BRIEF COMMUNICATION

THE EFFECTS OF TEMPERATURE AND ULTRAVIOLET IRRADIATION ON MULTIPLICATION OF BACTERIOPHAGE $\phi 29$

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ABSTRACT The effects of temperature and of ultraviolet radiation on the multiplication of bacteriophage $\phi 29$ were studied. Samples of $\phi 29$ that had been irradiated to surviving fractions of 0.44 or 0.10 were propagated at 37°C, 42°C and 43.5°C. Latent periods and burst sizes were obtained from one-step growth curves. At a particular temperature, as the dose delivered to the virus was increased, the latent period was extended and the burst size was decreased. For unirradiated virus, the burst size was the same at 42°C as at 37°C, but decreased dramatically at 43.5°C. For virus subjected to a particular dose, the burst size decreased as the temperature was raised. A statistical technique for improving the reliability of parameters obtained from one-step growth curves is presented.

INTRODUCTION

Ultraviolet radiation is known to cause the biological inactivation of virus particles and to result in increased rates of mutagenesis. However, the extent to which such effects are manifested by an irradiated virus sample depends upon the ability of viral or host cell enzyme systems to repair such lesions.

Luria (1) observed an extension of the latent period for viruses exposed to UV. A similar effect for irradiated coliphage T1 was reported by Setlow et al. (2). In the latter system the action spectrum for latent period extension was shown to resemble the spectrum for nucleic acid absorption more closely than that for protein absorption. Thus the extended latent period observed after irradiation might be attributed, at least in part, to the time required for repair of the phage genome; and the degree of extension might be correlated with the kinetics of repair.

Thermal perturbations of the enzymes in the UV-dark repair system might be followed by comparing the latent periods observed for viruses subjected to the same doses of UV but grown at different temperatures. A viral system could, then, provide the possibility of studying thermal effects on a specific coupled enzyme system in vivo.

Bacteriophage ϕ29, a small virulent virus of *Bacillus subtilis*, is well suited to such studies. This virus has a molecular weight of 19×10^6 daltons (3); its DNA has a molecular weight of 11×10^6 daltons (4) and an adenine-thymine content of 66% (5). Host cell DNA replication and transcription are not significantly inhibited by ϕ29 infection (6).

Ultraviolet irradiation of ϕ29 results in a biphasic survival curve (7). For coliphage T1 a similar type of survival curve has been explained by a dependence of the irradiated virus upon host cell enzymes for repair of UV-produced lesions in its DNA (8). Therefore ϕ29 probably depends upon its *B. subtilis* host for at least some of the enzymes necessary for dark repair.

We have used ϕ29 subjected to known doses of UV to investigate the effects of temperature on: (*a*) the length of the latent period, and thus we hope on the rate of repair of viral DNA, and (*b*) the burst size—a parameter probably correlated with the efficiency of the repair system.

METHODS

Phage and Bacterial Strains

Bacillus subtilis HSR was used as the host for growth and assay of bacteriophage ϕ29. Cultures of both ϕ29 and its host were generously provided by Dr. Frank Young and Dr. Ronald Yasbin of the University of Rochester School of Medicine and Dentistry.

Media

Stock cultures of bacteria were maintained on Tryptose blood agar base (TBAB, Difco Laboratories, Detroit, Mich.) slants. Broth cultures were grown in Penassay broth (PB), also referred to as antibiotic medium 3 (Difco).

Purified viruses were stored in Hirokawa's buffer, composed of 0.1 M Tris-HCl, 0.1 M NaCl, and 0.01 M $MgCl_2$ (9). Irradiation was done in Spizizen's minimal salts solution (10). In phage assays, TBAB was used as the bottom agar. The top agar overlay was that described by Reilly and Spizizen (10).

Preparation of Phage Stocks

The virus was grown and purified by a variation of the method of Rubio et al. (3). *B. subtilis* HSR were grown to a density of about 1.0×10^7 cells/ml, as determined with a Petroff-Hauser counting chamber, and were infected to give a multiplicity of infection (MOI) of 0.1. After lysis, NaCl, $MgSO_4$, lysozyme, RNase, and DNase were added to give final concentrations of 0.1 M, 0.01 M, 5.0 μg/ml, 0.1 μg/ml, and 0.1 μg/ml, respectively. This mixture was incubated at 37°C for 1 h.

The lysate was centrifuged at 8,000*g* for 20 min at room temperature. DNase and RNase were added to the supernatant liquid to give additional final concentrations of 0.1 μg/ml and 1.0 μg/ml and the suspension was again incubated for 1 h at 37°C. It was then cooled to 4°C and polyethylene glycol (mol wt 6,000, Union Carbide Corp., New York) was added to a concentration of 100 g/liter. This mixture was held at 4°C overnight and was centrifuged at 8,500*g* for 15 min at room temperature. The pellets were resuspended in PB containing 0.1 M NaCl and 0.01 M $MgSO_4$ and centrifuged at 44,400*g* for 100 min. Pellets from this centrifugation were resuspended in Hirokawa's buffer. Low and high speed centrifugations were repeated.

Cesium chloride was added to a density of 1.375 and the solution was centrifuged at 57,000*g*

for 25.5 h in a Beckman SW 27 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). 1-ml fractions were collected and their absorbance was monitored at 254 nm. Fractions around the absorbance maximum were dialyzed into Hirokawa's buffer and assayed. 98% of the virus was found in one fraction near the center of the tube. Virus from this fraction was used in all of the experiments reported here.

As a check on the homogeneity of the virus preparation, a sample of this preparation was analyzed by sedimentation velocity at 20,000 rpm, 20°C, in the AN-D rotor of the Beckman model E analytical ultracentrifuge. Virus concentrations of 0.32 and 0.16 mg/ml were used. Sedimentation was followed by the UV absorption optical system. Sedimentation profiles contained only one boundary with symmetry characteristic of a homogeneous preparation. The sedimentation coefficient extrapolated to zero virus concentration was 260, in good agreement with the value of 256 reported by Rubio et al. (3).

Irradiation of the Phage

A low-pressure mercury vapor germicidal lamp (G15T8, Atlantic Ultraviolet Corp., Bay Shore, N. Y.) with a sharp emission maximum at 254 nm was used for UV inactivation of the phage. The dose rate was measured with a Blak-Ray ultraviolet meter (Ultra-Violet Products, Inc., San Gabriel, Calif.). All irradiations were done at an incident dose rate of 20 ergs/mm^{2}s. The dose required to obtain the desired surviving fraction was determined from a ϕ29 survival curve obtained under the same conditions (7). After a virus sample at a concentration of approximately 2×10^9 phage/ml had been exposed to this dose, the actual surviving fraction was determined by assay.

One-Step Growth Experiments

One-step growth experiments were done as described by Eisenstark (11). Overnight cultures of bacteria were grown at the temperature to be used in the experiment. These were used to inoculate pre-warmed PB at a density of about 10^6 cells/ml. When the density reached $1–2 \times 10^8$ cells/ml, 0.1 ml of the appropriate concentration of phage was added to 0.9 ml of bacterial culture to give a final MOI of 0.2. After 10 min had been allowed for adsorption, 9.0 ml of prewarmed PB were added and further dilutions were made as prescribed by Eisenstark.

Starting at 15 min after mixing of the virus and cells, 0.1 ml samples were withdrawn and assayed. Assays were done in triplicate. When irradiated samples were used, virus growth and assays were done under yellow light to minimize the possibility of photoreactivation.

Least-squares fits to the one-step growth curve data were done on the Clemson University IBM 360–65 Computer (IBM Corp., White Plains, N. Y.). The integrated form of the Gaussian probability function was approximated by a polynomial expansion (12, Eq. 26.2.16).

RESULTS

One-step growth curves were obtained for unirradiated phage and for phage irradiated to 44% survival or 10% survival at the temperatures 37, 42, and 43.5°C. In each case the experimental data have been fit by the integrated form of the Gaussian distribution. The idealized case is that in which infection would occur at the same time ($t = 0$) for all bacteria infected, and in which each infected bacterium would have the same latent period as every other. In this case one would observe a growth curve that would be a true step function. In reality, however, the time of infection can be expected to be randomly distributed about an average time after mixing and the latent periods of the individual bacteria can also be expected to be randomly distributed about a certain

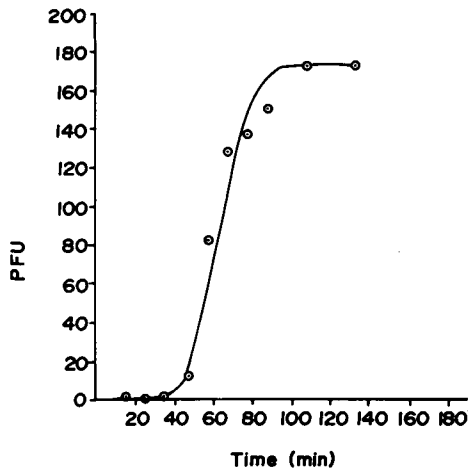

FIGURE 1 One-step growth curve for unirradiated $\phi 29$ in *B. subtilis* HSR at 42°C. Points represent experimental data normalized to the number of PFU before lysis began. The curve represents an integrated Gaussian probability function calculated to fit the data by a least-squares criterion.

average latent period. As a result, one can expect the distribution of new virus particles released as a function of time to be randomly distributed, or the total new virus particles released to be represented by an integrated form of the normal distribution. This is what one actually measures in a one-step growth experiment. We have, therefore, fit the experimental data to the curve represented by:

$$(\text{PFU})' = \frac{\text{BS}}{\sqrt{2\pi}} \int_{-\infty}^{t-t_{1/2}/\sigma} e^{\frac{-x^2}{2}} dx$$

Where $t_{1/2}$ represents the time required for the curve to reach one-half of its maximum, σ is a parameter proportional to the rise time,[1] (PFU)′ is the number of new virus particles released at time t, and BS represents the burst size. In this approach, the burst size is calculated as usual from the ratio of the number of plaques obtained after lysis has been completed to the number obtained before lysis began; (PFU)′ represents the average number of new virus particles released per infected cell at time t and is given by: $(\text{PFU})' = (N_t/N_0) - 1$, where N_t is the number of plaques measured at time t and N_0 is the number measured during the latent period. The normalized measured number of PFU is: PFU = (PFU)′ + 1. The parameters $t_{1/2}$ and σ were varied to minimize the sum of the squares of the fractional differences between the calculated value of (PFU)′ and the experimental value of (PFU)′ at each experimental point. That is, the quantity $\sum_i [(\text{PFU})'_i \text{ experimental} - (\text{PFU})'_i \text{ calculated}/(\text{PFU})'_i \text{ calculated}]^2$ was minimized by varying the parameters $t_{1/2}$ and σ. The summation is taken over all experimental points measured at various times after infection.

[1]The rise time is defined as the interval between the time at which the curve first begins to rise and the time at which it first begins to level off at its maximum value.

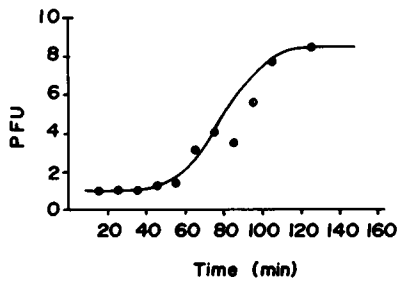

FIGURE 2 One-step growth curve for $\phi 29$ irradiated to a surviving fraction of 0.10 and multiplying in *B. subtilis* HSR at 43.5°C. Symbols as for Fig. 1.

One-step growth curves for the experiments yielding the highest and the lowest burst sizes are presented in Figs. 1 and 2. The ordinates of these plots have linear scales. Note that single-step growth curves are frequently reported as semi-log plots both in the literature and in virology texts. While such plots accurately reflect the appearance of new viruses within cells, for those viruses not released until the cell lyses, semi-log plots are of no advantage. In fact, on semi-log plots the plaque counts observed at later times are compressed by the ordinate scale. As a result, with such a plot one can be led to believe that for points observed at times after the rise period, the number of PFU has become constant, while actually this number is still increasing.

The values of $t_{1/2}$ and σ yielding best-fit curves are presented in Table I. In Table II, burst sizes are given relative to the burst size for unirradiated virus at 37°C.

DISCUSSION

Figs. 1 and 2 indicate that the one-step growth curves can be fit by a normal probability function. We have chosen to fit the data by the best function of this type because experimental fluctuations in the data points can cause large errors in determining the time when the curve begins to rise—the traditional definition of the end of the latent period. For comparison with latent periods determined by other methods, we have calculated the times required for 5% of the cells to lyse ($t_{.05}$, Table III).

The data in Tables I and III indicate that UV damage to the virus causes an increase

TABLE I

HALF-RISE TIMES FOR BEST LEAST-SQUARES FITS TO $\phi 29$ GROWTH CURVES

	Surviving fraction		
T	1.00	0.44	0.10
°C		*min*	
37	86 (16)*	99 (18)	101 (16)
42	63 (13)	57 (8)	81 (14)
43.5	58 (11)	78 (20)	78 (19)

*Values of σ are given in parentheses.

TABLE II

BURST SIZES FOR CELLS INFECTED WITH UV-IRRADIATED ϕ29 RELATIVE TO UNIRRADIATED VIRUS AT 37°C.

	Surviving fraction		
T	1.00	0.44	0.10
°C			
37	1.00*	0.67	0.53
42	1.02	0.59	0.17
43.5	0.11	0.08	0.05

*The burst size for cells infected with unirradiated virus at 37°C was 169.

in the time required for lysis. This is the result reported by Luria for coliphages α, γ, and T7 (1) and by Setlow et al. for coliphage T1 (2). We could not detect a clear functional correlation between latent period extension and dose; however, at all temperatures the latent period (defined in our work as $t_{.05}$) or the half-rise time ($t_{1/2}$) was significantly extended as a result of UV irradiation. Also, the burst size decreased as the extent of radiation damage was increased.

For virus not irradiated and for virus irradiated to a surviving fraction of 0.10, the half-times (and latent periods) decreased as the temperature was increased. In each case the data fit an Arrhenius plot when the reciprocal of $t_{1/2}$ was used as a measure of the rate constant for lysis (Fig. 3). The data for a surviving fraction of 0.44 do not fit a clear trend, but for this dose the measured growth curve characteristics represent an average for a mixed population, consisting of some bacteria infected with damaged phage and others infected with undamaged phage. A significant fraction of the phage had not suffered any UV lesions and did not require repair. At a surviving fraction of 0.10, however, essentially all of those phage that survived were damaged and subsequently repaired.

For unirradiated virus, an increase in temperature from 37°C to 42°C did not affect the burst size, but for irradiated virus the burst size was decreased. If the effects observed here result from thermal perturbation of a dark repair system, this would indicate that one of the enzymes in the repair system is more thermally sensitive than most

TABLE III

"LATENT PERIODS" FOR CELLS INFECTED WITH UV-IRRADIATED ϕ29

	Surviving fraction		
T	1.00	0.44	0.10
°C		*min*	
37	60	69	74
42	42	43	58
43.5	41	45	48

The latent period is defined as the time in minutes required for the number of plaque-forming units to increase by 5%.

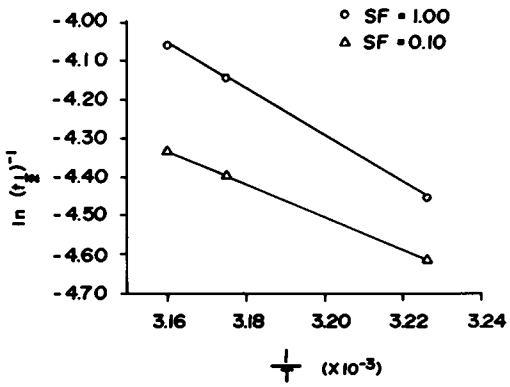

FIGURE 3 Arrhenius plot for the lysis of ϕ29-infected *B. subtilis*. The reciprocal of the half-time ($t_{1/2}$) obtained from least-squares fits to the experimental data was used as a measure of the rate constant for release of infective virus. Host cells were infected with unirradiated virus (○) or virus irradiated to a surviving fraction (SF) of 0.10 (△).

other enzymes involved in phage morphogenesis. Such an explanation is supported by the fact that the decrease in burst size for irradiated virus at 42°C is greater than predicted by simple additivity of the thermal and UV damage effects (Table II). The additional thermal stress of increasing the temperature to 43.5°C led to significant decreases in burst size for both irradiated and unirradiated viruses.

Whether the decrease in burst size reflected a decrease in the number of cells lysing, each releasing a normal burst, or a decreased burst size for each infected cell is unclear. Although theoretically this could be resolved by single burst experiments, such experiments are complicated by the fact that in the ϕ29 system the host cells were observed to grow in chains several cells long under all conditions used in this study.

Thus UV irradiation of ϕ29 results in an increased latent period and a decreased burst size. For virus that has received a particular amount of UV damage, increasing the growth temperature causes a decrease in burst size, indicating either a decrease in the efficiency of the repair system at higher temperature or premature lysis of the cells. Premature lysis could occur if the rate of lysozyme production has not been delayed as much as the rate of virus maturation. The mechanism by which virus multiplication is delayed in cells infected by irradiated viruses is not understood. Further investigation of this phenomenon could elucidate the poorly understood mechanisms for regulation of DNA-repair enzyme systems.

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