TEMPERATURE AND THE REPRODUCTION OF LAMBDA-PHAGE MUTANTS

NEAL B. GROMAN

Department of Microbiology, School of Medicine, University of Washington, Seattle, Washington

Received for publication April 4, 1962

ABSTRACT

GROMAN, NEAL B. (University of Washington, Seattle). Temperature and the reproduction of two lambda-phage mutants. J. Bacteriol. **84**:438-445. 1962.—A comparative study of phage λ, and mutants λ*tem* and λ*11*$_2$, was made, with particular emphasis on the effect of elevated temperature (44 C) on phage reproduction. Phage λ*tem* was selected at 44 C and λ*11*$_2$ was isolated from the late-lysing fraction of bacteria at 37 C. All three phages are similar in their host range, immunity pattern, and in the rate of inactivation of free phage by anti-λ antibody and heat. Differences were observed in their plaque size and in their relative plating efficiency at 37 and 44 C. One-step growth curve studies showed that phages λ and λ*tem* are similar in the time intracellular phage appears and in their rate of maturation at 37 C. These time and rate parameters were unchanged at 44 C. Both λ and λ*tem* exhibited a reduced latent period at 44 C. However, the latent period of λ*tem* was longer than that of λ at both 37 and 44 C, and its relative 44 C/37 C yield was about 40% while that of lambda is about 3%. Phage λ*tem* may be characterized as a λ mutant with an altered latent period. Phage λ*11*$_2$ was similar to λ and λ*tem* at 37 C, but at 44 C behaved quite differently. The time of appearance of intracellular phage was delayed, compared to 37 C, and the rate of maturation was slower. Phage production at 44 C was about 16% that at 37 C. On further investigation, it was observed that induced *Escherichia coli* K-12 (λ*11*$_2$) failed to lyse at 44 C, although it did lyse at 37 C. Lysis inhibition was imposed almost immediately by transferring cells from 37 C to 44 C at any time during the reproductive cycle, including the lytic phase. The behavior of λ*11*$_2$ at 44 C disposed of the possibility that the lytic step was the only step in the phage cycle sensitive to variations in temperature. However, it appeared that the lytic step was a consistant target for its action.

It was previously shown (Groman and Suzuki, 1962) that production of λ phage is virtually abolished at 44 C, owing primarily to earlier initiation of lysis than at 37 C. Attempts to artificially delay lysis to determine whether other factors contributed to this inhibition failed, and as a result a search was undertaken for mutants of lambda capable of producing more phage at 44 C. It was hoped that a study of the kinetics of phage reproduction by such mutants would provide additional information on the temperature-inhibition effect.

A word about terminology. There is a need to differentiate between the ability of a mature virus to withstand exposure to a given temperature and subsequently reproduce, and the ability of a virus to reproduce during exposure to a given temperature. It is proposed that the term "thermoresistance" and its derivatives be restricted to the former class of events, and that the term "thermoefficiency" and its derivatives be applied to the latter class. Thus, a virus which has an altered yield at one temperature compared to another temperature can be referred to as being more or less thermoefficient, as the case may be, or having a greater or lesser thermoefficiency. The same terminology can be applied in comparing two viruses at the same or different temperatures.

The isolation of thermoresistant phage mutants is commonplace (D'Herelle and Sertic, 1930; Adams and Lark, 1950; Groman and Suzuki, 1962). The isolation of mutants with altered thermoefficiency is rare. Beumer-Jochmans (1951) reported that successive passage of a staphylophage at 44 C produced a phage which had greater thermoefficiency at 44 C than did parental phage, but which retained its ability to reproduce at 37 C as well. A detailed comparison of parent and mutant was not undertaken. We have also reported briefly (Groman and Suzuki, 1961) on a mutant of lambda phage, λ*tem*, which had a greater thermoefficiency at both 37 and 44 C than did parental λ. It was

isolated after successive transfers of λ at 44 C. When it appeared that λ*tem* was simply a mutant with an extended latent period, a second mutant, λ*11*$_2$, was isolated at 37 C from the late-lysing fraction of phage-infected cells, on the assumption that it should be similar to λ*tem*. The present paper is a comparative study of these mutants and parental λ. The focal point of the study was the relevance of the reproductive cycle of these phages to the mechanism(s) of temperature inhibition.

MATERIALS AND METHODS

Bacteria and phages. Escherichia coli K-12 and K-12(λ) and parental phage λ were obtained from Allan Campbell. A streptomycin-resistant mutant of K-12 was obtained from Francois Jacob. It was used as indicator strain, and, when required, streptomycin was incorporated into the overlay medium at 200 μg/ml. The isolation and characteristics of λ*tem* and λ*112* will be described in the Results. Lysogenic K-12 (λ*tem*) and K-12 (λ*112*) were isolated in our laboratory.

Media and methods. The Trypticase Soy-Yeast Extract (TSY) medium employed in the growth of bacteria and in some of the experiments, and the Tryptone media employed in the phage assays, were previously described, as were the techniques of chloroform lysis, induction by ultraviolet light, and the one-step growth curve (Groman and Suzuki, 1962). In some of the experiments, a medium containing 1% Tryptone and sodium chloride, 0.5% yeast extract, and 0.1% glucose, at a final pH of 7.4, was employed. This will be referred to as LB medium.

In many experiments, assays of λ*tem* and λ*112* were performed with indicator cells irradiated with ultraviolet light for 30 sec under conditions similar to those used for induction. Although there was no measurable loss of colony-forming units, the plaque size of both phages was increased on irradiated indicator.

All cultures were aerated on a reciprocal shaker delivering 100 (1½-in.) strokes per min. Optical density changes were measured in a Klett-Summerson colorimeter with a no. 54 filter. Temperatures were controlled in water baths to ±0.1 C.

RESULTS

Isolation of λ*tem.* Successive transfers of λ phage were carried out as follows. In the initial passage, approximately 10^8 cells were infected at a phage-bacterium ratio of about 6:1 with phage from a 37-C lysate. Adsorption was carried out for 10 min in 0.01 M $MgSO_4$, after which the infected cells were diluted twofold into TSY medium at 44 C. After a total of 80 min, cell debris and intact cells were removed by centrifugation, and the supernatant fluid was used as the phage inoculum for the next passage. Phage yield was determined at each passage, and, in some cases, estimates of burst size were made.

In the isolation of λ*tem*, seven consecutive passages were made at 44 C and six at 45 C. By the fifth passage, the phage yield and burst size began to rise, and by the 13th passage the number of tiny (0.25 to 0.5 mm) plaques was so large as to indicate an obvious selection. Plaques were selected and purified through three successive isolations, a phage stock produced, and a lysogenic strain, K-12 (λ*tem*), isolated.

Isolation of λ*112.* Phage from late-lysing bacteria was obtained in the following manner. Adsorption was carried out as above; after adsorption, the cells were centrifuged out, resuspended in 5 ml of TSY broth containing anti-lambda antibody, and incubated at 37 C. Incubation was continued for 80 to 90 min, at which time lysis was virtually complete. The culture was centrifuged, washed once in TSY medium, and the sediment resuspended in the same medium without antibody for an additional 30 to 50 min. Again the culture was centrifuged, and the supernatant fluid was used as the phage inoculum for the next cycle. By the fourth passage, a significant number of small plaques was observed, and some, including λ*112*, were purified and their lysogenic derivatives isolated.

Characteristics of λ *and mutants* λ*tem and* λ*112.* All three phages displayed similar host ranges and established similar patterns of immunity in the lysogenic state. They were all co-immune. All were inactivated by antiserum to lambda phage at identical rates, and had an identical half-life of 40 to 45 min at 44 C. Their relative plating efficiency at 44 C compared to 37 C differed; it was 0.7 for λ, 1 for λ*tem*, and 0.2 for λ*112*. Both λ*tem* and λ*112* produced tiny (0.25 to 0.5 mm diam) plaques at 37 C; the plaques of λ*tem* were the same size at 44 C, but those of λ*112* were minute. Lambda phage produced

plaques 1 to 2 mm in diam at 37 C; at 44 C, plaques were smaller, with almost half of those produced similar in size to those of λ*tem* and λ*112* at 37 C.

One-step growth curves of λtem at 37 and 44 C. The one-step growth curves of λ*tem* at 37 and 44 C are given in Fig. 1. These curves show that at 44 C the latent period of λ*tem* was materially shortened, but the rise period was not significantly altered. It is as if the time at which each cell lyses was speeded up by a constant amount, while the interval between the lysis of cells had not been altered. The shortening of the latent period was accompanied by a depressed yield at 44 C. The 44 C/37 C ratio of phage yields varied over the range of 11 to 84%, with an average ratio of 39%. The average burst size of λ*tem* was 600 at 37 C and 250 at 44 C. This burst at 44 C was most remarkable, since it was about the size of a lambda burst at 37 C. In contrast to these changes, it is evident from data to be presented (Fig. 3) that intracellular phage appeared at the same time at both temperatures, and the rate of intracellular phage development was similar at both temperatures.

One-step growth curves of λ112 at 37 and 44 C. The rationale which led to the isolation of late-lysing mutants was the conclusion that λ*tem* was apparently a mutant with an extended latent period. It seemed likely that similar mutants could be isolated, without using temperature as the selective factor, by isolating phages from late-lysing cells. After the isolation of λ*112*, extensive efforts were made to study the kinetics of its production at 37 and 44 C. Curves representative of eight experiments are given in Fig. 2. In general, the latent and rise periods of both temperatures were about 60 min. The burst size at 37 C was usually in the 400 to 600 range, and that at 44 C was in the 30 to 80 range. The yield at 44 C was about 16% of that at 37 C, although this value ranged between 6 and 48%. What is obviously different in comparing λ*112* with λ*tem* is the apparent lack of effect of 44 C on the latent or rise period of λ*112*, despite the significant lowering of the yield at the higher temperature. This suggested that, in contrast to λ*tem*, maturation of λ*112* was influenced by the increase in temperature.

It was apparent throughout the work with λ*112* that there was greater uncertainty in the assays during the rise period than had been encountered with λ and λ*tem*. Whereas the latent period and the time of appearance of intracellular phage was established with reasonable certainty, the

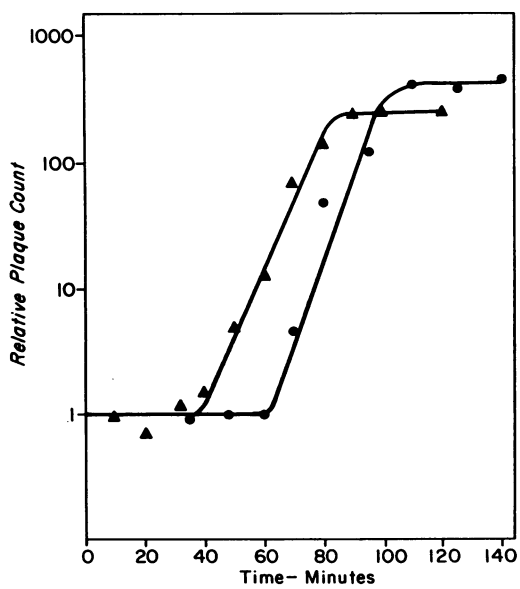

FIG. 1. *One-step growth curve of λtem. Total infective centers: direct assay, 37 C, ●; 44 C, ▲.*

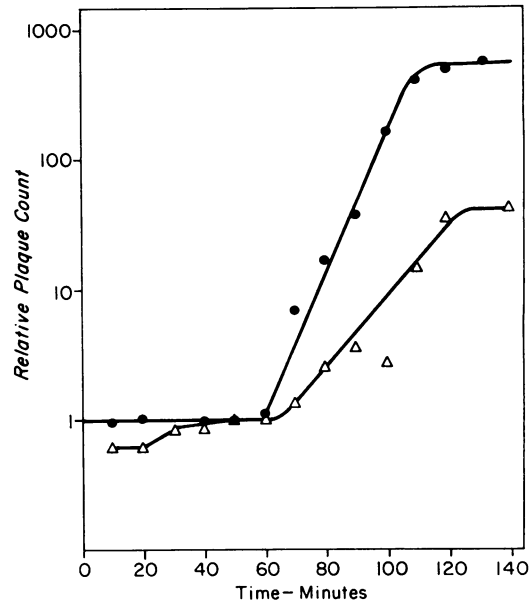

FIG. 2. *One-step growth curve of λ112. Total infective centers; direct assay, 37 C, ●; 44 C, △.*

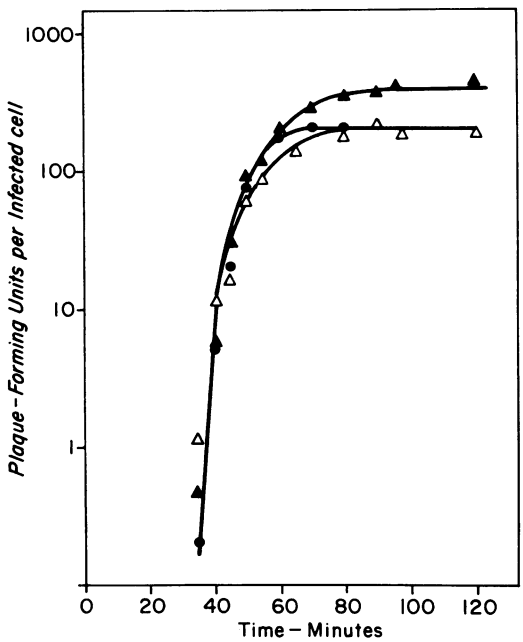

FIG. 3. *Comparison of the kinetics of phage production by λ and λtem. Total infective centers: chloroform lysates, λ, 37 C, ●; λtem, 37 C, ▲, 44 C, △.*

termination of the rise period and, hence, final yield and burst size were subject to much variation. In general, results at 37 C were better than those at 44 C, and total phage yields determined by the chloroform lysis technique were the most reliable. The reason for these observations undoubtedly resides in the unique response of the lytic mechanism of λ*112* to temperature. This will be described in a later section.

Comparison of the maturation rates of phage λ, λ*tem, and* λ*112*. In comparing phage maturation rates, an accurate estimate of the number of cells producing phage, i.e., infective centers, is of course essential. Assays for infective centers at 37 C posed no problem since, for all three phages, the counts were stable during the latent period. However, it was apparent, (*see*, e.g., Fig. 1 and 2) that at 44 C the infective-center counts of λ*tem* and λ*112* fluctuated during the latent period. After more extensive investigation, it was established that during the first 20 min of the latent period there was an apparent drop in the count at 44 C relative to that at 37 C in both cases, but that before lysis began the counts returned to the 37-C level. Therefore, in all comparisons of maturation rates at 37 and 44 C, the infective-center level established at 37 C was also used for 44 C. It was shown previously (Groman and Suzuki, 1962) that the infective-center counts of lambda phage are similar at both temperatures.

In comparing the maturation rates of λ, λ*tem*, and λ*112*, the chloroform lysis technique was employed. Each experiment was designed so that the maturation rate of λ*tem* or λ*112* was compared to that of lambda at 37 C. Representative data for λ*tem* are given in Fig. 3, and for λ*112* in Fig. 4. The rates of maturation of λ*tem* at both 37 and 44 C were identical with each other and with that of parental lambda phage at 37 C. The period over which this comparison was valid was 50 min after infection. Up to this time, less than 10% of the phage had been released by lysis, and therefore the average number of phage-producing units had not been significantly altered. As already indicated, the data also show that the time of appearance of intracellular λ*tem* was similar at both temperatures.

The data for λ*112* differ from those for λ*tem*. Although the rate of λ*112* maturation at 37 C

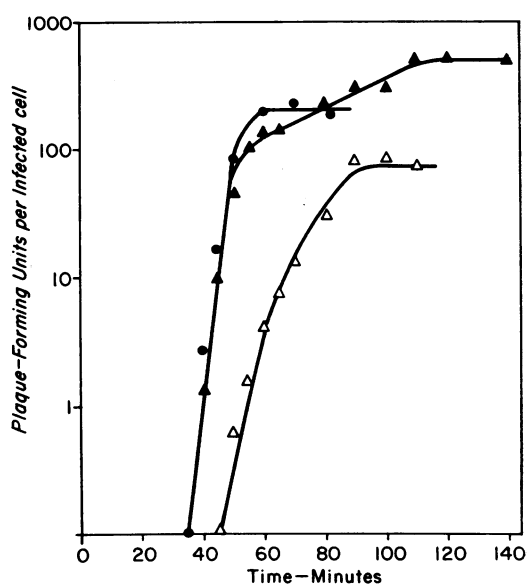

FIG. 4. *Comparison of the kinetics of phage production by λ and λ112. Total infective centers: chloroform lysates, λ, 37 C, ●; λ112, 37 C, ▲, 44 C, △.*

was similar to that of λ and, hence, λ*tem* at 37 C over the critical prelytic period, its rate of maturation was definitely slowed at 44 C. Furthermore, the time at which the first mature λ*112* appeared was delayed at the higher temperature. It is apparent from these data that there are significant differences in the response of λ*tem* and λ*112* to 44 C.

Effect of 44 C on the early stages of λtem and λ112 infection. It was demonstrated (Groman and Suzuki, 1962) that the initial stages of infection with λ phage are carried out at 44 C. Complete cycles of infection at 44 C were also demonstrated for λ*tem* and λ*112*. The burst size under these conditions was similar to that produced by cells infected at 37 C and then transferred to 44 C after the 10-min adsorption period. These experiments showed that for λ*tem* and λ*112*, as for λ, the early steps of the reproduction cycle can be carried out at 44 C, and that there are no steps which are completely blocked by the higher temperature. They do not show whether some fraction of the infections is aborted in the early or later stages. However, as was shown in the preceding section, massive abortion probably does not occur during the latent period. What happens subsequently is of course a key issue in the case of λ*112*, whose maturation is delayed and slowed at 44 C.

Low yield of λ112 after induction. In an effort to obtain a second estimate of the burst size of λ*112*, the relative 44 C/37 C yield of induced K-12 (λ*112*) was determined. In contrast to the 16% value obtained from infections of K-12 with this phage, the relative yield from induced cells, based on seven experiments, was about 1%. In some cases, this amounted to having a nonproductive infection at 44 C. There are two obvious explanations for this observation: the ultraviolet irradiation used in induction, or the fact that induced cells were maintained at 44 C throughout the reproductive cycle while in external infections the 10-min adsorption period was carried out at 37 C. There is no indication from experiments performed to date that either of these variables alone can account for the reduced relative yield in induced cells. Since induced cells incubated at 37 C produced bursts within the range obtained from K-12 cells infected with λ*112*, it appears that it is the combination of ultraviolet light and heat that produces the reduction in yield at 44 C.

Studies on induced K-12 (λ), K-12 (λtem), and K-12 (λ112). To determine whether the temperature effects observed in the one-step growth cycles were reflected in the lysis of more concentrated cell suspensions, strain K-12 was infected with λ at a multiplicity of about eight, incubated at 37 C and at 44 C and observed for changes in optical density. Lysis was not observed at 37 C but was evident at 44 C; it was reasoned that, under the conditions of infection, a lysogenic response was produced in a significant number of cells at 37 C. It was for this reason that induced lysogenic derivatives of the three phages were employed in this series of experiments. Induction was carried out as usual, but after induction the cells were diluted 1:1 into double-strength LB medium and incubated at 37 C and at 44 C. The use of LB medium was dictated by its potential usefulness in other phases of our investigation.

The induction curves for all three strains are given in Fig. 5. As anticipated from the one-step growth experiments, lysis of both K-12 (λ) and K-12 (λ*tem*) was initiated more rapidly at 44 C than at 37 C. Most surprising was the observation that lysis of K-12 (λ*112*) was inhibited at 44 C for an indefinite period, whereas lysis was observed at 37 C. Lysis inhibition was observed for at least 6 hr after induction.

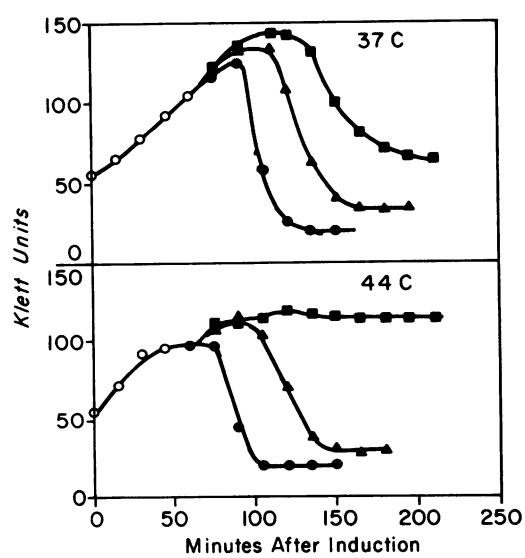

FIG. 5. *Induction of lysogenic strains of K-12: K-12* (λ), ●; *K-12* (λ*tem*), ▲; *K-12* (λ*112*), ■; *common points*, ○.

Further study of K-12 (λ*112*) centered about two questions. At what time after induction could lysis inhibition be imposed, and could lysis inhibition be overcome by lowering the temperature? To answer the first question, cells were induced as before, dispensed into a series of calibrated tubes, and incubated with shaking at 37 C. At various times, tubes were shifted to 44 C. The results (Fig. 6) show that transfer to 44 C at any time, whether in the prelytic or lytic period, resulted in the institution of lysis inhibition. Whatever the mechanism, it was imposed rapidly.

To answer the second question, i.e., whether lysis inhibition could be overcome by lowering the temperature, cells were induced as before, dispensed into tubes, and incubated at 44 C. At various times, tubes were shifted to both 37 and 30 C. Only a limited number of the curves are plotted in Fig. 7, to retain clarity of the graph. Transfer of cells from 44 C to 37 C, 60 min after induction (Fig. 7B), was followed by an increase in optical density prior to lysis. By 90 min and beyond, there was no rise in optical density after transfer and only negligible amounts of lysis This was also true for transfers at 120, 180, and 240 min. When cells were transferred from 44 C to 30 C at 60 min (Fig. 7C), there was a rise in optical density, followed by a significant amount of lysis. The pattern was similar for transfer at 90 min. In contrast to the shifts to 37 C, the later shifts to 30 C at 120, 180, and 240 min were followed by an immediate decrease in optical density. Thus, relative to 30 C, lysis inhibition was not imposed completely at any time. The difference between the responses before and after 90 min undoubtedly reflected a transition toward a completed process at 44 C. Once completed, there was no residual growth at 30 C prior to lysis. It should be noted that cells held at 30 C over the entire 6-hr period did not lyse. It seems unlikely that the absence of lysis in the 30-C control was due to the same lysis-inhibiting mechanism as at 44 C, since in some cases we observed small amounts of lysis at 30 C late in the experiment. It was probably due to

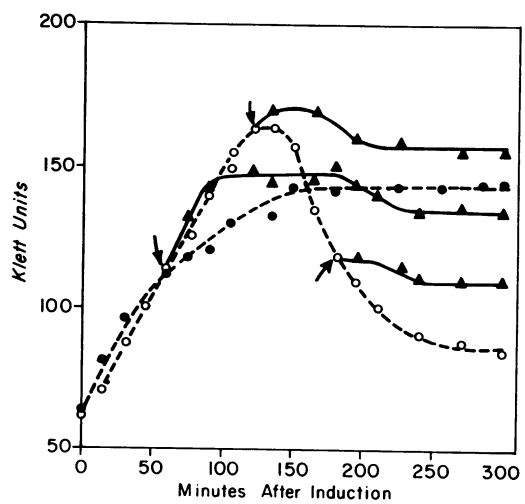

FIG. 6. *Effect of temperature shift-up (37 C to 44 C) on induced K-12 (λ112): 37 C, ○; 44 C, ●; points after shift up, ▲. The arrows indicate the shift-up times.*

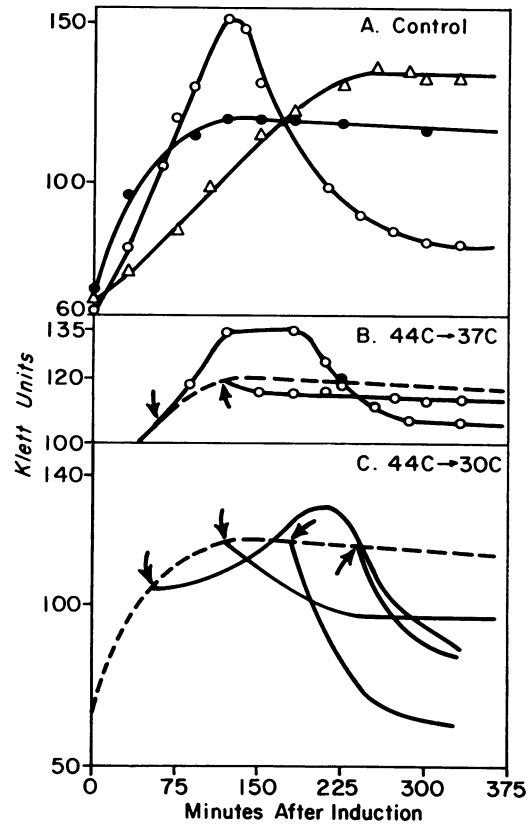

FIG. 7. *Effect of temperature shift-down on induced K-12 (λ112): 30 C, △; 37 C, ○; 44 C, ●. The arrows indicate the shift-down times. In parts B and C, the 44-C curve from part A is repeated as a dotted line. Because there was much overlapping, the points were omitted for the 30-C curve in part C.*

the very slow development of the reproductive cycle at this temperature, a development which was speeded up by a period of incubation at 44 C, as suggested by the 60-min transfer sample.

DISCUSSION

In the study of λ phage (Groman and Suzuki, 1962), it was concluded that the depression of phage yield at 44 C relative to that at 37 C was due to the earlier initiation and more rapid completion of lysis. There was no change in the time of appearance of intracellular phage at the higher temperature nor was there evidence for an alteration of maturation rate. With mutant λ*tem*, the evidence also indicates that the primary effect of increasing the temperature to 44 C is to initiate lysis earlier. As with λ, the time of appearance of intracellular phage and the rate of maturation are apparently unchanged at 44 C, and in fact these parameters are identical in the two phages. In total, these results suggest that the major difference between λ and λ*tem* is the length of their latent periods. This single difference seems to account for increased burst size of λ*tem* at both 37 and 44 C, for altered plaque size, and for the low plating efficiency at 44 C. One other difference observed in the one-step growth curve of these phages is the shortening of the rise period at 44 C in the case of λ but not in the case of λ*tem*. It is possible that the λ rise period is not really shortened but that during this period λ-infected cells are more fragile than those infected with λ*tem* and lyse during the assay for total phage. Whatever the cause, there appears to be another difference between these phages, but whether it is related to the altered latent period remains to be determined. We have in effect accomplished with λ*tem* what could not be done artificially with λ, i.e., delayed lysis so that the synthesis of phage could be observed for a longer period at 44 C. With λ*112* it was established with greater certainty than with λ that the steps involved in phage synthesis are not affected by the higher temperature.

The possibility of a generalization regarding elevated temperatures and phage reproduction was disposed of with the isolation of λ*112*. It now appears that, in appropriate mutants, a variety of processes will prove susceptible to temperature variations. At 37 C, λ*112* resembles λ*tem*. Once the temperature is raised, the similarity disappears. The appearance of intracellular phage is delayed, the rate of phage maturation is slowed, and there is evidence that lysis inhibition occurs. The relative 44 C/37 C yield of λ*112* is less than one-half that of λ*tem*. The one general similarity between these phages is the fact that the lytic mechanism is a consistent target of temperature action. It has not been determined yet what happens to λ*112* at 44 C. The most likely explanations are either a loss of infective centers, which the evidence indicates would have to come at least 60 min after infection, or a sharp curtailment of phage maturation in each infected cell.

The finding of lysis inhibition in induced K-12 (λ*112*) at 44 C suggests this as an explanation for the uncertainty observed in the total phage assays, particularly at 44 C. Erratic lysis as a result of temperature variations during pipetting and plating seems quite possible. This finding does not invalidate the values obtained for time of appearance of the first phage or the rate of maturation, since these were obtained from chloroform-lysed samples. The greater reliability observed with chloroform determinations was already noted. It has not been established as yet that infected cells undergo lysis inhibition in the same manner as induced cells. The nonidentity of induced and infected cells is a possibility, as was demonstrated by the difference in the 44 C/37 C yield of λ*112* under these two sets of conditions.

It is premature to speculate on the mechanism of lysis inhibition in induced K-12 (λ*112*). The phenomenon is being studied in relation to endolysin and phage synthesis. It should be noted that a phage with apparently similar properties was isolated by Campbell (1961). Whereas Campbell indicated that the inhibited mutant produces less endolysin than wild-type phage, our initial experiments with λ*112* indicate that it produces large amounts of endolysin and phage under conditions of lysis inhibition. Study of the inhibition mechanism may lead to a better understanding of the steps involved in lysis by phage.

ACKNOWLEDGMENTS

This investigation was supported in part by a research grant (E-3391) from the National Institute of Allergy and Infectious Diseases, U.S. Public Health Service, and in part by State of Washington Initiative 171 funds for Research

in Biology and Medicine. The technical assistance of Grace Suzuki is gratefully acknowledged.

LITERATURE CITED

ADAMS, M. H., AND G. LARK. 1950. Mutation to heat resistance in coliphage T 5. J. Immunol. **64**:335–347.

BEUMER-JOCHMANS, M. P. 1951. Etude, comparative du comportement a 44° de deux phages staphylococcique. Ann. inst. Pasteur **80**:536–544.

CAMPBELL, A. 1961. Sensitive mutants of bacteriophage λ. Virology **14**:22–32.

D'HERELLE, F., AND V. SERTIC. 1930. Formation par adaptation, de races de bacteriophages thermo-resistantes. Compt. rend. soc. biol. **104**:1256–1258.

GROMAN, N. B., AND G. SUZUKI. 1961. The mechanism of inhibition of lambda phage production at elevated temperatures. Virology **13**:144–146.

GROMAN, N. B., AND G. SUZUKI. 1962. Temperature and lambda phage production. J. Bacteriol. **84**:431–437.