ACRIDINE-RESISTANCE IN PHAGE T4D[1,2]

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AT least two developmental processes of bacteriophage T4 are sensitive to the acridine dyes, 9-aminoacridine (9AA) and proflavine. One of these is a nearly terminal step in the assembly of mature phage (FOSTER 1948; DEMARS, LURIA, FISHER and LEVINTHAL 1953; DEMARS 1955; KELLENBERGER and SÉCHAUD 1957; SUSMAN, PIECHOWSKI, and RITCHIE 1965). The second is an earlier process that we have called an "acridine-sensitive maturation clock" on the basis of the kinetics of recovery of phage-bacteria complexes from 9AA treatments (SUSMAN, PIECHOWSKI, and RITCHIE 1965). This clock starts 7 minutes after infection (at 30°C) and signals the start of infective phage formation at 23 minutes. A third acridine-sensitive process is the lysis of phage-infected bacteria. The effect of 9AA on lysis is probably distinct from the effect on the maturation clock (COUSE 1966).

The concentrations of acridine dyes required for inhibition of phage growth are not sufficient to prevent the growth of the host bacteria. This was at first regarded as evidence that the dyes specifically interfered with phage functions rather than host functions. This interpretation seems weak, however, in the light of SILVER's observation (1965) that bacteria infected with T-even phages will take up more acridine dye from the growth medium than is taken up by uninfected cells. On the other hand, the existence of acridine-resistant mutants of the T-even phages suggests that the functions that are inhibited are indeed phage-controlled functions.

Two mutations affecting the acridine-sensitivity of the T-even phages have been described. For T4, these are known as *ac* (EDGAR and EPSTEIN 1961) and *q* (SUSMAN and EDGAR, unpublished; cited in PRATT, STENT, and HARRIMAN 1961). The homologous mutations in T2 are known as *pr* and *q* (HESSLER 1963).

Studies of these mutants have been made in hopes that the mutations would help in the identification of the acridine-sensitive developmental processes. Studies of *ac* (*pr*) mutants, however, have not yielded such information. SILVER (1965) has shown that bacterial cells infected with *ac* or *pr* mutants take up less acriflavine from the culture medium than do cells infected with wild-type phage. SILVER concludes that the *ac* gene of the phage regulates the permeability properties of host cell. This is a trivial kind of resistance in the sense that it depends on

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the alteration of the effective concentration of the inhibitor rather than on an alteration of the intrinsic sensitivity of the target process.

HESSLER (1965) has studied the effects of the *pr* mutation on the induction of mutations and on the photosensitization of phage by proflavine present in the medium during phage growth. Her results are consistent with SILVER's conclusion that the mutation affects the uptake of the dye by the infected cell.

Both SILVER and HESSLER have presented evidence that the *q* mutation does not affect the intracellular concentration of acridine dyes.

In this paper, we describe a *q* mutation of phage T4. We argue that the gene containing the mutation is involved in a nearly terminal step in phage assembly.

MATERIALS AND METHODS

Phage strains: All phages used are derived from the wild-type of phage T4D (DOERMANN and HILL 1953).

The mutation designated *q*ED41 is the original *q* mutation isolated by SUSMAN and EDGAR (unpublished; cited in PRATT, STENT, and HARRIMAN 1961) from a doubly mutant strain. The origin of the mutation is as follows: An acridine-resistant mutant of T4 was selected from a wild-type stock by its ability to form a plaque on agar plates containing 0.25 μg/ml of acriflavine neutral in the bottom layer of agar. This mutant was called T4D *ac*41 (EDGAR and EPSTEIN 1961). The *ac*41 mutants could not form plaques on agar containing 1.25 μg/ml of acriflavine, but the *ac*41 stock contained doubly mutant phages that could. A cross of one of these doubly mutant phages to wild-type T4 yielded a class of recombinant progeny that proved to be less acriflavine-resistant but more quinacrine-resistant than *ac*41. This mutation was therefore designated *q*, or, in full, *q*ED41. Crosses between *ac*41 and *q*ED41 yielded *ac q* recombinants that would grow on agar plates containing 1.25 μg/ml of acriflavine.

The origin of other *q*-ish mutations will be described in RESULTS.

The amber mutations, *am*N66, *am*N56, and *am*E18, grow on the host strain *E. coli* CR63 but not on host strains B/5 or S/6/5. These mutations lie in genes 16, 17, and 18, respectively. "Gene" here refers to the complementation groups described in T4 by EPSTEIN *et al.*, 1963.

It should be noted that *am*N56 is not a true amber mutation. Its most significant peculiarity is its ability to grow on Hfr 4 (WOLLMAN and JACOB 1959), a strain which is restrictive for all of the other amber mutants that we have tested on it. This suggests that the suppressor of *am*N56 is not an amber or ochre suppressor. REBECCA HILL KRIEG (personal communication) has made an extensive study of this suppressor. She finds that the suppressor of *am*N56 is located very close to or is coincident with the locus determining the streptomycin-sensitive phenotype of many K-12 strains, and that the suppressor does not suppress true amber mutants.

We have used eight temperature-sensitive mutations, all in gene 17. These form plaques on plates incubated at 30°, but not on plates incubated at 42°.

For the *am* and *ts* mutants we are grateful to R. S. EDGAR and J. FLATGAARD.

Bacterial strains: Escherichia coli strain B/5 was used as host for all experiments except for crosses involving two amber mutants, (whether the two ambers were in the same gene or not), in which CR63 was the host. Strain B/5 or S/6/5 were used as plating bacteria and as selective indicators for *am*+ phages. Strain CR63 was used as the nonselective plating bacterium for experiments involving amber mutants.

Crosses: Crosses were done in H broth according to usual phage techniques. The procedures follow closely those described by STEINBERG and EDGAR (1962). For crosses in which recombination was stimulated by ultraviolet irradiation of the parents, the mixture of parental phage was irradiated in buffer to a dose of about eight phage lethal hits per phage. Test crosses showed that this dose increased frequencies of recombination between closely-linked markers from six- to tenfold.

Plaques to be spot tested were picked with sterile hardwood applicators. The applicators were

washed in 1 ml of sterile broth. This broth was then spotted onto test plates with capillary melting point tubes. Picked plaques of *q* phage can be distinguished from *q*+ by their ability to form a clearing on a plate seeded with *E. coli* B/5 and containing 0.8 μg/ml of proflavine in the bottom layer of EHA agar. Picked *am*+ plaques can be distinguished from *am* plaques by their ability to form a clearing when spotted on a plate seeded with *E. coli* B/5 or S/6/5.

Media and growth: The phosphate glucose growth medium supplemented with Casamino acids, broth, and the preparation of host and indicator bacteria and phage stocks are described by SUSMAN *et al.* (1965). One modification has been introduced on the basis of the observation that cells which have spent a longer time in the log phase have higher yields of phage. Therefore, the overnight cultures were diluted 1000-fold into fresh medium and grown under vigorous aeration at 36° for about 2.5 hours until the titer reached 6 to 8 $\times$ 10^7 cells/ml. After centrifugation and resuspension at about 1.5 $\times$ 10^9 cells/ml the host bacteria were distributed to two or three tubes and kept on ice for several hours (sometimes as long as 7 hours). The length of time while on ice does not measurably affect phage growth so that one can do two or three separate experiments on the same batch of bacteria and obtain results that agree as well as if they came from a single experiment.

Chemicals: Puromycin dihydrochloride Grade II was purchased from Sigma Chemical Company. Chloramphenicol donated by Parke, Davis and Company gives results identical to those obtained with puromycin as previously described (PIECHOWSKI and SUSMAN, 1966a), while chloramphenicol from other sources fails to do so.

9-Aminoacridine hydrochloride (9AA) was obtained from Mann Research Laboratories, Inc. This acridine compound was used because of its stability and lack of photosensitization of the infected complexes.

Proflavine dihydrochloride was from Mann Research Laboratories. Distilled water solutions were stored in the cold in brown glass bottles and were discarded when they were two weeks old.

RESULTS AND DISCUSSION

Mapping of q: It appears that *q*-type acridine resistance of T4D results from alterations in gene 17. We have not yet been able to show that the reference *q* mutation, *q*ED41, lies in gene 17, but the following evidence shows that it lies very close to gene 17.

Crosses between *q*ED41 and various turbid (*tu*) mutants showed that *q* was 6 map units from *tu*43 (SUSMAN, unpublished). This *tu* mutation lies in the map region occupied by genes 15–20. Representative *am* mutants in these genes were crossed to *q* in order to produce *am q* recombinant phages. (These doubly marked phages can be used in crosses in which recombinant progeny can be selectively scored.) All the crosses except the *am*N56 $\times$ *q* cross yielded the desired recombinants. Repeated efforts to isolate an *am*N56 *q* recombinant failed even in crosses in which recombination was stimulated by ultraviolet irradiation of the parent phage or of the infected bacteria. These results meant either that *am*N56 recombined at a low rate with *q*ED41 or that the desired recombinant did not have the predicted phenotype. In fact, we later discovered that *am*N56 is inhibited at a lower 9AA concentration than is required to inhibit the growth of wild-type phage; so it is likely that the double mutant will not be detected by our usual selection techniques.

*am*N56 in gene 17 lies between *am*N66 in gene 16 and *am*E18 in gene 18. On the basis of the crosses 1 and 2 in Table 1, we place *q*ED41 between *am*N56 and *am*N66. Of the progeny selected as recombinants between *am*N56 and *am*N66,

TABLE 1

*Crosses to locate q*ED41 *and to implicate gene 17 in the determination of 9AA resistance*

Cross No.	Parents	UV	Selected genotype	Number tested	Unselected genotype	Number observed
1	*am*N56 × *am*N66*q*ED41	No	*am*+(1.2%)	133	*q*+	25
2	*am*N56 × *am*E18*q*ED41	No	*am*+(2.5%)	152	*q*+	3
3	N56R1* × *am*N56	Yes	*am*+	600	*q*+	0
4	N56R1 × wild-type	No	....	294	*am*	0
5	N56R1 × wild-type	Yes	....	590	*am*	0
6	N56R1 × *q*ED41	Yes	....	365	*q*+	6
7	N56R1 × *q*ED41	No	....	747	*q*+	1
8	*ts*N20 × *am*N66*q*ED41	No	*ts*+*am*+(1.8%)	472	*q*+	18
9	*ts*N20 × *am*E18*q*ED41	No	*ts*+*am*+(4.0%)	484	*q*+	17

Parents of the indicated genotypes are crossed under the standard conditions for phage crosses. Where the parent phage have been ultraviolet irradiated, the dose used was sufficient to give a 6- to 10-fold increase in recombination frequencies for close markers. In some crosses, progeny phage of a particular genotype were selected from the mass of progeny for further characterization. Where no such selection is indicated, the progeny was sampled at random for further characterization. Where it is interesting, the frequency of the selected recombinant type is shown in parenthesis after the selected genotype. The indicated number of picked plaques of the selected genotype was then tested by appropriate spot test for the presence of the unselected marker indicated. The number of plaques carrying the unselected marker is shown in the column farthest to the right.

* Phenotype of N56R1 is like that of *q*.

20% are also recombinants between *q* and *am*N56. Of progeny selected as recombinants between *am*N56 and *am*E18, only 2% are also recombinants between *am*N56 and *q*. If we assume that negative interference is moderate in these crosses (CHASE and DOERMANN (1958) observed a 7- to 20-fold excess of double crossover progeny in crosses involving markers 2 to 3 map units apart), we can use these data to establish the position of *q*ED41 with respect to the three amber mutations. The data indicate that *q* lies between *am*N56 and *am*N66 and that the distance from *q* to *am*N56 is less than the distance from *q* to *am*N66. The following rough map is consistent with the data[4]:

In an effort to find a gene-17 mutation that lies to the left of *q*ED41, we crossed nine temperature-sensitive (*ts*) mutations in gene 17 to *am*E18 and to *am*N66 to determine which of the nine lay farthest to the left. The leftmost *ts* mutant, *ts*N20, was then crossed to the doubly mutant phages, *am*N66*q*ED41 and *am*E18-*q*ED41. The results of these latter crosses are shown as crosses 8 and 9 in Table 1. The results show that *ts*N20 is not allelic to *q*ED41, but that it is very close to *q*ED41. The sort of reasoning that led us to conclude that *q*ED41 lies between *am*N66 and *am*N56 would lead us to conclude from crosses 8 and 9 that *q*ED41 lies on both sides of *ts*N20[5]. This observation suggests, but does not justify, the

[4] It is possible by invoking high negative interference to reconcile the results of crosses 1 and 2 with a map on which *q*ED41 is to the right of *am*N56. This reconciliation, however, requires that in at least one of the crosses the number of double-crossover recombinants exceed the number of the less frequent single-crossover type. We think this is an unacceptable requirement. For this reason, we prefer the map order shown.

[5] Crosses 8 and 9 can be easily fitted to a map on which *ts*N20 is to the left of *q*ED41 and only with difficulty (see footnote 4) fitted to a map on which *ts*N20 is to the right. Since a choice between these two map orders affects the strength of our claim that *q*ED41 lies in gene 17, we must emphasize that neither map order is excluded by our data.

amusing speculation that one of the two mutations (*ts*N20 or *q*ED41) is a duplication mutation and that the other is a point mutant, with the duplicated region containing the site of the point mutant.

We have not succeeded, then, in finding a gene-17 mutation to the left of *q*ED41. We have not attempted to find gene-16 mutations to the right of it. At this time, there are no other mutations in gene 16 to use in such an attempt. We can only conclude, therefore, that *q*ED41 is either the leftmost known mutation in gene 17 (with the possible exception of *ts*N20), or that it lies in gene 16, or that it identifies a new gene between 16 and 17.

Other q-*ish mutants:* Two mutations to 9AA-resistance can be shown to lie in gene 17. One of these was isolated from *am*N56 as an am^+ revertant. It is designated N56R1. Crosses 3 to 7 in Table 1 show that N56R1 is allelic to *am*N56, that the acridine resistance of N56R1 was not acquired as the result of a second mutation at a site that recombines with *am*N56, and that N56R1 is distinct from *q*ED41 but very close to it on the genetic map.

The other 9AA-resistant mutation in gene 17 is *ts*N20, which we have already discussed. Crosses 8 and 9 in Table 1 show that the acridine-resistance of *ts*N20 is not the result of a mutation allelic to *q*ED41. To determine if the temperature-sensitivity and the acridine-resistance of *ts*N20 were acquired as a single mutation, we isolated eight independent ts^+ revertants from *ts*N20. Six of these were found to have lost their acridine-resistance along with their temperature-sensitivity. Two of the ts^+ revertants were still 9AA-resistant. Crosses of three of the acridine-sensitive revertants to wild-type T4D yielded fewer than 0.01% acridine-resistant recombinants. From these observations, we conclude that the two phenotypic differences between *ts*N20 and wild type result from a single reversible mutational event.

The function of gene 17: Lysates of gene-17 mutants grown under restrictive conditions contain structures that in electron microscope pictures appear to be normal head membranes and normal tail parts (EPSTEIN *et al.* 1963). EDGAR and WOOD (1966) using their *in vitro* phage-assembly system, have shown that the tail structures in gene 17 "extracts" can be used in the assembly of infective phage but that the head structures are defective. It might be concluded, therefore, that gene 17 is involved in some step in phage head production and, by association, that the *q* mutation affects that process in head production.

Acridine dyes seem to mimic the effect of the amber and temperature-mutations in gene 17. DEMARS (1955) showed that when maturation of T2 is inhibited by proflavine all the tail antigen (SBP) is found in the supernatant of lysates spun at speeds high enough to sediment whole phage. This indicates that the tail fibers, at any rate, are not attached to the phage heads that might be produced in the presence of the acridine. Head membranes free of tail structures were also found in proflavine lysates of T-even phages (DEMARS *et al.* 1953; KELLENBERGER *et al.* 1959). (DNA is usually lost from unattached heads during preparation for electron microscopy (KELLENBERGER, personal communication).)

Lysates of wild-type phage grown in the presence of 9AA do not contain functional heads but do contain functional tails when assayed in the EDGAR-WOOD system. Lysates of *q* grown under identical conditions do contain usable heads.

9AA does not interfere with the *in vitro* assembly of infective phage in mixtures of head- and tail-donor extracts (SUSMAN, unpublished). Since the defective heads will not attach to normal tails and since the point of attachment of tail to head is at one of the vertices of the head membrane (MOODY 1965), we might speculate that 9AA (and *am*N56) result either directly or indirectly in the defectiveness of these vertices.

In addition to gene 17 there are 11 genes known in T4 that play an essential role in the completion of the phage head. Amber mutations in genes 2, 64, 50, 65, 4, 13, 14, 16, and 49 cause the production under restrictive growth conditions of structures that look like phage heads in electron micrographs but that do not act like completed heads in the *in vitro* phage assembly system (EDGAR and WOOD 1966, and personal communication). The conclusion that 9AA affects the function governed by gene 17, therefore, must rest on the finding that *q* mutations are located in gene 17 and not on the similarity between the effect of 9AA and the effect of *am*N56 on phage development.

Phage yield in the presence of 9AA: To compare the acridine-sensitivity of *q* and q^+ phages, one can measure the phage yield from infected cells incubated throughout the cycle of infection in medium containing 9AA. Such results are summarized in Table 2. The relative acridine-resistance of *q* is conspicuous. We can see from the results how *q* manages to produce plaques on acridine-containing agar that will not support wild-type plaque formation.

It is still more informative to add 9AA to infected cells at various times during the latent period and to measure the amount of mature phage that can be made after the addition of the dye. Experiments of this type are shown in Figure 1. Cells infected with phage are transferred at intervals into medium containing 9AA. They are incubated until 70 minutes after infection, well after the time when the untreated cells have lysed (about 55 minutes) and are then lysed by shaking them with chloroform. The control consists of chloroform samples taken at intervals from the culture to follow the accumulation of intracellular phage.

As can be seen from Figure 1A, 1.0 μg/ml of 9AA is sufficient to give maximum inhibition of the growth of wild-type T4. The vertical distance between the 9AA

TABLE 2

Phage yield from cells incubated in the presence of 9AA

9AA concentration μg/ml	Burst size		Burst size as percent of control	
	T4D wild type	T4D*q*ED41	wild type	*q*
0.00	328	217	100	100
0.25	104	164	32	76
0.50	3.9	52	1.2	24
0.75	0.15	14	0.046	6.4
1.00	0.019	5.2	0.006	2.4

A log phase culture of *E. coli* B/5 was centrifuged and resuspended in fresh broth at 8.9×10^8 cells/ml. KCN was added to give a 0.002M solution. The cells were then infected with T4D wild-type phage at a multiplicity of infection (moi) of 6.9 phage particles/cell or with T4D *q*ED41 at moi of 6.3 phage particles/cell. After 16 minutes the infected cells were diluted 5000-fold in fresh broth free from cyanide but containing 9AA at the concentrations shown in the table. After an additional 104 minutes of incubation at 30° without aeration, the cells were lysed with chloroform. The burst sizes are expressed as phage particles/infected bacterium.

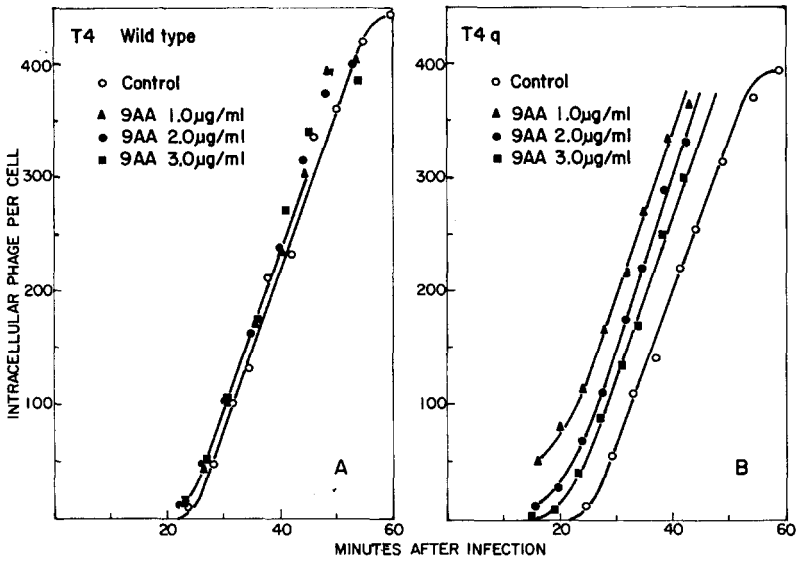

FIGURE 1.—Inhibition of maturation of T4 wild type and T4*q* by 9AA. Two separate experiments on the same batch of cells: the bacteria at a titer of 1.5×10^9 cells/ml were infected with T4D wild type at a multiplicity of 7 in the first experiment, and with T4*q* at a multiplicity of 5 in the second experiment. After 4 minutes of adsorption (with bubbling) and 4 minutes of serum treatment (k of the serum in adsorption tube about 2 min^{-1}) the cultures were diluted 1×10^4 and incubated at 30° with vigorous aeration. At intervals samples of the growth tubes were diluted 100-fold into broth saturated with chloroform and then plated to measure intracellular phage (control). Other samples, removed from the growth tubes at the times shown on the abscissa were diluted 100-fold into tubes containing medium with 1.0, 2.0, and 3.0 μg/ml of 9AA. The 9AA tubes were treated with chloroform at 70 minutes after infection and assayed for the yield of phage. The T4D wild-type experiment is shown in A; the *q* experiment in B.

curves and the control intracellular phage curve is equal to 12 phage particles/cell. This number is very close to the number (14 in this experiment) of phage particles produced per minute by the untreated cells. It could be concluded from this observation either that 9AA takes about one minute to penetrate the cell or that the nascent phage takes one minute after passing the acridine-sensitive step in maturation to become infective.

Figure 1B shows the results of a similar experiment involving phage carrying the *q* mutation. At 1.0 μg/ml of 9AA, the distance between the 9AA curve and the control intracellular phage curve is 120 phage particles/cell, ten times the distance found with wild-type phage at the same 9AA concentration. It might be supposed either that the *q* mutation alters the 9AA-sensitive step or that it alters the size of the pool of precursor phage en route from the 9AA-sensitive step to the final attainment of infectivity. The response of *q*-infected cells to higher concentrations of 9AA favors the first supposition. If there were a pool of immature phage that could become infective in the presence of 9AA, we might expect that with increasing concentrations of 9AA the distance between the 9AA curve and the chloroform lysis curve would approach the size of that pool. What is actually found in a series of experiments to test 9AA concentrations between

0 and 6 μg/ml is that the distance between the 9AA curve and the control curve diminishes with increasing 9AA concentration until maximum inhibition is achieved at 5 μg/ml of 9AA. At that concentration, the distance between the two curves is 12 phage particles/cell, the same as the minimum distance in experiments with wild type. Thus, in *q*-infected cells, there does not appear to be an abnormally large pool of immature phage particles that have passed the acridine-sensitive maturation step and are simply waiting to become infective phage.

Does the q *mutation affect the maturation clock?* No. Two sorts of evidence support this assertion. The first comes from observations on the kinetics of maturation following pulse treatments of infected cells with 9AA. Cells infected with *q*ED41, when subjected to pulse treatments with 9AA during the critical period from 7 to 23 minutes after infection, show the same delay in the beginning of maturation as do cells infected with wild-type phage. With 1.5 or 2.0 μg/ml of 9AA, these experiments give results nearly identical to those shown in Figures 5, 7, and 8 of a previous paper (SUSMAN *et al.* 1965). Therefore, we shall not present the results here in detail. At lower concentrations of 9AA, the delay in maturation of *q* and of q^+ phage is less than the maximum observed at 1.5 μg/ml, but it is *the same delay* for mutant and for wild type (J. D. MCLAREN, personal communication).

The second sort of evidence comes from observations on the kinetics of phage

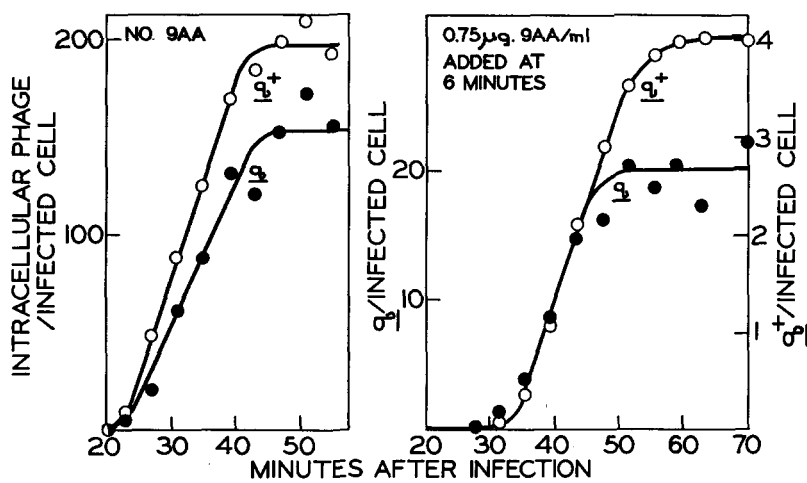

FIGURE 2.—Phage maturation in the presence of 0.75 μg/ml of 9AA. Log phase *E. coli* B/5 at a titer of 8.6×10^8 bacteria/ml in H broth was infected with T4D wild-type phage at a multiplicity of infection (moi) of 3.3 or with T4D *q*ED41 at a moi of 5.2. After 2 minutes of adsorption and 4 minutes of treatment with anti-T4 cow serum (violent aeration throughout), the cultures were diluted 10^4-fold into fresh H broth containing 0.75 μg/ml of 9AA and 10^4-fold into parallel control tubes containing acridine-free broth. Intracellular phage production was followed by removing samples from the growth tubes at intervals and blowing them into chloroform. The graph on the left shows phage production in the control tubes for the *q* and q^+ cultures. The graph on the right shows phage production in the two acridine-treated cultures. It must be noted that the ordinates in the right-hand graph are drawn to different scales for *q* and q^+ to facilitate comparison of the two curves.

production in the presence of low concentrations of 9AA. In the experiments shown in Figure 2, cells infected with T4D wild type or with T4D *q*ED41 were transferred at 6 minutes after infection into medium containing 0.75 μg/ml of 9AA. Samples from these cultures and from acridine-free controls were shaken at intervals with chloroform and assayed in order to measure intracellular phage. The *q*-infected cells yield more phage in the presence of 9AA than do the wild-type infected cells, but the beginning of maturation occurs at *the same time* in both cultures. Maturation begins about 11 minutes later in the presence of the acridine than in the controls. The ordinates in the right frame of Figure 2 are adjusted to emphasize the kinetic similarities between the *q* and q^+ curves. Similar experiments using 0.25 and 0.5 μg/ml of 9AA yield similar results. The appearance of phage is delayed in proportion to the concentration of dye added and is delayed by the same amount for the mutant and for wild-type phage.

These observations support the following two assertions: (a) The acridine-sensitive clock is distinct from the nearly terminal step in maturation whose acridine sensitivity is affected by the *q* mutation. We think that in wild-type phage the *q*-affected process is more sensitive than the clock, so that phage production is blocked almost completely at concentrations of acridine that do not give maximum inhibition of the clock. We suppose that the *q* mutation reverses this relationship so that in the mutant the terminal event will proceed in the presence of clock-stopping concentrations of 9AA. (b) The *q* mutation does not seem to affect the intracellular concentration of 9AA, since at least one process (the clock) is as sensitive to a given 9AA concentration in *q* infections as in q^+ infections. This agrees with SILVER's observation (1965) that *q*-infected cells take up as much acriflavine fluorescence from the growth medium as do q^+-infected cells. HESSLER (1965) arrives at similar conclusions on the basis of experiments on proflavine mutagenesis. COUSE (personal communication) finds that 9AA at a given concentration causes an identical delay of lysis in *q*- and q^+-infected cells.

The kinetics of phage accumulation in the presence of 9AA and after removal of 9AA: Figure 3 presents the results of an experiment in which the rate of maturation of T4D *q*ED41 was followed in the presence of three different concentrations of 9AA. Acridine was added to the infected cells at 24 minutes after infection. Intracellular phage was followed as usual by removing samples of the cultures at intervals and shaking them with chloroform. The addition of 9AA immediately establishes a new rate of maturation. The rate is characteristic for a given concentration of 9AA and is almost null in complexes that are maximally inhibited (Figure 3C). The rate of maturation in the presence of 9AA remains constant until the final level of phage is attained. Lower concentrations of 9AA allow a higher rate of phage formation over longer periods of time than do higher concentrations of the acridine.

Note that the speed with which 9AA alters the rate of maturation again argues that the dye affects a nearly terminal step in phage assembly.

Figure 3 also shows what happens if the complexes are diluted into fresh medium 6 minutes after the addition of 9AA. The level of acridine after dilution

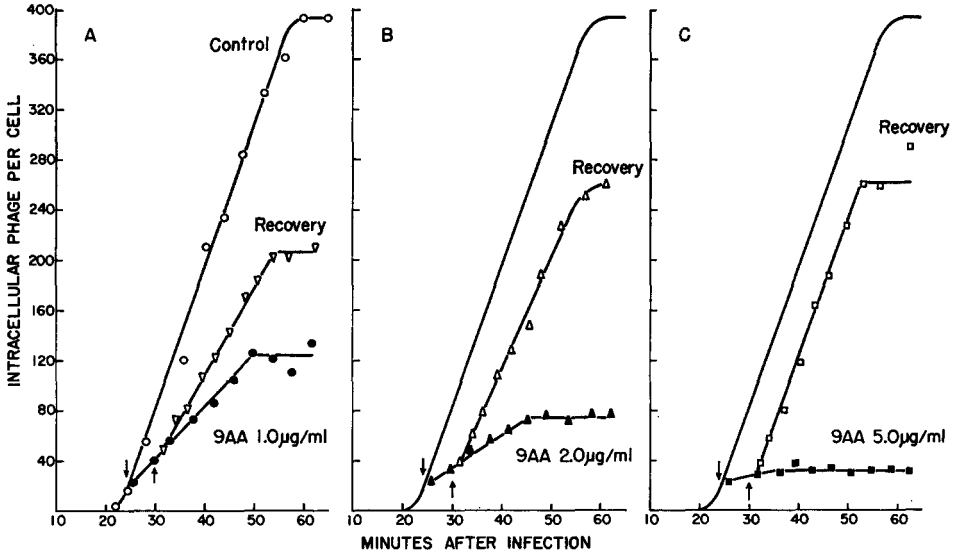

FIGURE 3.—Maturation of T4*q* in the presence of 9AA and recovery from a 6-minute pulse of 9AA. Bacteria at a titer of 1.2×10^9 cells/ml were infected with T4*q* at a multiplicity of 6 in three adsorption tubes (A, B and C). After 4 minutes of adsorption and 4 minutes of serum treatment the tubes were diluted 100-fold and bubbled at 30°. This growth tube was again diluted 100-fold to provide samples for the intracellular phage growth control curve. At 24 minutes after infection dilutions were made into 1.0, 2.0 and 5.0 μg/ml of 9AA. At intervals samples were taken from the 9AA tubes and diluted into broth saturated with chloroform and assayed for phage. At 30 minutes after infection (arrow) the 9AA tubes were again diluted (50-fold from 1.0 μg/ml, 100-fold from 2.0 μg/ml and 250-fold from 5.0 μg/ml). From these dilution tubes, samples were removed at intervals into chloroform and assayed to follow the accumulation of intracellular phage in bacteria recovering from 9AA treatment.

was 0.02 μg/ml in each case, a concentration that has a negligible effect. We find that the rate of phage production after removal of the acridine is inversely related to the rate in the presence of the acridine. Maximum inhibition secures maximum recovery. In Figure 3C, where maturation is almost completely stopped in the presence of the acridine, maturation resumes at the control rate after removal of the acridine. This does not differ from the situation with wild type, in which a much lower concentration of 9AA added shortly after the beginning of maturation is sufficient to give maximum inhibition of phage production and in which phage production resumes at the control rate immediately upon removal of the inhibitor (Figure 9 in SUSMAN *et al.* 1965).

The least inhibition (1.0 μg/ml) leads, after removal of the dye, to a rate of phage production that is only 60% of that of the control. These results are summarized in Table 3.

Infected bacteria seem to recover better from treatments with high concentrations of 9AA than from treatments with low concentrations, as if the struggle against the lesser adversary were more exhausting than complete surrender to the larger. Lasting damage is done to the cell in proportion to the cell's ability

TABLE 3

Comparison of maturation rates of T4q in the presence of 9AA and after a 6-minute pulse of 9AA

	Concentration of 9AA in μg/ml		
	1.0	2.0	5.0
In the presence of 9AA	37	24	8
In recovery from 6-minute pulse of 9AA	60	78	98

Rates of maturation given in the body of the table are expressed as percent of the control rate.

to produce infective phage in the presence of 9AA. We might imagine, for example, that production of infective phage in the presence of 9AA irreversibly diminishes the supply of some important maturation material. Or we might suppose that in the presence of the dye some stable inhibitory material accumulates along with the infective phage.

Maturation of T4q in the presence of 9AA and puromycin: In the experiment shown in Figure 4, bacteria infected with T4 *q*ED41 are divided into four cultures. The first is an untreated control. At 24 minutes after infection, puromycin at 250 μg/ml (final concentration) is added to the second culture. After addition of the puromycin, infective phage continues to appear at the same rate as in the untreated culture until the supply of maturable precursor protein (in this experiment, about 60 phage equivalents) is exhausted. This continued maturation after protein synthesis has been arrested is the subject of a previous paper (Piechowski and Susman 1966). At 24 minutes, 9AA at 1.0 μg/ml is added to the third culture. Following addition of the acridine, maturation continues at a rate lower than that of the control culture until about 100 additional phages have appeared. To the fourth culture, both puromycin (250 μg/ml) and 9AA (1.0 μg/ml) are added. In this last culture, the *rate* of maturation is the same as in the 9AA-

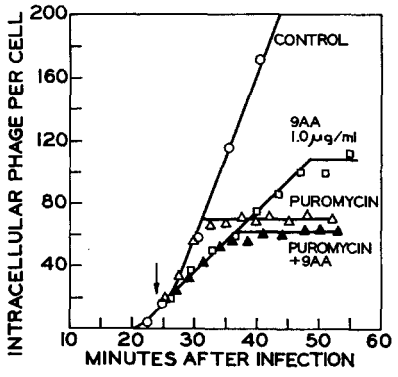

Figure 4.—Maturation of T4*q* in the presence of 9AA and puromycin. The bacteria at a titer 1.5×10^9 cells/ml were infected with T4*q* at a multiplicity of 7. After 3 minutes of adsorption and 3 minutes of serum treatment the culture was diluted 2×10^4-fold and divided into four batches. At 24 minutes after infection the first received 9AA (1.0 μg/ml final concentration), the second puromycin (250 μg/ml), the third 9AA and puromycin, and the fourth served as control.

treated culture and the *final yield* of phage is almost the same as in the puromycin-treated culture. Almost all of the precursor protein that *can* be converted into mature phage in the presence of puromycin alone *is* converted into phage in the presence of puromycin plus 9AA, but the rate of conversion is reduced. Thus, the acridine does not reduce the rate of maturation by causing fabrication of inviable phage particles along with good ones. Since inhibition of protein synthesis by puromycin does not affect the rate of phage production, we must conclude that 9AA exerts its influence by interfering with the function of the gene product rather than with the synthesis of the gene product.

It might be worthwhile to mention at this point that a specific interaction of an acridine dye (proflavine) with a biologically active protein (chymotrypsin) has been described recently (BERNHARD and GUTFREUND 1965). It was further shown that one molecule of proflavine binds with one active site of the enzyme (BERNHARD *et al.* 1966). In addition, WALLACE *et al.* (1966) discovered that 9AA acts as an activator of chymotrypsin-catalyzed hydrolysis.

Dominance studies: Cells were mixedly infected with q and q^+ phage at various input ratios. At 24 minutes after infection, 9AA (1.0 μg/ml) was added to the cultures. As can be seen in Figure 5, the rate of phage production after the addition of 9AA depends on the input ratio of the two phage types. Infection with wild type alone serves as the control for zero rate of maturation in the presence of the acridine; infection with q alone serves as the control for the

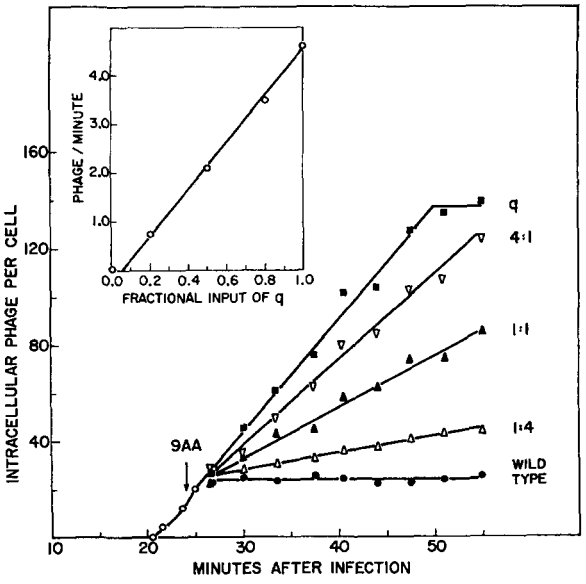

FIGURE 5.—Maturation in the presence of 9AA in cells mixedly infected with T4q and wild type. Bacteria at a titer of 1.0×10^9 cells/ml were infected with T4q, wild type or a mixture of both at an average total multiplicity of 9.4. The ratios of input phage in mixed infections are given with each curve. Adsorption and serum treatment as before, 9AA 1.0 μg/ml was added at 24 minutes after infection. The insert shows the plot of the rate of maturation in the presence of 9AA versus the fractional input of q.

maximum rate in the presence of the dye. The insert gives the rate of maturation in the presence of 9AA as a function of the fractional input of the *q* parent. The rate of maturation in the presence of the dye is directly proportional to the gene dose of *q*.

These results agree with those of HESSLER (1963), who showed that mixed infections with *q* and q^+ mutants of T2 H gave yields in the presence of proflavine that were lower than the yields from *q* infections and higher than the yields from q^+ infections. She also noted that the phage progeny contained both parental types.

The dominance properties of *q* differ from those of *ac*, as described by EDGAR and EPSTEIN (1961). Mixed infections with *ac* and ac^+ show that the ac^+ allele is dominant in the sense that the infected cells will not form plaques on plates containing acriflavine at a concentration that will permit growth of the *ac* mutant alone. (It might be noted here that the *ac* mutant is not at all resistant to 9AA.) Ultraviolet irradiation of the ac^+ parent used in a mixed infection increases the frequency of complexes that will form plaques on acriflavine plates. This presumably means that it is the abolition of the function of the *ac* gene that is responsible for the acridine-resistance of the mutants.

On the other hand, both HESSLER (1963) and SILVER (personal communication) have found that in mixed infections with T2 *pr* and T2 pr^+ the infected cells show an intermediate acridine sensitivity. Both the phage yield and the uptake of dye from the medium reflect this lack of dominance.

The existence of conditional lethal mutations in gene 17 demonstrates that this gene's function is not dispensable. We must conclude, therefore, that the *q* mutation alters the function of gene 17 but does not abolish the function. In this connection it is interesting to recall that a temperature-sensitive mutation, *ts*N20, alters the gene-17 product in such a way that it becomes *both* temperature-sensitive and acridine-resistant. Since the acridine-resistant form of gene 17 must make a functional gene product, it is not surprising that neither allelic form of *q* is dominant.

The competition between *q* and q^+ in mixed infection could simply mean that the concentration of the *q*-type gene-17 product becomes limiting in the presence of 9AA and that the presence of wild-type gene product proportionately reduces the amount of *q* product. On the other hand, it could be that the *q* and q^+ gene products interact functionally in one of several ways. If, for example, the gene product were an enzyme, we might suppose that *q* and q^+ proteins compete for substrate in the presence of 9AA but that q^+ enzyme is inactive. Or we might imagine that the gene product is a substrate for an enzyme and that in the presence of 9AA the q^+ product is a competitive inhibitor of the enzymatic reaction. If the gene product is a multimeric protein, we might argue from the data that the acridine-sensitivity of the multimer is directly proportional to the number of q^+ subunits that it contains or that *q* and q^+ subunits do not combine to form mixed multimers. Other assumptions about the nature of the multimeric product predict that one allelic form or the other will tend to be dominant.

There is a class of very specific models that predict that the observed degree

of dominance of q would depend on the time of addition of the acridine. For example, we might suppose that the product of gene 17 is a component of the phage head and that its high affinity for 9AA accounts for the acridine-sensitivity of head assembly. We might explain the q mutation as an alteration in the 9AA binding-site on the gene 17 product. We might also imagine that by adding 9AA before the gene 17 product is incorporated into phage heads, we might discourage the incorporation of q^+ gene product into phage heads and favor the incorporation of q product. This might cause q to appear to be more dominant the earlier 9AA was added. Figure 6 shows the results of an experiment done to test this possibility. The input ratio of $q:q^+$ is fixed at 1:3 but the time of adding 9AA (1.0 μg/ml) is varied. The rates of maturation and the final yields of phage,

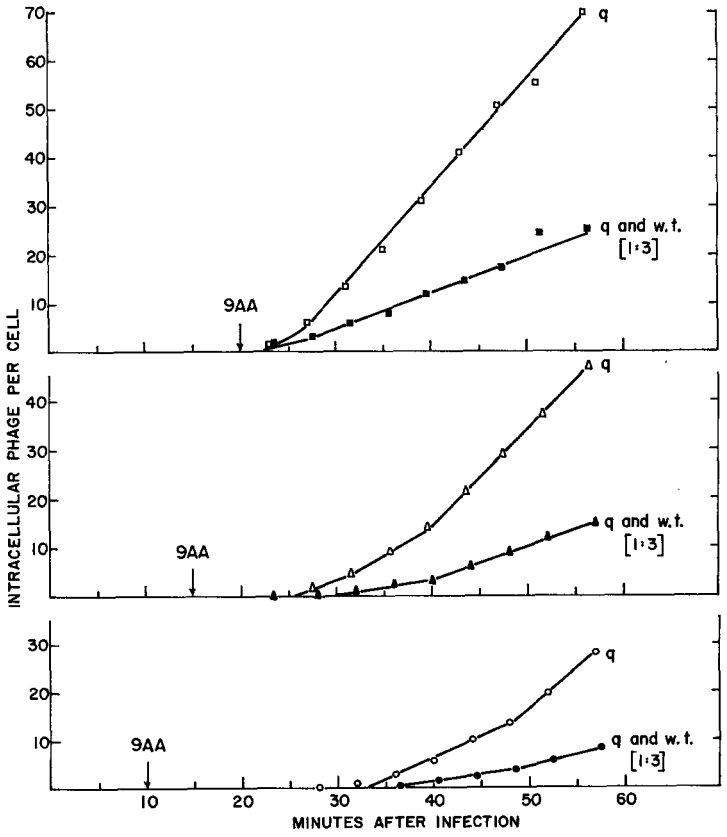

FIGURE 6.—Comparison of maturation in cells infected with T4q alone or with a 1:3 mixture of q and wild type when 9AA is added before the beginning of maturation. Bacteria at a titer of 1.4×10^9 were infected with T4q at a multiplicity of 16 and separately with a mixture of T4q and wild type at a total multiplicity of 11 (3.4 of q and 7.7 of wild type). After 3 minutes of adsorption with bubbling and 3 minutes of serum treatment the cultures were diluted 2×10^4. Each culture was divided into 5 ml batches and aerated at 30°. 9AA at 1.0 μg/ml (final conc.) was added at 10, 15 and 20 minutes after infection. The yield of phage was followed by sampling into broth with chloroform.

whether 9AA is added at 10, 15, or 20 minutes after infection, are very nearly one-third the rates and yields in the controls (q alone). The delay in maturation with earlier additions of 9AA results from partial inhibition of the maturation clock. Thus, the yield of phage and the rate of maturation in the mixedly-infected cells, as compared to q-infected cells, depends only on the gene dose of q and not on the time of addition of the dye.

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SUMMARY

9-Aminoacridine inhibits at least two processes in the development of bacteriophage T4. One of these is a nearly terminal step in the assembly of infective phage; the second is an acridine-sensitive maturation clock. The mutation qED41 affects only the acridine-sensitivity of the former process. It does not affect the intracellular concentration of acridine. The q mutations (qED41 and similar mutations) lie in gene 17, a gene that is involved in a nearly terminal step in the assembly of phage heads. In q-infected cells, 9-aminoacridine blocks phage maturation, not by causing dead phage to be made, but by hindering the assembly process itself. There is no dominance in mixed infections with q and q^+. The q mutations alter the function of the gene product rather than abolish the function of the gene.

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