Application of Arrhenius Kinetic Theory to Viral Eclipse: Selection of Bacteriophage ϕ X174 Mutants

N. L. INCARDONA

*Department of Microbiology & Immunology, University of Tennessee Center for the Health Sciences,
Memphis, Tennessee 38163*

Received 17 February 1981/Accepted 15 April 1981

Analysis of the bacteriophage ϕ X174 eclipse period in terms of Arrhenius kinetic theory suggests the following hypothesis: mutants should exist with two concomitant physiological characteristics as their phenotype. These are an eclipse rate lower than that of the wild type at permissive temperatures for plaque formation and an eclipse rate too low at lower temperatures to permit plaque development. Thus, enrichment of a mutagenized virus population for mutants that fail to eclipse during a short period at permissive temperatures should yield eclipse mutants with the cold-sensitive (*cs*; nonpermissive temperature, 25°C), and not the temperature-sensitive (*ts*; nonpermissive temperature, 42°C), plaque phenotype. In several trials, the frequency of the *cs* phenotype in the population increased from less than 0.2% to between 2 and 4% after the enrichment step, whereas the frequency of the *ts* phenotype remained unchanged (less than 0.2%). Moreover, 80% of these *cs* mutants have eclipse rates that are 3- to 40-fold lower than that of the wild type at both 37°C and 25°C. The successful application of the Arrhenius theory to ϕ X eclipse may provide insights into the molecular mechanism whereby the ϕ X174 genome is delivered into the host cell. Since the eclipse kinetics of other nonenveloped viruses are similar to those of ϕ X174, kinetic theory may be broadly applicable in the selection and characterization of viral eclipse mutants.

Since viral nucleocapsids are constructed from protein subunits and nucleic acid molecules, noncovalent interactions make a significant contribution to the structural stability of viruses and play an important role in their replication. The importance of noncovalent bonds in virus assembly has been extensively documented (3), and the principles that govern their formation are beginning to emerge (21). However, the disruption of these interactions must also play a part in virus replication at the point where viral genomes gain entry into host cells. Yet evidence for the precise role played by noncovalent interactions in this delivery of viral genomes remains very sketchy (1, 6, 12-15, 18, 27, 29, 30). This is partly due to the dearth of adequate means for interrupting the process to determine the number and nature of the intermediate steps. Advantage has been taken of the fact that, at low temperatures, many viruses adsorb but fail to enter the eclipse phase (17, 18, 22, 24) and that some viruses require metabolically active cells for complete entry into the cytoplasm (4, 16, 19). However, there is a need for selection techniques specific for conditionally lethal mutants that are defective in the steps subsequent to adsorption

and associated with the entry into the eclipse phase of the replication cycle. Since the viral genome is dissociated from at least some of the capsid proteins during eclipse, it is likely that mutants with altered noncovalent interactions will be generated by such procedures.

Structural and kinetic studies on the eclipse of bacteriophage ϕ X174 provide a background for the design of selection procedures that are specific for eclipse mutants. One can single out for study the steps associated with the loss in infectivity that occurs at the beginning of the eclipse period by synchronizing the infection in starvation buffer at 15°C and shifting to a higher temperature to initiate the entry into the eclipse phase. Moreover, since EDTA partially disrupts the outer wall of the cell, viral nucleocapsid structures can be recovered from infected cells between the attachment and complete DNA injection steps, inclusively. Use of these techniques has revealed that at the low temperature, ϕ X174 adsorbs to starved cells but does not begin to eject its DNA. Therefore, the recovered virus remains infective. Shifting these virus-cell complexes to temperatures above 20°C in starvation buffer results in the partial ejection of the

DNA with the concomitant loss of infectivity that defines the beginning of the eclipse period. Addition of nutrients to the starvation buffer is required for complete injection of the viral genome (22).

The kinetics of the loss in infectivity associated with the partial ejection process (for convenience, termed the eclipse reaction) can be followed by diluting virus-cell complexes made at 15°C into starvation buffer at higher temperatures and then stopping the reaction by diluting into EDTA at 0°C (23). The rate of this eclipse reaction for wild-type ϕ X and a cold-sensitive (*cs*) eclipse mutant, *cs70*, has been examined over a wide temperature range. At the nonpermissive temperature (25°C) used to select the *cs* mutants of ϕ X, *cs70* has an eclipse rate that is essentially zero. This is the defective step that is responsible for its *cs* plaque phenotype since its eclipse and latent periods, as well as its burst size, are the same as those of the wild type when virus-cell complexes made at 37°C in starvation buffer are shifted to nutrient broth at 25°C to complete the infection (28). However, the unusual property of this mutant is the lower eclipse rate at all temperatures between 30 and 40°C, the permissive range (8). Analysis of these kinetic properties in terms of Arrhenius theory provides a theoretical basis for the lower eclipse rate of *cs70* at permissive temperatures. Furthermore, since the theory requires that such eclipse mutants have the *cs* plaque phenotype, specific procedures have been devised for selecting these mutants. The *cs70* mutation is believed to be in gene F (26).

MATERIALS AND METHODS

Viral and bacterial strains. Wild-type ϕ X174 originally came from the laboratory of R. L. Sinsheimer and was subsequently plaque purified. High-titer stocks are routinely grown by infecting 25-ml cultures of *Escherichia coli* C/1 at a cell density of 10^8 /ml with randomly selected single plaques. Lysis is inhibited by the addition of 3 ml of sterile 2 M $MgSO_4$ at the time of infection (8). After 3 h of incubation at 37°C, the cells are collected by centrifugation, suspended in 5 ml of pH 9.5 saturated sodium borate containing 0.05 M EDTA, and stored at 2°C overnight for lysis. Stocks prepared in this manner always give plaques at 42°C.

E. coli C/1 and C/ ϕ X have been previously described (11).

Plaque assay. The procedure is the same as that previously described (10), with modifications to reduce the size of the plaques for counting with a New Brunswick Biotran II Automatic Colony Counter. For the wild type, the top agar concentration is 1.1%; 1.0 ml of a late-log-phase culture of *E. coli* C/1 is used per plate, and the plates are incubated for 1 h at 37°C and then overnight at 23°C. With the *cs* mutants, only 0.2 ml of

bacteria is used with 0.7% top agar. The plates are incubated at 37°C until plaques are faintly visible (between 2 and 4 h) and then shifted to 23°C for overnight incubation.

Chemical mutagenesis. Several high-titer stocks of wild-type ϕ X174 were diluted to 10^{10} PFU/ml and mutagenized with hydroxylamine as outlined by Tessman (31). After incubation at 37°C with 0.10 M hydroxylamine for 7 min, the survivors (20%) were diluted at least 100-fold in ice-cold medium to quench the chemical mutagenesis. Then an appropriate volume of the diluted survivors was added to a culture of *E. coli* C/1 at a multiplicity of less than 0.01. The conditions of the infection and lysis were identical to those described above for the high-titer stocks. The low multiplicity was used to reduce the probability of multiply infected cells, and an additional 50-fold dilution of the mutagen was also accomplished at this step.

Selection of mutants. The above-described lysates of the survivors' progeny (10^{10} to 10^{11} PFU/ml) were diluted with starvation buffer (23) to 10^9 PFU/ml and incubated at 37°C for 10 min. At that time, a 0.1-ml sample was added to 0.9 ml of *E. coli* C/1 in starvation buffer at 15°C and stirred for a 30-min adsorption period. The cells were grown to 2×10^8 /ml in KC broth (23), washed once with starvation buffer, and concentrated to 10^9 /ml. At the end of the adsorption period, the reaction flask was transferred to a water bath shaker at 37°C for a 10-min eclipse period, after which the reaction was quenched by a 100-fold dilution into ice-cold EDTA elution buffer (22). After 10 min, the cells were removed by centrifugation at $12,000 \times g$ for 10 min, and the supernatant solution was carefully removed and stored at 2°C. At a convenient time, the solution was plated on *E. coli* C/1 at 37°C, and individual plaques were transferred with sterile toothpicks to plates to test for the *cs* (25°C) and *ts* (42°C) phenotypes. Each mutant was plaque purified several times and retested for the two phenotypes.

In vitro transformation reaction. Virus stocks with titers between 5×10^9 and 1×10^{10} PFU/ml were diluted 100-fold into starvation buffer maintained at $37.00 \pm 0.01^\circ\text{C}$ to initiate the transformation to the form which adsorbs at low temperatures. At various time intervals, samples were withdrawn and diluted 100-fold into tubes containing *E. coli* C/1 in starvation buffer at either 2 or 15°C at a concentration of 2 to 3×10^9 /ml. After a 15-min adsorption period, each sample was centrifuged for 1 min in an Eppendorf 3200 centrifuge. The supernatant solutions were maintained in ice until the concentration of unadsorbed virus was measured by plaque assay. The zero time point was obtained by diluting the stock into buffer maintained in ice. A sample from the zero time flask and the reaction flask (after the last time point was taken) was also diluted into starvation buffer without cells as a measure of thermal inactivation of the virus. No significant inactivation was detected in any mutant examined, so these two values were averaged and used to calculate the unadsorbed fraction.

In vivo eclipse kinetics. The procedure of Newbold and Sinsheimer (23) was used with two modifi-

cations. First, the virus-cell complexes were not collected after the adsorption step. However, the level of unadsorbed virus was always checked by centrifugation of a sample at the end of the adsorption period and maintained below 25% by an appropriate choice of the preincubation time at 37°C and adsorption time at 15°C. Second, the eclipse reaction was initiated by either a 10- or 100-fold dilution of the adsorption mixture. The corrected rate constant for the faster component of the eclipse rate curve was obtained as previously described (8).

RESULTS

Theoretical analysis. Historically, Arrhenius kinetic theory was the first successful attempt to account for the fact that chemical reaction rates increase with increasing temperature. The relationship between the rate constant, k , and the absolute temperature, T , (expressed in its logarithmic form) is:

$$\ln k = \ln A - \frac{E_a}{R} \left(\frac{1}{T} \right)$$

Here E_a , the activation energy, is obtained from the slope of the linear plot of $\log k$ versus $1/T$, and the preexponential factor, A , comes from the intercept with the y axis at infinite temperature (R is the ideal gas constant). Although recent theories have attempted to relate the two fundamental parameters, E_a and A , to the structural properties of atoms and molecules, the above form will be sufficient for application to viral eclipse. One needs only to realize that numerical values for these two parameters are determined by the molecular structure of the reactants and the activated complex, as well as the mechanism of the reaction. The Arrhenius equation, therefore, reveals the contributions that these structural properties make to the reaction rate.

In the case of ϕ X174, the kinetics associated with the eclipse period of wild-type virus and the eclipse mutant, *cs*70, can be analyzed in terms of the Arrhenius equation. Concentrating first on the viral eclipse step, the parallel solid lines in Fig. 1 are the Arrhenius plots for the wild-type and *cs*70 eclipse reactions, whose rate constants have been measured between 30 and 40°C (8). For the purpose of this analysis, the experimental lines have been extended over a wider temperature range to include the nonpermissive temperatures of the *ts* and *cs* plaque mutants of ϕ X. In terms of the Arrhenius equation, the *cs*70 mutant eclipse reaction has the same activation energy (slope) as does the wild type, but it has a threefold-lower preexponential factor (y intercept) (8). This means that the threefold-lower eclipse rate of the mutant observed in the permissive temperature range

should also apply at 25°C, the nonpermissive temperature for plaque formation (Fig. 1).

Also important to the analysis are the similarities between *cs*70 and wild-type intracellular development after the eclipse step. Since the mutant and the wild type have the same eclipse and latent periods at 40°C, and at 25°C when a shift from the permissive temperature occurs after the eclipse reaction (28), no intracellular steps after complete DNA injection are significantly impaired in the mutant. Thus, the rate-limiting step in the intracellular development of the mutant is the same as that for the wild type at all temperatures between 40 and 25°C, and only one Arrhenius plot is required to define the temperature dependence of its rate (dashed line in Fig. 1). A comparison of the rates for the intracellular and eclipse reactions is necessary to understand why the *cs* plaque phenotype is theoretically linked to the lower eclipse rate at permissive temperatures. The activation energy for this intracellular reaction was estimated from the three- to fourfold increase in the length of the eclipse period between 40 and 25°C (28). However, since there is not an absolute value for the rate of this reaction at even one temperature, one must postulate a value for the preexponential factor. This will determine where the plot intercepts the y axis and the position of the plot in Fig. 1 relative to the two plots for the eclipse reactions.

The value for A can be established by making one simple postulate: the intracellular reaction is the rate-determining step for plaque formation. If the eclipse rate is greater than the rate of the intracellular reaction, plaques will form. If it is less, the uninfected cells surrounding the infected cell can establish surviving colonies before the virus burst, and no plaques will be visible. Therefore, the rate of the intracellular reaction must be less than both the *cs*70 and wild-type eclipse rate at 37°C since both give plaques at this temperature. However, at 25°C, the rate of the intracellular reaction must be less than only the wild-type eclipse rate since only the wild type gives plaques at this nonpermissive temperature. This reasoning then requires that the Arrhenius plot for the intracellular reaction in Fig. 1 must cross below both eclipse reaction lines at the 37°C ordinate but between the two eclipse lines at the 25°C ordinate, thus establishing an approximate value for A .

To summarize the experimental basis of Fig. 1, the two Arrhenius plots for the *cs*70 and wild-type eclipse reactions were established from experimentally measured rate constants so that no assumptions are required to establish the values for E_a and A (8). An estimate for the activation energy of the rate-limiting reaction for the intra-

FIG. 1. Theoretical basis for the *cs* plaque phenotype of ϕ X174 eclipse mutants. The solid lines are extrapolations of the experimentally determined Arrhenius plots for the eclipse reactions of the wild type and the *cs*70 eclipse mutant (8). The dashed line is a postulated Arrhenius plot for the rate-limiting intracellular reaction based on the measured eclipse periods at 40 and 25°C (28). Since the *cs*70 eclipse rate is greater than the rate of the hypothetical intracellular reaction at 37°C, plaques are formed at this temperature. However, at 25°C, the eclipse reaction of *cs*70 becomes rate limiting, and the uninfected cells outgrow the virus so that the plaques are not visible. *wt*, Wild type.

cellular development of both mutant and wild-type virus was obtained from experimental data at two temperatures (28). Only the preexponential factor for this intracellular reaction has a postulated value, based on the hypothesis that the rate of intracellular development of the virus is the rate-determining step in plaque formation.

Test for predicted plaque phenotype. Since the validity of applying Arrhenius theory to ϕ X eclipse rests on a simple, but crucial, assumption, one must find logical consequences that can be tested by experimental data. The first prediction can be illustrated by an examination of Fig. 1 in light of the above postulate. Mutants should exist with eclipse rates that are less than wild-type values at permissive temperatures, such as 37°C. For this to occur, the mutation must result in a change in E_a or A or both such that the Arrhenius plot for the mutant eclipse reaction crosses the 37°C ordinate below the wild-type eclipse line. Those that cross both

the 37 and 25°C ordinate above the plot for the intracellular reaction will give plaques at all temperatures between 25 and 42°C and will be indistinguishable from the wild type in the typical screen for the *cs* and *ts* plaque phenotypes. However, those that cross above the intracellular line at 37°C but below it at 25°C must also cross the 42°C ordinate above the intracellular reaction plot. Thus, these latter mutants will yield plaques at 37°C and have the *cs*, but not the *ts*, plaque phenotype. Those that cross both the 37 and 25°C ordinate below the intracellular line will be lost if 37°C is used to obtain the plaques for screening at the other two temperatures.

A test of this prediction is a procedure which enriches a mutagenized population of ϕ X for eclipse mutants that form plaques at 37°C, followed by a screen for both the *cs* and *ts* phenotypes. If the prediction is valid, there should be a larger increase in the frequency of the *cs*

phenotype in the population. Two trials of the following scheme were performed. After chemical mutagenesis, the survivors were grown at a low multiplicity to express the mutant genotype in the capsid proteins. An enrichment for mutants with a lower eclipse rate was accomplished by adsorbing the mixed population of progeny onto sensitive cells at 15°C and shifting the reaction flask to 37°C for a 10-min eclipse period. The complexes were then diluted into ice-cold EDTA-borate elution buffer to dissociate the uneclipsed virus from the cells. Over 99% of the wild-type virus, and those mutants with an eclipse rate the same as that of the wild type, are inactivated in this time interval at 37°C (8), but a significant fraction of the potential eclipse mutant population is still infectious due to a lower rate of eclipse. After centrifugation to remove all cells that may contain completely injected ϕ X DNA, the uneclipsed population was then screened for the *cs* and *ts* phenotypes by plating at 37°C and testing individual plaques at 25 and 42°C. The results (trials A1 and A2 in Table 1) clearly show at least a 10-fold increase in only the *cs* phenotype. In a third trial, the enrichment step was a 45-min eclipse period at 25°C (the reason for selecting this temperature will be discussed below), followed by a screen of plaques obtained at 37°C for the plaque phenotypes. Since the desired mutants have a lower eclipse rate than the wild type at 25°C (Fig. 1), they can be enriched in the population by inactivating the wild type during an eclipse period at 25°C, as well as at 37°C. Of course, they can be recovered by plating the survivors at 37°C (Fig. 1). Trial B in Table 1 shows that the results are again a 10-fold enrichment of only the *cs* phenotype. Thus, the prediction concerning the plaque phenotype is confirmed.

Test for predicted classes of eclipse mutants. A second prediction arises when one considers all of the possible ways of altering the values of E_a and A to obtain the Arrhenius plots for possible eclipse mutants. In Fig. 2, four of the eight possibilities are illustrated and represent the cases where only one of the Arrhenius pa-

rameters is changed from the wild-type value. Since *cs*70 is an example of a single mutation altering only one of the two parameters, there is precedent for initially considering only the simplest cases in Fig. 2. For the two classes above the wild-type line, one can design an enrichment step that takes advantage of their increased eclipse rate over that of the wild type; but neither class is expected to have either the *ts* or *cs* plaque phenotype for screening the enriched population (compare Fig. 1 and 2). As for the two classes with the eclipse rates slower than that of the wild type, the enrichment and screening procedures used to obtain the *cs* mutants in Table 1 should select for both classes. Thus, examples of both the "higher E_a " and "lower A " mutants should be present in each population of *cs* mutants (compare Fig. 1 and 2).

To test this prediction, a preliminary classification was obtained by measuring the eclipse

FIG. 2. Theoretical basis for the existence of different classes of ϕ X174 eclipse mutants. The solid line is an extrapolation of the experimentally determined Arrhenius plot for the eclipse reaction of the wild-type virus (8). The dashed lines are examples of some postulated classes of eclipse mutants with altered values of either the Arrhenius activation energy, E_a , or preexponential factor, A . Wt, Wild type.

TABLE 1. Effectiveness of eclipse mutant screen

Trial	Procedure		No. tested	Frequency of phenotype		No. of eclipse mutants/no. of <i>cs</i> mutants tested
	Growth temp (°C)	Enrichment		<i>cs</i> (25°C)	<i>ts</i> (42°C)	
Control	37	None	550	0.002	<0.002	ND ^a
A1	37	Uneclipsed at 37°C	100	0.02	<0.01	1/2
A2	37	Uneclipsed at 37°C	600	0.037	<0.002	4/6
B	37	Uneclipsed at 25°C	450	0.040	<0.002	6/6
C	37	Unadsorbed at 15°C	400	0.032	0.002	ND

^a ND, Not determined.

rate at 37 and 25°C for a representative group from each of the screening procedures. Six of the *cs* mutants from the 37°C enrichment (trial A2 in Table 1) are compared with the wild type in Table 2. The kinetic measurements were made with high-titer crude lysates since a preliminary experiment with wild-type ϕ X174 (data not shown) gave an Arrhenius plot with only a slightly higher *A* but with an *E_a* identical to that obtained with purified virus. For the most part, a single kinetic run was made at each temperature so that more mutants could be examined. This necessitates a conservative approach in comparing mutant and wild-type eclipse rates, since uncontrolled variations in cell properties occasionally lead to large differences in rates for the same virus sample. Although a reproducibility of 10 to 20% can be obtained from multiple determinations (8), tentative classification can be made by considering only ratios of wild-type to mutant rates that are greater than two as significantly different. By this criterion, at least two-thirds of the *cs* mutants in Table 2 are defective in the eclipse reaction. Moreover, examples of both classes are present. C33-25 and -30 probably belong to the higher *E_a* group, since their ratios at 25°C are significantly greater than those observed at 37°C. On the other hand, C33-27 and -28 probably represent the lower *A* class in that their ratios at both temperatures are within a factor of 2. In Table 3, six of the mutants generated by the enrichment at 25°C are compared. All mutants of this group had a lower eclipse rate than did the wild type at both temperatures, and again, both the higher *E_a* (C32-11 and -13) and lower *A* (C32-12, -14, and -18) are represented. Thus, the second prediction of the Arrhenius theory is also fulfilled.

If one considers the possibility that a single mutation might simultaneously alter both Arrhenius parameters, two other classes should be present in the *cs* populations. Mutants with both a lower *E_a* and a lower *A* would be selected by enrichment at either 37 or 25°C, since an Arrhenius plot with the same *y* intercept but with a smaller slope than the lower *A* mutant in Fig. 2 would fall between the wild-type and lower *A* lines. More detailed kinetic data at several temperatures will be required to distinguish this class. However, certain mutants with both a higher *E_a* and a higher *A* could be revealed by this data. For example, an Arrhenius plot with a higher *y* intercept and a steeper slope than the wild-type line in Fig. 2 could cross the 37°C ordinate at the same point as the wild-type line but below the wild-type line at the 25°C ordinate. Since this mutant would have the same eclipse rate as does the wild type at 37°C, it

TABLE 2. Kinetic characterization of eclipse mutants^a

Mutant	In vivo eclipse (single component)				Tentative class
	<i>k</i> (min ⁻¹)		<i>k</i> (wild type)/ <i>k</i> (mutant)		
	37°C	25°C	37°C	25°C	
	37°C	25°C	37°C	25°C	
Wild type	3.4 ^b	0.42 ^b			
C33-15	3.5	0.20–0.25	1	2	Wild type
C33-29	2.8	0.23–0.29	1	2	Wild type
C33-25	1.0 ^c	0.030	3	14	Higher <i>E_a</i>
C33-30	0.60 ^c	0.02	6	20	Higher <i>E_a</i>
C33-27	0.66 ^c	0.040	5	10	Lower <i>A</i>
C33-28	0.69	0.041	5	10	Lower <i>A</i>

^a Trial A2 of Table 1. Virus was grown and enriched at 37°C.

^b Biphasic first-order plots; the rate constant is for the corrected faster component.

^c These showed both a biphasic plot and a single component plot on repeat kinetic runs. However, the corrected rate constant for the faster component was within experimental error of the rate constant obtained from the single-component curve.

TABLE 3. Kinetic characterization of eclipse mutants^a

Mutant	In vivo eclipse (single component)				Tentative class
	k (min ⁻¹)		k (wild type)/ k (mutant)		
	37°C	25°C	37°C	25°C	
Wild type	3.4	0.42			
C32-11	0.69	0.03	5	14	Higher E_a
C32-13	0.46	0.01	7	42	Higher E_a
C32-14	0.35	0.02	10	21	Lower A
C32-16	0.20	— ^b	17	— ^b	?
C32-12	0.23	0.02	15	21	Lower A
C32-18	0.39	0.03	9	14	Lower A

^a Trial B of Table 1. Virus was grown at 37°C and enriched at 25°C.

^b The first-order plot showed a loss in PFUs at early times but a regaining of PFUs at later times.

would, therefore, be lost during the enrichment step at 37°C but not at 25°C. Yet this mutant does not seem to be represented in Table 3, since none of the six examined have a rate at 37°C that is close to that of the wild type.

Transformation of ϕ X174 from inactive to active form. In the course of this study, a certain feature of the ϕ X adsorption properties was observed to be altered in these eclipse mutants. To insure that this change in adsorption characteristics did not introduce an artifact into the measurement of the eclipse rates exhibited by these mutants, a preliminary study of the wild-type and eclipse mutant adsorption was made. Since the measurement of the eclipse reaction rate requires synchronized infections, the adsorption step had to be carried out at

15°C. When care was taken to maintain the virus samples in ice from the time they were removed from the refrigerator until the beginning of the adsorption period, only 10% of the PFUs attached to sensitive cells. If, however, the samples were inadvertently left at room temperature for a short period of time, the level of adsorbed virus was much higher. This variation was never observed in adsorption studies at 37°C, suggesting that ϕ X can exist in an inactive form during storage at low temperatures and is transformed into the active form at higher temperatures. If this is the case, the problem would only be encountered in studies requiring adsorption at low temperatures.

In a previous report (2), a transformation from a thermally stable form of ϕ X, incapable of adsorbing to cells at low temperatures, to a more labile form that could adsorb at 0°C was observed when the virus was incubated in pH 7 phosphate buffer at 37°C. To determine whether this or a similar transformation could account for the variable levels of adsorption encountered at the beginning of this study, experiments were designed to test for such a transformation in the starvation buffer used for the eclipse reaction. Anticipating a possible rapid rate of this transformation at 37°C, the reaction was initiated by a 100-fold dilution of ice-cold virus stocks into a reaction flask containing starvation buffer. At various time intervals, samples were removed and diluted 100-fold into tubes containing sensitive cells for a 15-min adsorption period at either 2 or 15°C. To insure that adsorption was rapid and complete in this time period, the cell concentration was above 10^9 cells per ml and the multiplicity was below 0.01. After centrifugation, the concentration of unadsorbed PFUs was determined in each supernatant solution. Since control experiments showed that incubation alone did not lead to virus inactivation, these titers represent the amount of virus that has failed to attach to the cells. Over 95% of the wild-type and *cs70* stocks were converted within 10 min at 37°C to an active form capable of adsorbing to cells at low temperatures (Fig. 3). However, the one eclipse mutant from trial A1 in Table 1 showed a significantly lower rate for this transformation, and a 90-min incubation was needed to convert 90% of this mutant to an adsorbable form. When the 12 *cs* mutants, whose eclipse rates are shown in Tables 2 and 3, were examined by this procedure, a possible correlation emerged. All of the mutants with lower eclipse rates than that of the wild type also had lower rates for this transformation reaction. The differences in the rates among the mutants varied from 25- to 200-fold lower than that of the wild type. The two *cs* mutants whose eclipse

FIG. 3. Comparison of the *in vitro* transformation reaction for wild-type ϕ X174 and its eclipse mutants. Virus stocks maintained continuously at 2°C were diluted 100-fold into starvation buffer at 37°C. At each time point, sample was diluted 100-fold into starvation buffer containing *E. coli* C/1 cells at 2°C. The conditions of adsorption for this experiment are 15 min at a cell density of 3×10^9 /ml and at a multiplicity of infection of less than 0.001. The zero time point was obtained by diluting the virus stock into starvation buffer at 2°C, and after 10 min, the sample was diluted into buffer containing cells at 2°C. wt, Wild type.

rates are not significantly different from that of the wild type (C33-150 and -29 in Table 2) also had transformation rates that were similar to that of the wild type.

This suggests that a slower transformation rate may be a property of eclipse mutants selected by the procedures described in this report and could be the reason for their enrichment during the eclipse step at 37°C. If this is the case, these same mutants should be selected by screening the unadsorbed mutagenized population of ϕ X. Trial C in Table 1 shows the expected enrichment of only the *cs* phenotype. Thus, the *cs* mutants selected in this study survived the eclipse step because they have either a slower eclipse rate after attachment at 15°C or a slower transformation rate to the adsorbable form.

The reversibility of this transformation reaction was tested during the initial experiment with wild-type ϕ X. A sample of virus was diluted at the end of the 37°C incubation period in

starvation buffer at 2°C for a 60-min period before cells were added. The level of free virus in this case was the same as that at the 8-min time point in Fig. 3. This means that no significant transformation back to the inactive form occurred in 1 h at low temperatures, and the data in Fig. 1 represent the concentration of active virus present at 37°C and during the adsorption period at lower temperatures.

Although the molecular basis for the interconversion between these forms is not understood, the above results were taken into account in the design of the selection procedures and the characterization of the mutants that were generated by them. For example, the transformation reaction was examined with each mutant so that an appropriate preincubation period at 37°C could be used before the adsorption step to insure that more than 90% of the virus was attached to cells at the beginning of the eclipse kinetic experiments. Moreover, the level of free virus was usually measured at the end of the adsorption period. As for the selection procedures, the mutagenized wild-type stock was always preincubated at 37°C for 15 min before the addition of cells. These precautions were necessary to insure that the majority of the infectious virus were attached to cells during the enrichment step.

DISCUSSION

The preliminary characterization of the eclipse kinetics for these mutants clearly establishes the selectivity of the combined enrichment and screening procedures. In all four trials, there was at least a 10-fold increase in the frequency of the predicted *cs* phenotype. Moreover, in two trials, the eclipse kinetics of six mutants were examined at two temperatures, and over two-thirds of the mutants had the predicted lower eclipse rate. In measuring the eclipse kinetics, one begins the reaction with a 100-fold dilution of virus-cell complexes formed at 15°C. This design excludes any effect of altered adsorption kinetics since sufficient time is allowed for adsorption to be completed before the eclipse reaction is begun. Thus, the observed lower eclipse rate in the mutants is not a result of altered adsorption properties. Additional experiments are required to determine whether some of the mutations may be pleiotropic and may impart altered adsorption kinetics as well. If a reversible adsorption step precedes the irreversible eclipse reaction, more detailed studies are required to determine what contribution, if any, an increase in the detachment rate makes to the observed decrease in the rate at which these mutants enter the eclipse phase. Because of this possibility, the adsorption kinetics at low temperatures must be carefully examined to estab-

lish whether the first step leading to ϕ X infection is reversible. However, it is clear that the procedures described in this report are highly selective for mutants with defects in steps that are subsequent to adsorption.

The selectivity of the technique and the preliminary characterization of the mutants suggest that the *in vivo* eclipse reaction of ϕ X174 may, indeed, be understood in terms of kinetic theory. Two of the three detectable classes predicted by the theory have been selected. As pointed out earlier, mutants with a higher E_a and a higher A were not represented in the group of six that were examined in detail (Table 3). However, a single base change which results in an altered value for both Arrhenius parameters may not exist or may occur so infrequently that a large number of mutants may have to be examined for one to be found. The initial success of the theory in selecting these mutants warrants more detailed kinetic and structural studies, with the hope of revealing the molecular properties that determine the values of both Arrhenius parameters. Although kinetic analysis of biochemical reactions has yielded values for the transition-state equivalents of E_a and A , the ϕ X eclipse reaction is the first system to demonstrate that one can alter the values of these parameters by genetic mutation. With the availability of the amino acid sequence of the capsid proteins and complete nucleotide sequence of the ϕ XDNA (26), rapid sequencing methods for locating base changes in these mutants will provide a beginning in identifying the domains in the DNA and capsid structures that are involved in forming the activated intermediate for the reaction.

As for the applicability of these selection procedures to other viruses, key features of adsorption and eclipse kinetics of ϕ X are shared by many nonenveloped viruses. For example, whereas the adsorption rate exhibits only a slight dependence on temperature (9), the eclipse kinetics show an almost 100-fold decrease between 37 and 15°C (8, 23). That this may be a general feature is suggested by the diversity of the viruses in which it has been observed. These include R17 (4), T1 (25), T4 (16), lambda (20), poliovirus (7), rhinovirus (18), and adenovirus (17, 24). The same holds true for the dissociation of certain capsid proteins during the beginning of the eclipse period. In addition to ϕ X174, the phenomenon occurs with the phages M13 (13), R17 (15), T4 (29), lambda (27), P22 (12), and ϕ 29 (6) and the following animal viruses: human rhinovirus (18) and adenovirus (30). Thus, a genetic selection technique that is based on such a common property as the temperature dependence of the eclipse rate could be applicable to a wide range of viruses.

ACKNOWLEDGMENTS

This work was supported in part by General Research Grant RR05423 from the National Institutes of Health and grant PCM77-15982 from the National Science Foundation.

Technical assistance was provided by Marsha L. Brown. The helpful discussion and suggestions of James Kane, Robert Reeves, and David Kingsbury are greatly appreciated.

LITERATURE CITED

1. Arscott, P. G., and E. B. Goldberg. 1976. Cooperative action of the T4 tail fibers and baseplate in triggering conformational change and in determining host range. *Virology* **69**:15-22.
2. Bleichrodt, J. F., and E. R. Van Abkoude. 1967. The transition between two forms of bacteriophage ϕ X174 differing in heat sensitivity and adsorption characteristics. *Virology* **32**:93-102.
3. Casjens, S., and J. King. 1975. Virus assembly. *Annu. Rev. Biochem.* **44**:555-611.
4. Danziger, R. E., and W. Paranchych. 1970. Stages in R17 infection. III. Energy requirements for the F-pili mediated eclipse of viral infectivity. *Virology* **40**:554-564.
5. Gschwender, H. H., and P. H. Hofschneider. 1969. Lysis inhibition of ϕ X174-, M12-, and Q β -infected *Escherichia coli* bacteria by magnesium ions. *Biochim. Biophys. Acta* **190**:454-459.
6. Hirokawa, H. 1972. Transfecting deoxyribonucleic acid of *Bacillus* bacteriophage ϕ 29 that is protease sensitive. *Proc. Natl. Acad. Sci. U.S.A.* **69**:1555-1559.
7. Holland, J. J., and B. H. Hoyer. 1962. Early stages of enterovirus infection. Cold Spring Harbor Symp. Quant. Biol. **27**:101-112.
8. Incardona, N. L. 1974. Mechanism of adsorption and eclipse of bacteriophage ϕ X174. III. Comparison of the activation parameters for the in vitro and in vivo eclipse reaction with mutant and wild-type virus. *J. Virol.* **14**:469-478.
9. Incardona, N. L. 1981. The chemical nature of virus-receptor interactions, p. 157-167. In K. Lonberg-Holm and L. Philipson (ed.), *Virus receptors part 2, receptors and recognition*, series B, vol. 8. Chapman and Hall, London.
10. Incardona, N. L., R. Blonski, and W. Feeney. 1972. Mechanism of adsorption and eclipse of bacteriophage ϕ X174. I. In vitro conformational change under conditions of eclipse. *J. Virol.* **9**:96-101.
11. Incardona, N. L., and L. Selvidge. 1973. Mechanism of adsorption and eclipse of bacteriophage ϕ X174. II. Attachment and eclipse with isolated *Escherichia coli* cell wall lipopolysaccharide. *J. Virol.* **11**:775-782.
12. Israel, V. 1977. E proteins of bacteriophage P22. I. Identification and ejection from wild-type and defective particles. *J. Virol.* **23**:91-97.
13. Jazwinski, S. M., R. Marco, and A. Kornberg. 1973. A coat protein of the bacteriophage M13 virion participates in membrane-oriented synthesis of DNA. *Proc. Natl. Acad. Sci. U.S.A.* **70**:205-209.
14. Jazwinski, S. M., R. Marco, and A. Kornberg. 1975. The gene H spike protein of bacteriophages ϕ X174 and S13. II. Relation to synthesis of the parental replicative form. *Virology* **66**:294-305.
15. Krahn, P. M., R. J. O'Callaghan, and W. Paranchych. 1972. Stages of phage R17 infection. VI. Injection of a protein and RNA into the host cell. *Virology* **47**:628-637.
16. Labedan, B., and E. B. Goldberg. 1979. Requirement for membrane potential in injection of phage T4 DNA. *Proc. Natl. Acad. Sci. U.S.A.* **76**:4669-4673.
17. Lawrence, W. C., and H. S. Ginsberg. 1967. Intracellular uncoating of type 5 adenovirus deoxyribonucleic acid. *J. Virol.* **1**:851-867.
18. Lonberg-Holm, K., and B. D. Dorant. 1972. Early interaction of rhinoviruses with host cells. *J. Virol.* **9**:29-40.
19. Lonberg-Holm, K., and N. M. Whiteley. 1976. Physical and metabolic requirements for early interactions of poliovirus and human rhinovirus with HeLa cells. *J. Virol.* **19**:857-870.
20. Mackay, D. J., and V. C. Bode. 1976. Events in lambda injection between phage adsorption and DNA entry. *Virology* **52**:154-166.
21. Murialdo, H., and A. Becker. 1978. Head morphogenesis in complex double-stranded deoxyribonucleic acid bacteriophage. *Microbiol. Rev.* **42**:529-576.
22. Newbold, J. E., and R. L. Sinsheimer. 1970. Process of infection with bacteriophage ϕ X174. XXXII. Early steps in the infection process: attachment, eclipse, and DNA penetration. *J. Mol. Biol.* **49**:49-66.
23. Newbold, J. E., and R. L. Sinsheimer. 1970. Process of infection with bacteriophage ϕ X174. XXXIV. Kinetics of attachment and eclipse steps of the infection. *J. Virol.* **5**:427-431.
24. Philipson, L. 1967. Attachment and eclipse of adenovirus. *J. Virol.* **1**:868-887.
25. Puck, T. T., A. Garen, and J. Cline. 1951. The mechanism of virus attachment to host cells. I. The role of ions in the primary reaction. *J. Exp. Med.* **93**:65-88.
26. Sanger, F., A. R. Coulson, T. Friedmann, G. M. Air, B. G. Barrell, N. L. Brown, J. C. Fiddes, C. A. Hutchison III, P. M. Slocombe, and M. Smith. 1978. The nucleotide sequence of bacteriophage ϕ X174. *J. Mol. Biol.* **125**:225-246.
27. Scandella, D., and W. Arber. 1976. Phage λ DNA injection into *Escherichia coli* pel^- mutants is restored by mutations in phage genes V or H. *Virology* **69**:206-215.
28. Segal, D. J., and C. E. Dowell. 1974. Cold-sensitive mutants of bacteriophage ϕ X174. II. Comparison of two cold-sensitive mutants. *J. Virol.* **14**:1115-1125.
29. Silverstein, J. L., and E. B. Goldberg. 1976. T4 DNA injection. II. Protection of entering DNA from host exonuclease V. *Virology* **72**:212-223.
30. Sussenbach, J. S. 1967. Early events in the infection process of adenovirus type 5 in HeLa cells. *Virology* **33**:567-574.
31. Tessman, I. 1968. Mutagenic treatment of double- and single-stranded DNA phages T4 and S13 with hydroxylamine. *Virology* **35**:330-332.