QUANTITATIVE EVIDENCE FOR ENZYMATICALLY-
INDUCED DNA DOUBLE-STRAND BREAKS AS
LETHAL LESIONS IN UV IRRADIATED
pol+ AND polA1 STRAINS OF E. COLI K-12

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(Received 28 April 1975; accepted 25 August 1975)

Abstract – We have recently reported that DNA double-strand breaks arise enzymatically during the
course of excision repair in uvr+ strains of Escherichia coli K-12. Survival curves for ultraviolet (UV)
irradiated E. coli K-12 pol+ (JG139) and polA1 (JG138) strains have a pronounced shoulder region.
The regions of the survival curves at which killing approaches exponential correspond to the fluences
at which DNA double-strand breaks (assumed to be lethal events) accumulate linearly. Reducing the
number of UV photoproducts either by photoreactivation or fluence fractionation results in an increase
in survival and a decrease in the yield of DNA double-strand breaks in both strains. These data
support the hypothesis that enzymatically-induced DNA double-strand breaks may be the lesion ultima-
tely responsible for UV-induced cell killing in the pol+ strain of E. coli K-12, and perhaps also in
the polA1 strain.

INTRODUCTION

The dark repair process which removes damaged sequences in the DNA of UV irradiated organisms has
been characterized in considerable detail (for reviews see Setlow and Setlow, 1972; Howard-Flanders, 1968).
The use of mutants lacking particular repair enzymes has been a valuable tool in elucidating the pathways
of dark repair.

Most radiation sensitive mutants of E. coli lack a
pronounced shoulder on their UV survival curves
(Howard-Flanders and Boyce, 1966). Their radiation
sensitivity can be attributed to the partial or total
lack of repair of the radiation insult as a result of
the absence of critical gene products. Wild-type E.
coli strains, which are considered to contain a full
complement of repair enzymes, have a pronounced
shoulder on their UV survival curves. One interpre-
tation of an increase in the slope of the survival curve
with an increase in UV fluence (i.e. a shoulder) is
that inactivation is a two-hit process. That is, the
probability of forming a lethal event increases non-
linearly with fluence in the region of the shoulder. In
this regard Harm (1968) suggested that the progressi-
ve loss of the shoulder on the survival curve of the
repair proficient E. coli B/r may be due to a decreas-
ing repair proficiency with increasing UV fluence, pre-
sumably by interference in the repair process by over-
lapping excised regions. By utilizing very low UV fluence rates, Harm showed that the survival of E.
coli B/r could be dramatically increased; however, the
excision repair deficient strain B-14 exhibited no in-
crease in survival under these conditions.

Moss and Davies (1974) have investigated liquid
holding recovery in a rec− uvr+ strain of E. coli and
have also hypothesized that cell killing may be a con-
sequence of overlapping excised regions leading to the
production of DNA double-strand breaks.

We have reported that strains of E. coli K-12 profi-
cient in excision repair (uvr+) exhibit DNA double-
strand breaks during post irradiation incubation
(Bonura and Smith, 1975). Although these lesions ap-
peared in rec− and exp− strains, they only occurred
after supralethal UV fluences. Only in a wild-type
strain and a mutant deficient in DNA polymerase I
could DNA double-strand breaks be qualitatively
related with cell killing. We provide here quantita-
tive evidence that enzymatically-induced DNA
double-strand breaks may be of primary importance
in the killing by UV irradiation of wild-type (pol+)
cells, and possibly also in cells deficient in DNA
polymerase I (polA1).

MATERIALS AND METHODS

Bacteria and culture conditions. Two thymine requiring
strains of Escherichia coli K-12 (W3110) were used. They
were originally obtained from J. D. Gross and designated
JG138 (polA1) and JG139 (pol+). A minimal salts medium
(Ganesan and Smith, 1968) was used supplemented with
thiamine (0.5 µg/ml) and thymine (10 µg/ml) for overnight
cultures. The overnight cultures were diluted 1:50 into
fresh medium supplemented with 3H-methyl thymine (New
England Nuclear Corp.; > 12 Ci/mmol) at 200 µCi/ml and
2 µg/ml unlabelled thymine. Cultures to be used for sur-

vival curves contained only unlabelled thymine at 10
µg/ml. Cells were grown at 37°C for several generations
in exponential phase to a density of ~2 x 10⁸ cells/ml.
It was determined that 5H-methyl thymine at a concen-
tration of 200 µCi/ml did not affect survival during the
labelling period indicated.
Irradiation conditions. Cells were harvested in exponential phase by filtration onto 0.45 μm membrane filters (Millipore Corp.) and were resuspended in minimal medium at a concentration of ~2 × 10^8 cells/mL. The suspension was then placed in a glass petri dish on a rotary shaker and irradiated at room temperature with an 8-W General Electric germicidal lamp. Fluence rate at 254 nm was determined with an International Light, Inc. germicidal photometer (No. II-254). Cells were assayed for viability by diluting in 0.067 M phosphate buffer at pH 7 and plating on minimal medium plates supplemented with 10 μg/mL thymine (Difco Noble agar). Experiments were carried out under General Electric 'gold' fluorescent lamps to prevent photoactivation.

Photoactivation. An aliquot of the UV irradiated cell suspension was placed in a covered glass petri dish on a rotary shaker and exposed to two General Electric 15-W blacklight lamps. The distance between the lamp and surface of the suspension was 6 cm and the temperature of the cell suspension was ~32°C. For the UV fluences used in this study, the maximum photoreactivation usually required 40-60 min exposure to photoreactivating light.

Neutral sucrose gradients. After irradiation and incubation, 0.2 mL of the cell suspension was added to 0.3 mL of ice-cold 0.07 M Tris pH 7.16 containing lysozyme (Worthington Biochemicals) at 200 μg/mL. After 10 min on ice, 0.1 mL of the spheroplast suspension was layered on a 5-20% (w/v) neutral sucrose gradient containing 0.5% sodium dodecyl sulfate (recrystallized), 5 × 10^{-3} M Tris, 10^{-3} M sodium citrate, 10^{-2} M sodium chloride and pronase at 0.1 mg/mL. Stock solutions of 10 and 40% sucrose, 10 mM sodium citrate and 0.1 M NaCl were saturated with CHCl₃ and diluted to the final concentration described. The resulting gradient was about 70% saturated in chloroform. The chloroform was added to aid in the delipidization of the DNA. After 90 min at room temp, gradients were centrifuged for 16 h at 8000 rpm in a SW50.1 rotor in a Beckman model L2 or L265B ultracentrifuge. After centrifugation, the bottom of each tube was pierced and 0.16 mL fractions were pumped onto Whatman no. 3 MM filter paper discs which were then dried, washed in 5% trichloroacetic acid, 95% ethanol, acetone, and finally dried and counted in a Packard Triac or Nuclear Chicago liquid scintillation spectrometer. Number average mol wts were calculated directly from the sedimentation profiles using only the main peak and excluding the radioactivity near the meniscus. The DNA mol wt marker used was from 14C-thymine labeled bacteriophage T2 and was sedimented in a separate gradient with each set of E. coli DNA samples. Further details of the neutral sucrose gradient method and mol wt analysis have been described (Bonura et al., 1975).

RESULTS

UV survival. The kinetics of cell killing by UV irradiation of JG138 (polA) and JG139 (pol^-) are shown in Fig. 1. Both survival curves exhibit a pronounced shoulder; the D₅₀ values (point of extrapolation of the exponential portion of the survival curve to a surviving fraction of 10⁻⁵) were ~30 J m⁻² and ~13 J m⁻² for the pol^ and polA1 strains, respectively. The survival curves indicate that exponential killing occurs at UV fluences >60 J m⁻² for the pol^ and >20 J m⁻² for the polA1. At these fluences the survival of both strains was reduced to about 3-5 × 10⁻². By assuming that cell killing can be described as being approximately exponential between 60-120 J m⁻² in the pol^ and 20-35 J m⁻² in the polA1 strains, the UV fluences required to introduce one lethal hit in these regions of the curves (D₅₀) were calculated to be 9.7 J m⁻² and 2.5 J m⁻² for the pol^ and polA1 strains, respectively. These values are in agreement with those reported by Boyle et al. (1970) for the same strains.

Figure 1. UV survival curves for log phase E. coli K-12 JG138 (polA) (O — O) and JG139 (pol^-) (● — ●). Cells were irradiated in minimal medium and plated on minimal medium agar.

Figure 2. DNA sedimentation profiles showing the appearance of double-strand breaks as a function of incubation time in minimal medium at 37°C after UV irradiation. The pol^ cells were exposed to a UV fluence (254 nm) of 120 J m⁻²; the polA1 cells to 35 J m⁻². After incubation, cells were transformed to spheroplasts and lysed on neutral sucrose gradients as described in Materials and Methods. Brackets indicate the regions of the DNA distributions used in the calculation of M₅₀. Panel A, JG139 (pol^-); panel B, JG138 (polA).
DNA double-strand breaks. We have recently reported that DNA double-strand breaks appear as a result of excision repair in \textit{uer} \textsuperscript{+} strains of \textit{E. coli} K-12 (Bonura and Smith, 1975). Fig. 2 shows the change in the DNA sedimentation profiles as a function of time for the \textit{pol} \textsuperscript{+} and \textit{polA1} strains at UV fluences which result in approximately the same survival (~10\textsuperscript{-4}). The sedimentation profiles exhibit the most pronounced shift to lower mol wt during the first 30 min of post irradiation incubation. Thereafter, the mol wt distribution decreased slightly up to 60 min and further incubation did not result in a decrease in DNA mol wt. The \textit{polA1} strain shows a more rapid decrease in mol wt than the \textit{pol} \textsuperscript{+} strain after UV irradiation.

Quantitative aspects. The technique which we have described allows us to isolate DNA no larger than about 4.2 \times 10\textsuperscript{8} daltons. The difference between the mol wt of the intact chromosome and that which we measure in our unirradiated samples has been attributed to a shear event introducing \sim 7 double-strand breaks into the genome (Bonura \textit{et al}., 1975). Thus, in order to quantitate the fluence required to introduce a DNA double-strand break, our observations must center on those fluences which result in a sufficient number of DNA double-strand breaks to reduce the DNA to a size insensitive to shear. This unfortunately prevents us from quantitating DNA double-strand breakage on the shoulder region of the survival curves.

In an attempt to measure the rate at which DNA double-strand breaks appear as a function of UV fluence we reincubated irradiated cells at 37°C in minimal medium for a fixed period of time judged sufficient to allow for the complete expression of DNA double-strand breaks (80 min). Figures 3 and 4 show the increase in UV induced DNA double-strand breaks in the \textit{pol} \textsuperscript{+} and \textit{polA1} strains, respectively. In both figures, the dashed horizontal line intersecting the ordinate at \textit{y} = 0.36 represents the reciprocal mol wt of the intact genome. The point at which this and the extrapolated regressed line intersect is an estimate of the UV fluence at which DNA double-strand breaks begin to appear at a constant rate. These estimates are 15 J m\textsuperscript{-2} and 20 J m\textsuperscript{-2} for the \textit{polA1} and \textit{pol} \textsuperscript{+} strains, respectively, in reasonable agreement with the \textit{D}_0 values indicated above.

Fluences beyond which exponential killing occurs (~20 J m\textsuperscript{-2} in \textit{polA1} and ~60 J m\textsuperscript{-2} in \textit{pol} \textsuperscript{+}) correlate with the UV fluences which result in a linear increase in DNA double-strand breaks (~20 J m\textsuperscript{-2} in \textit{polA1} and ~50 J m\textsuperscript{-2} in \textit{pol} \textsuperscript{+}). The rate at which DNA double-strand breaks appear was calculated from the slopes of Figs. 3 and 4 to be 0.17 \pm 0.02 double-strand breaks per genome per J m\textsuperscript{-2} above 50 J m\textsuperscript{-2} in the \textit{pol} \textsuperscript{+} strain and 1.83 \pm 0.24 double-strand breaks per genome per J m\textsuperscript{-2} in the \textit{polA1} strain above 20 J m\textsuperscript{-2}. We will allude to certain difficulties with this type of quantitation in the discussion. These values probably represent upper limits to the numbers of DNA double-strand breaks present after UV irradiation.

Fluence fractionation studies. It has been theorized that UV-induced DNA double-strand breaks arise as a result of an unsuccessful attempt at excision repair of lesions occurring on opposite strands of the DNA twin helix such that an overlap of excised regions occurs (Harm, 1968; Moss and Davies, 1974). Our previous paper (Bonura and Smith, 1975) gave experimental support for this hypothesis. It is reasonable to predict that a higher survival and a decreased number of DNA double-strand breaks would be observed after fluence fractionation compared with a single acute exposure since repair processes could act.

![Figure 4](image4.png)  
**Figure 4.** Appearance of DNA double-strand breaks as a function of UV fluence (254 nm) in \textit{E. coli} K-12 \textit{polA1} cells. The reciprocal of the number average molecular weight of the DNA from UV irradiated \textit{polA1} cells after 80 min of post irradiation reincubation in minimal medium at 37°C is plotted against UV fluence. The straight line drawn through the 20–40 J m\textsuperscript{-2} data is a least squares fit.

![Figure 3](image3.png)  
**Figure 3.** Appearance of DNA double-strand breaks as a function of UV fluence (254 nm) in \textit{E. coli} K-12 \textit{pol} \textsuperscript{+} cells. The reciprocal of the number average molecular weight of the DNA from UV irradiated \textit{pol} \textsuperscript{+} cells after 80 min of post irradiation reincubation in minimal medium at 37°C is plotted against UV fluence. The straight line drawn through the 50–140 J m\textsuperscript{-2} data is a least squares fit.
Table 1. Fractionated irradiations for pol⁺ and polAI strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>First irradiation (J m⁻²)</th>
<th>Minutes of incubation between irradiations</th>
<th>Second irradiation (J m⁻²)</th>
<th>Surviving fraction</th>
<th>Calculated equivalent UV fluence (J m⁻²)</th>
<th>[1/M₄] × 10⁸ (double-strand breaks per 2.8 × 10⁹ dalton)</th>
<th>Calculated equivalent UV fluence (J m⁻²)</th>
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<tr>
<td>pol⁺</td>
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<td>9.60 × 10⁻³</td>
<td>24</td>
<td>5.91 (8.62)</td>
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</table>

*The pol⁺ and polAI strains were irradiated at room temperature with the fluences indicated and assayed for survival and DNA double-strand breaks as described in Materials and Methods. An aliquot was removed and reincubated for 20 or 40 min at 37°C before the second irradiation, after which DNA double-strand breaks and survival were determined as for the acute exposure.

†Estimated from the UV survival curve shown in Fig. 1.
‡Estimated from the regression line given in Figs. 3 and 4.

during the interval between exposures to reduce the amount of damage present at any given time. With this in mind we irradiated both strains with either a single acute exposure or two exposures separated by a repair interval and then measured survival and DNA double-strand breakage.

Table 1 shows the results of a typical fluence fractionation experiment in terms of survival and reciprocal number average mol wt (1/M₄). It is evident that both the pol⁺ and polAI strains show an increase in survival and a decrease in 1/M₄ when the UV fluence is fractionated rather than given as a single acute exposure. That is, fluence fractionation decreases the number of DNA double-strand breaks and increases the surviving fraction relative to a single acute exposure.

**Photoreactivation studies.** If the lesions which give rise to double-strand breaks are photoreactivable we should observe a decrease in double-strand breaks if UV irradiation is followed by photoreactivation. Also, the magnitude of the increased survival and decrease in DNA double-strand breaks should be similar in both strains assuming approximately equal amounts of photoreactivating enzyme exist in both strains. Table 2 summarizes the results of an experiment in which cells were photoreactivated for various times after UV irradiation. Note that both pol⁺ and polAI strains showed a much reduced yield of DNA double-strand breaks and an enhancement in survival after photoreactivation.

**DISCUSSION**

DNA double-strand breaks are produced in urr⁺ cells by enzymatic processes after UV irradiation and appear to be the consequence of an attempt at excision repair of photoproducts lying at approximately opposite positions on the twin helix (Bonura and Smith, 1975). DNA double-strand breaks resulting from ionizing irradiation have been considered to be lethal events in a number of systems (Freifelder, 1965; Kaplan, 1967; Bonura et al., 1975) and since they occur enzymatically in E. coli K-12 urr⁺ strains after UV irradiation, they could be a significant lesion in cell killing after UV irradiation as well.

The pol⁺ and polAI strains both exhibit a shoulder on the UV survival curve. The region of the shoulder extends to ~30 J m⁻² and ~13 J m⁻² in the pol⁺ and polAI strains, respectively, correlating with the approximate UV fluence required before DNA double-strand breaks appear at a constant rate (~20 J m⁻² and 15 J m⁻² for the pol⁺ and polAI strains, respectively). The UV fluences beyond which exponential killing occurs are ~60 J m⁻² in the pol⁺ and ~20 J m⁻² in the polAI strains and above these fluences DNA double-strand breaks appear to increase linearly.

Our calculations show that the D₀ fluence of 9.7 J m⁻² for the pol⁺ strain produces 1.6 ± 0.2 double-
strand breaks per genome. Since we have not observed the rejoining of DNA double-strand breaks in the pol+ strain, even after extended post-irradiation incubation (e.g. see Fig. 2), and since double-strand breaks appear to be lethal events after ionizing irradiation (Kaplan, 1967; Bonura et al., 1975) it seems likely that we may be overestimating the yield of DNA double-strand breaks in the pol+ strain to a small degree. Similarly, calculations for the polAI strain show that at the D₀ fluence there are 4.58 ± 0.6 double-strand breaks produced per genome, which would constitute a much larger overestimate for DNA double-strand breakage than for the pol+ strain. One mechanism by which such an overestimate could occur would be via DNA degradation. That is, if the DNA on either side of a strand break is degraded and not replaced, the number of breaks calculated on the basis of the size of the remaining pieces will exceed the actual number of breaks present by a factor proportional to the length of the excised gap. Because the pol+ strain is proficient in the gap filling steps of excision repair (Boyle et al., 1970; Youngs and Smith, 1973; Youngs et al., 1974), we would not expect the overestimate for DNA double-strand breakage to be very great. The polAI strain, however, degrades its DNA extensively after UV irradiation (Boyle et al., 1970; Paterson et al., 1971). Furthermore, the polAI strain does not fill the resulting gaps as efficiently as the pol+ strain (Kanner and Hanawalt, 1970; Boyle et al., 1970; Youngs and Smith, 1973). Each of these responses to UV irradiation could contribute to overestimating the yield of DNA double-strand breaks in the polAI strain.

The importance of DNA double-strand breaks to cell inactivation is further evidenced by fluence fractionation and photoactivation. Decreasing the number of UV photoproducts present per unit of time, either by photoenzymatic repair or by fluence fractionation, results in a decrease in the number of DNA double-strand breaks as well as an increase in survival (Tables 1 and 2). The pol+ and polAI strains are capable of excision of pyrimidine dimers (Boyle et al., 1970) and can fill in the excised regions to differing degrees (Youngs et al., 1974). The growth medium dependent branch of the excision repair pathway (Youngs et al., 1974) probably accounts for a good deal of the recovery of the polAI strain observed in the fluence fractionation experiments (Table 1). We have also observed that, upon incubation after an acute exposure, the final level of DNA degradation is greater than that attained when the exposure is fractionated (data not shown). Pollard and Randall (1973) have observed a similar response using a combination of UV and γ-irradiation. They suggest that the first exposure to UV radiation may induce an inhibitor of DNA degradation. It seems reasonable then that the increase in survival and decrease in DNA double-strand breakage after fractionated exposure to UV radiation may be due to an inhibition of DNA degradation as well as the successful repair of pyrimidine dimers resulting from the first exposure. Both mechanisms would reduce the probability of forming a DNA double-strand break after the second exposure, the first by reducing the number of dimers present at any one time and the second by reducing the length of the excised region.

Our results suggest that double-strand breaks may be the major lesion responsible for UV-induced killing of the pol+ and perhaps polAI strains of E. coli K-12. If there were a direct correlation between DNA double-strand breaks and lethal events after UV irradiation it should be possible to construct a theoretical relation between breaks (or 1/Mₘ) and UV fluence based upon the survival curve in Fig. 1. The surviving fraction (N/N₀) is defined to be:

\[ N/N_0 = e^{-x} \]

where x is the average number of lethal events per cell. Assuming that all lethal events are DNA double-strand breaks and solving this equation for x at various UV fluences gives us the average number of double-strand breaks per cell. However, since we cannot measure pieces of DNA larger than about 4.2 × 10⁶ daltons (Bonura et al., 1975) the number of breaks per genome (B) must be transformed to an expected 1/Mₘ as follows

\[ B = \frac{2.8 \times 10^6}{4.2 \times 10^6} \left( \frac{4.2 \times 10^6}{M_m} - 1 \right) \]

or

\[ 1/M_m = (B/6.67) + \frac{1}{4.2 \times 10^6} \]

where 1/Mₘ is the reciprocal number average mol wt which should be observed based upon the average number of lethal events per cell.

We have constructed such a theoretical relation for the pol+ strain using data only from the exponential portion of the survival curve (Fig. 5). A similar treatment for the polAI strain was not undertaken because of the problem of overestimating the true value of 1/Mₘ due to excessive DNA degradation. It is evident that the experimental data approximate the theoretical curve. The deviation of the theoretical curve from

![Figure 5. Comparison of the observed increase in 1/Mₘ as a function of UV fluence with the expected increase in 1/Mₘ based upon the survival curve for the pol+ strain (from Fig. 1). The vertical bars represent the 95% confidence interval for the regression line shown in Fig. 3. The straight line is derived from the exponential portion of the pol+ survival curve by the analysis given in the Discussion.](image-url)
the observed data at the higher UV fluences may be a result of DNA breakdown resulting in a value of \(1/M_h\) somewhat larger than would be expected on the basis of double-strand breakage alone. The limitation on the maximum size of the DNA which we can measure makes it impossible to extend the correlation to fluences less than 50–60 J m\(^{-2}\). However, if we were able to measure DNA molecular weights up to the size of the genome, the survival curve would predict that the yield of DNA double-strand breaks would be non-linear between 0 and 50 J m\(^{-2}\), having an increasingly positive slope.

Our results support the hypothesis that DNA double-strand breaks, which result from repair processes, may be the most significant lesions in determining UV-induced cell killing in wild-type *E. coli* K-12. They also exemplify a particularly important concept, namely, that the type of DNA damage which ultimately results in cell death, may be quite different from the initial radiation-induced lesion. In the present case, excisable base damage (e.g. pyrimidine dimers) were converted to DNA single-strand breaks by repair enzymes, and subsequently to lethal DNA double-strand breaks.

Acknowledgements We thank Ms. Helen Kompfner for her excellent technical assistance. The work was supported by Public Health Service Grant CA-06437 and Research Project Grant CA-10372 from the National Cancer Institute.

REFERENCES


