cytoplasmic membrane.

MATERIALS AND METHODS

Phage and bacterial strains. Phage and bacterial strains were from the collection of R. S. Edgar. AS19, a lysozyme sensitive strain of E. coli B restrictive for phage amber mutants, was obtained from M. Sekiguchi through R. L. Sinsheimer. The rII mutant phage rdf41 carries a deletion including both the A and B cistrons. Phage amber mutants are defined by their ability to form plaques on the permissive host, Escherichia coli CR63, but not on the restrictive host, E. coli S/6. CR63(λ) was used as a host permissive for amber mutants but restrictive for rII mutants. G(λ) was used as a host restrictive for both amber and rII mutants. Phage and bacteria were prepared as described previously (Josslin, 1970).

Media and reagents. The media and reagents were as described previously (Josslin, 1970). Bovine albumin (BSA) was obtained from Sigma Chemical Corporation, St. Louis, Missouri.

RESULTS AND DISCUSSION

Cell Wall Disintegration Is Not the Only Function of the t Gene Product

Incorporation of egg white lysozyme into solid medium allows e mutant (lysozyme-defective) phage to form plaques (Emrich, 1968). If both the e gene and the t gene products control cell wall disintegration, disruption of the wall with egg white lysozyme should

also result in the lysis of t mutant-infected cultures. To see if this was true, one step growth experiments were performed with AS19 bacteria, a lysozyme-sensitive strain of E. coli B (Sekiguchi and Iida, 1967), infected with am^+, amH26 (gene e-defective) or amtA3 (gene t-defective) phage, and grown in broth under isotonic conditions in the presence and absence of egg white lysozyme. The results (Figures 1A and 1B, collated in Table 1) show that under the conditions used, addition of egg white lysozyme allowed a normal lysis of cells infected with lysozyme-defective phage suggesting that the infected-cell wall must disintegrate before progeny phage can be released. This disintegration, normally caused by phage lysozyme presumably just prior to lysis (Mukai et al., 1967), can result from treatment early in infection with exogenous egg white lysozyme. Similar treatment had no significant effect on the lysis phenotype of cells infected with t mutant phage suggesting that cell wall disintegration is not the sole function of the t gene product. However, both of these results can be explained if the t gene product induces the disruption of the cell membrane allowing the e gene product to initiate digestion of the cell wall.

Fig. 1. One step growth curves of phage-infected cells in the presence and absence of egg white lysozyme. AS19 bacteria at 4 x 10^8/ml in 4 mM KCN were infected with an equal volume of phage at 4 x 10^7/ml. The reaction mixture was incubated for 10 min then diluted by at least 4 x 10^4-fold into growth tubes containing 0.5% BSA (time = 0). At 5 min sucrose was added to the growth tubes to a final concentration of 20%. For lysozyme addition, at 15 min egg white lysozyme was added to the growth tubes to a final concentration of 0.1 mg/ml. At 25 min $MgSO_4$ was added to the growth tubes to a final concentration of 0.05 M. Control experiments showed that under these conditions, $MgSO_4$ stopped the lysozyme reaction. Platings from appropriate tubes represent infective centers. A: no lysozyme added; B: egg white lysozyme added. O: *am*$^+$; Δ: *am*H26; □: *am*tA3.

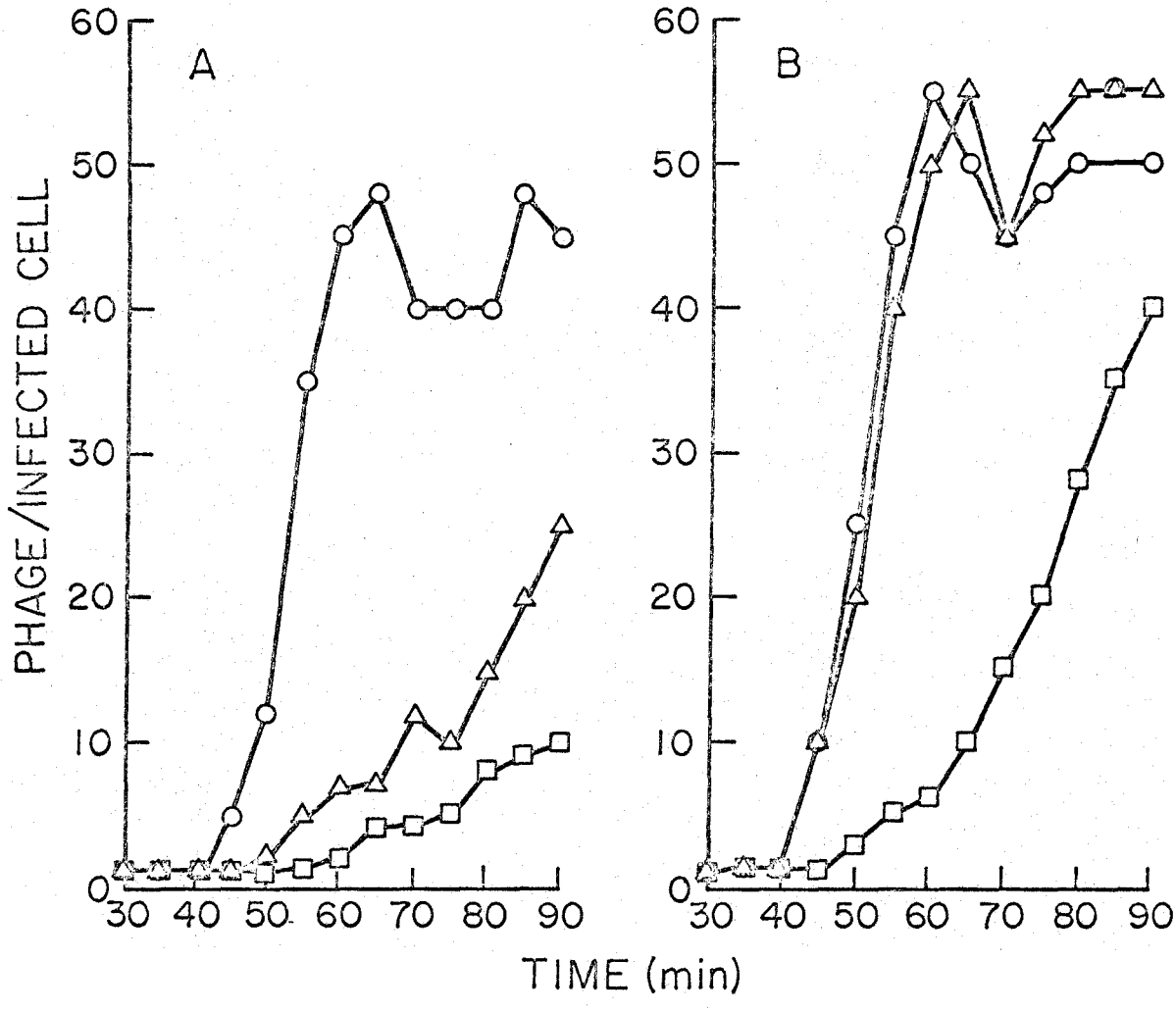

A
B
PHAGE/INFECTED CELL
TIME (min)
0
10
20
30
40
50
60
30
40
50
60
70
80
90

TABLE 1

Growth Parameters of Phage-Infected Cells Grown in the Presence and Absence of Egg White Lysozyme. (Summary of Experiment Shown in Fig. 1).

	No Lysozyme			Lysozyme Added		
	Lysis Time (min)	Intracellular Phage Infected Cell		Lysis Time (min)	Intracellular Phage Infected Cell	
		50 min	90 min		50 min	90 min
am$^+$	52	60	60	50	60	65
*am*H26	>90	50	50	52	50	50
*am*tA3	>90	60	180	>90	60	120

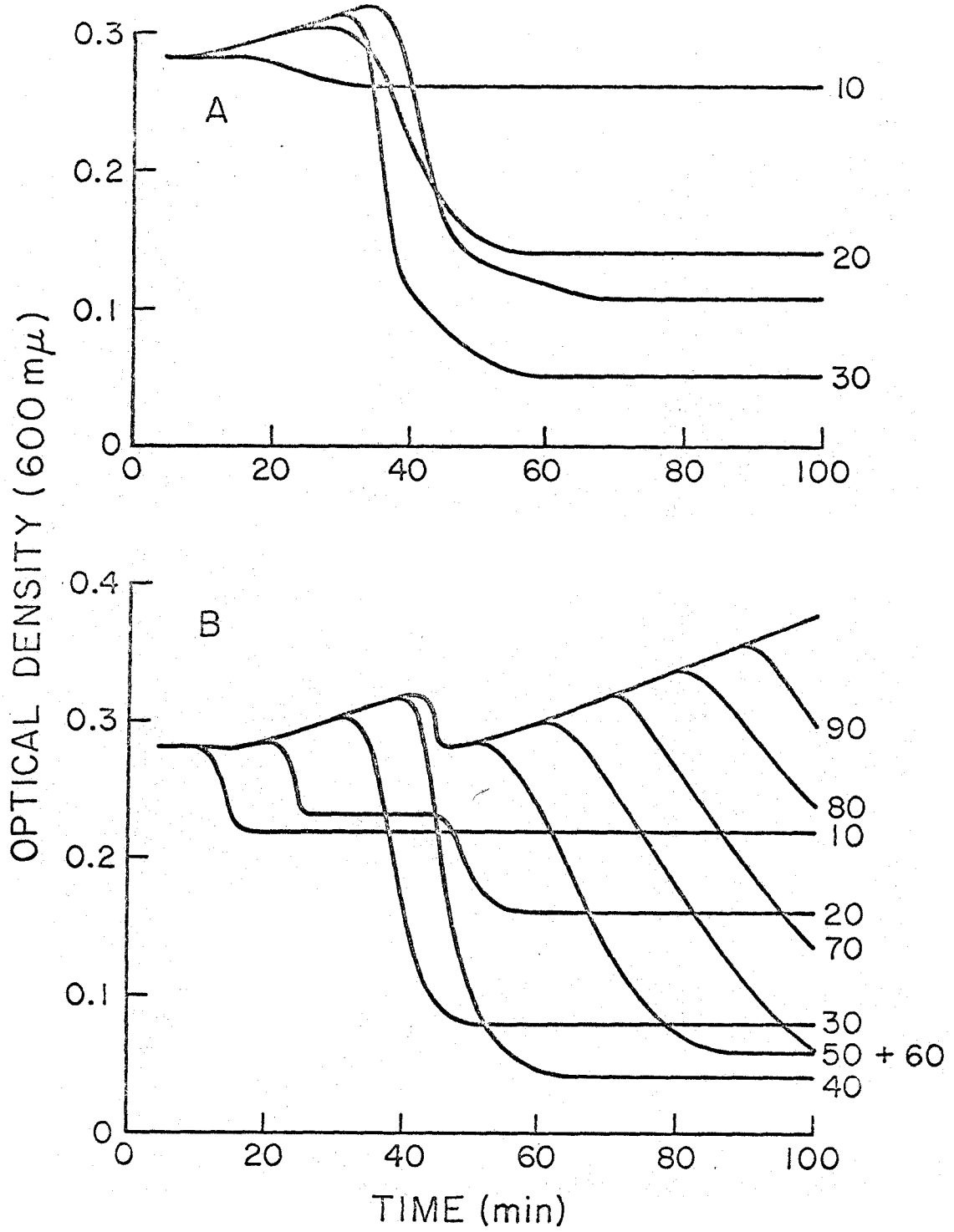
Lysis time is measured as the time at which half of the progeny phage have been spontaneously released. Intracellular phage were titered by treating growth tube aliquots with $CHCl_3$ or sonic oscillation prior to plating for infective centers.

Cessation of Metabolism Is Not the Only Function of the *t* Gene Product

Three observations suggest a causal relationship between metabolic inhibition and lysis during T4 infection. Doermann (1952) showed that during the last half of the latent period, cyanide induces premature lysis. Mukai *et al.* (1967) found that cells infected with lysozyme-defective phage fail to lyse, yet cease oxygen uptake at the normal lysis time. Finally, *t* gene mutants synthesize phage lysozyme yet fail to either cease metabolism or lyse at the usual lysis time (Josslin, 1970). Thus when both the *t* gene and the *e* gene products are functional, cessation of metabolism causes lysis, whether at the normal time or prematurely upon cyanide addition. These observations suggest that the primary function of *t* gene product might be to block metabolism. The following experiments were designed to determine if induced cessation of metabolism would cause premature lysis of *t*-defective infected cells.

Control experiments showed that cyanide induced premature lysis of t^+-infected cultures. For these experiments S/6 cultures were infected with either *am*B25 (gene 34, tail fiber defective) or am^+ phage and treated with 4 mM cyanide at various times after infection. As shown in Figure 2A, the *am*B25-infected culture lysed at 42 min. When cyanide was added at 30 min, the culture lysed prematurely at 37 min. As shown in Figure 2B, the am^+-

Fig. 2. Optical density of $\underline{t}^+$-infected cultures after addition of cyanide. S/6 bacteria at 2 x 10^9/ml were infected with an equal volume of phage at 6 x 10^9/ml. After 10 min of incubation, the reaction mixture was diluted 10-fold into a growth tube and aeration was begun (time t = 0). Every 10 min thereafter, at the times indicated in the margin to the right, an aliquot was treated with KCN to a final concentration of 4 mM. A: $\underline{am}$B25; B: $\underline{am}^+$

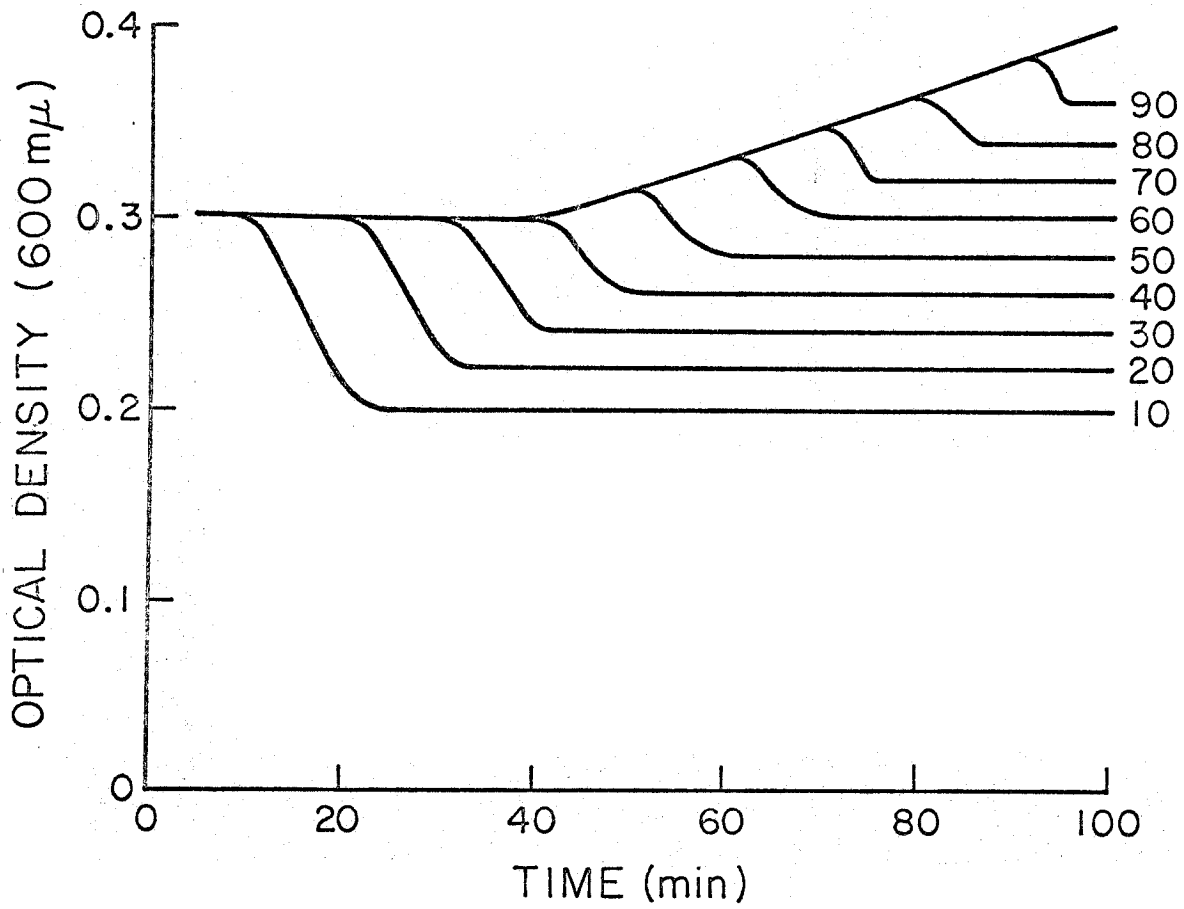

A
B
0.3
0.2
0.1
0
0.4
0
20
40
60
80
100
10
20
30
90
80
10
20
70
30
50 + 60
40
OPTICAL DENSITY (600 mμ)
TIME (min)

infected culture was lysis inhibited presumably as a result of superinfection by infective progeny phage (Doermann, 1948). However, cyanide added at 30 min or later also resulted in premature lysis.

When S/6 cultures were infected with amtA3:amB25 phage or amtA3 phage (data not shown) and treated with cyanide at various times after infection, results were obtained as shown in Figure 3. In all cases addition of cyanide resulted in at most a slight decrease in optical density as compared to that found in cultures of t^+-infected cells. These observations suggest that in contrast to t^+-infected cells, cyanide fails to induce lysis of t-defective infected cells.

Presumably cyanide induces lysis by blocking metabolism. This idea was supported by experiments similar to the above in which metabolism was blocked by oxygen deprivation rather than cyanide. For these experiments S/6 cultures were infected with either am^+ or amtA3 phage and bubbled with nitrogen instead of air at various times after infection. The resulting changes in optical density of these cultures before and after the shift to anaerobic growth were analogous to those shown in Figures 2B and 3. At 30 min or later, nitrogen bubbling resulted in an almost total loss of optical density of the am^+-infected culture but only a

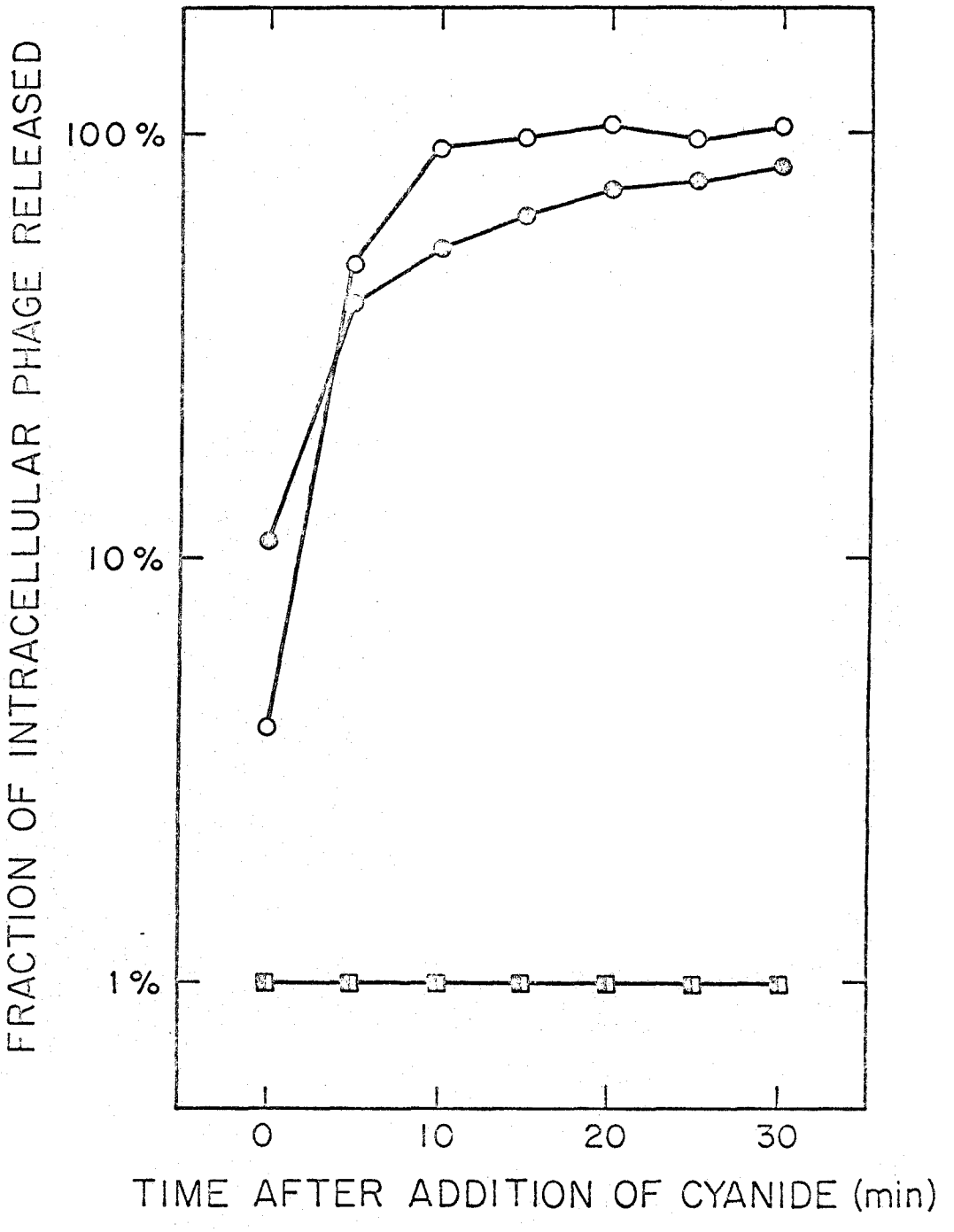
Fig. 3. Optical density of a *t*-defective infected culture after addition of cyanide. Protocol as in Figure 2 except with *amt*A3:*am*B25 phage.


OPTICAL DENSITY (600 mμ)
0.4
0.3
0.2
0.1
0
0
20
40
60
80
100
TIME (min)
90
80
70
60
50
40
30
20
10

slight decrease of optical density of the amtA3-infected culture. In the above experiments the t-defective infected cultures were successfully infected since in all cases the optical densities of the cultures decreased from >0.3 to <0.05 after addition of chloroform. Therefore, after 30 min of phage growth, metabolic inhibition induced by either cyanide or nitrogen bubbling results in premature lysis only in the presence of functional t gene product.

To independently show that cells infected with t mutant phage fail to lyse when their metabolism is blocked, the release of progeny phage was measured following cyanide addition. For these experiments S/6 cultures were infected with either am^+ or amtA3 phage and grown at 10^8/ml. At both 30 and 90 min, culture aliquots were diluted 200-fold into broth containing 4 mM cyanide and further diluted and plated at various times. Culture aliquots were also diluted into broth containing both 4 mM cyanide and chloroform at 30 and 90 min to titer intracellular phage. Figure 4 shows the fraction of intracellular phage released with respect to time after cyanide addition. As expected, cyanide added at either 30 or 90 min resulted in the release of am^+ progeny phage but failed to induce release of amtA3 progeny phage. In the same experiment a Petroff-Hauser counter was used to measure the concentration of infected cells before and after cyanide addition.

Fig. 4 Increase of infective centers in infected cultures after addition of cyanide. S/6 bacteria were infected as described in the legend to Figure 2. At 30 min (○, □) and at 90 min (●, ■), culture samples were diluted 200-fold into H broth containing KCN at a final concentration of 4 mM (time t = 0). Aliquots of these samples were immediately treated with $CHCl_3$ to titer intracellular phage. The fraction of intracellular phage released was calculated as 100X the ratio of infective centers in the KCN-treated sample at time t to that in the aliquot treated with both KCN and $CHCl_3$. ○ and ●: *am*$^+$; □ and ■ *amt*A3.


FRACTION OF INTRACELLULAR PHAGE RELEASED
100 %
10 %
1 %
0
10
20
30
TIME AFTER ADDITION OF CYANIDE (min)

Cyanide addition to am^+-infected cells resulted in a loss after 30 min of 89% and 98% of cells which received cyanide at 30 and 90 min, respectively. Nevertheless, the concentration of cells infected with amtA3 phage remained stable 30 min after cyanide addition at either 30 or 90 min after infection (102% recovery in both cases).

Therefore, inhibition of metabolism induces both lysis and release of progeny phage in cultures of t^+-infected cells but fails to do so in cultures of cells infected with t mutant phage. Consequently T4-infected cell lysis is not induced solely by blocking metabolism but requires functional t gene product as well. Thus the function of the t gene product must not solely be to block metabolism. However, these results can be explained if the t gene product induces the disruption of the cell membrane which results in cessation of metabolism in the host.

Mutations in the rII Genes Partially Suppress the t Gene Defect

Lysis of T4-infected cells entails the cessation of metabolism followed by the immediate degradation of the cell wall and release of progeny phage (Mukai et al., 1967). The cessation of metabolism and consequently lysis itself are delayed when cells are repeatedly infected (Mukai et al., 1967). T4 can sport mutants which are

defective in any of these phenotypes. The t gene mutants have lost the ability to block metabolism (Josslin, 1970). The e gene mutants have lost the ability to degrade the cell wall (Streisinger, 1961). The mutants in the r genes have lost the ability to delay lysis after repeated infection (Mukai et al., 1967). To investigate the relationship between these various functions, double mutant phage were constructed of amtA3 together with an e mutant and with one representative from each of the three classes of r mutants. Then one step growth experiments were performed with S/6 infected with each of the possible phage types.

Figure 5 shows the growth cycle during infection with either amH26 (gene e-defective) or amtA3:amH26 phage. In neither case did the infected cells lyse as measured by a significant increase in infective centers in the culture. However, when culture aliquots were treated with both chloroform and egg white lysozyme, progeny phage were released in both cases. Synthesis of amH26 phage stopped at 45 min and yielded an average of 200 intracellular phage/infected cells. However, synthesis of amtA3:amH26 phage continued past 45 min, so that by 90 min, each infected cell contained on the average 1000 phage. Thus the t mutant phenotype is not affected by the loss of function of gene e.

Figure 6 shows the growth cycle during infection with rdf41, amtA3, and amtA3:rdf41 phage. Cells infected with rdf41 (class rII)

Fig. 5. One step growth curves of cells infected with lysozyme-defective phage. S/6 bacteria at 4 x 10^8/ml in 4 mM KCN were infected with an equal volume of phage at 4 x 10^7/ml. The reaction mixture was incubated for 10 min then diluted by at least 4 x 10^4-fold into growth tubes. Intracellular phage was titered by plating $CHCl_3$-treated aliquots of the growth tubes before (O, □) and after (●, ■) addition of egg white lysozyme at a final concentration of 1 mg/ml. At all times the amH26-infected culture had less than 0.5 infective centers/infected cell while the amtA3:amH26-infected culture had less than 0.1 infective centers/infected cell. O and ●: amH26; □ and ■ :amtA3:amH26.

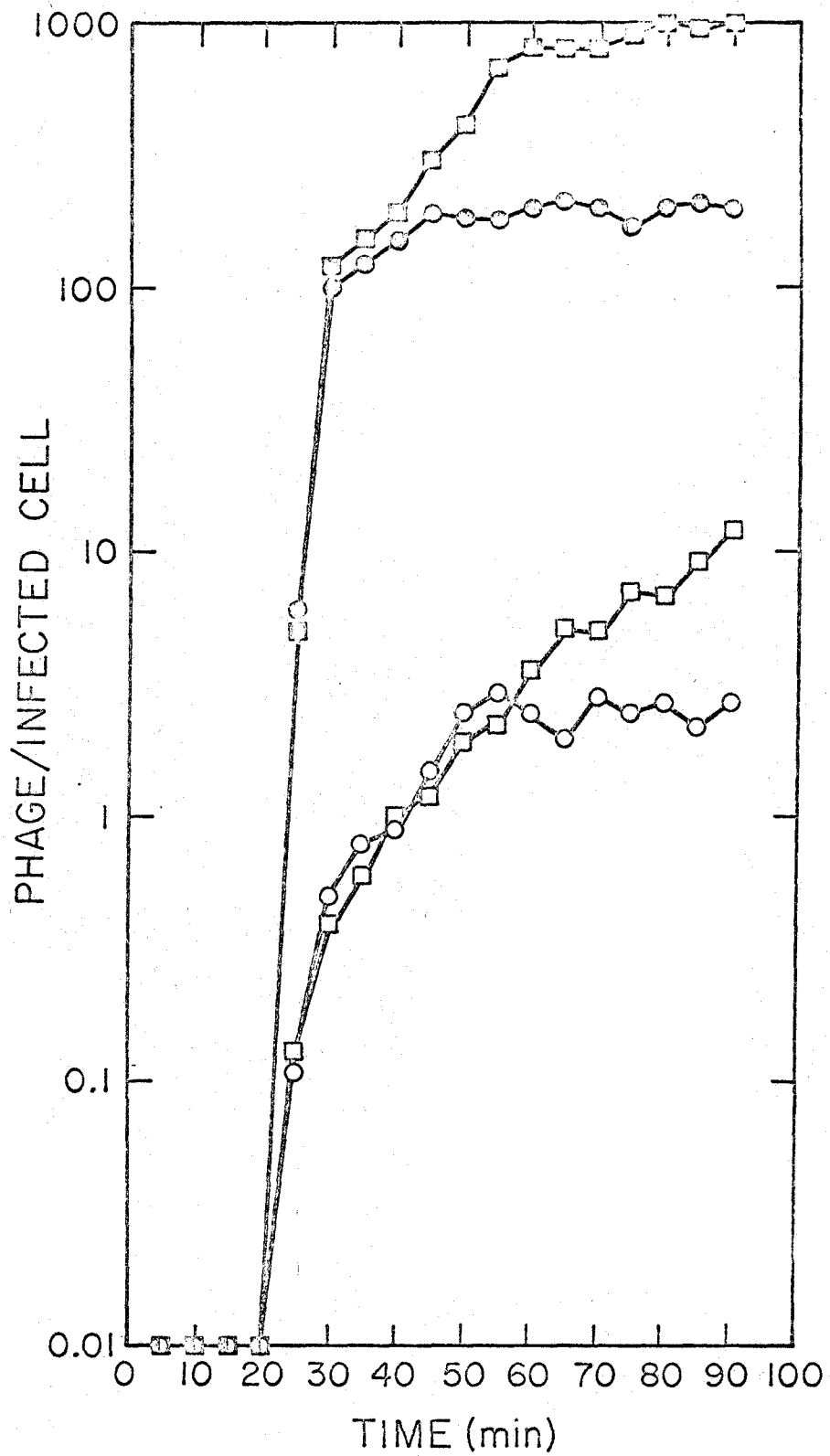
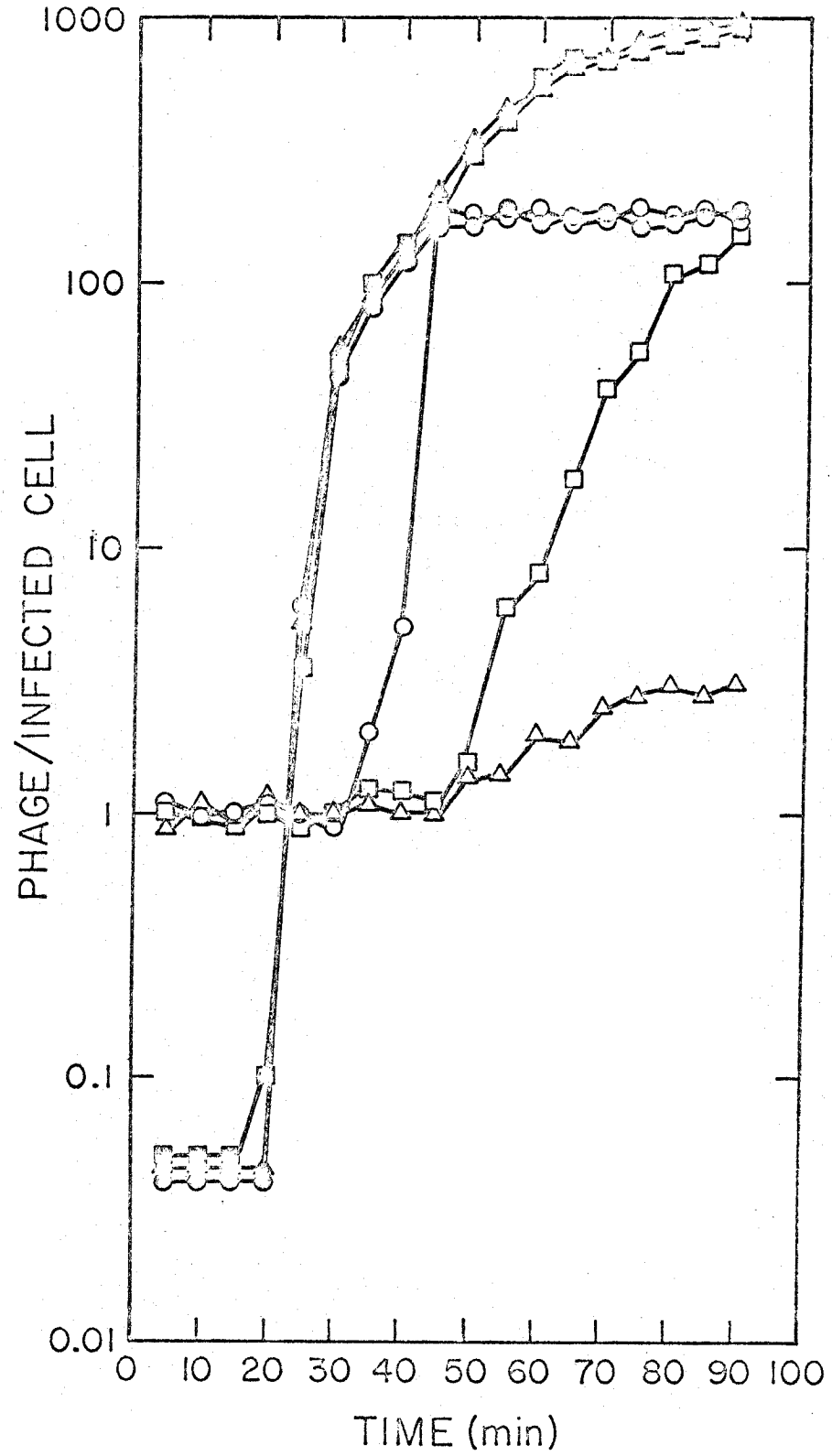

1000
100
10
1
0.1
0.01
PHAGE/INFECTED CELL
0
10
20
30
40
50
60
70
80
90
100
TIME (min)

Fig. 6. One step growth curves of cells infected with r- and t-defective phage. S/6 bacteria were infected as described in the legend to Figure 5. Platings from appropriate tubes before (O, Δ, □) and after (●, ▲, ■) treatment with $CHCl_3$ represent infective centers and intracellular phage, respectively.
O and ●: rdf41; Δ and ▲: amtA3; □ and ■ : amtA3:rdf41.


1000
100
10
1
0.1
0.01
PHAGE/INFECTED CELL
0
10
20
30
40
50
60
70
80
90
100
TIME (min)

phage or with either r48 (class rI) or r67 (class rIII) phage (data not shown) had a wild-type lysis phenotype. In each of these cases, lysis occurred at 45 min yielding a burst of 200 phage. Those infected with amtA3 phage or with either amtA3:r48 or amtA3:r67 phage (data not shown) had the lysis-defective, t mutant phenotype. In each of these cases, phage synthesis continued past the normal lysis time so that by 90 min, each infected cell contained on the average 1000 phage. Furthermore, at 90 min the titer of infective centers in the culture was only 3 per infected cell. Nevertheless, during infection with amtA3:rdf41 phage, while phage synthesis continued past the normal lysis time, the rate of increase in infective centers in the culture was markedly greater than that during infection with amtA3 (r^+) phage; by 90 min, the titer of infective centers in the culture was 150/infected cell. Furthermore, while amtA3:r48, amtA3:r67, as well as amtA3 itself, fail to form plaques on S/6, amtA3:rdf41 does so and the plaques are indistinguishable from those formed by rdf41. Thus the t mutant phenotype is not affected by either the r48 (class rI) or the r67 (class rIII) mutations. Nevertheless the addition of an rII mutation to t mutant phage partially suppresses the lysis-defective, t mutant phenotype. Although the infected cells continue to synthesize phage past the normal lysis time, phage are released at an increased rate in liquid culture and plaques can form on solid media.

Revertants of t Mutant Phage Are Often t:rII Double Mutants

To further investigate the suppression of the t mutant defect, revertants of t mutant phage were isolated. Ten independent plaques of amtA3 and ten of amtB5 grown on CR63 were replated on S/6 and grown at 18°. Revertants were recovered from all twenty clones with a frequency of approximately 10^{-5}. One revertant plaque was selected from each of the original twenty clones of t mutant phage. These phage were called tR phage for "t revertant." On the expectation that many of these tR phage were t:rII double mutants, each was tested for the rII^+ ability to form plaques on a lambda lysogen, CR63(λ). Although both amtA3 and amtB5 formed plaques on CR63(λ), seventeen of the twenty tR phage failed to do so suggesting that these seventeen phage carry an rII mutation.

For confirmation of the t:rII genotype of these seventeen tR phage, each was backcrossed to its respective am t parent and to the rII mutant phage rdf41. No t^+r^+ recombinants were found in any of the thirty-four crosses. Therefore all of these seventeen phage carry their original t mutation together with an rII mutation. Finally, each of these seventeen tR phage was tested for the ability to complement r61 and r65 phage carrying point mutations in the rIIA and rIIB genes, respectively. As shown in Table 2, each of the seventeen failed to complement one rII

TABLE 2

Complementation Between *tR* Phage and *rII* Phage

tR Phage	Alone	r61	r65	Defective *rII* gene
*amt*A3R1	0	0	+	A
2	0	+	0	B
3	0	0	+	A
4	+	+	+	
5	+	+	+	
6	0	+	0	B
7	0	+	0	B
8	0	0	+	A
9	0	+	0	B
10	0	0	+	A
*amt*B5R1	0	+	0	B
2	0	+	0	B
3	0	+	0	B
4	0	+	0	B
5	0	+	0	B
6	0	0	+	A
7	0	0	+	A
8	0	+	0	B
9	+	+	+	
10	0	+	0	B

TABLE 2 (continued)

tR Phage	Alone	r61	r65	Defective rII gene
rII Phage				
r61	0	0	+	A
r65	0	+	0	B

One drop of tR phage was mixed with one drop of rII mutant phage both at 10^7/ml on a plate seeded with G(λ) bacteria. Complementation was detected by confluent lysis after overnight incubation.

mutant but did complement the other. Therefore all of the seventeen phage are defective in only one of the two rII gene products. Furthermore, the failure to complement an rII mutant phage was not correlated with respect to either rII gene or t mutant.

The three tR phage which formed plaques on CR63(λ) did so on G(λ) as well; however, two, amtA3R5 and amtB5R9, formed minute plaques on G(λ), readily distinguishable from those of t^+ phage, when incubated at 42°. This made possible the enumeration of $t^+ r^+$ recombinants in a background of t and rII phage. No $t^+ r^+$ recombinants were found when these two tR phage were backcrossed with their respective am t parent. However, when they were crossed with rfd41 phage, 9% and 8% of the progeny, respectively, scored as $t^+ r^+$ recombinants. Therefore, these two tR phage have their original t mutation together with a mutation at a second site not closely linked to rII. Since the last tR phage, amtA3R4, behaved as $t^+ r^+$ under all conditions tested, its genotype was not investigated.

Thus revertants of t mutant phage are often double mutant phage carrying the original t mutation as well as a mutation in either of the two rII genes or, less frequently, at a site not closely linked to rII.

ACKNOWLEDGMENTS

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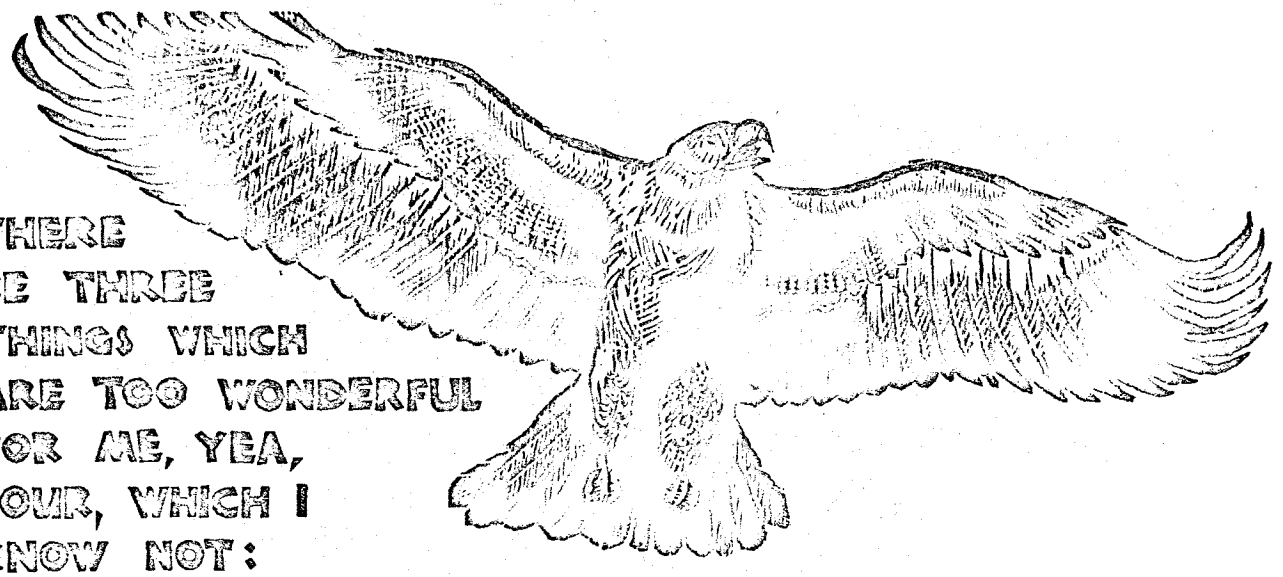

THERE
BE THREE
THINGS WHICH
ARE TOO WONDERFUL
FOR ME, YEA,
FOUR, WHICH I
KNOW NOT:
THE WAY OF AN EAGLE IN THE AIR,
THE WAY OF A SERPENT ON A ROCK,
THE WAY OF A SHIP IN THE MIDST OF THE SEA,
AND
THE WAY
OF A MAN
WITH
A MAID.

The Effect of Phage T4-Infection on Phospholipid Hydrolysis in *E. coli*

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SUMMARY

Cronan and Wulff (1969) proposed that phospholipid hydrolysis is a phage T4-induced function involved in the phage lysis mechanism. The experiments reported here show that phage infection does not necessarily alter the rate of phospholipid hydrolysis detected in uninfected cells. Furthermore, uninfected cells contain a latent phospholipid hydrolyzing activity which becomes activated upon lysis of the cells by a variety of treatments, including phage infection. It is concluded that phospholipid hydrolysis may play no role specific to the phage life cycle.

INTRODUCTION

Under normal conditions, T4-infected bacteria lyse at a characteristic time after infection, each infected cell releasing a few hundred progeny phage. The mechanism of this lysis is not understood. Mukai et al. (1967) found that cells infected with lysozyme-defective mutant phage fail to lyse, yet their respiration stops at the usual lysis time. One interpretation of this observation is that the phage genome directs the synthesis of two lysis factors -- one which degrades the host cytoplasmic membrane, resulting in the cessation of infected cell metabolism and allowing the other, phage lysozyme, to degrade the cell wall and release progeny phage.

The existence of a second lysis factor was supported by the isolation of a novel class of lysis-defective mutants of phage T4, the T4 amber t mutants (Josslin, 1970). Su^- bacteria infected with an amber t mutant synthesize both phage and phage lysozyme yet fail to either cease metabolism or lyse at the usual time.

Concurrently, Cronan and Wulff (1969) observed that T4 phage infection induces hydrolysis of host membrane phospholipid. From a variety of experiments, they proposed that phospholipid hydrolysis is a phage-induced function involved in the phage lysis mechanism.

If phage T4 induces a lysis factor which promotes hydrolysis of membrane phospholipid, the t gene product would be a likely

candidate since it is required for cessation of metabolism at the normal lysis time. To investigate this idea, phospholipid hydrolysis was measured in $\underline{Su}^-$ bacteria infected with either $\underline{t}^+$ or lysis-defective, amber $\underline{t}$ mutant phage. The results indicated an alternative explanation for the findings of Cronan and Wulff (1969) and necessitated a reevaluation of the role of phospholipid hydrolysis in the phage life cycle. Further experiments showed that the ability to hydrolyze phospholipid is present but inactive in growing cells and that any of a variety of treatments, including normal phage infection, which result in cell lysis induce phospholipid hydrolysis. No evidence was obtained for the idea that host phospholipid hydrolysis plays any role specific to the phage life cycle.

MATERIALS AND METHODS

Phage materials and method. The T4D phage and E. coli bacterial strains, the preparation of phage and bacteria, and the media and reagents except bovine albumin have been previously described (Josslin, 1970). Bovine albumin (BSA), added to the phage growth medium to bind FFA (Davis and Dubos, 1947; Goodman, 1958), was obtained from Sigma Chemical Company, St. Louis, Missouri. To purify phage, a Millipore-filtered phage stock was centrifuged at 18×10^3 rpm for 2 hr and the pellet was resuspended in buffer

containing CsCl at a final density of 1.5 g/ml. This suspension was centrifuged at 48 x 10^3 rpm for 16 hr and the opaque fraction in the center of the gradient was collected. 50% of the plaque formers in the phage stock were recovered in this fraction. Phage were titered by their ability to kill S/6 bacteria (Delbrűck and Luria, 1942). For the phage used in these experiments the ratio of the CR63 plating titer to the S/6 killing titer varied from 0.60 to 0.65.

Radioactive labeling. Na-acetate-1-C-14 (50 mc/mM) was obtained from International Chemical and Nuclear Corporation, Irvine, California. To prepare bacteria labeled with C^{14}-acetate, a fresh stationary-phase culture of S/6 was diluted 10^3-fold into broth containing Na-acetate-1-C-14 at a final concentration of 5 µc/ml and grown at the appropriate temperature.

Lipid analysis. Lipids were extracted, fractionated, and assayed for radioactivity as described by Cronan and Wulff (1969).

RESULTS

During T4 Infection, Phospholipid Hydrolysis Begins at Lysis

Cronan and Wulff (1969) showed that the free fatty acid (FFA) produced during infection arises from hydrolysis of host membrane phospholipid, and that FFA production therefore reflects phospholipid

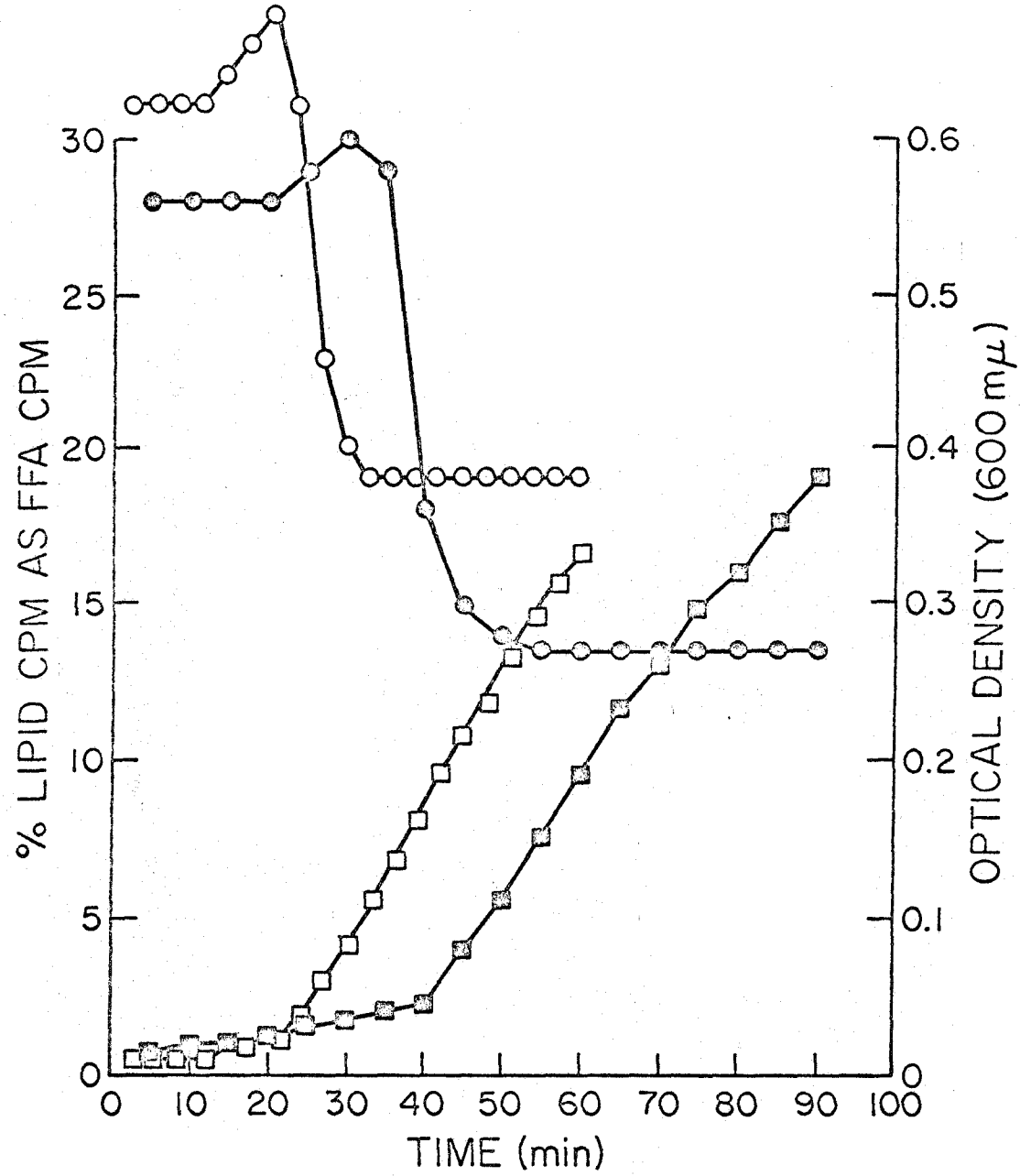
hydrolysis.

The rate of FFA production during T4 infection is shown in Fig. 1. In this experiment a culture of E. coli S/6 which had been grown at 37° in T broth in the presence of C^{14}-acetate was infected with T4D amB25 mutant phage. In S/6 amB25 (gene 34-defective) produces noninfective particles which lack tail-fibers and therefore cannot induce lysis inhibition, so that the need for treatment with anti-T4 serum is eliminated. If the time of lysis is defined as the time at which the optical density of a culture is halfway between its maximum and final values, then the amB25-infected culture grown in T broth at 37° lysed at 25 minutes. Production of C^{14}-FFA, expressed as % of total lipid radioactivity, began at the time of lysis instead of at 8 minutes as found by Cronan and Wulff (1969). A similar result was obtained during phage growth in H broth at 30° as shown in Fig. 1. The absence of FFA production under both conditions suggests that host phospholipid is stable throughout infection until lysis.

During T4 Infection, Anti-T4 Serum Induces Phospholipid Hydrolysis

These experiments must be reconciled with those of Cronan and Wulff (1969) who found FFA production began not at lysis but early during infection. The experimental conditions of Cronan and Wulff are different in five ways from those reported here (see Fig. 1):

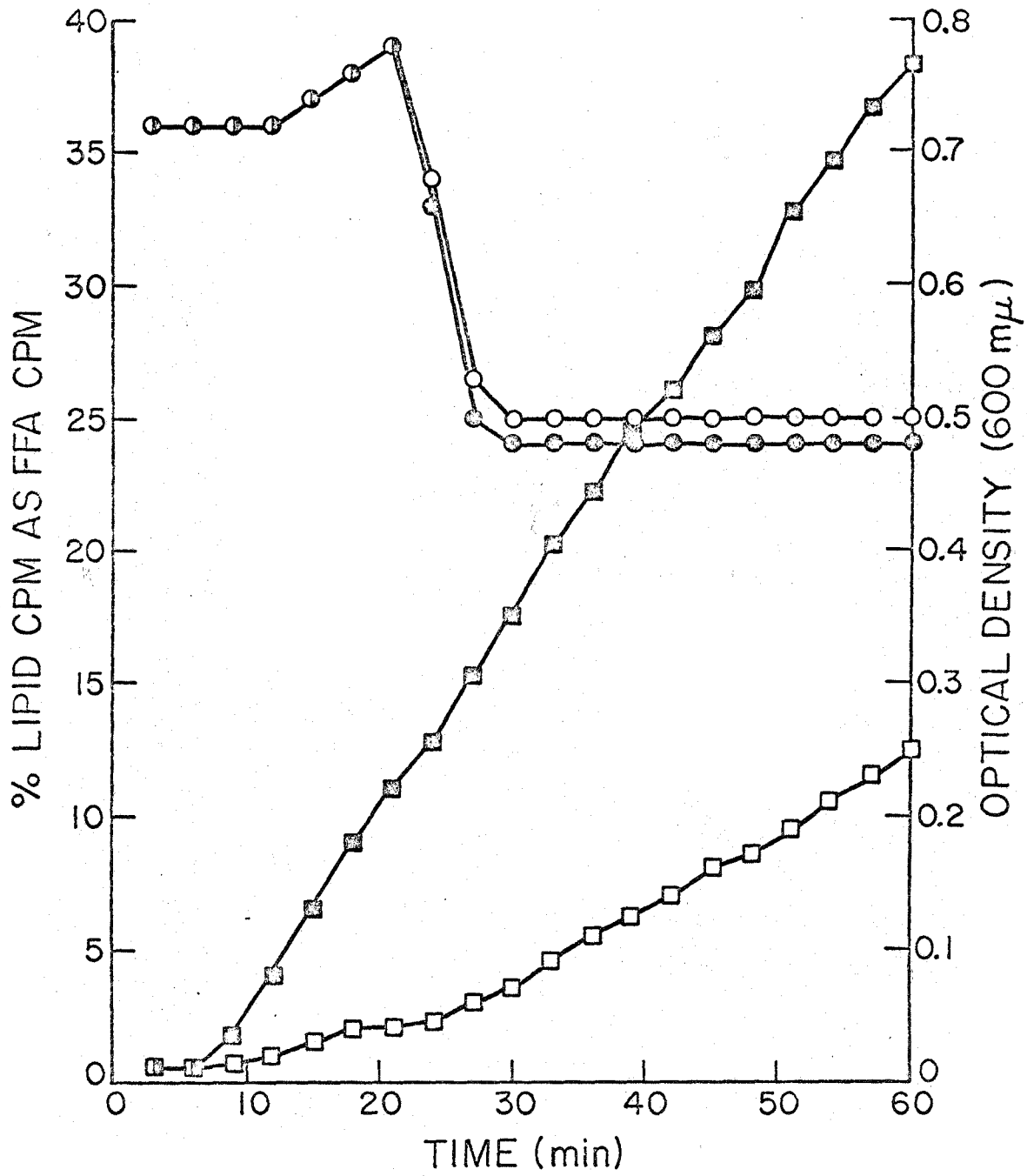
Fig. 1 FFA production in an S/6 culture infected with amB25 mutant phage. S/6 bacteria were grown in either T broth at 37° (O, □) or H broth at 30° (●, ■) to 3×10^8/ml in the presence of C^{14}-acetate, then amB25 phage and BSA were added at a multiplicity of 5 and at a final concentration of 0.1%, respectively (time t=0). O and ●: optical density; □ and ■: % lipid as FFA.


% LIPID CPM AS FFA CPM
OPTICAL DENSITY (600 mμ)
TIME (min)
0
5
10
15
20
25
30
0.1
0.2
0.3
0.4
0.5
0.6
0
10
20
30
40
50
60
70
80
90
100

Cronan and Wulff	Josslin
1. E. coli B	E. coli S/6
2. T4B	T4D
3. wild type	amber mutant
4. anti-T4 serum	no serum
5. no BSA	BSA

In their experiment Cronan and Wulff added anti-T4 serum at 5 min and observed FFA production began within 3 min. To test the possibility that anti-T4 serum might induce FFA production, the effect of serum addition was determined in an experiment performed with amB25-infected cells grown in T broth without BSA at 37°. As shown in Fig. 2, the infected cultures lysed at 25 min, and FFA production in the untreated control began at that time. In the culture which received anti-T4 serum, FFA production began at 6 min, the time of addition of anti-T4 serum. Additional experiments showed that anti-T4 serum failed to induce FFA production when added to either an uninfected culture or a phage-infected culture grown in the presence of 0.1% BSA. These results strongly suggest that FFA production observed early in infection by Cronan and Wulff was caused by anti-T4 serum rather than by phage infection itself. In any event, these results rule out the possibility that lysis results when some threshold value of FFA is produced. In the experiments shown, lysis occurred over a 25-fold range of values of

Fig. 2 Effect of anti-T4 serum on FFA production in an S/6 culture infected with amB25 mutant phage. S/6 bacteria were grown in T broth at 37° to 3 x 10^8/ml in the presence of C^{14}-acetate, then amB25 phage was added at a multiplicity of 5 and the culture was divided into two equal aliquots (time t=0). At 6 min, anti-T4 serum was added to one aliquot to a final activity of 10/min. 0, □ : untreated culture; ●, ■ : culture treated with anti-T4 serum. 0, ●: optical density; □ , ■ : % lipid as FFA.

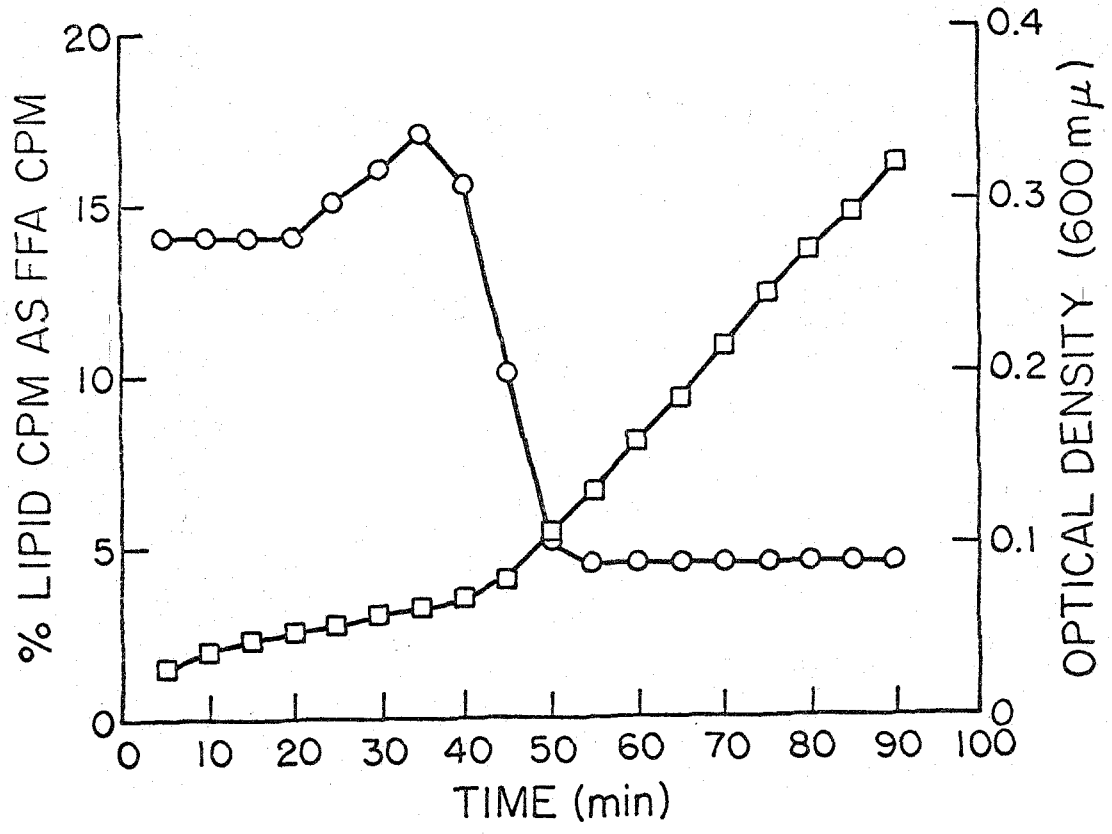

% LIPID CPM AS FFA CPM
OPTICAL DENSITY (600 mμ)
TIME (min)
0
5
10
15
20
25
30
35
40
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0
10
20
30
40
50
60

% lipid as FFA: from 0.5% during growth in T broth with BSA shown in Fig. 1 to 12.5% during growth in T broth with anti-T4 serum shown in Fig. 2.

On the possibility that phospholipid hydrolysis might nevertheless play a role in the phage lysis mechanism, further experiments were undertaken to determine more carefully the effects of T4 infection in general and t gene function in particular on FFA release from phospholipid. To simplify the interpretation of results, two changes were made in the experimental procedure. First, to assume synchronous infection, the phage were preadsorbed onto concentrated bacteria under anaerobiosis for 10 min, after which growth was initiated by diluting the adsorption mixture 10-fold and beginning aeration. Second, the radioactive label was removed from the culture at the time of infection. Therefore, in these experiments all radioactivity measured is derived from C^{14}-acetate incorporated before infection.

The effect of synchronous phage infection on prelabeled bacterial lipid is shown in Fig. 3. This experiment was performed with amB25 phage purified by CsCl density gradient centrifugation. Since purified phage are used, changes in % lipid as FFA result solely from phage rather than from possible contaminants in the phage stock such as lipases. In this experiment lysis occurred at 45 min as did an increase in the rate of FFA production. Before lysis, the % lipid as FFA increased linearly from 1.8% (extrapolated to

Fig. 3 FFA production in an S/6 culture infected with purified amB25 mutant phage. S/6 bacteria were grown in H broth at 30° to 3 x 10^8/ml in the presence of C^{14}-acetate, then chilled, centrifuged at 5 K/min for 10 min, and resuspended in unlabeled 0° H broth. The bacteria, adjusted to 2 x 10^9/ml, were incubated at 30° with an equal volume of purified amB25 phage at 10^{10}/ml. After 10 min the reaction mixture was diluted 10-fold into H broth containing BSA at a final concentration of 0.1% and aeration was begun (time t=0). O: optical density; □ : % lipid as FFA.


% LIPID CPM AS FFA CPM
OPTICAL DENSITY (600 mμ)
TIME (min)
0
5
10
15
20
0.1
0.2
0.3
0.4
0
10
20
30
40
50
60
70
80
90
100

1.6% at 0 min) to 3.5% at 45 min. After lysis, the % lipid as FFA increased linearly to 16.2% at 90 min. Therefore the rate of FFA production after lysis increased 6.7-fold over the rate prior to lysis.

To show that phage are produced under conditions identical to those of the above experiment, a control experiment was performed with $\underline{am}^+$ phage at a multiplicity of 5. Aliquots of the am^+-infected culture were treated with chloroform at 45 and 90 min, then titered for infective centers. Burst sizes were calculated from these titers and that of the input bacteria. The burst size was 150 at 45 min, a normal burst of progeny phage. Furthermore the burst size increased to 360 by 90 min which shows that the $\underline{am}^+$-infected culture was lysis inhibited, presumably due to superinfection by the infective progeny phage (Doermann, 1948).

Phage Growth is Not Required for Phospholipid Hydrolysis

When an infected culture lyses, FFA production begins. Lysis represents the end of the phage growth cycle. To see if a successful cycle of infection is required for FFA production, the FFA experiment was performed with $\underline{am}$B25 phage at various multiplicities of infection. Infection at a multiplicity less than approximately 10 to 20 (killing particles/input cell) should result in phage growth terminated by lysis from within. Infection at higher multiplicities should result in abortive infection and immediate lysis from

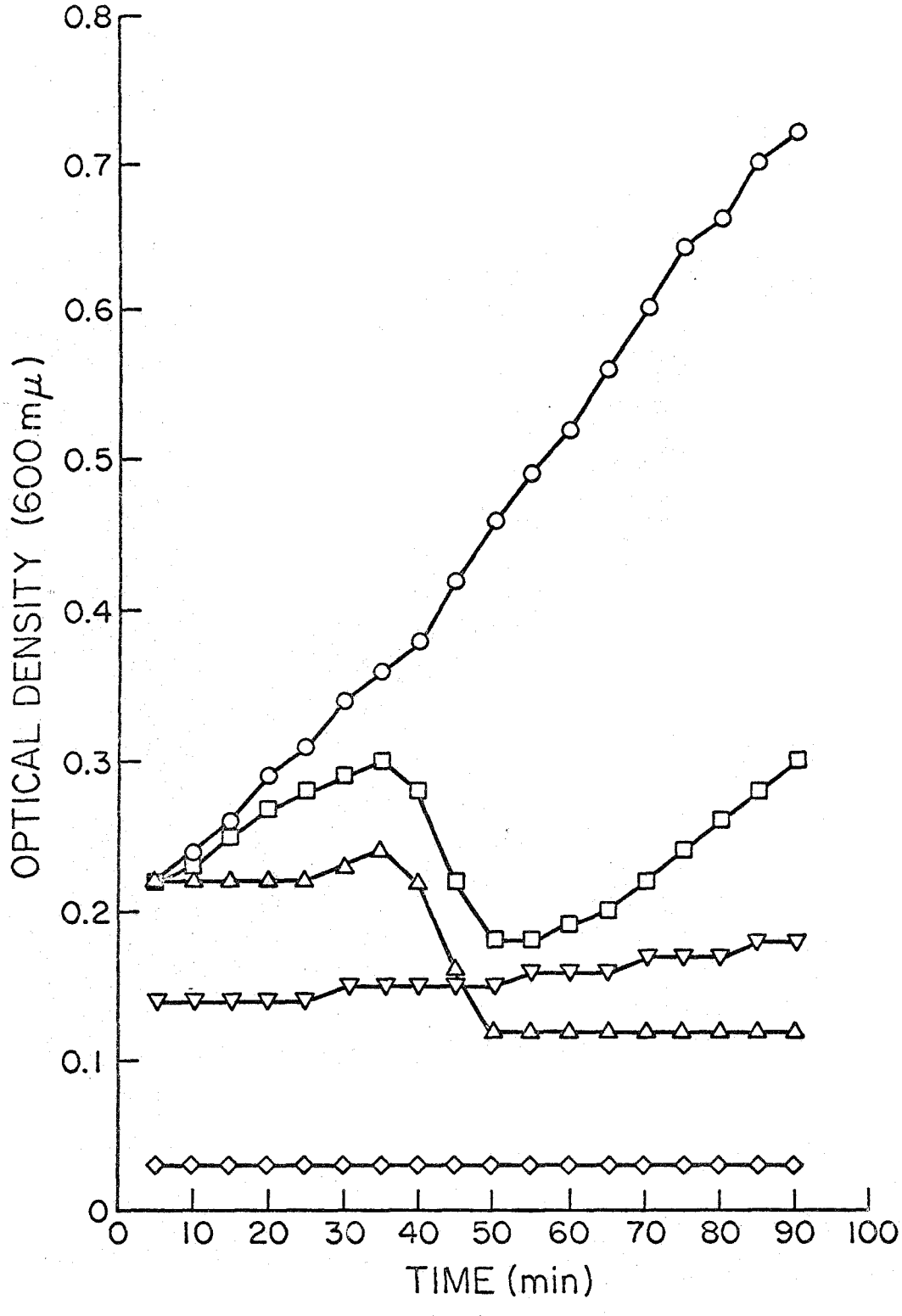
without. As shown in Fig. 4, uninfected cells failed to lyse and had a constant low rate of FFA production. Cells infected at multiplicities of 1.5 and 5 lysed at 40 min, at which time FFA production began. Cells infected at multiplicities of 15 and 40 lysed immediately and FFA production then began. Since FFA production is observed after lysis from without, phage growth is not required for phospholipid hydrolysis.

This conclusion was further supported by the results of an FFA experiment performed with uninfected cells lysed by treatment with sonic oscillation as well as with cells infected with either amB25 or amtA3:amB25 phage, grown for 30 min, then lysed by treatment with sonic oscillation. As shown in Fig. 5, artificial lysis of all three cultures resulted in a marked increase in the rate of FFA production. Therefore the ability to hydrolyze phospholipid is present but inactive in growing cells and is not the function of t gene product.

During T4 Infection, Phospholipid Hydrolysis Depends on the Event Promoted by Functional t Gene Product

The causal relationship between lysis and FFA production was investigated with lysis-defective mutant phage. Studies with these mutants have shown the T4 lysis mechanism to be a sequential process of at least two steps (Josslin, 1970): function of phage t gene product resulting in the cessation of host metabolism followed by function of phage e gene product causing the degradation of host cell wall. As shown in Fig. 6, when cell wall degradation

Fig. 4 FFA production in S/6 cultures infected with amB25 mutant phage at various multiplicities. C^{14}-acetate labeled S/6 cultures were infected with amB25 phage as described in the legend to Fig. 3. A: optical density; B: % lipid as FFA. O: multiplicity = 0; □ : multiplicity = 1.5; Δ: multiplicity = 5; ∇: multiplicity = 15; ◇ : multiplicity = 40.


0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
OPTICAL DENSITY (600 mμ)
0
10
20
30
40
50
60
70
80
90
100
TIME (min)

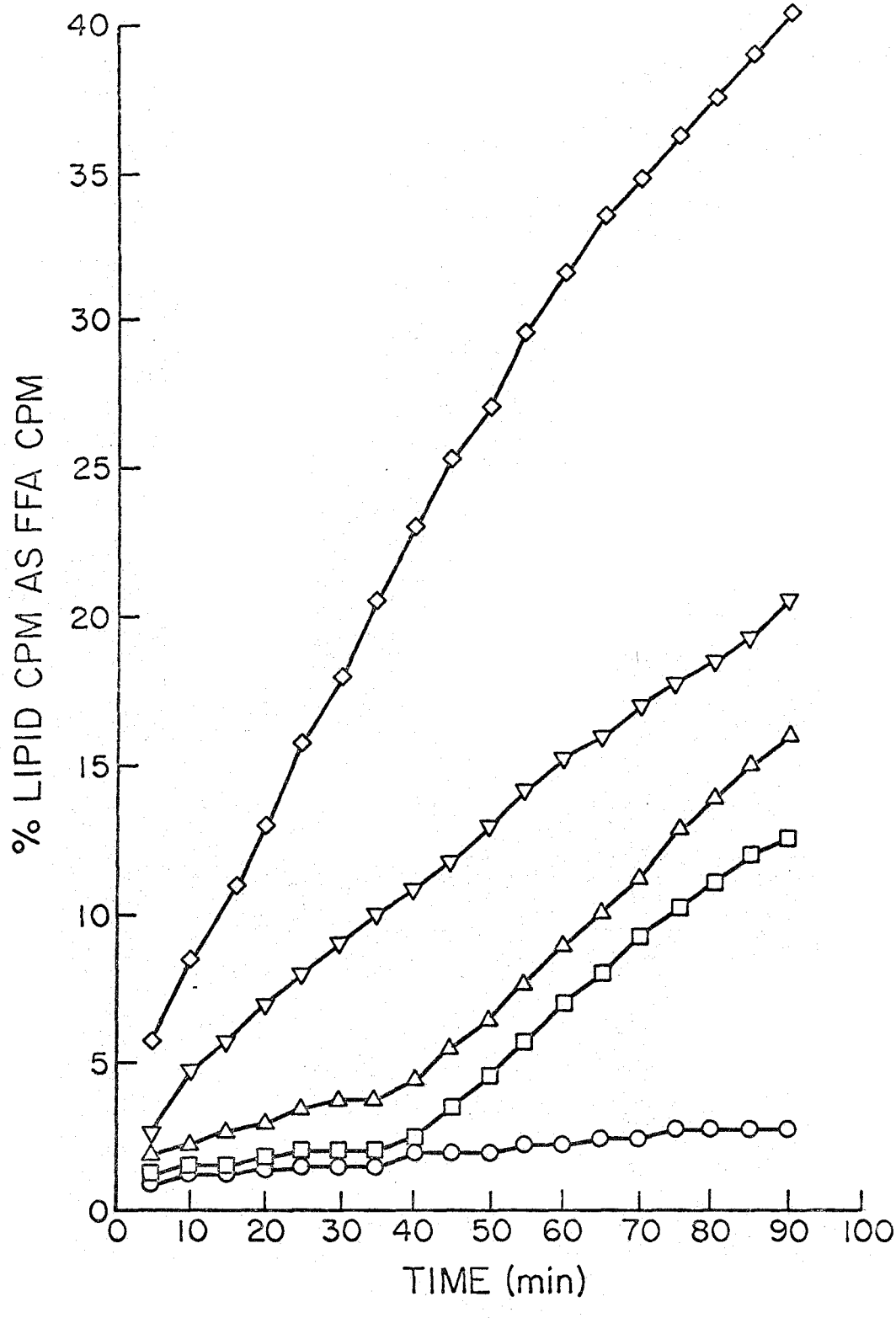

% LIPID CPM AS FFA CPM
TIME (min)
0
5
10
15
20
25
30
35
40
0
10
20
30
40
50
60
70
80
90
100

Fig. 5 FFA production in uninfected and phage-infected S/6 cultures following treatment with sonic oscillation. C^{14}-acetate labeled S/6 cultures were treated with phage at multiplicities of 0 (uninfected) or 5 (phage-infected) as described in the legend to Fig. 3. The cultures were grown for 0 min (uninfected) or 30 min (phage-infected), then chilled. After treatment with 5 amp of sonic oscillation for 3 min, each culture was returned to growth conditions. 0: uninfected; Δ: amB25-infected; ∇: amtA3:amB25-infected.

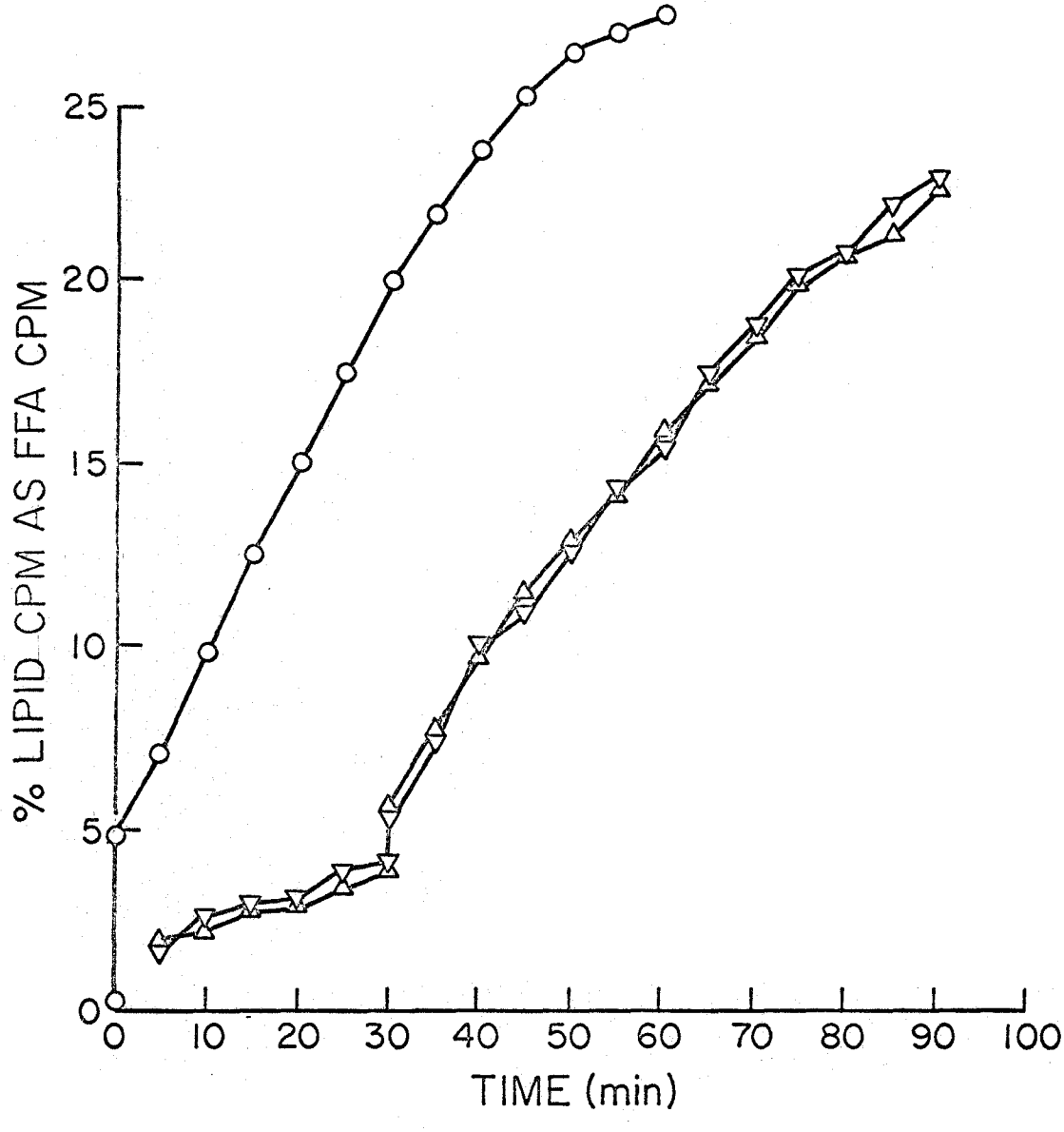
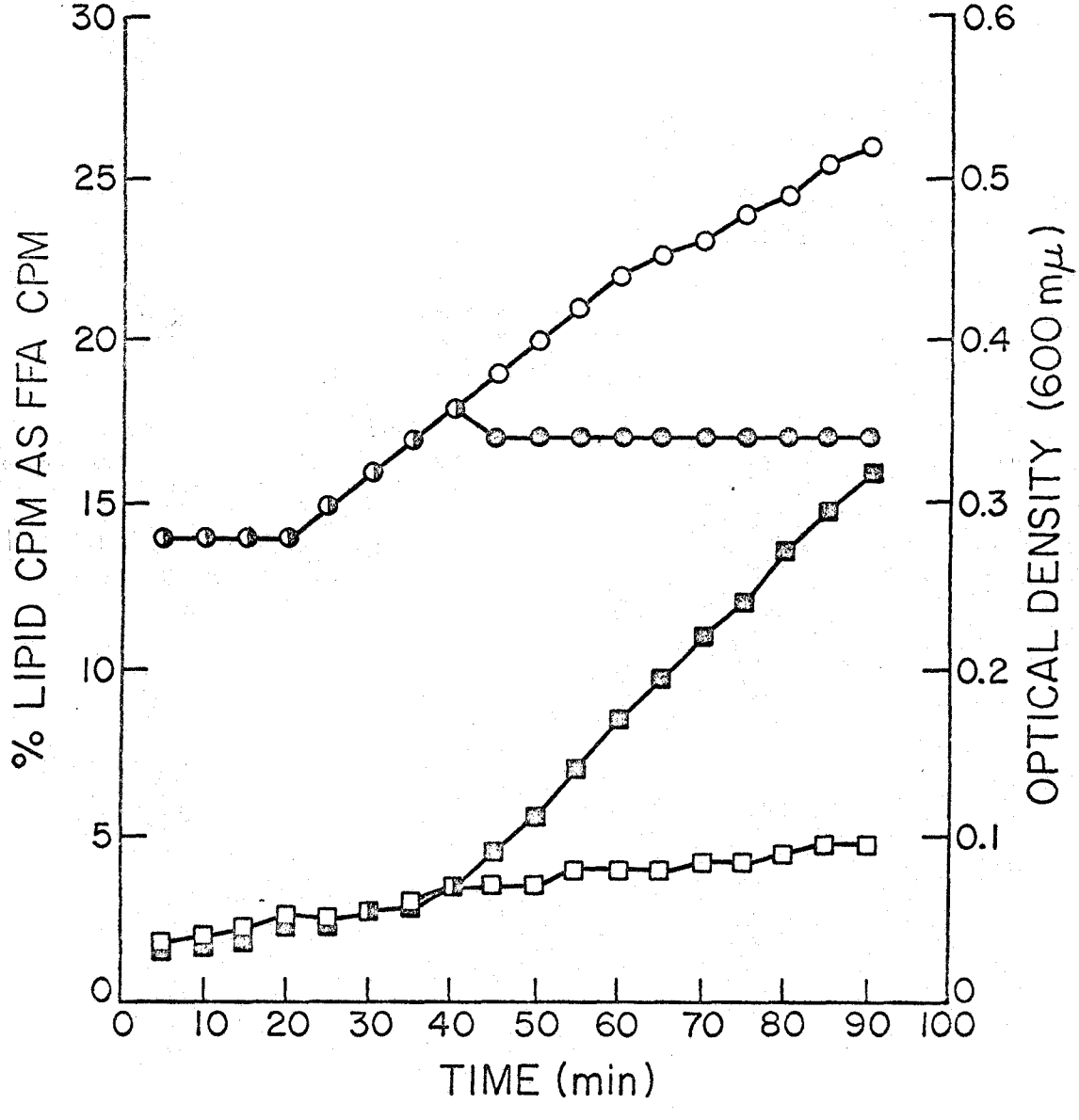

% LIPID CPM AS FFA CPM
25
20
15
10
5
0
0
10
20
30
40
50
60
70
80
90
100
TIME (min)

Fig. 6 FFA production in S/6 cultures infected with lysis-defective mutant phage. C^{14}-acetate labeled S/6 cultures were infected with either amtA3:amB25 (O, □) or amH26:amB25 (●, ■) phage as described in the legend to Fig. 3. O and ●: optical density; □ and ■ : % lipid as FFA.


% LIPID CPM AS FFA CPM
OPTICAL DENSITY (600 mμ)
TIME (min)
0
5
10
15
20
25
30
0.1
0.2
0.3
0.4
0.5
0.6
0
10
20
30
40
50
60
70
80
90
100

was mutationally blocked during infection with amH26: amB25 (gene e: gene 34-defective) phage, FFA production increased at the usual lysis time, indicating that lysis is not required for FFA production. Nevertheless, when both cessation of metabolism and cell wall degradation were blocked during infection with amtA3:amB25 (gene t:gene 34-defective) phage, FFA production was not observed. Therefore, during T4 infection phospholipid hydrolysis depends upon the event promoted by functional t gene product.

Phospholipid Hydrolysis is Not Required for Lysis

T4-induced lysis requires function of t gene product followed by function of e gene product. Function of t gene product causes FFA production as shown above. Does phospholipid hydrolysis necessarily accompany lysis?

Fig. 4B shows that in the uninfected culture and in the culture infected at a multiplicity of 1.5, there was no detectable difference in FFA production until lysis. This observation suggests that FFA production is not required before lysis. To provide further evidence for this interpretation the following experiment was performed under conditions which allowed normal lysis but inhibited FFA production.

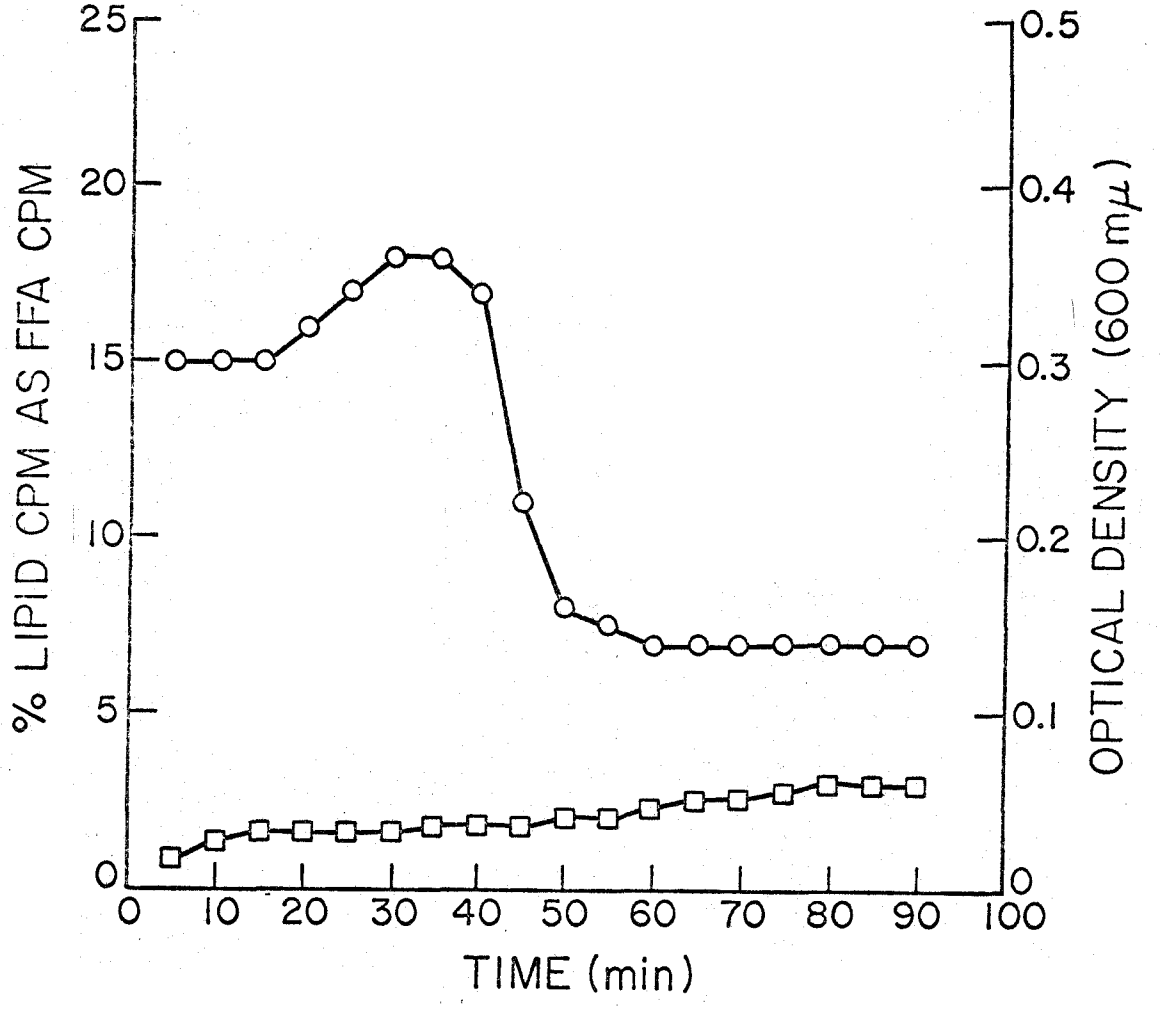
Presumably FFA production is catalyzed by phospholipase A, an endogenous enzyme of E. coli (Fung and Proulx, 1969; Okuyama

and Nojima, 1969). Phospholipase A from snake venom requires Ca^{++} for activity (Long and Penny, 1957; Magee and Thompson, 1960; Dawson, 1963). To test whether FFA production in an infected culture could be inhibited by a chelating agent, an FFA experiment was performed with amB25 phage and 10^{-3} M EDTA in the phage growth medium. As shown in Fig. 7, the infected culture lysed normally at 40 min. However under these conditions, FFA production increased at the same rate as observed in the uninfected culture shown in Fig. 4. A control experiment showed that addition of EDTA up to 10^{-2} M to a lysate of an infected culture containing C^{14}-labeled lipids did not detectably affect the lipid assay. Therefore the presence of EDTA in the infected culture allows normal lysis but inhibits FFA production. Thus phospholipid hydrolysis is a by-product of the event controlled by the t gene and does not necessarily accompany lysis.

Phage Infection Inhibits Host Lipid Synthesis

The kinetics of FFA production, shown in Fig. 1 (for H broth at 30°), are similar to those shown in Fig. 3. Nevertheless in the latter experiment C^{14}-acetate was absent from the phage growth medium. This observation shows that the majority of FFA produced is derived from phospholipid present in the cell before infection and suggests that phage infection may inhibit host lipid synthesis.

Fig. 7 Effect of EDTA on FFA production in an S/6 culture infected with amB25 mutant phage. A C^{14}-acetate labeled S/6 culture was infected with amB25 phage as described in the legend to Fig. 3. At time t=0 EDTA was added to the culture to a final concentration of 10^{-3} M. O: optical density; □ : % lipid as FFA.

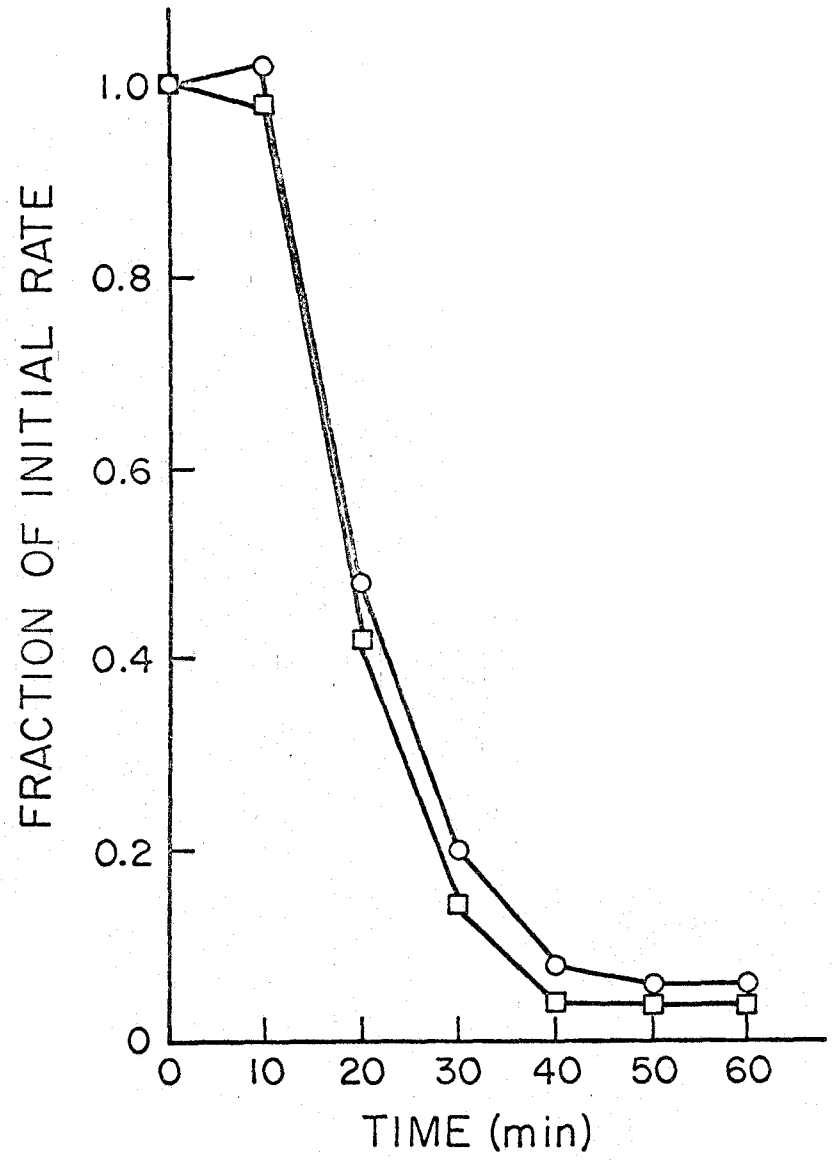

% LIPID CPM AS FFA CPM
OPTICAL DENSITY (600 mμ)
TIME (min)
0
5
10
15
20
25
0.1
0.2
0.3
0.4
0.5
0
10
20
30
40
50
60
70
80
90
100

Buller and Astrachan (1968), Furrow and Pizer (1968), and Peterson and Buller (1969) have shown that T4 infection results in a decreased rate of incorporation of P^{32} into phospholipid. To test this more directly, the rate of incorporation of C^{14}-acetate into lipid (chloroform-soluble radioactivity) was measured in pulse experiments throughout infection with both amB25 and amtA3:amB25 phage. The results (Fig. 8) show that the rate of incorporation of C^{14}-acetate into lipid decreases after infection and is independent of the t genotype of the infecting phage. A similar experiment was performed to measure the rate of incorporation of P^{32} into phospholipid (chloroform-soluble radioactivity). By 20 min the rate of incorporation of P^{32} into phospholipid was less than 30% of that found at the time of phage addition. The observation that phage infection results in a decreased ability to incorporate either C^{14}-acetate or P^{32} into lipid strongly suggests that phage infection results in inhibition of net lipid synthesis in the host.

The rII Gene Products May Affect Host Lipid Metabolism

Buller and Astrachan (1968) stated that infection with rII mutant phage resulted in an increased incorporation of P^{32} into diphosphatidylglycerol. This suggests that perhaps the products of the rII genes concern host lipid metabolism. To test whether the lack of rII gene products affects phospholipid hydrolysis

Fig. 8 Rate of incorporation of C^{14}-acetate into lipid in phage infected cultures of S/6. An S/6 culture was grown in H broth at 30° to 3 x 10^8/ml and divided into two equal samples. One was infected with amB25 phage, the other with amtA3:amB25 phage, each at a multiplicity of 5. Every 10 min C^{14}-acetate was added to aliquots of both cultures to a final concentration of 5 µc/ml. 2.5 and 5 min later samples were taken for determination of lipid raioactivity. The rate of incorporation of C^{14}-acetate into lipid at time t is normalized to that found at time 0 (2700 cpm incorporated after 5 min). 0: amB25; □ : amtA3:amB25.

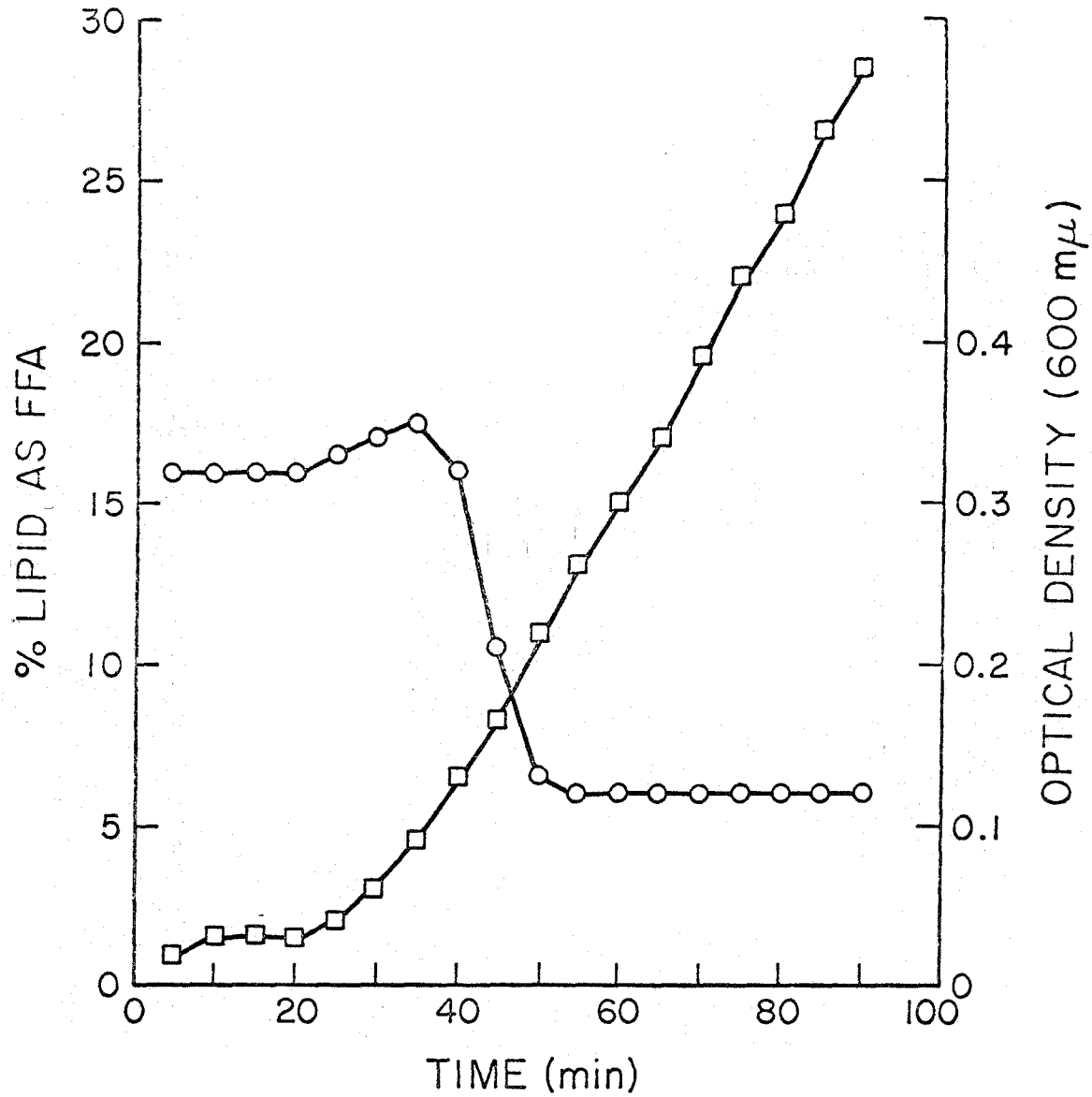

FRACTION OF INITIAL RATE
1.0
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
60
TIME (min)

in the host, the FFA experiment was performed with rdf41:amB25 phage. (The rII mutant phage rdf41 carries a deletion including both the A and B cistrons.) As shown in Fig. 9, the rII-mutant infected culture lysed at 45 min. However, in contrast to r^+-infected cultures (see for example Fig. 3), FFA production began at 30 min, 15 min before lysis. Furthermore FFA production occurred at a rate almost twice that found in r^+-infected cultures. This observation suggests that the products of the rII genes are required for the continued low rate of FFA production throughout infection.

DISCUSSION

E. coli grown in the presence of excess C^{14}-acetate (Fig. 4B) incorporate 0.5% of the lipid radioactivity as free fatty acid (FFA), the remainder being phospholipid. Following removal of label from the medium there is a slow increase in percent lipid radioactivityas FFA, suggesting that phospholipid is slowly hydrolyzed during cell growth. Presumably this hydrolysis is catalyzed by endogenous phospholipase A (Fung and Proulx, 1969; Okuyama and Nojima, 1969) which becomes activated upon lysis. While the physiological significance of phospholipase A and of phospholipid hydrolysis are not known, a variety of treatments have been shown to induce phospholipid hydrolysis in E. coli: addition of colicins E1 or K or of chloramphenicol, penicillin,

Fig. 9 FFA production in an S/6 culture infected with rII mutant phage. A C^{14}-acetate labeled S/6 culture was infected with rdf41:amB25 phage as described in the legend to Fig. 3. 0: optical density; □ : % lipid as FFA.


30
25
20
15
10
5
0
% LIPID AS FFA
0.4
0.3
0.2
0.1
0
OPTICAL DENSITY (600 mμ)
0
20
40
60
80
100
TIME (min)

formaldehyde, toluene, or chloroform (Cavard et al, 1968), heating at 48° or above for 5 min (Rampini et al., 1969), and addition of serum complement (Barbu and Lux, 1969). Presumably all these treatments result in the activation of phospholipase A.

In addition phospholipid hydrolysis is induced by lysis from without (Fig. 4B), sonication (Fig. 6), and lysis from within (Fig. 3) as shown by the experiments reported here. Nevertheless, while the t gene event is required for lysis from within and causes phospholipid hydrolysis (Fig. 5), lysis can occur in the absence of phospholipid hydrolysis (Fig. 7). Therefore phospholipid hydrolysis is a by-product of the t gene event and is not required for lysis.

The experiments reported here do not support Cronan and Wulff's proposal (1969) that phospholipid hydrolysis is a phage-induced function involved in the phage lysis mechanism. Their conclusion was based on the observation that FFA production begins soon after phage addition. It was shown above that this effect was most likely caused by anti-T4 serum rather than by phage infection itself.

Nevertheless this explanation of Cronan and Wulff's results fails to account for those of the lysis inhibition experiment shown in Fig. 2 of Cronan and Wulff (1969). They state: "A culture of E. coli B was infected with T4B eG502. At 5 min ... an aliquot was removed and added to a warm aerated flask containing 'anti T4-serum.'

In a similar manner, aliquots were removed at 7, 13, and 18 min ... and added to flasks containing T4B eG502 at a multiplicity of 5." They observed that in the aliquot which received serum, a high rate of FFA production began at 10 min as usual. However in the aliquots which received additional phage, the amount of FFA produced at the time of addition of secondary phage was the same as that in the aliquot which received serum. Although they failed to measure FFA production in an infected culture which received neither anti-T4 serum nor additional phage, these data imply that FFA was being produced during infection in the absence of anti-T4 serum. The experiments reported here are inconsistent with this implication and I have no explanation for this dilemma.

Unanswered is the question "Does the phage genome code for products which modify lipid metabolism in the host?" The observation that infection results in a decreased rate of radioactive label incorporation into lipid (Fig. 8) suggests that phage infection inhibits net lipid synthesis. The experiments reported here did not differentiate between various species of either phospholipids or fatty acids. Buller and Astrachan (1968), Furrow and Pizer (1969), and Peterson and Buller (1969) have found evidence which suggests that phage infection inhibits synthesis of phosphatidylethanolamine (PE), the major species of host phospholipid, but not of phosphatidylglycerol (PG). It is not known whether changes in

rates of synthesis or degradation of the various species of host phospholipid are promoted by bacterial or by phage gene products. Evidence consistent with host-dependent modification of lipid metabolism is that an increased ratio of PG:PE was observed in both stationary-phase cultures (Furrow and Pizer, 1968) and cultures grown under partial anaerobiosis (Peterson and Buller, 1969). Evidence consistent with phage-dependent modification of lipid metabolism is that infection with rII mutant phage results in both an increased incorporation of P^{32} into diphosphatidylglycerol (Buller and Astrachan, 1968) and the beginning of FFA production significantly before lysis (Fig. 9).

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APPENDIX

Lipid Analysis

Extraction: *E. coli* lipids were extracted by the method of Bligh and Dyer (1959) as modified by Kates *et al.* (1964). 1 ml of cell culture was added to a mixture of 2.5 ml of methanol plus 1.25 ml of chloroform. After overnight incubation, 1.25 ml of chloroform followed by 1.25 ml of water were added. After centrifugation, the lower phase was withdrawn and evaporated to dryness at 40° by blowing nitrogen over the miniscus. A few drops of chloroform: methanol (2:1, v/v) were added to dissolve the sample for application to a chromatographic plate.

Fractionation: The sample was spotted on a chromatographic plate layered with silica gel G (Analtech, Inc., Wilmington, Del.). The plate was chromatographed in the solvent system of Lepage (1964): diisobutylketone:acetic acid:water, 40:25:5 (v/v/v). Diisobutylketone (2,4-dimethyl-4-heptanone) was obtained from Aldrich Chem. Co., Milwaukee, Wisconsin. During chromatography, the solvent front was allowed to move approximately 8 cm beyond the point of sample application. Afterwards, the plate was dried by evaporation.

Assay for radioactivity: The silica on the plate was scribed 1 cm to each side of the point of sample application in the direction of solvent migration. This strip was then divided into two rectangles by lines at Rf values of 0.1, 0.7, and 1.0. The two rectangles of silica were scraped into separate scintillation vials and to the vials were added 10 ml of toluene scintillation solution containing approximately 5% Cab-O-Sil (Cabot Corp., Boston, Mass.). The vials were assayed for radioactivity in the C^{14}-H^3 channel of a Beckman LS-200B liquid scintillation counter at a gain of 4.50.

Test of the lipid assay: Three independent observations with phospholipid and fatty acid standards showed that in this assay, fatty acids migrate at Rf values between 0.8 and 0.9 while phospholipids migrate at Rf values between 0.3 and 0.6. The tests were

1) Visualization of fluorescent spots upon spraying with Rhodamine 6G,
2) Autoradiography of C^{14}-labeled lipids, and
3) Scintillation counting of C^{14}-labeled lipids.

These observations suggest that in this lipid assay, fatty acids are found in the small silica rectangle containing the fast moving counts while the remaining lipids are found in the large silica rectangle containing the slower moving counts.

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DISCUSSION

To study the lysis mechanism of phage T4, I isolated lysis-defective mutants of the phage. This work, described in Manuscript I, resulted in the discovery of a new class of lysis-defective T4 mutants, which define a new gene called *t*. Bacteria infected with *t* mutant phage synthesize both phage and phage lysozyme yet fail to either cease metabolism or lyse at the usual time. These properties indicate that T4-induced lysis entails a sequence of at least two events: function of *t* gene product, which results in the cessation of host metabolism and allows *e* gene product, phage lysozyme, to degrade the host cell wall and release progeny phage.

The event promoted by *t* gene product may be the primary cause of not only T4-induced lysis but also of lysis induced by other coliphages. The functions of λ gene S (Harris *et al.*, 1967; Goldberg and Howe, 1969), ØX174 gene I (Hutchison and Sinsheimer, 1966), or S13 gene V (Baker and Tessman, 1967) appear identical to that of T4 gene *t*.

My approach to the mechanism of *t* gene product action was based on the following ideas. Lysis destroys the structural stability of the host. The rigid cell wall is primarily responsible for this structural stability, and phage lysozyme,

the enzyme which destroys it, is presumably separated from its site of action by the cell membrane, whose integrity is also necessary for continued metabolism. The finding that a t gene defect prevents both cessation of metabolism as well as lysozyme action led to the idea that t gene product acts to disrupt the host membrane, halting metabolism and allowing phage lysozyme to attack the host cell wall.

The results reported in Manuscript II are consistent with this mechanism in that treatment with neither a metabolic poison nor exogenous lysozyme will phenotypically revert the t gene defect. These results show that the normal function of gene t involves something besides cessation of host metabolism and disruption of the host cell wall.

If t gene product disrupts the host membrane, perhaps it acts by degrading membrane phospholipids. This idea was tested by measuring hydrolysis of host membrane phospholipid during T4 infection. As shown in Manuscript III, lysis, although normally accompanied by phospholipid hydrolysis, can occur in the absence of this reaction. Thus the phospholipid hydrolysis observed is a by-product of the t gene event rather than a requirement for lysis.

Therefore, if t gene product is involved in disruption of the host membrane, its mechanism involves a reaction which would not

have been detected by measuring gross phospholipid hydrolysis. Possibilities would include, for example,

1) degradation of a minor species of membrane phospholipid,

2) degradation of membrane protein,

3) shift in membrane structure to an unstable configuration.

Alternatively, the site of t gene product action may be an unknown layer of the host envelope instead of either the membrane or the lysozyme-sensitive layer.

Further studies are required to determine the mechanism of t gene product action. Such studies would be aided by an *in vitro* assay for t activity which involves as few assumptions as possible about its mechanism. A possible approach might be based on the following observations.

Lysis involves disruption of the structural stability of the cell. This stability is due, at least in part, to the lysozyme-sensitive layer of the cell envelope, but perhaps not solely, since cells must be treated with chloroform (Brown, 1956; Weidel and Katz, 1951; Sekiguchi and Cohen, 1964), with a chelating agent (Repaske, 1958), or with lyophilization (Tsugita *et al.*, 1968) before treatment with exogenous lysozyme causes lysis. Unpublished experiments of my own show that disruption of the lysozyme-sensitive layer does not necessarily lead to lysis, suggesting that lysis requires the disruption of more than

one layer of the cell envelope. In these experiments lysozyme treatment of a lysozyme-sensitive mutant of E. coli B, AS19 (Sekiguchi and Cohen, 1967) rendered >90% of the cells sensitive to osmotic shock but had no effect on either viable count or culture turbidity. These observations support the idea that while the lysozyme-sensitive layer may be responsible for resistance to osmotic shock, it is not solely responsible for the structural stability of the cell.

This result suggests a possible assay for t gene product activity. Using egg white lysozyme-treated AS19/4 bacteria as the substrate, extracts of lysozyme-defective, T4-infected cells could be assayed for lysin activity leading to a decrease in either viable count or turbidity. If such a lysin activity were detected, extracts of cells infected with e:t double mutant phage could be assayed to see if the new lysin activity depended on functional t gene product.

Evidence for a T4 lysin activity independent of phage lysozyme has been found by other investigators. Barrington and Kozloff (1956), Katz and Weidel (1961), and Katz (1964) found a lysin activity associated with T2 phage particles which could be dissociated by freezing and thawing, a process that disrupts phage (Weidel and Primosigh, 1957). To ask whether this activity was due to phage lysozyme, Emrich and Streisinger (1968) measured the

lysin activity of disrupted T4 $\underline{e}^+$ and $\underline{e}$ mutant phage particles. Both preparations contained lysin activity although the level of activity per particle in the $\underline{e}$ preparations was only 3% of that in the $\underline{e}^+$ preparations. No activity was found in the phage preparations prior to disruption. To verify that the lysin activity associated with $\underline{e}$ mutant particles was not due to phage lysozyme, both preparations were treated with antiserum prepared against purified phage lysozyme. The lysin activity associated with $\underline{e}$ mutant particles was not destroyed by antiserum treatment while antiserum treatment destroyed 95% of the lysin activity associated with $\underline{e}^+$ particles. Emrich and Streisinger (1968) concluded that phage particles have a lysin activity which is not due to the $\underline{e}$ gene product, phage lysozyme. It would be interesting to assay preparations of disrupted $\underline{e}$:$\underline{t}$ double mutant particles for this new lysin activity.

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I remember my youth
and the feeling that will never come back anymore
- the feeling that I could last forever,
outlast the sea,
the earth,
and all men.

Joseph Conrad