The Effects of Ultraviolet Irradiation on Mycobacteriophages and their Infectious DNAs

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SUMMARY

Mycobacteriophages D4, D29, D29A and D32, their isolated DNAs and their host, Mycobacterium smegmatis, ATCC 607, are highly resistant to u.v. irradiation. Infective DNA appeared more resistant than intact phage but this difference was in part due to shielding by the greater u.v. absorbency of the DNA solutions. It was, however, also due to the occurrence of non-reversible u.v. damage to phage protein. D29, D29A and D32 and their DNAs showed two-component dose-survival curves, D4 and its DNA were inactivated exponentially, while M. smegmatis showed a non-linear semilog curve with a distinct shoulder in the low-dose region. M. smegmatis possesses a mechanism for reversing u.v. damage to the bacterial genome and to the genome of infecting phage by photoreactivation. Specific dark-repair mechanisms were not identified, except in the case of a significant level of acriflavin-sensitive host-cell reactivation demonstrated for D29. The observed enhancement of survival of heavily irradiated D29 and D4 by pretreatment of the assay bacteria with iodoacetate was interpreted as evidence that a delay of growth and division of the host, and/or a delay of phage replication, contributed indirectly to a dark-repair mechanism.

INTRODUCTION

Numerous investigators have employed bacteriophages to study the biological effects of ultraviolet (u.v.) irradiation and photoreactivation. The effects of irradiation on infectious DNA isolated from intact phage have also been studied (Sinsheimer et al. 1962; Harm & Rupert, 1963; Okubo & Romig, 1965) and the direct effects of u.v. irradiation or photoreactivation, as indicated by loss or recovery of plaque-forming ability, have been observed. Four different transfecting DNAs, together with the parent mycobacteriophages D4, D29, D29A, and D32 from which they were isolated, could be investigated in a single host, Mycobacterium smegmatis, ATCC 607 (Tokunaga & Sellers, 1964; Sellers & Tokunaga, 1966). Thus, any observed differences in u.v. sensitivity or photoreactivation could be attributed to the infectious agent, as opposed to the host cell.

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METHODS

Mycobacteriophages. D4, D29, D29A, and D32 were obtained originally from Dr Seymour Froman (Froman, Will & Bogen, 1954). D29A is a spontaneously occurring host-range mutant of D29 which has acquired a new antigen(s) as shown by cross-neutralization techniques and is more heat-labile than wild-type D29 (Mizuguchi & Sellers, 1970). Methods of propagation and assay were as previously published (Sellers & Runnals, 1961; Sellers & Tokunaga, 1966).

Bacteria. Mycobacterium smegmatis ATCC607, and Mycobacterium sp ATCC 9033 were obtained from Dr Froman's collection. Maintenance and routine propagation of these bacteria were described by Sellers & Runnals (1961). Micrococcus lysodeikticus ATCC 4698 and Escherichia coli B were used as reference strains in some experiments.

Transfecting DNA. Preparation of high-titre phage lysates, concentration of phage and extraction of transfecting DNA by water-saturated phenol were performed by methods previously described (Tokunaga & Sellers, 1964; Sellers & Tokunaga, 1966) with the following exceptions: (1) three successive extractions by phenol were required to release the DNA of phage D29A; and (2) the buffer was 0·01 M tris + HCl pH 7, plus 0·15 M NaCl and 0·002 M CaCl₂. When assayed on cultures containing a relatively high proportion of competent bacteria our best preparations of D4, D29 and D32 DNA produced between 100 and 150 plaques/µg, while the best preparations of D29A DNA produced only 20 plaques/µg of DNA.

U.v. irradiation. The source of u.v. was a General Electric germicidal lamp connected to a voltage stabilizer (output, 115 v), emitting light predominantly at 253·7 and having an intensity of 80 ergs/mm²/sec. at a distance of 20 cm. (as measured directly with a YSI model 65, YSI-Kettering Radiometer, manufactured by Yellow Springs Instrument Company, Yellow Springs, Ohio). Routinely, suspensions of DNA (10 /µg/ml.) in tris buffer in open Petri dishes were placed on an oscillating platform at a distance of 20 cm. from the u.v. source, and were stirred gently during irradiation. At intervals, 0·1 to 1·0 ml. samples were withdrawn and assayed for plaque-forming activity. The total DNA sample was mixed with an excess of competent bacteria and plated directly in soft agar. Intact phage was added to a log-phase culture of bacteria (m.o.i. = 0·1), allowed to adsorb for 15 min. at 37° and then appropriately diluted and plated. To compare whole phage and its DNA, phage was mixed with an excess of log-phase bacteria and plated directly; the u.v. sensitivity of phage and its photoreactivability were similar regardless of which assay method was used. As a precaution against photoreactivation, all operations were performed in a dark room illuminated with a dim yellow light. Bacteria were washed and resuspended (10⁸ cells/ml.) in tris containing Tween 80 (0·06 %). After irradiation samples were plated either in soft agar or spread over the surface of agar plates and incubated at 37° for 5 days. Plaque (or colony) counts represented the mean of three or four plates per radiation dose and the results were expressed as a percentage survival of the unirradiated population.

Photoreactivation. Radiant energy was supplied by lamps containing two 15-watt 'cool white' fluorescent tubes with less than 2 % emission of wavelengths shorter than 380 nm. (General Electric F15T8-CW). A series of lamps was set up for use in a walk-in incubator. Duplicate sets of samples for each irradiation dose, plated in covered pyrex Petri dishes, were illuminated at a distance of 20 cm. from the light source. One hr of illumination at 37°, starting immediately after plating, resulted in maximum photoreactivation. The dark control samples were treated like the illuminated samples, except that the plates were wrapped in
opaque aluminium foil. Plaque (or colony) counts represented the mean of three or four plates per radiation dose in both the illuminated and the dark control series and were expressed as the percentage survival of the unirradiated samples or as the photoreactivable sector as described in the results.

**Production of competent bacteria for assay of DNA activity.** Middlebrooks 7H9 broth (Difco) consisted of (NH₄)₂SO₄, 0.5 g.; glutamic acid, 0.5 g.; NaHPO₄, 2·5 g.; KH₂PO₄, 1·0 g.; ferric ammonium citrate, 0.4 g.; MgSO₄, 0·05 g.; CaCl₂, 0·0005 g.; ZnSO₄, 0·001 g.; CuSO₄, 0·001 g.; glycerol, 2 ml.; and H₂O, 900 ml. with Tween 80 added to a final concentration of 0·06 %.

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Bacteria propagated in 7H9 broth were diluted in 30 ml. of fresh medium in a 300 ml. Klett side-arm flask to give a concentration of 1 to 5 × 10⁷ cells/ml. The flasks were placed on a horizontal shaker and shaken at 40 excursions/min. at 37®, and at intervals the extinction at 540 nm. was determined in a Klett colorimeter. At the same time, 1 ml. samples were withdrawn and exposed to an excess (0·1 ml. containing 20 to 30 μg./ml.) of DNA preparation and assayed for plaques. In preliminary experiments, additional samples of the cultures were withdrawn for viable bacterial counts on heart infusion agar, and total bacteria were counted in a Petroff Hauser counting chamber. The number of competent cells was expressed as the number of infectious centres per total number of bacteria. Although grown in apparently the same way, batches of cells often varied widely in competence; in general, cultures within the E₅₄₀ range from 2·5 to 3·5 were usually the most satisfactory. Because of the variability, cultures were removed from the shaker when they reached a desired E₅₄₀ and frozen immediately at −30°. If test samples proved satisfactorily competent, the culture was stored at −30° for up to 2 weeks for use in subsequent experiments.

**Cellular DNA.** Calf thymus DNA was purchased from Worthington Biochemical Co., Freehold, New Jersey, *Escherichia coli* DNA was prepared according to the method of Marmur (1961) and *Mycobacterium smegmatis* DNA was prepared by the method previously published (Sellers & Tokunaga, 1966).

**Preirradiation chemical treatment of Mycobacterium smegmatis.** The method was essentially that described by Elder & Beers (1965). Bacteria grown in heart infusion broth with aeration to a density of 1 to 5 × 10⁸ cells/ml. were centrifuged, washed twice in tris containing Tween 80 (0·06 %), and brought back to one-half the original volume in the same buffer. Iodoacetate (Fisher Scientific Co., Pittsburg, Pa.) was added to a final concentration of 0·1 mM to one sample and 0·2 mM to another, while a third remained as a control. Incubation was continued, and at various intervals samples were withdrawn and used for assay of u.v.-irradiated phage.

**RESULTS**

**Competent bacteria.** Competence increased in parallel with the increase in growth until the mid-log phase (E₅₄₀ = 0·4 or approximately 5 × 10⁸ colony formers/ml.) (Fig. 1). At this point, the numbers of transfectants decreased somewhat but then increased again until the stationary phase of growth was reached (E₅₄₀ = 0·6 or approximately 10⁹ colony formers/ml.), when they decreased precipitously. Thus, only a small proportion of the culture was competent at any one time, suggesting that the long duration of competence in the culture as a whole was due to a continuous turnover of the population. Similar findings have been reported for pneumococci where the competent state is relatively independent of growth rate or phase of growth of the culture (Tomasz & Hotchkiss, 1964). Efforts to prepare competent mycobacteria in more complex media were only partially successful; even the
addition of 7H9 enrichment to the chemically defined 7H9 broth proved deleterious. In the most competent culture, only 1 or 2 cells in $10^6$ bacteria were competent. However, cultures containing 500 to 1500 competent cells/ml were quite adequate for the present experiments.

**U.v. inactivation and photoreactivation of phage and isolated DNA.** Both D29 and D29A as well as their isolated DNAs have a two-component survival curve with about 50% of the phage and 80% of the DNA being inactivated at a slower rate than the remainder of the population. D32 phage and its infectious DNA showed a two-component survival curve with about 40% of the infectivity of intact phage, 60% of the DNA infectivity being lost at a slower rate. D4 phage and D4 DNA were inactivated exponentially (Fig. 2). The sensitivity of the different phages and of an intact phage and its isolated DNA were compared (Table 1). The sensitivity of each infectious agent was taken as the u.v. dose ($D_o$) in ergs/mm.$^2$ required to reduce survival on the exponential part of the curve to 37%, for example, the dose required to reduce the surviving fraction from $10^{-1}$ to $0.37 \times 10^{-1}$ plated in the dark ($D_{od}$); the second component of the two-component curves was arbitrarily chosen for measurement of this sensitivity. Analogous measurements were made of the u.v. sensitivities after maximal photoreactivation ($D_{op}$), and the photoreactivable sectors (Dulbecco, 1950) calculated from the resultant values; the mean of four experiments was recorded (individual values did not fluctuate by more than 10%). The isolated DNAs were from 1.3 to
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2.4 times more u.v.-resistant than the corresponding intact phage. With the exception of D32, intact phage was more efficiently photoreactivated than DNA; this could reflect a difference in the physiological state of the host cell, competent bacteria being somewhat deficient in photoreactivating enzyme (Spizizen, Reilly & Evans, 1966). The difference in sensitivity between phages was less than twofold. There was a small but consistent difference between D29 and its host-range mutant, D29A; even the isolated DNAs show some difference with 1.23 times more irradiation being required to inactivate the same fraction of D29 as of D29A DNA (Table 1). The average Dod of 1285 ergs of the mycobacteriophages was 32 times greater than the Dod of 40 ergs found for T2, used as a reference and irradiated under the same conditions (Fig. 5).

Table 1. Mean lethal dose Do*

<table>
<thead>
<tr>
<th></th>
<th>D29</th>
<th>D29A</th>
<th>D32</th>
<th>D4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Phage DNA</td>
<td>Phage DNA</td>
<td>Phage DNA</td>
<td>Phage DNA</td>
</tr>
<tr>
<td>Dod</td>
<td>1400</td>
<td>2160</td>
<td>1160</td>
<td>1840</td>
</tr>
<tr>
<td>Dopr</td>
<td>2000</td>
<td>2720</td>
<td>1600</td>
<td>2240</td>
</tr>
<tr>
<td>PR sector†</td>
<td>0.3</td>
<td>0.21</td>
<td>0.28</td>
<td>0.18</td>
</tr>
</tbody>
</table>

* Values are stated as ergs/mm² per hit. Dod refers to plating in the dark and Dopr to plating under conditions of maximal photoreactivation.
† The photoreactivated sector is an expression for the maximum fraction of lethal u.v. lesions that can be photoreactivated.
‡ Irradiated after infection.

Effect of extinction of the suspending medium on survival of intact phage. Relatively high concentrations of DNA were irradiated to provide sufficient numbers of surviving plaques for reliable assay counts. The u.v. extinction of these solutions may have provided a measure of protection against u.v. damage so that the apparently greater u.v. sensitivity of intact phage could have been due to the relatively greater transparency of the phage suspensions. To test this possibility, phage suspensions were irradiated in tris containing various concentrations of DNA (Fig. 3). The slope of the survival curve was 0.75 for phage only, 0.88 in the presence of 1 µg. DNA and 0.91 when the DNA concentration was increased to 10 µg./ml. Thus, addition of the higher concentration of DNA resulted in a significant change in slope. However, the difference in extinction of the solutions accounted for much less than half of the difference between the u.v. sensitivity of intact phage and the isolated DNA.

Mode of infection as a factor in intact phage survival. The greatest difference between the u.v. sensitivity of phage and its isolated DNA was found for D32, and photoreactivation of D32 DNA was 3.8 times greater than that of intact phage (Table 1). Experiments were conducted to determine the influence of e.o.p. on these results.

The propagating host for D32 was Mycobacterium sp. ATCC 9033, on which plating efficiency is two to three times greater than on Mycobacterium smegmatis, with clear instead of hazy plaques. U.v. inactivation and photoreactivation of D32, substituting the Mycobacterium sp. for Mycobacterium smegmatis as host, did not alter the results. Apparently, the differences between phage and DNA sensitivity could not be lessened, nor could photoreactivation of the irradiation damage be facilitated, by providing an environment in which D32 can adsorb and penetrate the host more efficiently (Sellers & Tokunaga, 1966).

Further support for the proposition that the greater u.v. sensitivity of intact phage was indirectly related to the mode of infection was provided by the second photoreactivable
sector recorded in Table 1 for D32; phage was irradiated after infection and then photoreactivated. The fraction of the irradiated population which was reactivated under these conditions increased from 0.05 (phage irradiated before infection) to 0.12. One interpretation of these results is that under these conditions, more phage genomes reached the internal environment of the host and thus, after u.v. irradiation, were accessible to the action of photoreactivating enzyme.

The effect of u.v. irradiation on DNA release from intact D32 phage. Irradiation of high concentrations (10^13/ml.) of D32 caused the release of DNA into the suspending buffer; after irradiation with increasing doses of u.v. and digestion with DNase, followed by high-speed centrifugation, the E_max of the supernatant increased significantly. Measuring the extinction of the irradiated samples against the non-irradiated control sample set at zero,
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E = 0.06 was found after 180 sec. u.v., which increased to 0.57 after three times this dose. This suggested that u.v. irradiation of D32 produced a lesion similar to that in T4D phage protein which causes DNA release (Winkler, Johns & Kellenberger, 1962). Similar treatment of suspensions of D4 and D29 phages yielded results of questionable significance.

![Graph showing the effect of u.v. dose on percent survival of phage D29](image)

**Fig. 3.** The effect of optical density of the phage suspension, contributed by the addition of 1 µg and of 10 µg DNA/ml of suspension, on the u.v. inactivation of D29. ○, DNA only, 10 µg/ml; O, DNA only, 1 µg/ml.; ▲, DNA + DNA, 10 µg/ml.; △, DNA + DNA, 1 µg/ml.; ■, DNA only.

**Multiplicity reactivation as a factor in DNA survival.** The infectivity of mycobacteriophage DNA in proportion to input DNA concentration was determined. As illustrated in Fig. 4 for D29 (competent cells in the assay culture have become saturated as the DNA concentration reaches 10 µg/ml., causing the curve to level off at this point), all the phage DNAs employed in the present investigation yielded titres of infectious centres strictly proportional to DNA concentration. Furthermore, irradiation of DNA from two temperature-sensitive mutants of D29, ts-5 × ts-8, failed to enhance the yield of wild-type progeny resulting from genetic recombination or functional cooperation among fragments of phage genomes (Mizuguchi & Sellers, 1970). These results do not support an assumption that a multiplicity reactivating mechanism, similar to that found for SPhO1 subtilis phage DNA (Okubo, Stodolsky & Strauss, 1964), accounted for the relatively higher survival of irradiated phage DNA.

**U.v. inactivation and photoreactivation of host cells.** U.v. sensitivity, based solely on survival, does not distinguish between inactivation and inactivation and repair. While the u.v. sensitivity of Mycobacterium smegmatis was investigated, the bacteria were thus examined at the same time for the presence of host-cell repair processes. Survival after u.v.
irradiation of *M. smegmatis* is shown in Fig. 5. For purposes of reference, *Micrococcus lysodeikticus* and *Escherichia coli* B (Haynes, 1964; 1966) were irradiated under the same conditions. The survival curve for *M. smegmatis* showed a distinct shoulder in the low-dose region and, although not shown, the linear portion of the curve extended through the fifth decade of survival and then tended to bend upward; the D20 was 200. The survival curves for *E. coli* and *M. lysodeikticus* agree relatively well with the data published by Haynes.

There was a modest increase in survival in the irradiated population of *Mycobacterium smegmatis* exposed to photoreactivating light (Fig. 5); the photoreactivable sector is 0.13; we therefore assume that *M. smegmatis* possessed photoreactivating enzyme which is able to repair u.v. damage not only in phage DNA but in its own genome as well.

**Fig. 4.** The development of infectious centres as a function of D29 DNA concentration. •, actual yield; --- ---, theoretical 1:1 yield.

**Fig. 5.** U.v. inactivation and photoreactivation (PR) of *Mycobacterium smegmatis* and reference survival curves for *Micrococcus lysodeikticus*, *Escherichia coli* B and coliphage T2. ○, *M. smegmatis* + PR; ●, *M. smegmatis*; ▲, *M. lysodeikticus*; ■, *E. coli* B; □, T2.

**Acriflavin-sensitive dark repair of u.v. damage.** The u.v.-induced lag observed in the appearance of either colonies or plaques among irradiated populations has been interpreted as the time required to complete dark-repair mechanisms before successful replication can take place (Witkin, 1947; Rupert & Harm 1966). The addition of acriflavin to the assay medium prevents dark repair of bacteria (Witkin, 1963) and of phage (Feiner & Hill, 1963); this acriflavin-sensitive repair has been designated host-cell reactivation (hcr) and is a direct enzymic repair mechanism (Sauerbier, 1962a). When u.v.-irradiated D4 was assayed in the dark in the presence of up to 5 µg. acriflavin/ml. of assay agar (the threshold of toxicity for the host cells), there was no significant difference between the numbers of survivors and the controls without acriflavin; however, acriflavin affected the number of survivors of irradiated D29 and D32 (Fig. 6). A concentration of 2 µg./ml. was not inhibitory for unirradiated D29, but 1 µg./ml. just inhibited D32. The effect of acriflavin indicated that hcr accounted for the survival of a measurable fraction (F) of the u.v.-damaged phage. The efficiency of
repair may be defined as the ratio of the number of hits repaired ($D_2$) to the number produced by irradiation ($D_1$) which gives reactivable sectors of 0.3 and 0.1 for D29 and D32, respectively (Harm, 1963a). The surviving fraction of the irradiated D29 population was constant over the dose range up to 4000 ergs/mm²; apparently her functioned relatively efficiently at this dose range, but less so at higher doses. The curve shows that the fraction of lethal u.v. lesions in D29 which is subject to her was not independent of dose; apparently, the chances for a lesion being repaired become smaller as the number of additional lesions in the genome increases. This result can be explained if we postulate that the number of her-enzyme molecules per cell is very small, and that when they become exhausted, her no longer functions (Harm, 1963b).

![Graph showing survival and repair of D29 and D32 phages](image)

**Fig. 6.** The effect of acriflavin on the survival of D29 and D32 phages after exposure to increasing doses of u.v. irradiation. ○, control; ● + acriflavin.

Other mechanisms of dark repair. When certain phages are lightly irradiated, for example, T1 or Hemophilus phage 1, survival is increased by plating on irradiated host cells. This is not a direct repair process but an enhancement of her due to inhibition of antagonistic processes (Weigle, 1953; Harm, 1963b; Harm & Rupert, 1963). To investigate a possible association of UVR with her in D29, *Mycobacterium smegmatis* was exposed to various doses of u.v. irradiation and used to assay D29 which had been exposed to u.v. for 60 sec. There was no evidence of enhancement by the lightly irradiated bacteria; the titres were comparable on cells receiving up to 10 sec. of u.v., at which dose the numbers of infectious centres began to decline (Fig. 7). Experiments made with *M. smegmatis* failed to reveal either her or UVR of irradiated bacteria (Rupert & Harm, 1966). The addition of up to 5 μg. of acriflavin/ml of plating agar did not decrease the colony-forming ability of irradiated bacteria, nor did the addition of irradiated DNA to the same population enhance colony-forming activity.

Effect of u.v.-irradiated DNA on transfection. Transfection of *Bacillus subtilis* can be enhanced by exposing the bacteria to u.v.-irradiated cellular DNA (Epstein & Mahler, 1968). In analogous experiments, pre-exposure of competent *Mycobacterium smegmatis* to irradiated
Fig. 7. Survival of D29 phage after irradiation for 60 sec. as a function of u.v. dose applied to the assay bacteria. ●—●, bacteria irradiated with the indicated dose of u.v.; ○—○, unirradiated bacteria.

Table 2. The effect of pre-exposure of competent Mycobacterium smegmatis to u.v.-irradiated* cellular DNA on transfection by D29 DNA

<table>
<thead>
<tr>
<th>DNA species</th>
<th>Transfection in liquid culture</th>
<th>Transfection in soft agar</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. plaques per ml.</td>
<td>Relative no. plaques</td>
</tr>
<tr>
<td>None</td>
<td>85</td>
<td>1.0</td>
</tr>
<tr>
<td>Calf thymus</td>
<td>80</td>
<td>0.94</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>57</td>
<td>0.67</td>
</tr>
<tr>
<td><em>M. smegmatis</em></td>
<td>56</td>
<td>0.66</td>
</tr>
</tbody>
</table>

* 2 × 10^4 ergs/mm^2.

Table 3. Survival of u.v.-irradiated D29 assayed on iodoacetate-treated and non-treated Mycobacterium smegmatis

<table>
<thead>
<tr>
<th>U.v. dose (sec.)</th>
<th>Control (tris only)</th>
<th>1 mM/ml.</th>
<th>2 mM/ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>90 min.</td>
<td>40 min.</td>
</tr>
<tr>
<td>0</td>
<td>7.3 × 10^4</td>
<td>7.7 × 10^4</td>
<td>8.4 × 10^4</td>
</tr>
<tr>
<td>30</td>
<td>5.9 × 10^4</td>
<td>6.5 × 10^4</td>
<td>9.4 × 10^4</td>
</tr>
<tr>
<td>60</td>
<td>8.6 × 10^4</td>
<td>9.1 × 10^4</td>
<td>1.7 × 10^5</td>
</tr>
<tr>
<td>120</td>
<td>1.9 × 10^5</td>
<td>2.0 × 10^5</td>
<td>3.4 × 10^5</td>
</tr>
</tbody>
</table>

Titres represent plaques formed by u.v.-irradiated phage plated on host cells preincubated for the various time periods indicated, either in tris buffer or in buffer containing iodoacetate.
DNA from a variety of sources failed to enhance transfection by D29 DNA. In fact, both Escherichia and M. smegmatis DNAs reduced the relative level of transfection by 0.3 (Table 2). Competent M. smegmatis was pre-exposed to the irradiated DNA (15 μg./ml.) for 50 min., exposed to D29 DNA (10 μg./ml.) and divided into two portions; one was plated directly in soft agar and the other placed on a horizontal shaker, shaken very gently at 37° for 90 min. and then plated. Similar attempts to enhance D4 transfection were also unsuccessful.

Enhancement of survival of u.v.-irradiated phage by iodoacetate treatment of host cells. Preincubation of Mycobacterium smegmatis at 37° in tris containing 1 mM-iodoacetate/ml. for 140 min. before infection gave about a twofold increase in survival of phage irradiated for 30 sec. and a three- to fourfold increase with irradiation for 60 and 120 sec. (Table 3). A similar effect on the survival of irradiated D4 phage was produced by iodoacetate treatment of M. smegmatis but experiments with D32 showed only a minimal degree of drug-induced enhancement. Because of the extreme tendency of M. smegmatis to clump when incubated in the tris + iodoacetate medium, it was impossible to measure the true u.v. inactivation rate of drug-treated bacteria.

DISCUSSION

One of the variables which affect the types of photoproducts formed in the DNA of micro-organisms by u.v. irradiation is the base composition of the DNA. Haynes (1964) found a correlation between A-T content and u.v. sensitivity among a variety of bacteria, the greatest sensitivity being found in DNA containing the highest proportion of A-T. The importance of thymine dimers among the various u.v. photoproducts and the role they play in the relative u.v. sensitivity of various DNAs has been established (Beukers, Ilijkstra & Berends, 1959; Setlow & Setlow, 1963). Conversely, X-ray sensitivity may be correlated with a higher G-C content of DNA (Kaplan & Zavarine, 1962; Haynes, 1964). In this connection, Mycobacterium smegmatis DNA contains 32 % A-T and 68 % G-C (identical to Micrococcus lysodeikticus; Schildkraut, Marmur & Doty, 1962). The A-T content of D4 and D29 is 36 % with a G-C content of 64 %; the base ratio of D32 is identical to that of M. smegmatis (Sellers & Tokunaga, 1966). Thus, on the basis of the base composition of the DNA alone, the relative u.v. resistance of these agents might have been predicted. It is interesting to note that among a variety of phages examined, Bertani (1959) found D32 to be the most sensitive to ionizing radiation.

Undoubtedly, the presence and efficiency of repair mechanisms, whether direct or indirect, are very important in determining the u.v. sensitivity of both mycobacteria and phage, and the final outcome or loss in biological activity is highly conditional on the balance achieved between injurious consequences of damage and their reversal. Post-irradiation exposure of Mycobacterium smegmatis to visible light increased the numbers of surviving colonies, or plaques in the case of phage-infected bacteria, indicating a functional photoreactivating enzyme. In this respect mycobacteria are unique; bacteria of other species which can be rendered competent appear to be deficient in this repair mechanism (Spizizen et al. 1966). The activity of host-cell reactivating enzyme(s) which functions in the dark is considerable for D29, D29A and D32 phages; calculated in the same way as for phages T1, T3 and λ (Feiner & Hill, 1963; Harm & Rupert, 1963b), between 40 to 50 % of the survivors have been repaired. Since less than half of the repairable lesions were subject to dark repair by the hcr acriflavin-sensitive mechanism, other types of dark-repair mechanisms as yet unknown must be operative in mycobacteria. The fact that acriflavin did not affect the recovery of u.v.-irradiated M. smegmatis might indicate that the amount of hcr repair enzymes in the
Reactivating mechanisms other than photoreactivation and hcr which convert potentially lethal u.v. damage into non-lethal events by direct enzymic repair processes, have been described. These include photoprotection, liquid-holding recovery and other phenomena in which an alteration in the physiological state of the host cell indirectly facilitates repair of u.v. damage (Rupert & Harm, 1966). Elder & Beers (1965) reported that the high resistance of \textit{Micrococcus lysodeikticus} to u.v. irradiation was the result of a very efficient dark-cell repair mechanism that could be blocked by iodoacetate or dinitrophenol. The chemicals also inhibited repair of u.v.-damaged \textit{M. lysodeikticus} phage. Analogous experiments made with the mycobacteriophage system showed that dinitrophenol, up to a concentration of 10 \(\mu g./ml\) was ineffective; survival rates of either irradiated host or phage were unchanged by the action of this inhibitor; moreover, iodoacetate treatment of the bacteria caused a significant enhancement in the survival of u.v.-irradiated phage. This latter phenomenon can most reasonably be interpreted as a drug-induced division or growth delay of the host which in turn provides time for repair mechanisms to operate (Sauerbier, 1962b; Rupert & Harm, 1966). Among its other effects, iodoacetate inhibits both RNA and protein synthesis in bacteria and it may be that one or both of these effects serve to lock the replicative cycle of the genome of \textit{Mycobacterium smegmatis} in a stage analogous to that described for starved \textit{Escherichia coli} (Sauerbier, 1962b). The effect of iodoacetate was more pronounced at the higher irradiation doses. Perhaps in the low dose range, sufficient enzyme is available to complete repair but, as the dose is increased, an increasing deficit of enzyme develops, and under these conditions enhancement by iodoacetate becomes more demonstrable. An alternative explanation is that inhibition of RNA and protein synthesis by iodoacetate makes a small critical class of lesions, predominantly present in the heavily irradiated phage genome, more available to repair enzymes. Although not compelling, the data recorded for 40 min. pre-exposure of \textit{M. smegmatis} to 2 mM/ml. perhaps give some degree of support for this hypothesis; the e.o.p. of unirradiated or zero-time samples of phage is reduced by about one-half when drug-treated bacteria are employed as the assay host. At the 60 sec. dose, the number of infectious centres in the test samples is about one-third the number found in the comparable control assay, but in the population of phage which has received 120 sec. of u.v. irradiation, iodoacetate treatment of the host suddenly elicits a fourfold increase in the numbers of infectious centres which develop.

A phenomenon related to UVR has been described, in which transfection of \textit{Bacillus subtilis} can be enhanced by the exposure of competent cells to u.v.-irradiated homologous or heterologous cellular DNA. The most probable explanation of these results is that the irradiated DNA binds cellular nucleases which would otherwise inactivate the incoming transfecting DNA (Epstein & Mahler, 1968). Pre-exposure of competent \textit{Mycobacterium smegmatis} to irradiated DNA from a variety of sources not only failed to enhance transfection but reduced the relative level of transfection. The latter is probably due to non-transfecting DNA competing for uptake and/or replicative sites, thereby decreasing the probability for transfection of the cell to occur (Spizizen \textit{et al.} 1966). Apparently, transfection in mycobacteria is not modified by the same environmental factors that modify nuclease activity in \textit{Bacillus subtilis}. Enhanced recovery of irradiated phage by UVR was also not demonstrated in the mycobacterial system. 

\textit{spo2} phage, when assayed on a wild-type sensitive strain of \textit{Bacillus subtilis}, was several times more resistant to u.v. inactivation than its isolated infective DNA, irradiated at a concentration of 10 \(\mu g./ml\). However, when assayed on an \textit{hcr} \textsuperscript{−} mutant, the u.v. sensitivity
of the phage was about 0.7 that of the isolated DNA (Okubo & Romig, 1965); approximately
the same difference between phage and DNA was found for the u.v. sensitivity of the \textit{Haemophilus} phage HPI (Harm & Rupert, 1963). The infective DNA of mycobacteriophages was
from 1.3 to 2.4 times more resistant to inactivation than the intact phages. Some of this
difference can be attributed to the shielding effects provided by the relatively greater u.v.
extinction of the DNA solutions at the time u.v. is applied; however, more than half of the
difference appears to be real. A comparison between the u.v. sensitivity of a given phage and
its isolated DNA at concentrations of DNA more nearly comparable to the concentration
of DNA in the intact particle was not feasible. For example, D29 contains roughly \(1.6 \times 10^{-16}\) g. of DNA/p.f.u. (Sellers & Tokunaga, 1966) and testing the u.v. sensitivity in this
range of concentration was not possible because of limitations imposed by the level of
competency in the assay culture. Moreover, a family of curves obtained from irradiation of
relatively low concentrations of DNA with extrapolation to higher concentrations yielded
questionable results. As a first approximation, one might expect that DNA packed inside
the phage head would be more resistant to irradiation than when randomly distributed in
aqueous solution; however, our results did not support this supposition. One explanation
for the data is that, in addition to producing lesions in DNA, u.v. irradiation causes func-
tional lesions in phage protein which are not subject to repair. Winkler, Johns & Kellenberger
(1962) found that u.v. irradiation of T4D coliphage at 235 nm. produced two distinct lesions
in the phage protein: (1) a rupture of the head membrane causing DNA release, and (2) tail
lesions accompanied by tail sheath contraction. Light of 265 nm. wavelength did not produce
these effects, and in the range 245 to 265 nm. protein damage was negligible. The lamp
employed for the mycobacteriophages emitted u.v. of 253.7 nm. predominantly; however,
the beam was not filtered and it is possible that a small fraction of the incident radiation
occurred at a shorter wavelength which specifically damaged phage protein. Alternatively,
it was quite possible that even at 253.7 nm. irradiation, damage to mycobacteriophage
protein occurred, since absorption for protein is large at this wavelength. Moreover, indirect
evidence was obtained that u.v. irradiation of D32 produced lesions similar to those in
T4D which caused DNA release.

It has become increasingly clear that a great variety of factors determines the loss or
survival of biological activity following u.v. irradiation. The importance of gene-controlled
mechanisms such as the \(v\)-gene in T4 (Harm, 1963a) and the \(uvr\)-gene in \textit{Escherichia coli}
K12 (Clark & Margulies, 1965) has been documented. In the case of the mycobacterial
system, information concerning such control mechanisms is totally lacking. The relatively
small but consistent differences noted among the different phages probably reflect differ-
ences among phage-dependent control mechanisms, since one and the same host was used.
That the host mechanisms also play a role is indicated by the finding that survival of
irradiated phage could be increased appreciably by treatment of the assay host with
iodoacetate.

Many of the phenomena concerned with u.v. damage and its reversal in other micro-
organisms are operative in the mycobacterial system, while certain other mechanisms appear
to be absent. A considered advantage of the mycobacterial system is the high content of
G–C in the transfecting DNA; theoretically, this should facilitate the isolation and identifi-
cation of the molecular lesion(s), rendering this DNA species so sensitive to ionizing radia-
tion (Bertani, 1959; M. I. Sellers, unpublished results). Efforts are under way to isolate
mutants of \textit{Mycobacterium smegmatis} with diminished and with increased sensitivity to
irradiation, in order to provide means for identification and characterization of the specific
repair mechanisms for u.v. and/or ionizing radiation damage in these micro-organisms.
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REFERENCES


**U.v. effects on phage and DNA of mycobacteria**


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