The Relation Between Radiation Stability and DNA Replication of Phage T4†

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SUMMARY

Two types of experiment have been made during the early part of the latent period on a culture of cells singly infected with phage T4.

First, samples taken from the infected cells at different times were irradiated with ultraviolet light in order to obtain a set of Luria-Latarjet curves. Secondly, and with the same culture of infected cells, the kinetics of phage DNA synthesis was followed using a density gradient technique.

A comparison of these results suggests that the radiation stability shown by the Luria-Latarjet curves is first detectable at about the time that phage DNA synthesis commences. The radiation stability, however, increases at a rate which is much faster than the rate of replication of the parental phage DNA.

INTRODUCTION

The capacity of bacteria singly infected with phage T4 (T4 monocomplexes) to liberate infectious progeny phage is destroyed by exposure to radiation. During the early stages of phage growth the radiation resistance of T4 monocomplexes follows a distinct pattern. Throughout the first quarter of the latent period the resistance is constant and similar to that of T4 phage particles. The resistance then increases rapidly until mid-latent period when it is more than 20 times greater than its initial value. This pattern is independent of the type of radiation employed, being observed in experiments with ultraviolet light, X-rays and 32P decay products (Luria & Latarjet, 1947; Benzer, 1952; Stent, 1955; Symonds & McCloy, 1958). This increase in radiation resistance acquired by monocomplexes, referred to as 'stabilization' or 'radiation stability', was observed as early as 1947 in experiments with phage T2 (Luria & Latarjet, 1947). Although a number of explanations of radiation stability have been proposed, its interpretation remains obscure (Benzer, 1952; Stent, 1955).

There are four characteristics of the monocomplex survival curves which any theory of radiation stability must explain: (1) the stage during the latent period at which stabilization commences, (2) the exponential slope of the curves during the first half of the latent period, (3) the rate at which stability develops, and (4) the loss of stability during the latter part of the latent period.

The last point has a straightforward explanation which follows from two experimental observations. First, the lytic process in T4-infected cells is initiated midway through the
latent period and the cells lyse at a definite time later. Secondly, there is a considerable
time lag in the production of viable phage particles by complexes that survive irradiation.
Therefore, complexes irradiated during the second half of the latent period appear more
sensitive because they tend to lyse before phage production has occurred (Symonds,
1957).

It is the first three characteristics of the survival curves which relate to the nature of
stabilization, and about which this paper is concerned. They are in fact closely interrelated.
The onset of stabilization must herald the initiation of some step in phage development.
That the survival curves remain exponential is a reflexion of the degree of synchrony attained
in phage development in the complexes but, more important, it shows that some gradual
process is involved in stabilization. If some abrupt change occurred, say the sudden appear-
ance of a new repair enzyme, some complexes would suddenly acquire maximum resistance
and, inevitably, the survival curves would display two components: this is not observed.

A simple and plausible explanation of radiation stability relates stabilization to the
occurrence of multiplicity reactivation (Benzer, 1952; Stahl, 1959). On this interpretation
any radiation damage occurring in duplicated portions of the parental genome is efficiently
repaired by recombination. The sensitive ‘target’ in the infected cells is therefore the non-
replicated part of the phage genome which is present only as a single copy. If, after ultra-
violet irradiation by dose \( D \), the surviving fraction of the monocomplexes at the start of
the latent period is represented by \( S = e^{-kD} \), then at times up to the completion of the first
duplication of the genome the surviving fraction can be represented by \( S = e^{-kp} \), where
\( k \) varies between 1 and 0, and is a measure of the sensitive target as the fraction of the genome
which has not replicated. Stabilization and DNA replication start simultaneously, and the
extent of stabilization at any time reflects the fraction of the phage genome which has
replicated. Maximum resistance marks the completion of the first duplication of the parental
phage genome. This explanation is attractive because it is known that the intracellular
synthesis of phage DNA commences at about the same time as stabilization; and that
multiplicity reactivation does occur between irradiated T4 phage particles (Stahl, 1959).

There have been a number of attempts to test the multiplicity reactivation hypothesis
experimentally with results which tend to be unfavourable, although inconclusive (Pratt,
Stent & Harriman, 1961; Kozinski & Bessman, 1961; Fisher & Pardee, 1968). We have
attempted a further test of this hypothesis by comparing closely the acquisition of radiation
stability in a culture of T4 monocomplexes with the time course of replication of the
parental phage DNA. In order that the test should be sufficiently sensitive, the replication
of the parental DNA was followed by a technique involving density gradient centrifugation
of fragmented replicating DNA.

**METHO**

**Phage and bacteria.** Phage T4Bo1, an osmotic-shock resistant mutant of T4B (Brenner &
Barnett, 1959) was used throughout this work. For the transfer experiments, *Escherichia coli*
strain R 2/1, 6 was employed as both host and plating indicator. This strain is a phage T1
and T6 resistant mutant strain R 2 (Hershey, Dixon & Chase, 1953). *E. coli* strain b/r/1,
a T1 resistant mutant of b/r, was used to grow the \(^{32}P\)-labelled phage.

**Media.** Broth, containing per l.: bacto peptone (Difco), 15 g. nutrient broth (Difco),
8 g.; NaCl, 8 g.; glucose, 1 g. Phosphate buffer, pH 7.0 is the adsorption medium of
Hershey & Chase (1952). The algal salts solution used in the ‘heavy’ growth medium con-
tained per l.: NaCl, 8 g.; MgSO\(_4\)-7H\(_2\)O, 0.25 g.; CaCl\(_2\cdot2\)H\(_2\)O, 0.015 g.; KCl, 0.001 g.
The algal hydrolysate, in which over 90% of the carbon and nitrogen consists of the ‘heavy’ isotopes, $^{13}$C and $^{15}$N, has been described previously (Boyle, Ritchie & Symonds, 1965).

**Ultraviolet light irradiation.** The source of ultraviolet light was a 15 W Hanovia Bactericidal Ultraviolet unit. Samples of 3 ml. in phosphate buffer, were irradiated in watch glasses at a distance of 34 cm. from the light source.

**Assay methods.** Ultraviolet light absorbence of DNA solutions was measured at 260 nm. with a Unicam SP 500 spectrophotometer. Radioactivity was counted with an end-window geiger counter (Isotope Developments Ltd.), having a counting efficiency of about 25% for $^{32}$P.

**Isolation of DNA.** Twenty ml. samples of an infected culture at $5 \times 10^8$ cells/ml. were centrifuged and suspended in 1 ml. of standard saline citrate ($0.15 M \text{NaCl}, \ 0.015 M \text{Na citrate}$) and the cells lysed by incubation with sodium lauryl sulphate (2.5% w/v) at 45°C for 5 min. The DNA was extracted by shaking for 30 min. with an equal volume of freshly distilled phenol containing sodium para-amino salicylate (6% w/v). The aqueous phase was shaken with ether and the nucleic acids precipitated with 2.5 volumes of ethanol. The precipitate was redissolved in standard saline citrate. Finally the DNA was fragmented by rapid passage three times through a no. 26 syringe needle.

**Density gradient centrifugation.** DNA solutions were prepared in aqueous CsCl solutions to give a density of about 1.71 g./ml. Samples of 2-8 ml. were overlaid with mineral oil and centrifuged at 35,000 rev./min. for 72 hr in the SW 39 rotor of a Spinco ultracentrifuge. Fractions were then collected in phosphate buffer through a hole pierced in the bottom of the centrifuge tube. The refractive index of several fractions from each gradient was measured and used to establish the density gradient by reference to a calibration curve.

**Preparation of $^{13}$C $^{15}$N $^{32}$P-labelled phage T4.** A broth culture of B/r/1 was washed twice in algal salts solution and resuspended in this medium at $5 \times 10^8$ cells/ml. This culture was diluted 100-fold into 1 ml. of algal salts solution supplemented with, 1-tryptophan, an adsorption cofactor (10 µg./ml.), algal hydrolysate (5% v/v) and carrier-free $^{32}$P orthophosphate (Radiochemical Centre, Amersham) at an activity of 500 µc/ml. The cells were grown with aeration at 37°C in this ‘heavy’ medium to a concentration of $2 \times 10^9$ cells/ml. and then infected with phage T4 at a multiplicity of 0.01 p.f.u./cell. Two hr later the cells were lysed with chloroform. The lysates were purified by filtration through an Oxoid membrane filter and centrifuged three times at 17,000g for 45 min. in the presence of $3 \times 10^{11}$ particles/ml. of carrier T6 phage. Phage pellets were resuspended in 1 ml. of phosphate buffer by standing at 4°C for 1 hr followed by gentle agitation. These lysates were assayed immediately and used 4 hr later when the assay plates could be counted.

The purified lysates gave T4 phage infectivities of about $1 \times 10^{10}$ p.f.u./ml. DNA extracted from these phage had a buoyant density of 1.74 g./ml. compared with 1.70 g./ml. for $^{13}$C $^{14}$N T4 DNA and 1.71 g./ml. for $^{13}$C $^{14}$N E. coli DNA.

**The transfer experiment.** It was necessary to remove samples from the infected cultures at frequent intervals during the early stages of phage growth. To make the times of sampling more accurate the latent period was extended by growing the infected cells at 30°C rather than at 37°C. At 30°C the latent period of T4 was 35 to 40 min. and the burst size about 100 p.f.u./cell.

A stationary-phase culture of R2/1,6 was diluted 100-fold in broth and grown with aeration to a concentration of $2 \times 10^8$ cells/ml. The cells were washed twice in phosphate buffer and resuspended at $1 \times 10^8$ cells/ml. in 60 ml. of phosphate buffer containing 1-tryptophan (10 µg./ml.) and then infected with $^{18}$C $^{15}$N $^{32}$P-labelled T4 at a multiplicity of 0.03 p.f.u./cell. Following adsorption for 5 min., synchronous phage development was started by the addition of an equal volume of warm broth and aeration at 30°C. At subsequent intervals, 20 ml.
samples were withdrawn from the culture into KCN (0.01 M) to inhibit further growth. Aliquots of each sample were exposed to each of three doses of ultraviolet light and assayed for infectivity to determine the radiation resistance. The DNA was extracted from the remainder for density gradient analysis. Fractions from each gradient were assayed for radioactivity to obtain the density distribution of the parental $^{32}$P label: the ultraviolet light absorbance was measured in order to fix the position of the non-radioactive bacterial DNA present in great excess. As the bacterial DNA has a density of 1.71 g./ml. this gave a useful density index for the gradient.

RESULTS

Preliminary considerations

The aim of this work was to make a careful comparison, through the early part of the latent period, between the development of the radiation resistance of T4 monocomplexes and the course of replication of the parental phage genomes. This required a sensitive method for following the replication of the parental phage DNA.

The method employed was a modification of the transfer experiments of Roller (1961, 1964) and Kozinski (1961). Bacteria growing in non-radioactive broth, which contains the normal or ‘light’ isotopes of carbon, $^{12}$C and nitrogen, $^{14}$N, are infected with radioactive parental phage labelled with the ‘heavy’ isotopes $^{13}$C and $^{15}$N. During phage growth in these cells only $^{12}$C$^{14}$N molecules are incorporated into the newly synthesized phage DNA. Since T4 DNA replicates semiconservatively (Kozinski, Kozinski & Shannon, 1963), the first daughter DNA molecules will be hybrids and will have a density intermediate between that of $^{13}$C$^{15}$N parental DNA and that of T4 DNA containing only $^{12}$C$^{14}$N isotopes.

Before completion of the first cycle of DNA replication, newly synthesized DNA will be associated with parental ‘heavy’ DNA. To determine the time at which phage DNA synthesis commences, it is necessary to fragment the intracellular DNA before density gradient analysis. For example, if replication of the parental DNA molecules has progressed along 10% of their length, the density of these structures will be only marginally lighter than that of the non-replicated parental molecules. If each of these partial replicas is now broken into ten fragments, the population will consist of non-replicated fragments of completely ‘heavy’ density and fragments containing newly incorporated ‘light’ precursors whose density will show a spread, the lightest of which will be hybrid. The appearance of hybrid molecules may be detected following separation from the heavy molecules in a cesium chloride density gradient.

The transfer experiment

This method for following DNA replication was tested in a transfer experiment in which samples were removed from a population of T4 monocomplexes during the entire latent period, and the fragmented intracellular DNA was analysed in a density gradient.

The density distribution of the parental radioactivity is shown in Fig. 1. In the 1 min. sample the parental radioactivity bands almost entirely at a density of 1.74 g./ml., the position of unreplicated parental DNA; there is also a small radioactive peak at 1.71 g./ml., the nature of which will be considered below. Hybrid DNA banding at 1.72 g./ml. is in all later samples. No appreciable radioactivity bands at densities lighter than that of hybrid DNA.

The percentage of parental $^{32}$P label in the hybrid DNA band of each sample is listed in Table 1 (Expt 3) with results from two similar experiments. The results are consistent, and indicate that at 30° the first cycle of phage DNA replication starts earlier than 10 min. after
Fig. 1. The density gradient distribution of $^{32}$P-labelled parental phage DNA during intracellular phage growth. Bacteria growing in non-radioactive, 'light' medium were infected with $^{32}$P-labelled, 'heavy' T4 (0.02 p.f.u./cell). At 1, 10, 15, 20, 30 and 40 min. after infection the intracellular DNA was extracted, fragmented and centrifuged in a density gradient.
infection and is virtually complete by 15 min. In all experiments the transfer of $^{32}$P label from parental to hybrid DNA is incomplete at 35 to 50% of the total. In experiments of this type using $^{32}$P-labelled T4, transfer of label from the parental to the daughter DNA molecules is never complete and does not exceed 50 to 70% (Kozinski, 1961; Kozinski & Bessman, 1961; Kozinski et al. 1963): our transfer values are comparable to these previous results.

Table 1. Kinetics of hybrid DNA formation during growth of T4 monocomplexes infected with 'heavy' parental phage

<table>
<thead>
<tr>
<th>Time after infection (min.)</th>
<th>Percentage of parental DNA in hybrid band</th>
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<tbody>
<tr>
<td></td>
<td>Expt. 1</td>
</tr>
<tr>
<td>10</td>
<td>21</td>
</tr>
<tr>
<td>15</td>
<td>51</td>
</tr>
<tr>
<td>30</td>
<td>47</td>
</tr>
<tr>
<td>35</td>
<td>52</td>
</tr>
<tr>
<td>40</td>
<td>52</td>
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Results were obtained from experiments of the type described in Fig. 1.

There are two main reasons for this incomplete transfer. First, there will be a contribution from inactive phage particles which are able to adsorb and inject their DNA into the cells. Inactive phages may result from the effects of $^{32}$P decay (Hershey et al. 1951; Stent & Fuerst, 1955) and calculations indicate that about 5% of the parental phage will be inactivated by the decay of incorporated $^{32}$P atoms. The mechanical resuspension of pelleted phage particles may also cause inactivation. Secondly, on adsorption in buffer, a certain proportion of cells infected with viable phage do not produce phage progeny and presumably do not support DNA replication. Benzer (1952) observed that one-third to one-half of the infected cells showed this 'abortive adsorption' and failed to yield. Unadsorbed phages are not a problem, as they are reduced to an insignificant level.

A further problem is the extraction of DNA from cell populations in which over 95% of the cells are uninfected. Consequently, before fragmentation, the DNA preparations contain a large excess of bacterial DNA of high molecular weight. In transfer experiments with non-fragmented DNA it was found that about 10% of the parental phage DNA and almost all of the replicated phage DNA banded at the density of the bacterial DNA. This trapping effect, also noted by Konrad & Stent (1964), is almost completely eliminated when the DNA is fragmented; however, the small amount of radioactivity banding at 1.71 g./ml. in the 1 min. sample of Fig. 1 is attributable to this cause.

The relation between stability and transfer

Our main problem was to follow in detail the first cycle of replication of the parental DNA during the period in which stabilization was developing. Preliminary experiments showed that at 30° radiation stability first appears about 8 min. after infection. A transfer experiment was therefore performed in which samples were removed from a culture of T4 monocomplexes at 1 min. intervals during the first half of the latent period. Aliquots of each sample were irradiated with several doses of ultraviolet light in order to follow the development of the radiation resistance. The DNA was isolated from the remaining bulk of the 7
U.v. stabilization and DNA replication of T4

The results are presented in Fig. 2 and 3. Fig. 2 shows the survival-dose curves. From 1 to 4 min. after infection the survival curves are identical (Fig. 2). Between 4 and 7 min. the curves show a small and increasing radiation resistance. The first abrupt change in resistance occurs between 7 and 8 min. after infection and resistance then increases rapidly to a maximum at 15 min. At later times the resistance decreases characteristically (Benzer, 1952; Symonds, 1957). The survival curves during the early period are approximately exponential

and show that stabilization is synchronized in the population of monocomplexes. The relative slopes of the survival exponentials at these different times are summarized in Table 2 Col. 2.

The density gradient analyses of these samples show (Fig. 3) identical patterns of radioactivity at 7 and 8 min. with most of the $^{32}$P banding at a density of 1.74 g./ml., corresponding to unreplicated parental phage DNA. No radioactivity is seen at the hybrid position. The small lighter peak banding with the bacterial DNA results from trapping and should not be confused with replicating phage DNA. Nine min. after infection a small peak has appeared at the hybrid position and indicates that replication has started. As phage development
Fig. 3. The density gradient distribution of $^{32}$P-labelled parental phage DNA, $\bullet$--$\bullet$ and non-radioactive bacterial DNA, $\bigcirc$--$\bigcirc$ during intracellular phage growth. Bacteria growing in non-radioactive, 'light' medium were infected (0.03 p.f.u./cell) with $^{32}$P-labelled, 'heavy' T4. At 7, 8, 9, 10, 11 and 12 min. after infection the intracellular DNA was extracted, fragmented and centrifuged in a density gradient. The results in Fig. 2 and 3 were obtained from the same population of monocomplexes.
progresses the amount of hybrid material increases until 12 min. after infection over 20% of the radioactivity is located at the hybrid density. There is evidence of trapped DNA in all samples. The fraction of the parental label found in the hybrid band at these times is listed in Table 2, Col. 4. As the growth conditions employed in this experiment are similar to those used in the experiments of Table 1, only 35 to 50% of the original label can be expected to be replicated. Allowing for this, the actual transfer of parental label from the replicating phage into the hybrid band can be calculated (Table 2, Col. 5).

Table 2. Comparison between the change in radiation sensitivity and parental DNA replication in T4 monocomplexes

<table>
<thead>
<tr>
<th>Time after infection (min.)</th>
<th>Relative radiation sensitivity</th>
<th>Replication predicted on multiplicity reactivation theory (%)</th>
<th>Total $^32P$ in hybrid band (%)</th>
<th>Transfer to hybrid band from viable parents (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5, 6, 7</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>0.77</td>
<td>23</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>0.33</td>
<td>67</td>
<td>5.0</td>
<td>10–15</td>
</tr>
<tr>
<td>10</td>
<td>0.28</td>
<td>72</td>
<td>15.8</td>
<td>31–47</td>
</tr>
<tr>
<td>11</td>
<td>0.16</td>
<td>84</td>
<td>17.1</td>
<td>34–52</td>
</tr>
<tr>
<td>12</td>
<td>0.09</td>
<td>91</td>
<td>20.7</td>
<td>41–62</td>
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</table>

The figures in Col. 2 are the relative slopes (k) of the radiation survival lines (Fig. 2). From the multiplicity reactivation theory, the survival can be represented by the expression; $S = e^{-kD}$, where k is the non-replicated fraction of the parental genome and D is the dose of ultraviolet light. The figures in Col. 3 are calculated as the remaining percentage of the genome. The figures in Col. 4, from Fig. 3, are corrected for trapped DNA amounting to about 7% of the total $^32P$ in each case. The figures in Col. 5 are calculated as two to three times those in Col. 4 on the assumption that only 35 to 50% of the parental DNA is capable of replication.

DISCUSSION

Any interpretation of radiation stability must explain three features of the survival curves for monocomplexes. (1) Their exponential shape; (2) the time at which stabilization first becomes apparent; (3) the rate at which stabilization proceeds.

The mechanism of multiplicity reactivation satisfies the first feature, since the sensitive 'target' in the monocomplexes is the unreplicated portion of the parental phage DNA molecules; this fraction would be expected to diminish gradually without abrupt changes and to give an exponential survival curve for a synchronously replicating population. Multiplicity reactivation also allows definite predictions concerning the last two features; the onset of stabilization should coincide with that of DNA replication, and the rate of increase in radiation resistance should reflect the rate of replication of the parental genome. The experiments reported were made to test these consequences of multiplicity reactivation.

Before discussing experimental results it is necessary to consider their limitations. Measures have been made, at different times after infection, of the transfer of the $^32P$ label from the density gradient band of 'heavy' parental DNA to the band of hybrid DNA. The complete phage genome will clearly not become hybrid until one cycle of DNA replication is complete. In order to follow the course of this first cycle of replication the intracellular DNA must be fragmented; the smaller the fragments, the nearer will the appearance of hybrid DNA herald the initiation of DNA synthesis. In these experiments the DNA was fragmented by rapid passage through a hypodermic needle. This method was found to break T-even phage DNA into fragments of about $10 \times 10^8$ daltons; a molecular weight
equivalent to about 10% of the intact molecule. Thus no $^{32}$P label will be observed in a hybrid band until a continuous region of about 10% of the phage genome has been replicated and the technique must underestimate by about 10% the amount of parental label in hybrid DNA.

The experiment of Werner (1968) strongly suggests that DNA replication is initiated at only one site/T4 DNA molecule. This result, taken with the limitation discussed above, indicates that replication of only 10% of each parental DNA molecule can be detected.

The experimental results can now be considered in terms of the two predictions of the multiplicity reactivation theory concerning the correlation between the onset of stabilization and DNA replication, and the relative rates at which stabilization and replication occur.

The first indication of radiation stability is at 8 min. (Fig. 2 and Table 2, Col. 2) after infection and 1 min. before the first appearance of hybrid DNA. However, bearing in mind the possible underestimate of 10% in the transfer values, the agreement between the times at which stabilization and replication begin are extremely close.

The results relevant to the second prediction are in Table 2, Col. 2, 3 and 5: by 9 min. after infection, for example, the slope of the monocomplex survival curve has decreased by a factor of three (Col. 2). In terms of the multiplicity reactivation theory this implies that 67% of the phage genome has replicated by this time (Col. 3). However, at 9 min. the fraction of $^{32}$P label transferred to the hybrid band from viable parents is 10 to 15% (Col. 5) or 20 to 25% after correction for underestimation: thus, at most only 25% of the phage genome could have replicated. Similar discrepancies between Col. 3 and 5 are apparent at other times. The predictions of the multiplicity reactivation theory are clearly not borne out in this test.

Recently Fisher & Pardee (1968) examined the radiation stability of a T4 amber mutant whose DNA synthesis was delayed for several minutes when infecting a non-permissive host: stabilization still commenced at the normal time and they concluded that radiation stability was probably not related to DNA replication. However their method for measuring DNA synthesis, may have failed to detect some replication taking place at the usual time.

The evidence presented in this paper, together with the results of Pratt et al. (1961) and of Fisher & Pardee (1968) point to the conclusion that the multiplicity reactivation theory of radiation stability in the form in which it has been propounded in this paper cannot explain all the experimental results. Either multiplicity reactivation has little to do with radiation stability, and the normally close agreement between the first appearance of stabilization and DNA replication is fortuitous, or the theory is basically correct but there is some implicit assumption in the present formulation which leads to incorrect predictions. One assumption, which is rarely stated but underlies the application of the theory, is that radiation damage in any part of the phage genome is equally likely to lead to inactivation. If damage in certain genes, for example those controlling early enzyme synthesis, was more likely to cause inactivation than damage elsewhere (Ginoza, 1967), then the predictions of the multiplicity reactivation theory would change.

REFERENCES


U.v. stabilization and DNA replication in T4


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