The Influence of Oxygen on the Yield of DNA Double-Strand Breaks in X-Irradiated *Escherichia coli* K-12

Thomas Bonura, Christopher D. Town,[1] Kendric C. Smith, and Henry S. Kaplan

Department of Radiology, Stanford University School of Medicine, Stanford, California 94305

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Using a procedure which includes lysis on neutral sucrose gradients containing pronase and detergent with sedimentation at low speeds, we have measured the rate of DNA double-strand breakage by X rays in *E. coli* K-12 in the presence and absence of oxygen. The number of DNA double-strand breaks increased linearly with dose in both air and nitrogen. The energy required to produce a double-strand break under aerobic irradiation conditions was 532 ± 28 eV and under anoxic conditions 1290 ± 126 eV giving an oxygen enhancement ratio for DNA double-strand breaks of 2.42 ± 0.39. The D_0 values based upon cell killing kinetics were determined to be 2.29 krad for irradiations under aerobic conditions and 6.38 krad for irradiations under anoxic conditions. The resulting oxygen enhancement ratio was 2.79. From these data we have calculated that 1.3–1.4 double-strand breaks per genome were produced per lethal event, suggesting that DNA double-strand breaks may play a primary role in the killing of wild-type *E. coli* K-12 by X-irradiation.

INTRODUCTION

Double-strand breaks in DNA have been implicated as lethal events in bacteriophage (*1*) and bacteria (*2*) exposed to ionizing radiation. In bacteriophage T7 under maximally protective conditions, Freifelder (*1*) showed that there were approximately the same number of surviving phage as unbroken double-stranded DNA molecules. For *E. coli* K-12, Kaplan (*2*) showed correlations between changes in radiosensitivity and DNA sedimentation profile in neutral gradients both for cells which were sensitized by the incorporation of 5-bromodeoxyuridine into their DNA, and cells which were protected by irradiation in the presence of mercaptoethylamine. Subsequently, several authors have investigated X-ray-induced DNA double-strand breakage in both bacterial and mammalian systems (e.g., *3–9*). Although some investigators have had only limited success in isolating freely sedimentating DNA from unirradiated cells, a considerable amount of data has been generated on the rate of production of DNA double-strand breaks in

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[1] Present address: Imperial Cancer Research Fund, Burtonhole Lane, London NW7 1AD, England.

irradiated cells, where fewer technical problems are encountered. Values for the efficiency of production of double-strand breaks in air range from about 500 eV/break [in *M. radiodurans* (7), metaphase Chinese hamster cells (4), and *B. subtilis* (8)] to about 3000 eV/break in cultured murine lymphoma cells, L5178Y (6).

We report in this paper a simple technique for the measurement of double-strand breaks in DNA of X-irradiated *E. coli* K-12. Using our technique we have observed an oxygen effect for the production of DNA double-strand breaks. At the dose required to introduce, on the average, one lethal event per cell we observed between 1.3–1.4 double-strand breaks. Our data support the hypothesis that in repair proficient *E. coli* K-12 cells, DNA double-strand breaks may be of primary importance in X-ray-induced cell killing.

MATERIALS AND METHODS

The bacterial strain used was JG139, a thymine requiring derivative of *E. coli* K-12 W3110, which was obtained from Dr. J. D. Gross, and has been used for our previous studies on DNA single-strand breakage and repair (10, 11).

Cells were grown in a minimal salts medium (12) supplemented with 10 µg/ml thymine. For the sedimentation experiments, 200 µCi/ml ³H-thymine (New England Nuclear, ~20 Ci/mmol) was included in the growth medium; for survival experiments, all the thymine was unlabeled. Cells were grown at 37°C in a gyrotory water bath (New Brunswick Scientific) to about 2×10^8 cells/ml. They were collected on 0.45 µm membrane filters (Millipore Corp.), washed, and resuspended in 0.05 *M* tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.6 at room temperature at a concentration of 4×10^7 cells/ml.

Cell suspensions were equilibrated with either air or nitrogen ($>99.996\%$ purity) 5 min before and during irradiation in Plexiglas sample holders. X-irradiation was performed using the twin-tube 50 kVp X-ray unit described by Loevinger and Huisman (13). The dose rate as determined by ferrous sulfate dosimetry was 7.21 krad/min with 0.25 mm Al added filtration.

For survival experiments cells were diluted after irradiation in 0.067 *M* phosphate buffer pH 7.0 and plated on minimal medium plates (supplemented with 10 µg/ml thymine, 1.6% Difco Noble agar). Plates were incubated 48–72 hr at 37°C for colony formation.

Neutral Sucrose Gradients

In order to effect lysis on the gradients, cells were transformed to spheroplasts by mixing 0.2 ml of cell suspension with 0.3 ml ice cold 0.07 *M* Tris-0.17 *M* ethylenediaminetetraacetate (disodium) containing 200 µg/ml lysozyme (Worthington Biochemicals). The suspension was kept on ice for 10 min. Samples of 0.1 ml ($\sim 2 \times 10^6$ cells) were layered directly onto the neutral gradients described below and were left undisturbed for 90 min at room temperature.

The stock solutions for the neutral gradients were made by mixing: 5% (w/v) sodium dodecyl sulfate (SDS); 10 or 40% (w/v) sucrose; 0.1 *M* Tris-HCl, pH 7.6; 0.01 *M* sodium citrate, pH 8.0; 0.1 *M* sodium chloride, and pronase at

1 mg/ml H_2O (B grade, Calbiochem) such that the resulting solutions contained 5 or 20% sucrose, 0.5% SDS, 5×10^{-3} M Tris, 10^{-3} M sodium citrate, 10^{-2} M sodium chloride, and 0.1 mg/ml pronase. The pronase and lysozyme solutions were freshly prepared before each experiment. The stock solutions of sucrose, Tris, sodium citrate, and NaCl were chloroform saturated, resulting in a final sucrose gradient approximately 70% saturated in chloroform. The addition of chloroform has been reported to aid in the delipidization of the cell membrane (*14*). The final pH values of the 5 or 20% sucrose solutions were 7.75 and 8.0, respectively. Linear 4.8 ml gradients were made at room temperature using a Buchler gradient maker with a peristaltic pump.

The gradients were centrifuged in an SW50.1 rotor in a Beckman ultracentrifuge Model L, L2, or L265B at 20°C. We have found that DNA from unirradiated cells sediments independently of rotor speed up to 10,000 rpm. At 16,500 rpm and 20,000 rpm the control DNA sediments with an apparent sedimentation coefficient which is smaller than that at 8000 rpm by factors of 1.54 and 5.9, respectively (data not shown). Therefore, to eliminate the problem of speed dependence and to insure that the DNA sedimentation distribution was well separated from the meniscus, rotor speeds from 7500-10,000 rpm were employed, depending upon the dose of radiation and gassing condition used.

In all experiments, ^{14}C-thymine labeled bacteriophage T2 DNA was included in a separate gradient for each centrifugation condition. After centrifugation the bottom of each tube was pierced and 0.16 ml fractions were pumped onto Whatmann No. 3MM paper discs. The discs were washed in 5% trichloroacetic acid, ethanol, and acetone and counted in a liquid scintillation counter, as described previously (*15*).

Analysis of the Sedimentation Data

The data from a typical sedimentation experiment show the distribution of DNA in terms of the fraction of the total gradient radioactivity, f_i, sedimenting to a point, d_i, from the meniscus of the gradient. For convenience, all of the experimental data have been normalized to gradients of unit length.

In order to evaluate molecular weight, we have utilized a modification of the empirical relation established by Burgi and Hershey (*16*) which relates the sedimentation coefficient with molecular weight as:

$$(S_1/S_2) = (M_1/M_2)^{\alpha} \qquad (1)$$

where S and M represent the sedimentation coefficient and molecular weight, respectively, for each sedimenting species. The exponent, α, has been evaluated to be 0.38 (*8, 17*). Using this exponent and since the sedimentation coefficient is proportional to distance sedimented (d), we rearrange Eq. (1) as:

$$M_1/M_2 = (d_1/d_2)^{1/0.38} = (d_1/d_2)^{2.63} \qquad (2)$$

where M_1 and M_2 are the molecular weights at points d_1 and d_2 from the meniscus.

In determining the number average molecular weight (M_n) of the *E. coli* distribu-

tion relative to bacteriophage T2 DNA (M_{T2}) Eq. (2) becomes:

$$\frac{M_n}{M_{T2}} = \frac{\left[\dfrac{\sum f_i}{\sum (f_i/d_i{}^{2.63})}\right]E.\,\bar{\,}coli}{\left[\dfrac{\sum f_i}{\sum (f_i/d_i{}^{2.63})}\right]T2} \tag{3}$$

where d_i is the distance sedimented by the ith fraction and f_i is the percent of total radioactivity in that fraction. Substituting 1.1×10^8 daltons for the molecular weight of phage T2 DNA (*17*) and since $\sum f_i = 1.0$, Eq. (3) may be rewritten:

$$M_n = 1.1 \times 10^8 \frac{[\sum (f_i/d_i{}^{2.63})]T2}{[\sum (f_i/d_i{}^{2.63})]E.\,coli} \tag{4}$$

Because the number average molecular weight calculation is sensitive to small amounts of low molecular weight DNA (*18*), any radioactivity within five to six fractions of the meniscus was not used in molecular weight calculations.

RESULTS

The technique which we have described gives reproducible DNA sedimentation profiles for irradiated *E. coli* K-12 cells, with virtually all the radioactivity layered on the gradient recovered. The reproducibility of the unirradiated sedimentation profiles was not as good as that with irradiated cells. Although the reason for this is not immediately clear, we feel that the unirradiated DNA occasionally remains bound to some cellular constituent or retains a configuration which prevents it from sedimenting normally. We have observed, however, that low doses of X rays (<5 krad) and of uv radiation (10–20 J/m²) consistently result in freely sedimenting DNA (data not shown).

In spite of the occasional difficulty in obtaining freely sedimenting unirradiated DNA, we have been successful in determining the kinetics and efficiency of DNA double-strand breakage in air and nitrogen in *E. coli* K-12. Figure 1 shows sedimentation profiles of native DNA from cells irradiated in either air or nitrogen in relation to a labeled bacteriophage T2 DNA marker. Unirradiated cells in Fig. 1 show a DNA sedimentation distribution with a number average molecular weight of 4.58×10^8 daltons (Fig. 1a) and 5.19×10^8 daltons (Fig. 1g). These molecular weights were typical of the unirradiated DNA which we observe with our procedure $(4.17 \pm 1.06 \times 10^8$ dalton)[2], or about one-seventh of the intact *E. coli* genome of molecular weight 2.8×10^9 daltons (*19*). There are at least two explanations for this discrepancy between the size of the whole genome, which is known to be uninterrupted (*19–22*) and the unirradiated molecular weight which we measure. First, there may be an enzymatic process occurring during the preparation of *E. coli* spheroplasts which reduces the size of the DNA to that which we observe. If this were the case, alkaline sucrose gradient analysis of DNA

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[2] Represents the mean ±1 standard deviation for nine observations.
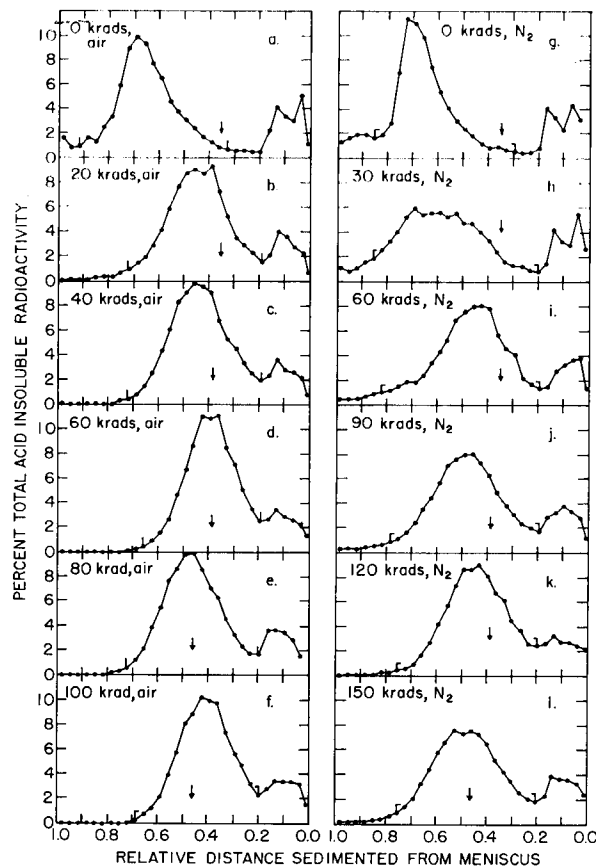
Fig. 1. Sedimentation profiles of ^{3}H-thymine labeled DNA from gently lysed *E. coli* K-12 spheroplasts. Sedimentation was for 16 h at 7600 rpm (a, b, g, h, i), 9000 rpm (c, d, j, k), and 10,000 rpm (e, f, l). The direction of sedimentation is from right to left. A ^{14}C-thymine labeled T2 bacteri-o phage DNA sample was sedimented in a separate tube and its position is shown in each gradient profile by an arrow. Brackets indicate the region of the sedimentation profile used in the calculation of M_n. The X-ray doses and irradiation conditions are shown on the graphs.

liberated from spheroplasts should indicate a lower DNA molecular weight than DNA liberated from intact cells lysed immediately on alkaline sucrose gradients. When we performed this experiment, however, both distributions were virtually identical. Furthermore, the inclusion of 10 mM sodium cyanide in the lysozyme solution did not change the sedimentation properties of the unirradiated DNA (data not shown). Because other investigators have isolated the intact, highly folded chromosome using a modification of this lysozyme procedure (*20–22*), we feel the breaks observed in the control DNA are probably not enzymatic in origin.

A more reasonable explanation is that shearing may occur during lysis which reduces the DNA molecular weight to about 4.2×10^8 daltons and prohibits us from obtaining DNA of larger size. The shear hypothesis is suggested in Fig. 2 where we have expressed the reciprocal of the number average molecular weight

(proportional to the number of double-strand breaks in the DNA) as a function of X-ray dose under air or nitrogen. At doses greater than 20 krad in air or 30 krad in nitrogen the reciprocal molecular weight varied linearly with dose within the dose range of our experiments. These data suggest that within the ranges specified, DNA double-strand breaks arise predominantly from single events. At low doses the deviation of the observed molecular weights from the extrapolated regressed lines is significant and is indicated by the dashed curves through the low dose points. If this deviation were due to shear of any molecule $>4.2 \times 10^8$ daltons, the extrapolation of the regressed lines to zero dose should approximate the true weight of the genome. This is indeed the case. The extrapolated intercepts are neither statistically different from one another nor are they significantly different from the reciprocal molecular weight of the genome (0.36×10^{-9}).

We may calculate the energy required to produce a DNA double-strand break from the linear portion of the curves shown in Fig. 2. The number of breaks per number average piece (N) is expressed as:

$$N = [(M_{n1}/M_{n2}) - 1]$$

where M_{n1} is the number average molecular weight after a dose in rads, D_1, and likewise for M_{n2} and D_2. The number of breaks per gram (B) is:

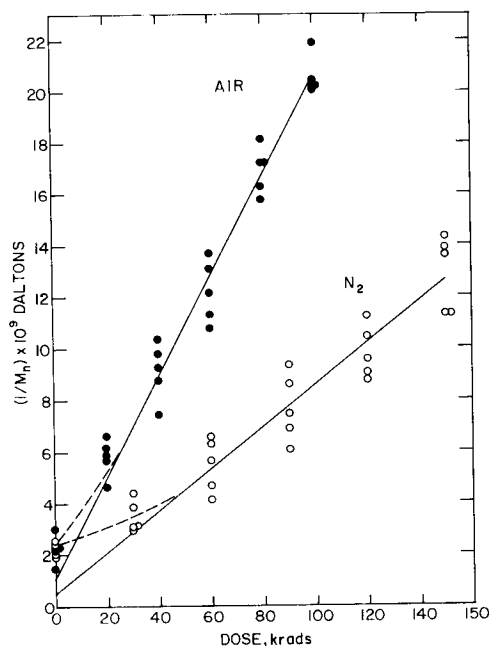$$B = N(6.03 \times 10^{23}/M_{n1})$$

FIG. 2. Reciprocal number average molecular weights (M_n) given as a function of X-ray dose. The solid lines were fitted to the data by the method of least squares within the ranges of 40–100 krad in air (closed circles) and 60–150 krad in nitrogen (open circles). Dashed lines represent an eye fit through the control and low dose points connecting with the regressed lines (see text).
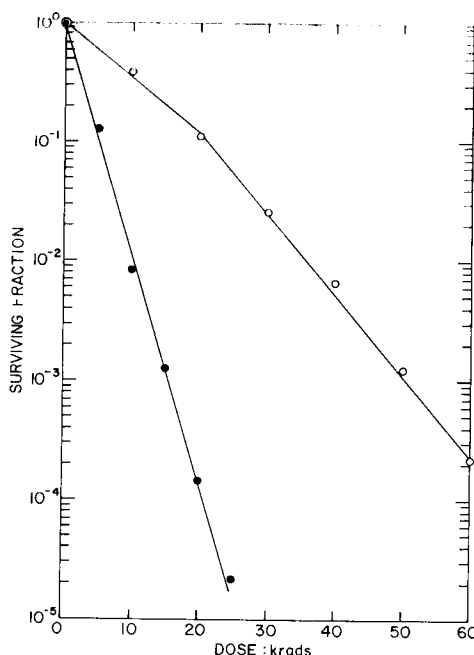
FIG. 3. Survival curves for *E. coli* K-12 (JG139) irradiated at room temperature in 0.05 *M* Tris pH 7.6 in air, ●, or nitrogen, ○. The points represent the average of three experiments. The lines were fitted to the data by the method of least squares through the most exponential portions of the survival curves (5–25 krad in air and 20–60 krad in nitrogen).

The energy required to produce a DNA double-strand break is therefore:

$$\text{eV/double-strand break} = 6.25 \times 10^{13}(D_2 - D_1)/B$$

Applying our data to this analysis we have determined an energy requirement of 532 ± 28 eV/double-strand break[3] in air and 1290 ± 126 eV/double-strand break in nitrogen. The resultant oxygen enhancement ratio is 2.42 ± 0.27. These mean values correspond to about 0.55 double-strand breaks per krad in air and 0.22 double-strand breaks per krad in nitrogen for the *E. coli* genome based upon a DNA molecular weight of 2.8×10^9 daltons.

The survival curves in air and nitrogen for *E. coli* K-12 irradiated under conditions identical to those used in measuring DNA double-strand breaks are shown in Fig. 3. D_0 values of 2.29 ± 0.065 krads[4] for air and 6.38 ± 0.32 krad for nitrogen were determined from these data. The oxygen enhancement ratio calculated from these data is approximately 2.79 ± 0.16 which is not statistically different from the OER for the production of DNA double-strand breaks. Thus, the dose required to introduce one lethal event per cell produces on the average 1.3 to 1.4 double-strand breaks per genome under either aerobic or anoxic conditions.

[3] Calculated from the slope of the regression lines shown in Fig. 2 ±1 standard deviation.

[4] D_0 values were determined from the slopes of the exponential portion of the survival curves shown in Fig. 3. The errors given are ±1 standard deviation of the slope of the regressed lines.

DISCUSSION

Until recently, studies of the production and quantitation of radiation-induced double-strand breaks have been beset with numerous technical problems, and agreement among published data has not been good. Perhaps the most significant problem in determining the molecular weight of very large DNA is the effect of rotor speed on the sedimentation velocity of macromolecules (*8, 23, 24*). Thus, one must use caution in evaluating data in the literature concerning the energy required for double-strand breakage in irradiated cellular DNA unless the speed dependence problem has been taken into account.

Recently Levin and Hutchinson (*8*) have developed a technique for the sedimentation of very large pieces of *B. subtilis* DNA. The major drawback of their technique is that it is rather time consuming, requiring many hours for deproteinization of the DNA. The technique which we have described is relatively simple to execute and gives highly reproducible results in irradiated cells. We have utilized the observation of Yamagishi (*25*) that pronase increases the recovery of DNA from the gradient. In addition we have included 0.5% SDS and have made the gradient approximately 70% saturated in chloroform to promote delipidization of the DNA-membrane structure. We have also utilized low speed sedimentation in order to obviate the problems of speed dependence.

Thus, the major benefit of our technique lies in its simplicity and ease of execution. The major drawback is that we cannot isolate DNA larger than about 4.2×10^8 daltons which may be due to shearing that occurs upon lysis of the spheroplasts. It is not possible then to quantitate DNA double-strand breakage at those radiation doses which do not result in sufficient damage to reduce the DNA to a size which minimizes shear. Therefore, our attempts to quantitate the energy required to produce double-strand breaks at biologically significant doses rely on our ability to extrapolate from superlethal doses. We feel that this is justified since the extrapolated molecular weight for unirradiated cells is very large ($>10^9$ daltons) and is not statistically different from that of the entire chromosome. The deviation in linearity of the curves shown in Fig. 2 points out a significant pitfall in quantitating the energy requirement for DNA double-strand breakage. By only utilizing data at low doses (<20 krad in air and <40 krad in nitrogen), the energy requirement would be considerably overestimated. Indeed, if we assume linearity at these doses, the energy required to produce a DNA double-strand break would be overestimated by a factor of 2 to 3 relative to calculations made at higher doses. A similar deviation in linearity of the dose response curve for the production of DNA double-strand breaks, as determined by viscosity measurements, was also observed by Dean *et al.* with *M. radiodurans* (*26*). In utilizing our present technique, therefore, one must use caution in evaluating absolute parameters such as eV/break at radiation doses which do not introduce sufficient damage to the DNA to minimize the contribution of shear.

We have utilized the hypothesis that double-strand breaks are the result of single events within the dose ranges in this study. This is predicated on the assumption that double-strand breaks arising from closely matched single-strand breaks do not contribute significantly to the total number of breaks which we observe. We feel this is reasonable since an analysis of variance of the regression

lines shown in Fig. 2 is consistent with the hypothesis that both regressions are linear ($p < 0.05$). Furthermore, we have calculated the number of double-strand breaks which might result from juxtaposed single-strand breaks. Based upon a value of 20 eV/single-strand break in air and 60 eV/single-strand break in nitrogen and sedimentation analysis at low ionic strength (*27*), at the maximum doses studied, less than 5% of the measured breaks could be due to multiple events.

We have determined the energy required to produce a DNA double-strand break under aerobic conditions to be 532 ± 28 eV. This result approximates the values established for DNA double-strand breakage in *M. radiodurans* (*7*), *B. subtilis* (*9*) and metaphase Chinese hamster cells (*4*). Other reported values for aerobic double-strand breakage are higher by factors of 2 to 5.5 (*5, 6, 14, 28, 29*).

Under our conditions of anoxia the energy required to produce a DNA double-strand break is 1290 ± 126 eV. The only other data available for anoxic irradiations *in vivo* are those of Lennartz *et al.* (*28*). These authors reported an energy requirement for the production of DNA double-strand breaks in thymocytes to be about three-fold higher than our values both for aerobic and anoxic irradiations.

Determinations of the energy required to produce DNA double-strand breaks may actually be underestimated by current techniques, since radiation-induced single-strand breaks could serve as shear sensitive sites leading to the production of double-strand breaks, as has been found using T5 DNA (*30*). If the contribution of single-strand breaks to shear induced double-strand breaks is significant, the extent to which our data might be affected is unknown at present.

The oxygen enhancement ratio of 2.4 for DNA double-strand breaks which we measure is similar to that reported for irradiated thymocytes (*28*), bacteriophage T7 DNA (*29*) T1, and calf thymus DNA (*31*).

We have determined that at the D_0 dose the number of double-strand breaks per genome is 1.3 to 1.4. Thus, it seems clear that DNA double-strand breaks appear to play a very significant role in cell killing of wild-type *E. coli* K-12 under conditions of both aerobic and anoxic irradiation.

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