

Influence of Ultrafast Repair Processes (Independent of DNA Polymerase I) on the Yield of DNA Single-Strand Breaks in *Escherichia coli* K-12 X-Irradiated in the Presence or Absence of Oxygen

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The rate of production of DNA single-strand breaks has been studied in several *polA1* derivatives of *Escherichia coli* K-12 x-irradiated in the presence or absence of oxygen. For log phase cells irradiated in phosphate-buffered saline, pH 7.3, the rate of production of breaks per single-strand genome per krad was 2.13 in the presence of air, and 0.66 in its absence [an oxygen-nitrogen breaks ratio (ONBR) of 3.2]. To determine if this oxygen effect was due to a difference in the absolute yield of breaks or to a differential ability for repair, the yield of single-strand breaks was studied in cells which had been inactivated either by heat treatment (52°C) or cold shock (0°C). In both cases there was a large increase in the yield of radiation-induced anoxic breaks (2.8-fold) and a small increase (1.25-fold) in aerobic breaks. Attempts were also made to inhibit repair using chemicals. Sodium cyanide (1 mM) used at 0°C had no effect on the anoxic yield of breaks. However, in the presence of quinacrine (0.2 mM) the level of anoxic breaks increased above that seen in untreated *pol⁻* cells, while the aerobic level was the same. *N*-ethylmaleimide, NEM (0.5 mM), had a similar effect, but another SH-enzyme inhibitor, iodoacetic acid (1 mM), had no effect. Hydroxyurea (10 mM) had an effect qualitatively similar to NEM. These results suggest that *E. coli* possess an ultrafast repair system, which operates mainly on anoxic breaks and can be inhibited by physical or chemical pretreatment of the cells. This inhibition permits the demonstration that the initial yield of x-ray-induced DNA single-strand breaks

in vivo is largely independent of the presence of oxygen, but that the yield of breaks in anoxic cells is very rapidly modified by repair.

INTRODUCTION

The modification of radiobiological effects by the presence of oxygen at the time of irradiation is one of the most striking and well documented effects of ionizing radiation. In living systems, the presence of oxygen at ambient partial pressure generally increases the sensitivity of the system by a factor of two to three over that seen under anoxic conditions. It has been postulated that the radioresistance of some tumors is due to a fraction of hypoxic cells which are less responsive to radiation (1). The mechanism of the oxygen effect *in vivo*, like the lethal effect of radiation itself, is not well understood. Information relating to the molecular basis for the oxygen effect should thus prove useful both for more effective radiation therapy, and also for a better understanding of the molecular basis of radiation lethality.

The repair of single-strand breaks appears to be an important process for bacterial cell survival, since cells which show a reduced capacity to repair such breaks either because of a mutational defect (2-4), altered growth conditions (5), or the presence of inhibitory compounds during (6, 7) or after (8, 9) irradiation are also more sensitive to the lethal effects of radiation. In reviewing the literature on DNA single-strand breakage *in vivo* in the context of the oxygen effect, we find a wide variation in both the efficiency of strand breakage in the presence of oxygen and in the reduction in the apparent yield of breaks in its absence (Table I). When examining these data, it is important to consider whether measurements were made before or after known repair systems might have operated, and also whether the system used admits the possibility of additional and as yet undescribed repair processes. Many of the rates of strand breakage reported previously for *E. coli* are erroneously low because the existence of the polymerase repair system (4) was not recognized. This type of repair rejoins many of the x-ray-induced breaks in the *E. coli* chromosome in buffer at room temperature. The reaction proceeds even at 0°C, albeit slowly at that temperature, and is almost absent in mutants of *E. coli* lacking DNA polymerase I. The number of breaks per rad observed will thus depend upon the degree to which the irradiation and prelysis conditions inhibit this repair process.

Table I has been divided into four sections on the basis of the efficiency of strand breakage and the size of the oxygen effect. The first section contains data which show both a high efficiency of strand breakage and no oxygen effect. The data in the second section show a fairly high efficiency of strand breakage, and also a significant oxygen effect in a system where enzymatic repair could not occur. The data in the third section show a high efficiency of strand breakage in the presence of oxygen and also a large oxygen effect. In this group, measurements were made before polymerase repair could occur; in all examples the possibility of the action of an undescribed repair system cannot be excluded. Data in the fourth section show a low efficiency

TABLE I
 EFFICIENCY OF DNA SINGLE-STRAND BREAKAGE BY X-IRRADIATION

| Test system | Modifying compounds present during irradiation | eV per Break ^a | | Oxygen-nitrogen breaks ratio (ONBR) | Reference |
|-----------------------------------|--|---------------------------|----------------|---|-----------|
| | | O ₂ | N ₂ | | |
| Group I | | | | | |
| Bacteriophage B3 | 1 mM histidine; 1 mM L-cysteine | 49 | 49 | 1.0 | 10 |
| Bacteriophage T7 DNA | "Dry" | ~57 | ~57 | 1.0 | 11 |
| <i>M. radiodurans</i> | 20 mM EDTA | 50 | 50 | 1.0 | 12 |
| Murine lymphoma cells (L5178Y) | | 66 | 66 | 1.0 | 12 |
| Group II | | | | | |
| Bacteriophage T4 | 20 mM guanylate | 83 | 125 | 1.5 | |
| | 100 mM thiourea | 62 | 125 | 2.0 | |
| Bacteriophage T7 | 20 mM guanylate | 67 | 83 | 1.25 | 13 |
| | 100 mM thioglycol | 100 | 143 | 1.4 | |
| Group III | | | | | |
| <i>M. radiodurans</i> | Without EDTA | 50 | 150 | 3.0 | 12 |
| Bacteriophage λ | Superinfecting <i>E. coli</i> K12(λ) | 74 | 207 | 2.8 | 14 |
| Bacteriophage λ | Superinfecting <i>E. coli</i> K12(λ) | 24 | 79 | 3.3 | 15 |
| <i>E. coli</i> B/r | 20 mM EDTA | 87 | 250 | 2.9 | 16 |
| Group IV | | | | | |
| <i>E. coli</i> B/r | Without EDTA | ~520 | ~2500 | 4.5 | 16 |
| <i>E. coli</i> B/r | | ~500 | | 3.5-4.0 | 17 |

^a Calculated on the basis of the direct physical energy absorbed in the DNA.

of strand breakage. Polymerase repair had certainly occurred before measurements were made as judged both by the experimental conditions and the low strand breakage efficiency. In this group the possibility of the action of other repair systems also exists.

Perhaps the best indication of the effect of oxygen on the initial yield of strand breaks comes from experiments on extracellular viruses and dry DNA which are free from the effects of repair enzymes or reactive cytoplasmic sulfhydryl compounds. The data in Table I can be summarized in the following general statement: when DNA is irradiated *in vivo* in the presence of oxygen, one strand break occurs for approximately every 60 eV (± 20 eV) deposited directly in the DNA. This same value also applies to isolated DNA irradiated in the dry state, or to DNA in phage irradiated in aqueous suspension in the presence of an excess of radical scavenging material. Thus, strand breakage *in vivo* requires the same energy as the average

energy loss event for the interaction of x-rays with matter (18). If every energy loss event in the DNA caused a strand break, and only directly absorbed energy was effective, the same number of breaks would result in the presence or absence of oxygen.

It is postulated that the molecular basis for the oxygen effect lies not in an increase in the initial number of lesions but rather, by modifying the chemical nature of the broken region, oxygen alters the extent to which the breaks are reparable by one or another enzymatic system. To test this hypothesis, it becomes important to ascertain what are the rates of DNA single-strand break production when cells are irradiated in the presence or absence of oxygen under conditions in which repair is minimized, and whether breaks produced under different irradiation conditions are repaired with the same efficiency (rate and extent). In this study we have attempted to answer the first question, using *polA* mutants which lack one rapid repair system (4), and have measured the rates of break production in chromosomal DNA by x-rays in the presence or absence of oxygen. Physical and chemical treatment of the cells has been used to inhibit any undefined repair systems which might otherwise modify our estimates of initial break yield.

MATERIALS AND METHODS

Bacterial Strains

Except where otherwise specified, all experiments were done with strains of *E. coli* K-12 having the *polA1* genotype. The majority of the experiments were done with JG138 *polA1*; a few used the otherwise isogenic strains JG136 *polA1 wrA6*, and JG139 *pol+*. These strains were kindly provided by Dr. Julian Gross. The primary mutant, P3478 *polA1*, a gift of Dr. John Cairns, was also used. All strains required thymine.

Culture Conditions

Cells were grown at 37°C with aeration to log phase ($\sim 2 \times 10^8$ cells/ml) in a glucose salts medium (19) supplemented with 2 $\mu\text{g/ml}$ thymine. For the sedimentation experiments 25 $\mu\text{Ci/ml}$ ^3H -thymine (New England Nuclear; 15.0–19.2 Ci/mole) was also added. (In later experiments where only $\sim 10^6$ cells were layered per gradient, 50 $\mu\text{Ci/ml}$ ^3H -thymine was used.) The medium for P3478 was also supplemented with 1 mg/ml Bacto Casamino Acids (BCA), as was the medium for the experiments with quinacrine.

Irradiation

For irradiation, cells were collected by Millipore filtration,¹ washed, and resuspended in phosphate-buffered saline pH ~ 7.3 (PBS) (NaCl 8.0 g, KCl 0.2 g, Na₂

¹ The Millipore filters were boiled for 15 min in distilled water to extract residual detergent, and presoaked in 0.1% Bovine Serum Albumin solution to minimize cell adherence.

HPO₄ 1.15 g, KH₂PO₄ 0.2 g, H₂O to 1.0 liter). In some experiments 50 mM phosphate buffer pH 8 (P/8) or pH 6.9 (P/7) was used. Cell suspensions were irradiated in Plexiglas irradiation vessels using the twin-tube 50 kVp x-ray unit described previously (20). The samples were bubbled vigorously with either air or nitrogen (>99.996% purity) for 5 min prior to and during the irradiation to ensure gas equilibration. Dosimetry was by ferrous sulfate solutions and the doses are expressed as those absorbed in comparable volumes of water (this being most nearly tissue equivalent). The dose rate was 7–8 krads/min, and depended upon the particular irradiation vessel and filtration being used. Room temperature irradiations were carried out using 0.2 mm Al added filtration. Irradiation at 0°C necessitated surrounding the samples with 3–4 mm ice. In order to maintain a comparable dose rate, filtration was reduced to 0.1 or 0 mm Al. Physically this ice layer should partly compensate in terms of radiation quality for the reduction in Al filtration. There was no detectable change in biological effectiveness of the radiation from 0 to 0.3 mm Al added filtration for either DNA strand breakage or cell survival.

Sedimentation of the DNA and Analysis of Data

Cells were lysed by layering them directly onto a 0.1-ml cap of 0.5% Sarkosyl (Geigy NL30) in 0.5 N NaOH (21) on top of a 4.6-ml gradient (5–20% w/v sucrose in 0.1 N NaOH). The cap of each gradient was gently stirred with a pin, and the gradients were allowed to stand for 1 hr at room temperature to permit cell lysis, the dissociation of DNA from other cell components and its complete denaturation. The gradients were then spun for 105 min at 30,000 rpm at 20°C using a SW50.1 rotor in a Beckman L2 or L2 65B centrifuge. After centrifugation, the bottom of each tube was pierced and fractions were collected from the bottom onto filter paper discs. These were washed and assayed for radioactivity in a liquid scintillation counter. Except for the Sarkosyl lysis procedure, the above methods and the numerical analysis of data have been described in more detail earlier (5).

In that paper (5), the number of breaks (n) per single-strand genome is calculated from the relationship $n = 6[(D_1/D_2)^{2.63} - 1]$. By comparison with sedimentation data for DNA from bacteriophage T2 (single-strand molecular weight of 6.0×10^7) the first-moment for DNA from unirradiated cells can be converted into a weight-average molecular weight of $4\text{--}4.5 \times 10^8$. If the number average molecular weight M_n is half M_w , then $M_n = 2\text{--}2.25 \times 10^8$ which corresponds to 6–7 pieces of DNA per *E. coli* single-strand genome of molecular weight 1.4×10^9 . Since M_w/M_n may be <2 for DNA from unirradiated cells, we are using the lower number of 6.

RESULTS

Effect of Oxygen on the Production of Single-Strand Breaks in polA1 Mutants

These experiments were carried out at 0°C in order to minimize the effect of any undescribed repair system. The three *polA1* strains described above were used, and

irradiations were carried out in several different buffers, with the idea that this might also influence repair [as we had observed earlier for polymerase repair (4)]. The results obtained with JG136 *polA1 wrA6* and JG138 *polA1 wr⁺*, irradiated at 0°C in PBS, are shown in Fig. 1.

Similar results were obtained with these strains and also P3478 *polA1* irradiated in 50 mM phosphate buffer pH 8. At higher pH, the yield of breaks increased more after irradiation under anoxic than under aerated conditions in all three *polA1* strains, thus causing a decrease in the observed ONBR. These results are shown in Table II. In other experiments, when cells were irradiated in Tris buffer, pH 8.0 or Veronal buffer at pH 8.0 or 9.0 (all buffers 50 mM), similar reductions in ONBR were obtained.

Production by X-rays of DNA Single-Strand Breaks in Cells Inactivated by Physical Pretreatment

Heat-treated cells. It has been reported that the yield of single-strand breaks in *E. coli* K-12 cells which were heated for 10 min at 52°C was about 5-fold higher than in unheated cells (22). This treatment produced no detectable DNA destruction or loss of viability. In view of our recent knowledge concerning the polymerase repair

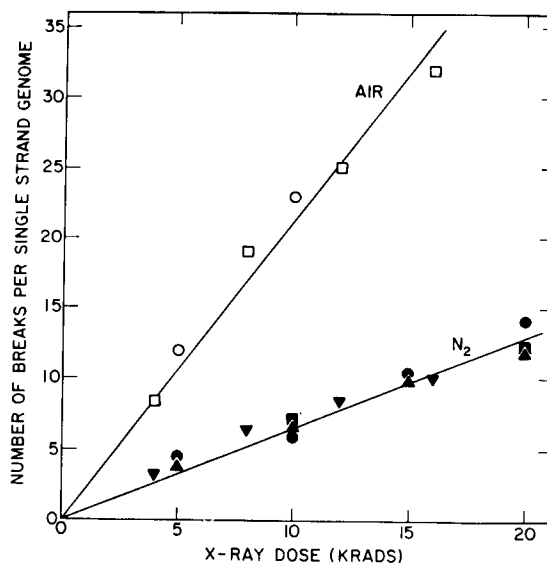


FIG. 1. Production of single-strand breaks in the DNA of *E. coli* K-12 *polA1* irradiated under aerobic or anoxic conditions. Cells were irradiated at 0°C in phosphate-buffered saline pH 7.3 and then lysed immediately by layering onto a Sarkosyl-capped alkaline sucrose gradient. Details of the procedure and analysis of results are described in the text. □, ○ JG138 *polA1* in air; ■, ● JG138 *polA1* in N₂; ▼, ▲ JG136 *polA1 wrA6* in N₂. Different symbols refer to data from independent experiments.

TABLE II
RATE OF PRODUCTION OF SINGLE-STRAND BREAKS IN THE DNA OF *E. coli* K-12 AFTER IRRADIATION OF CELL SUSPENSIONS IN EQUILIBRIUM WITH AIR OR NITROGEN^a

| Irradiation medium | Breaks per single-strand genome per krad after irradiation in the presence of | | Energy requirement per break (eV) ^b | | Oxygen-nitrogen breaks ratio (ONBR) |
|-------------------------------|---|----------------|--|----------------|-------------------------------------|
| | Air | N ₂ | Air | N ₂ | |
| PBS, pH 7.3 | 2.13 | 0.66 | 68 | 226 | 3.2 |
| 0.05 M PO ₄ , pH 8 | 2.38 | 0.94 | 61 | 154 | 2.5 |

^a Calculated from data pooled from three experiments on P3478, two on JG136, and five on JG138.

^b Calculated on the basis of the direct physical energy absorbed in the DNA.

system (4), these results with heated bacteria may be interpreted as due to the inactivation of enzyme(s) involved in this repair process. We therefore examined the influence of oxygen on x-ray-induced DNA strand breakage in heat-treated cells in the hope that enzymatic intervention would be largely eliminated.

Cells (either *polA1* or *pol*⁺) suspended in PBS were heated for 5 or 10 min at 52°C and then transferred to an ice bucket. For the rest of the experiment they were held at 0°C. Both treatment times caused a small decrease in the molecular weight of the DNA. Typical results of irradiation experiments in the presence or absence of oxygen are shown in Fig. 2, along with comparable data for untreated and cold-shocked cells. The data from all such experiments are plotted in Fig. 3. The level of strand breakage in anoxically irradiated heat-treated cells is raised almost to the level seen in untreated *polA1* cells irradiated aerobically. The level of breakage in aerobically irradiated cells also increased after heat treatment by a much smaller amount than in the case of anoxic cells, but to an extent significantly greater than in untreated cells.

Cold-shocked cells. During the course of experiments on the *in vivo* repair of radiation damage by DNA polymerase I, we observed that the sudden chilling of cells had an inhibitory effect which was more marked after anoxic than aerobic irradiation conditions. We therefore investigated the production of single-strand breaks by x-rays in cold-shocked cells. The following procedure gave good results with little reduction in the molecular weight of the unirradiated DNA. An exponentially growing culture at 37°C was poured into 50 vol of double-distilled water at 0°C. After approximately 5 min at 0°C, this dilute cell suspension was filtered, washed, and re-suspended in PBS at 0°C. It was then maintained (25–40 min) at this temperature until irradiation.²

² We originally observed cold-shocking when cells in growth medium were rapidly filtered and washed with buffer at 0°C. Under these conditions there was little change in ionic strength. The experimental conditions used here might also cause osmotic shock, but from our previous experience we feel that cold shock is the predominant effect.

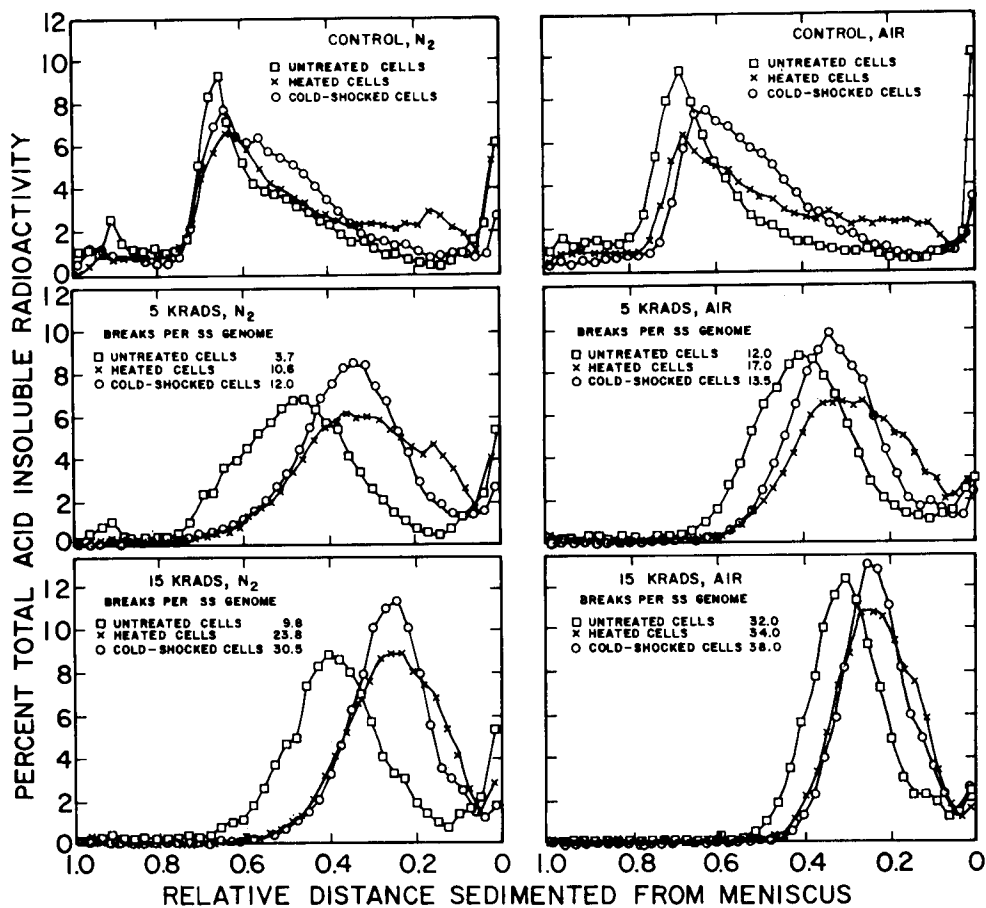


Fig. 2. Representative sedimentation profiles of cells, strain JG138 *po1A1* (untreated \square , heat-treated \times , or cold-shocked \circ) irradiated at 0°C in phosphate-buffered saline pH 7.3 in equilibrium with either air or nitrogen. Details of radiation dose, gas condition, and calculated number of breaks per single-strand genome are shown in each panel.

The results with cold-shocked cells are in every respect similar to those from the heat-treated cells (Figs. 2 and 3). Therefore, we have pooled these data and calculated that the rate of production of DNA single-strand breaks is 2.48 breaks per single-strand genome per krad under aerobic conditions, and 1.85 under anoxic conditions. The aerobic yield of breaks is thus increased by a factor of 1.2 and the anoxic yield by 2.8, so that the ONBR falls from 3.2 in untreated cells to about 1.3 in inactivated cells. These results suggest that a large proportion of the breaks produced under anoxic conditions and a small fraction of those produced under aerobic condi-

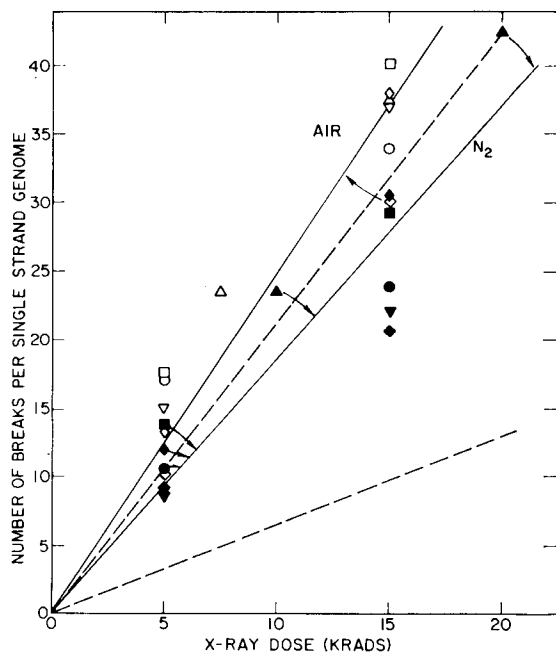


FIG. 3. Production of single-strand breaks in DNA of *E. coli* K-12 inactivated by either heat treatment or cold shock, and irradiated at 0°C in phosphate-buffered saline pH 7.3 in equilibrium with either air or nitrogen. ○, △, JG138 *polA1*, heat treated; □ JG139 *pol+*, heat treated; ◇, ▽, ◇ JG138 *polA1*, cold shocked. Symbols of different shapes refer to independent experiments, aerobic (open symbols) and anoxic (closed symbols) irradiations were performed with the same cells in each separate experiment. The broken lines are reproduced from Fig. 1 and show the apparent yield of breaks in untreated *polA1* cells irradiated in air or nitrogen. The solid lines are fitted through the origin for the pooled data from heat-treated and cold-shocked cells, since lines fitted to the two sets of data separately had essentially the same slope.

tions are subject to a rapid repair process not requiring DNA polymerase I, which we will refer to as ultrafast repair. The fact that the rate of production of breaks in inactivated cells under anoxic conditions does not quite attain the level in aerated inactivated cells is probably due to incomplete inactivation of the ultrafast repair system, as judged by the fact that the extent of inactivation (as measured by the yield of breaks) varied considerably from day to day (Fig. 3).

Chemical Modification of the Yield of DNA Single-Strand Breaks

As an alternative approach to studying the yield of breaks in cells inactivated by physical means, we also attempted to inhibit the postulated ultrafast repair system by chemical means. Because of the speed of this repair process the compounds were

TABLE III
EFFECT OF VARIOUS COMPOUNDS, PRESENT DURING IRRADIATION, ON THE YIELD OF SINGLE-STRAND BREAKS IN THE DNA OF *E. coli* K-12

| Drug | Strain and irradiation conditions ^c | Dose (krads) | Number of single-strand breaks per single-strand genome | | | |
|---------------------|--|--------------|---|-----------------------|-------------------|------------|
| | | | N ₂ | N ₂ + drug | Air | Air + drug |
| EDTA (20 mM) | P3478 in P/8 | 2 | 1.9 ^a | 0 | | |
| | | 6 | 5.6 ^a | 0.8 | | |
| | JG138 in PBS | 8 | 5.3 ^a | 3.2 | | |
| NaCN (1 mM) | JG138 in P/8 | 16 | 10.6 ^a | 7.7 | | |
| | | 5 | 4.6 ^a | 4.1 | | |
| | | 10 | 9.2 ^a | 10.4 | | |
| Quinacrine (0.2 mM) | JG139 in P/7 ^b | 15 | 13.8 ^a | 13.5 | | |
| | | 5 | 3.3 ^a | 6.7 | 10.7 ^a | 13.1 |
| | | 10 | — | — | 21.3 ^a | 19.0 |
| | | 20 | 13.2 ^a | 19.0 | 42.6 ^a | |
| NEM (0.5 mM) | JG138 in PBS | 16.3 | 10.8 ^a | 25.5 | 34.7 ^a | |
| | | 5 | — | — | 10.7 ^a | 10.0 |
| | | 10 | 6.6 ^a | 17.0 | 21.3 ^a | 22.5 |
| IA (5 mM) | JG138 in PBS | 20 | 13.2 ^a | 37.0 | 42.6 ^a | |
| | | 10 | 6.6 ^a | 4.9 | | |
| HU (10 mM) | JG138 in PBS | 20 | 13.2 ^a | 12.2 | | |
| | | 10 | 7.2 | 10.4 | 21.3 ^a | |
| | | 20 | 12.3 | 20.8 | 42.6 ^a | |

^a Values calculated from data in Table II, where such values were not determined in the same experiments.

^b Note that JG139 is a *pol*⁺ strain. The data without drug for *pol*⁻ strains are shown for comparison. The near equality of the breaks in the *pol*⁻ and the drug-treated *pol*⁺ cells in the Air columns indicates the effectiveness of quinacrine in inhibiting polymerase repair; only one to two breaks would have remained if polymerase repair had occurred. Irradiation was at room temperature which would normally have permitted complete polymerase repair.

^c All irradiations were performed at 0°C except those with quinacrine which were at room temperature. In all cases, cells were lysed on the gradients immediately after irradiation. Abbreviations: EDTA, ethylenediaminetetraacetic acid; NEM, *N*-ethylmaleimide; HU, hydroxyurea; IA, iodoacetic acid.

added before irradiation (generally at 0°C), and the cells were lysed on the sucrose gradient shortly after irradiation (within 20–30 sec).

Rather than increasing the number of anoxic breaks as had been observed for *M. radiodurans* (12) the presence of ethylenediaminetetraacetic acid (EDTA) (20 mM) increased the yield of single-strand breaks when *E. coli* K-12 was irradiated at 0°C under anoxic conditions (Table III).

We had observed previously that when sodium cyanide (1 mM) was used in conjunction with the 0°C-rapid lysis technique, DNA polymerase repair was more effectively inhibited than by the rapid lysis technique alone. Cyanide (1 mM) used

