RESISTANCE TO ULTRAVIOLET LIGHT AS AN INDEX TO 
THE REPRODUCTION OF BACTERIOPHAGE 

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Infection of a susceptible bacterium by a single phage particle initiates a series of events climaxed, after a time called the latent period, by bursting of the cell and the release of a number (burst size) of replicas of the initial phage. We are here concerned primarily with the intervening process of phage replication which takes place behind the cloak of the cell wall. By prematurely disrupting infected cells, Doermann (1948) found that infectious phage replicas are already present well before the time at which the bacterium bursts, i.e., about two-thirds of the way through the latent period. At earlier times, however, no plaque-forming particles are recovered, not even the initial phage. Our attention is, therefore, focused upon this "dark" period, during which the infecting phage must undergo some modification, and the key processes of phage reproduction come to pass.

Luria and Latarjet (1947) conceived the following experiment in an attempt to use target theory for an analysis of the intracellular developments. It had been shown by Anderson (1948) that a bacterium (Escherichia coli, strain B) could be subjected to rather heavy doses of ultraviolet light and still survive in its ability to support the growth of phage T2. Thus, if one were to infect cells of strain B with single particles of T2 and irradiate the phage-bacterium complexes, the survival of infectivity (the ability to release at least one phage particle, thereby forming a plaque) of the complex should be determined by the survival of the phage part. If the irradiation is done immediately after infection, one should obtain the same survival curve for complexes as for the free phage irradiated before addition to bacteria, i.e., an approximately exponential or "one-hit" curve. If complexes are allowed to develop to the point where several intracellular phage particles are present, the inactivation of the complex requires at least one "hit" in each phage, and a multiple-target survival curve should be obtained. The set of curves for samples irradiated at different stages in the latent period would be expected to resemble the theoretical curves of figure 1. These multiple-target curves, plotted on a semilogarithmic graph, are characterized by asymptotes of constant slope equal to that for the single phage particle. The intercept of the asymptote, extrapolated to zero dose, corresponds to the logarithm of the number of targets.

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In experiments with *E. coli*, strain B, and phage T2, Luria and Latarjet found that immediately after infection the survival curve agreed with that of free phage. However, at later times, instead of showing a progressive increase in multiplicity with constant slope, the curves during the first half of the latent period showed a progressive decrease in slope (i.e., a decrease in sensitivity to ultraviolet) while remaining essentially exponential in character. At mid-latent period the curves became multiple-target in character, and thereafter the sensitivity increased again. Since the results did not resemble the family of curves

![Theoretical survival curves for complexes (after Luria and Latarjet, 1947).](http://jb.asm.org/)

For an individual target the survival $y$ is given by $e^{-D}$, where $D$ is the dose in arbitrary units.

For a complex containing $n$ identical, independent targets, the survival of any one of which is sufficient for survival of the complex, $y = 1 - (1 - e^{-D})^n$.

In figure 1, it was not possible to perform a target-theory determination of the number of intracellular phage particles. Latarjet (1948) repeated these studies, using X-rays, and observed changes which were similar in character although differing in degree.

Further investigation of this problem was suggested by the discovery of "multiplicity reactivation" (Luria, 1947; Luria and Dulbecco, 1949). In this phenomenon, two ultraviolet inactivated phage particles, when infecting the same cell, can combine their resources, leading to the production of active phage.
This result may also be stated in the following way: complexes formed by infection of a cell with two phage particles do not have a survival curve corresponding to the two-target curve of figure 1; at any given dose of ultraviolet the probability of the complex being infective is greater than given by that curve. This effect occurs to a marked degree with phage T2 and would be expected to cause anomalous results in an experiment of the Luria-Latarjet type, where intracellular multiplication is going on. However, there are other phages, T7 for instance, with which multiplicity reactivation does not occur, and it seemed of interest to extend the experiments to such a phage.

Another possible cause of anomalous results is "photoreactivation" of phage (Dulbecco, 1950). Ultraviolet inactivated phage particles may be reactivated, after adsorption to a sensitive bacterium, by exposure to white light. Thus, we may expect the infectivity of a phage-bacterium complex to have a higher resistance to ultraviolet if exposed to light (after ultraviolet irradiation) than if kept in the dark. Precautions are therefore necessary in order to avoid this effect.

**MATERIALS**

Phages: T7, T2, and T2r, prepared from lysates in broth, purified by centrifugation, and resuspended in buffer.

Bacterium: *Escherichia coli*, strain B, grown in broth.

Growth medium (broth): bacto-tryptone, 1 per cent plus 0.1 m NaCl.

Buffer: 1/15 m phosphate buffer, pH 7, plus 0.1 m NaCl, plus 10⁻³ m MgSO₄.

**METHODS**

A Luria-Latarjet experiment involves the following steps:

1. Phage particles are added to a suspension of bacteria and time is allowed for infection of the cells to occur, then unadsorbed phage is eliminated.

2. The complexes are allowed to develop and samples are removed at various stages of the latent period.

3. Each sample is exposed to several doses of ultraviolet.

4. Aliquots are plated to determine the fraction of infective centers surviving each dose. These operations must be completed before the end of the latent period. Furthermore, since the radiation resistance of the infective centers changes rapidly with time, it is essential for accurate results that growth start almost simultaneously in all cells and that it be halted during irradiation.

In an attempt to best satisfy these requirements, the following procedure is used:

1. *Adsorption of phage without growth*. The bacteria are prepared from an aerated broth culture in the logarithmic phase (1 × 10⁸ cells per ml). The cells are centrifuged, resuspended in buffer, centrifuged again, resuspended in buffer at a concentration of 1 × 10⁶ per ml, and aerated by bubbling at 37 C for one hour in order to exhaust intracellular nutrients and bring the bacteria to a starved condition. A purified suspension of phage particles in buffer is then added. Under these conditions adsorption takes place, but no lysis or phage liberation
However, the absence of phage liberation is not sufficient to exclude the possibility that intracellular growth progresses to a fairly advanced intermediate stage without reaching completion.

We can test for this by making use of the large change in resistance to ultraviolet of T2r complexes. If intracellular development proceeds, the resistance should increase with time. In figure 2, it can be seen that so long as the infected cells are kept in buffer and no nutrient is added, the resistance remains constant. The value of the resistance is only slightly higher than that of the free phage (see later). Thus, the intracellular development does not progress beyond a very early stage. Even if the adsorption period extends over many minutes, each complex is arrested in its development, and adsorption is effectively simultaneous.

In experiments with bacteria grown in synthetic medium (instead of broth) there was a slow increase of resistance, even in buffer, as if considerable intracellular nutrient reserves remained even after starving for an hour. This resistance reached a maximum value corresponding to that attained after several minutes of growth in the presence of ample extracellular nutrient.
T2r is adsorbed to the extent of over 90 per cent in 10 minutes in buffer. At the same buffer concentration, T7 adsorption is extremely slow, but the rate is greatly increased by using buffer diluted 10 times with distilled water (Watson, personal communication). Therefore, throughout the experiments with T7 diluted buffer was used. The number of infective centers obtained (with either phage) was usually found to be less, by one-third to one-half, than the number of phages adsorbed. The cause of this "abortive adsorption" is not understood. To eliminate unadsorbed phage, the cells are washed by centrifugation and resuspended in fresh buffer.

Figure 3. One-step growth curve at 37 C for T2r complexes formed in buffer. Broth added at time zero. "Infective center" signifies a plaque-forming unit, either an infected cell or a free phage particle.

2. Growth. To the suspension of infected cells in buffer at 37 C, an equal volume of broth at the same temperature is added, and time is reckoned from this moment. In the case of phage T2r the radiation resistance promptly begins to rise (figure 2). From the one step growth curve in figure 3, it may be seen that the (minimum) latent period for T2r is 19 minutes, and the average burst size is 60. For T7, the latent period is 12 minutes, and the burst size is 150. To stop development at any chosen time during the latent period, a sample is removed and diluted rapidly (by blowing out of a pipette) into buffer chilled in an ice bath. The chilling brings the change in radiation resistance to an immediate
halt; the resistance remains constant for hours provided the sample is kept chilled. The pipettes used for addition of broth and removal of samples are previously equilibrated at 37 C in an incubator.

In this manner, growth may be started and stopped in all cells simultaneously, and the timing controlled to within a few seconds. Furthermore, many samples may be taken at close intervals within the same growth experiment and the irradiation conducted afterwards at leisure.

For samples chilled very close to the end of the latent period (after 16 minutes for T2r or 9 minutes for T7) lysis is not prevented by chilling. After a delay, there appears a gradual increase in plaque count, presumably due to slow lysis of cells containing completed phage particles.

3. Irradiation. One or two ml of the suspension to be irradiated are placed in a shallow watch glass. The suspension is transparent to ultraviolet by virtue of the large dilution (100X) of the broth. Ice in a small dish is used to chill the suspension from below (figure 4). This prevents growth during the irradiation. Chilling also serves to reduce greatly the rate of photoreactivation (Dulbecco, 1950) which might otherwise be caused by the visible light emitted by the ultraviolet lamp.

Ultraviolet is supplied by a 15 watt "germicidal lamp" (General Electric Company). The energy emitted in the 2537 A line accounts for almost all the antiphage activity. The intensity of ultraviolet is such that T2r survives to the extent of \(10^{-2}\) after an exposure of 40 seconds. This same intensity is used throughout the experiments, and the doses are, therefore, given in units of seconds. All manipulations after irradiation are conducted in dim yellow light to minimize the possibility of photoreactivation.

4. Plating. Aliquots of samples subjected to various doses of ultraviolet are plated in a top layer of soft agar, seeded with unirradiated B, on broth agar plates. The resultant plaques are counted to determine the fraction of complexes whose infectivity has survived the irradiation.

Note on multiplicity of infection. It is essential that the proportion of phage particles to bacteria be kept quite small in order that very few multicomplexes (i.e., bacteria infected with more than one phage particle) be formed. This is particularly important in the case of T2, where the phage exhibits multiplicity reactivation and the multicomplexes are much more resistant to ultraviolet than monocomplexes. Thus, if one assumes a Poisson distribution of phages per bacterium, and a survival value of \(10^{-2}\) is to be accurate within 10 per cent, the average multiplicity of infection must be \(2 \times 10^{-3}\) or less. In order to have
such a low multiplicity of infection and still have a measurable concentration of survivors after growth, dilution, and irradiation, it is necessary to start with a high concentration of bacteria (1 × 10⁶ per ml). Concentrations either half or twice this value were found to give the same results.

PRELIMINARY CONSIDERATIONS

1. Ability of irradiated bacteria to support phage growth. It is necessary to know the validity, under the conditions used, of the basic assumption that the irradiation of the bacteria does not affect their ability to support phage growth. For this reason Anderson's experiments were repeated, using phage T2r. Starved bacteria, prepared as previously described, were irradiated in buffer before addition of phage. The number of infective centers produced per adsorbed phage was determined by plating and compared to the result with unirradiated bacteria. At the intensity of ultraviolet here used, 4,000 seconds of irradiation are required to reduce to one-half the number of bacteria capable of liberating phage after infection with T2r. Since the largest doses used in irradiating infected cells were 1,000 seconds, inactivation of the bacterial part of the complex should have had a negligible effect upon the survival curve. It is conceivable, however, that the sensitivity of the bacterial part of the complex does not remain unchanged throughout the latent period. Indeed, it becomes difficult to separate the complex into phage and bacterial components once growth has started.

For T7, it is likewise found that the highest dose used (350 seconds) has negligible effect upon the ability of the bacteria to yield phage after infection.

2. Effect of irradiation of complexes upon the latent period and burst size of the survivors. It is found that a progressive lengthening of the latent period and decrease in burst size are produced by increasing doses of ultraviolet. For T2 monocomplexes irradiated at mid-latent period with a dose such that 5 per cent survive to form visible plaques, the latent period of the survivors is doubled and the burst size is reduced to 10 per cent of normal. This effect results in a smaller and more variable size of the plaques formed by surviving monocomplexes. The degree of the effect appears to be determined primarily by the dose of ultraviolet rather than the percentage inactivation of the complexes. Therefore, it is most apparent at times during the latent period when the largest doses are required for obtaining survival curves. In plotting survival curves and attempting to analyze them by target theory, we are assuming that inactivation of a complex is an all-or-none phenomenon. It must be realized that the delay in lysis and reduction in burst size of the surviving infective centers could also lead to failure of some of them to produce visible plaques. However, for the doses used in this paper, the plaques observed with T2r and T7 do not taper gradually down to zero in diameter; probably few infective centers fail to be counted for this reason.

RESULTS

Experiments with T2r. T2 (used by Luria and Latarjet) and its mutant T2r, which has the same sensitivity to ultraviolet as T2, gave similar results. It is
preferable to work with T2r because it produces larger plaques, thereby obviating in some measure the difficulty created by the decrease in plaque size at large doses.

In figure 5 a complete set of curves for T2r (latent period 19 minutes) is given. These qualitatively confirm the observations of Luria and Latarjet with T2. The free phage does not have a strictly exponential survival curve, but the changes in resistance during the latent period are so large that this may be ignored for our purposes. At \( t = 0 \) (i.e., for a sample irradiated before the addition of nutrient) the resistance of monocomplexes is slightly higher (by 20 per cent) than that of free phage. This may be partially due to a small amount of development of the phage which can take place in buffer.

As development proceeds, the resistance of all complexes increases. An especially rapid rise occurs between 6 and 8 minutes, the curves still remaining essentially exponential. The smallness of the bend (at large doses) in the survival curve taken at 7 minutes is very significant, since it implies that, at least up to this point (one-third of the way into the latent period), development has progressed rather uniformly in all infected cells. If, at 7 minutes, 10 per cent of the infective centers had progressed to a stage corresponding to 8 minutes (by virtue of 15 per cent more rapid development), the bend in the 7 minute curve would have been greater than observed.

At 9 or 10 minutes the resistance reaches a maximum phase, and the curve has now become "multiple-target" in character. In figure 6 data are given which were obtained at 10 minutes. The points fit very well a theoretical curve for a
double target. However, the fit may be fortuitous, since slight inhomogeneities in the stage of development are to be expected. At the time of maximum resistance, those complexes which are either slightly ahead or behind in stage of development have lower resistances, causing a distortion of the true curve at the moment of maximum resistance. Thereafter, the curve retains a multiple-target shape, but the resistance of the individual targets, as judged by the final slope, decreases progressively as the end of the latent period is approached.

Figure 6. Circles: Survival data obtained for T2r complexes irradiated at the phase of highest resistance to ultraviolet (t = 10 minutes).
Dashed curve: Theoretical survival curve for a double-target complex.

The late curves have slight "tails" which may be due to a small fraction of cells in which the development is retarded.

In some experiments, peculiar composite survival curves were obtained as though the development in about half the cells was arrested at a stage corresponding to around 8 minutes, while the remaining cells continued normally. It has not been found possible to clarify the conditions leading to this result.

According to Doermann's findings, there may be a few infective phage particles per cell at the latest time here studied (15 minutes). These would presumably have the resistance of free phages. If survival as an infective center depends upon survival of either the complex or at least one completed phage, the effect
of the phages upon the observed survival curve will be negligible, since at 15 minutes the complex has a much higher survival than have several free phages. By reference to figure 1 it can be seen that even a cluster of 100 phages would be inactivated more rapidly than the 15 minute complexes.

Experiments with T7. A complete set of survival curves for monocomplexes of T7 and strain B irradiated at intervals during the latent period (12 minutes) is given in figure 7. It will be observed that the resistance remains unchanged (at the same value as for free phage) for 3 minutes in the presence of nutrient.

As time goes on, the curves become multiple-target in character, the average multiplicity continually increasing, while the final slope changes only slightly.

This result stands in marked contrast to T2r and resembles that predicted by target theory. The exact shapes of these curves are not consistent with an assumption of equal multiplicities for all complexes at a particular time. However, the shape may be explained by a distribution of the number of targets, which may be expected as a consequence of different rates of phage multiplication in different cells. It is well known (Delbrück, 1945) that the number of phage particles released by lysis of individual cells has an extremely wide distribution.

![Figure 7](https://jb.asm.org/Downloaded from http://jb.asm.org/)
To illustrate the effect of such a wide distribution upon our survival curves, a composite curve is plotted in figure 8, assuming a population consisting of equal numbers of cells having the various multiplicities in figure 1. By extrapolation to zero dose of the asymptotic slope of such a curve, it is possible, in principle, to determine the *average* multiplicity. However, this requires precise data at low survival values and cannot be done accurately with the points in figure 7.

*Figure 8.* -- -- -- Theoretical survival curves for complexes containing various numbers of targets (as in figure 1).

--- Theoretical survival curve for a mixed population of complexes. The mixture is assumed to contain equal proportions of complexes having multiplicities 1, 2, 5, 10, 20, 50, and 100.

Note: It is possible, by assumption of a suitable population containing a very wide distribution of multiplicities, to obtain a composite theoretical survival curve which is exponential, simulating a "one-hit" curve (Dulbecco, personal communication).

**DISCUSSION**

The original intent of Luria and Latarjet's experiment, namely to observe the increase in the number of intracellular phage particles during the latent period, has been achieved with T7. The pattern of growth, so far as radiation resistance is concerned, does not exhibit the anomalies of (T2 and) T2r. There appears to be simply an increase with time of the average number of targets per cell, each target being similar radiologically to a T7 particle.

This result shows that irradiation of intracellular phage, in the case of T7,
enables us to discern the existence of multiple intracellular entities at a time when fully infective phages are not yet detectable with the Doermann technique.

The anomalous results with T2r may be of far greater interest, however. During the critical phase between the entrance of one infecting particle and the appearance of intracellular progeny, enormous changes are reflected by the survival curves. It seems likely that these resistance changes may bear a close relation to the phenomenon of multiplicity reactivation. While the resistance changes do not tell us what is actually happening, they at least give us something easy to measure which serves as an empirical index to development. The value of this index has already been demonstrated previously in the justification of the techniques used in these experiments (adsorption in buffer, chilling to stop growth).

In the case of T2r, the maximum change in slope is by a factor of over 20 (free phage compared with \( t = 10 \) minutes). As pointed out by Luria and Latarjet, intracellular accumulation of ultraviolet-absorbing materials (nucleic acid components) must be considered as a possible contributing factor.

By microspectrography of uninfected cells (at 2570 A) Hedén (1951) finds an average extinction through the thickness of a cell of around 0.1, corresponding to 80 per cent transmission. A phage particle which is adsorbed on the surface of a bacterium is therefore shielded from ultraviolet to the extent of 20 per cent in one direction. This should lead to an average increase by only 10 per cent in the resistance (at \( t = 0 \)) compared with free phage (since the cell rotates in all directions during exposure). Penetration of the phage into the cell should have little effect on the magnitude of this average shielding. In order to account for a subsequent change by a factor of 20 in the intensity of ultraviolet reaching the phage, a coating of nucleic acid, 1 \( \mu \) in diameter, would have to be produced. The measurements of Luria and Human (1950) and Cohen and Arbogast (1950) on optical density and nucleic acid content in suspensions of cells multiple infected with T2 reveal increases during growth, but these are far too small to account for the observed resistance changes during the first 10 minutes.

In the case of T7, however, the small change in slope may well be due to a screening effect.

A plausible interpretation of the increase in resistance of T2r complexes during the first half of the latent period may be the following: A T2r particle, after adsorption to a sensitive bacterium, must undergo a series of successive steps \( A \rightarrow B \rightarrow C \rightarrow D \rightarrow \), etc. in the course of reproduction. Each of these steps has a certain cross section for being blocked by ultraviolet (e.g., by inactivation of an enzyme which is concerned with the step). Blockade of any one of these steps prevents normal development and causes inactivation of the phage. At time zero, the total cross section of the phage is therefore the sum of these individual cross sections, and the survival curve is exponential. As development proceeds, the steps which have already been passed are no longer needed, and the effective cross section decreases progressively while inactivation of the remaining steps retains the characteristics of a one-hit phenomenon.

The absence of this behavior in the case of T7 suggests that there may be great differences in the mode of reproduction of T7 and T2r. These two phages are also dissimilar in other respects. T2r is a relatively large particle with a head and a tail and appears to have a kind of membrane (Anderson, 1949); it
shows multiplicity reactivation with other particles of T2r, and also undergoes genetic recombination with mutants of T2 and other closely related phages. T7, on the other hand, is much smaller in size, spherical in shape, does not appear to have a membrane, and does not show multiplicity reactivation. Whether it undergoes genetic recombination is not known.

Therefore, the sequence of events in the intracellular development of T2r, which is reflected in the resistance changes, may be characteristic for the multiplication of only certain types of phage.

We stand to learn a great deal about the growth of T2r by making use of the radiation resistance index. If conditions are suitable for phage development, the index (i.e., the survival of infective centers after a given standard dose of ultraviolet) goes up (or down, in the latter half of the latent period). If, for any reason, phage development is blocked, the index remains constant; its value marks the stage at which the block occurs. This makes it possible to study the effect, at any chosen part of the latent period (except near the end), of many factors, for example temperature, growth requirements, and specific chemical inhibitors. The results of these investigations will be reported separately.

This tool offers promise not only for studying phage growth but for certain problems in bacterial physiology as well. The growth of phage in a bacterium is dependent upon the metabolic well-being of the cell and on its ability to make use of the substrates supplied to it. By infecting the bacterium with a T2r particle and using the radiation resistance index, the metabolic capabilities of the bacterium under particular conditions can be measured. A unique feature of this technique is that one can determine not only the average rate of metabolism but the distribution of rates (from analysis of the survival curves) among the individual cells in the population. This idea is currently being applied to the problem of the kinetics of enzymatic adaptation in bacteria.

SUMMARY

Techniques are described for simultaneous starting and stopping of the growth of bacteriophage in all the host cells of a culture. Using these techniques, a comparison is made of the changes in resistance to ultraviolet of phages T2r and T7 during intracellular growth, following the method of Luria and Latarjet.

Phage T7 gives results similar to expectations from target theory, while the results with T2r confirm the (anomalous) behavior observed by Luria and Latarjet, indicating that there may be large differences in the modes of reproduction of different phages.

The utility of the change in resistance as a tool in studying bacteriophage reproduction and certain problems in bacterial physiology is pointed out.

REFERENCES


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