INDUCTION BY GAMMA IRRADIATION OF DOUBLE-STRAND BREAKS OF *ESCHERICHIA COLI* CHROMOSOMES AND THEIR ROLE IN CELL LETHALITY

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ABSTRACT Viscoelastometric measurements of DNA from γ-irradiated bacteria were used to identify the induction of double-strand breaks (DSBs) in the chromosome of *Escherichia coli*. It is shown by means of inhibitors of repair endonucleases and different repair mutants that most DSBs in DNA of *E. coli*, γ-irradiated in buffer, arise from enzymatic incision of primary γ-damages; therefore, previous conclusions regarding DSB repair must be reconsidered. Based on these results, much of the reparable damage is single-strand breaks, and this damage can initiate formation of gaps and ultimately, when repair is insufficient, generation of enzymatically caused DSBs. After extensive repair, the first *residual* DSB in the *E. coli* chromosome is generated at ~160 Gray (Gy), which corresponds to the D_{37} dose. We propose that DSBs induced directly by γ-irradiation are not repaired in wild-type strains. In a recently isolated γ-resistant strain, *E. coli* Gam^{r}444, the dose required for observation of DSB after postirradiation incubation is 1,000 Gy, which corresponds to the D_{37} of this strain. The resistance is proposed to be due to an ability to repair genuine DSBs.

INTRODUCTION

In our preceding papers (1, 2), dealing with the effect of chemical protectors on survival of bacteria after γ-irradiation, we come to the conclusion that double-strand breaks (DSBs) are the main cause of lethality in wild type cells of *E. coli*. We propose that they arise eventually as a consequence of unbalanced action of repair enzymes, the nucleases that enlarge the single-strand gaps in DNA strands and the polymerases that fill these gaps. Because our hypothesis was based on indirect arguments, we have approached this issue using direct measurements of DSBs, when the average value of the number of DSBs in the DNA of the bacterial chromosome was on the order of unity. We applied the viscoelastometer method of Zimm and co-workers (3) for this purpose, because it is able to measure molecular weights reliably on the order of 10^9–10^{11} or more. In the present work, we used this sensitive system to show how the induction of DSBs depends on the cell genotype and how the cell survival relates to the number of DSBs. The problem of DSB repair is discussed, and arguments against such repair in wild-type cells are presented. We propose, however, that in some γ-resistant mutant strains of *E. coli* actual DSBs are repaired (4).

MATERIALS AND METHODS

Bacteria

The strains of *E. coli* K-12 used in this report are listed in Table I together with genotype and origin.

DNA Preparation and Measurements

An overnight culture of *E. coli* cells in nutrient broth was grown to exponential phase (2). After 45 min of starvation at 37°C with aeration in M9 medium (0.025 M K, Na-phosphate buffer at pH 7.2, 0.01 M NaCl, 0.004 M NH_4Cl, 0.04 M $MgSO_4$ without supplements), the culture was irradiated at 4°C by a ^{60}Co γ-source at a dose rate of 70–75 Gy/min (Gy: Gray). In certain cases, the cells were irradiated in BA medium (0.1 M H_3BO_3, 0.01 M Na_2-EDTA, 0.034 M NaOH, 0.14 M NaCl, pH 8.3 [5]). In this medium, the action of nucleases was strongly inhibited. Greater inhibition of enzymatic cleavage of DNA was attained in LET medium (0.05 M Tris-HCl, pH 7.0 with 0.5 M Na_2-EDTA [6]), but this buffer could not be used as irradiation medium because of partial cell lysis. For the lysis and subsequent viscoelastometry we used the LET medium: 0.1 ml of lysozyme solution (2 mg/ml lysozyme "Serva" in LET medium) added to 1 ml cell suspension. After 10 min at room temperature, a spheroplast suspension was formed. Afterwards 1 ml of lysing solution (2% Na-laurylsarcosinate "Serva" in LET medium) was carefully overlaid on the spheroplast suspension, to avoid mechanical perturbations. After 10 min at room temperature, 1 ml of pronase solution (2 mg/ml Pronase E "Serva" in 60% glycerol and 2 M NaCl [7]) was added in the same way. The pronase solution was prepared as previously described (8). The measurements were started after 15–20 h following completion of all preliminary procedures. It was shown previously (9) that during this time interval no degradation of DNA takes place.

All procedures outlined above were performed in the viscoelastometer vessel. Then the rotor was set for ~0.2 rpm. At some arbitrary moment the field was switched off. The back movement of the rotor began and the angle θ was registered at equal time intervals. These intervals were chosen in such a way to enable the registration of some 10–20 values of the angle $\theta(t)$. The last point, $\theta(\infty)$, was chosen as the θ value which did not change during 5 time intervals. The interpolated logarithmic straight line

$$\ln [\theta(t) - \theta(\infty)] = -t/\tau_i \qquad (1)$$

was traced by a computer according to the least-squares algorithm and the retardation time τ_i computed from the slope. This is shown in Fig. 1. The rotor's angle of rotation, $\theta(t) - \theta(\infty)$, was measured by comparing

†Deceased.

TABLE I
ESCHERICHIA COLI STRAINS USED IN THIS WORK

Strain	Genotype of repair loci	Origin
CR34/45	wild type	Bonhoeffer
LC173	*dna*$_{ts}$*A*46	Caro
AB1886	*uvrA*6	Howard-Flanders
AB2463	*recA*13	Howard-Flanders
JC5547	*recA*13 *recB*21 *recC*22	Hart
Gam^{r}444	*gam*^{r}	Our laboratory

the phase shift in microseconds of an informative electric signal with a standard current. The generation of alternating electric signals was effected in a photomultiplier tube by the modulation of incident polarized light by means of traversing a polaroid plate built into the rotor. The frequency of rotation was in the range 0.1–0.4 rpm. Maximal values of $\theta(t) - \theta(\infty)$ usually were ~180°, corresponding to a phase shift $9 \cdot 10^4$ μs. Minimal values, depending on residual friction in the apparatus, were ~1° (corresponding to a phase shift $5 \cdot 10^2$ μs). A detailed description of the instrument has been given (2, 7, 9). Because the interaction of macromolecules in solution is a function of concentration of DNA, a whole series of τ_i values was measured in the concentration range 2–10 μg/ml of DNA, and the retardation time was extrapolated to zero concentration. This extrapolation to zero concentration is very important, but was neglected by some authors (10, 11). We see in Fig. 2, that the interaction of macromolecules in solution changes the value of τ_i in different ways in case of high or low τ_i. This is not surprising because the retardation time depends on η/E, the ratio of viscosity of solution to the elasticity modulus of the coiled molecules. Both these values, viscosity and elasticity, apparently increase with DNA concentration but in a different way in the case of large or small coils. Hence, the different concentration function τ_i (C) for native and γ-irradiated (degraded) DNA. To make the extrapolated data reliable we must measure our limiting points at concentrations low enough to be near the extrapolated value. This complication requires much additional work but is indispensable if we want to know the real dimensions of separated macromolecules in solution. Of course, it is important not to induce shear of DNA molecules during measurements. We adopted a typical velocity gradient used by Zimm and co-workers (3) with chromosomes of even bigger dimensions, and we made control experiments, which showed that the value of τ_i does not depend of the rate

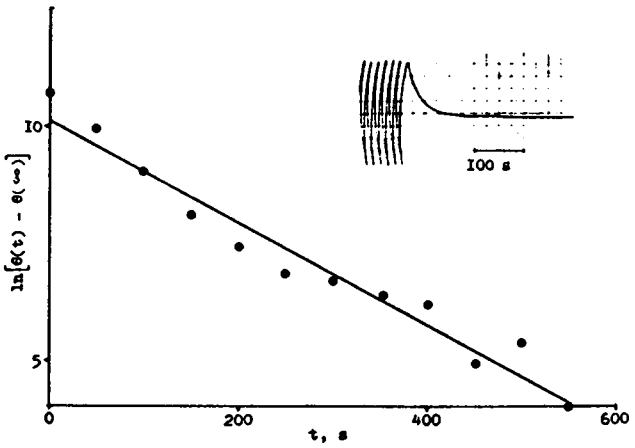

FIGURE 1 The relaxation curve of the DNA of wild-type strain *E. coli* CR34/45 in linear (inset) and semilogarithmic plots. Solid line gives the interpolated retardation time (see text).

gradient in the range 0.01–0.002 s^{-1}. The final number of revolutions of the rotor before switching off the magnetic field did not exceed 5, which is absolutely safe for molecular chains. In all our experimental data we use the zero concentration value of τ_i. This is denoted by the symbol τ^0.

The design of the instrument was similar to that of Zimm and co-workers (3). The absolute values found for τ^0 of many different native DNA samples (*E. coli, B. subtilis,* HeLa, murine fibroblasts) coincided to within 10% with the data of Zimm and co-workers (3) obtained in a similar instrument with analogous preparative procedures and in solutions of identical ionic strength.

Viscoelastometry Applied to DNA Degradation

Zimm and co-workers (3) applied their method to paucidisperse DNA molecules. However, after γ-irradiation, there is a continuous molecular weight distribution (MWD) that approximates a random (Poisson) distribution. The viscoelastometer consists of two cylinders. The inner cylinder (rotor) is freely floating and rotates at constant speed under the action of a rotating magnetic field. The outer cylinder (stator) does not move. Macromolecules in the solution between both cylinders undergo elastic deformation in the velocity gradient. When the magnetic field is

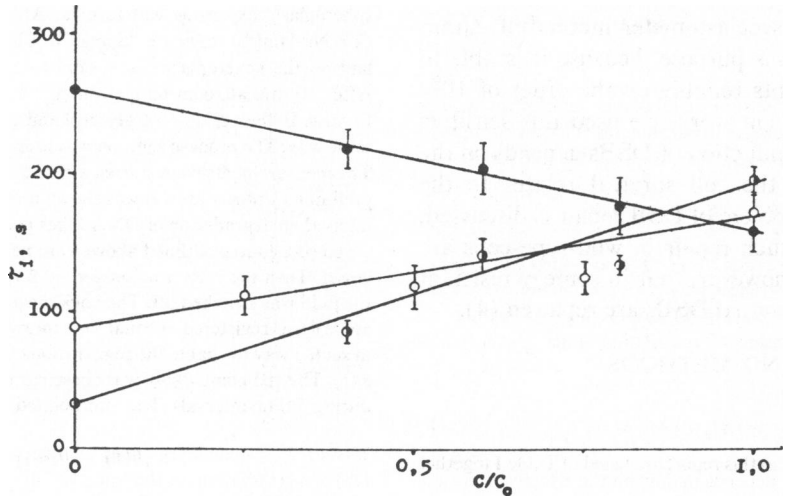

FIGURE 2 Dependence of the retardation time τ_i on the relative concentration C/C_0 (where C_0 corresponds to $2 \cdot 10^8$ cell/ml) of DNA and on the dose of γ-irradiation of cells CR 34/45: ●, without irradiation; ○, with irradiation of 25 Gy; ⊕, with irradiation 100 Gy.

switched off, the inner cylinder rotates some time in the opposite direction because of the elastic energy of the DNA molecules. According to the theory given in reference 12, the angle of rotation is a sum of relaxation terms:

$$\theta(t) - \theta(\infty) = \sum_i \gamma_i e^{-t/\tau_i} \tag{2}$$

$\theta(t)$ is the angle at time t; $\theta(\infty)$ is the angle at standstill of the rotor; τ_i is the relaxation time of the ith mode; and γ_i is the weight of the ith mode. Because the series is strongly convergent we can use one term:

$$\theta(t) - \theta(\infty) = \gamma e^{-t/\tau}. \tag{3}$$

τ is the so-called retardation time, dependent on the molecular weight of polymer molecules. Generally

$$\tau = \kappa \cdot M^{\alpha} \tag{4}$$

where $\alpha = 5/3$ in our cases. The constant κ is found by calibration of the instrument with well-known DNA of bacteria and phages. For a mixture of macromolecules of different molecular weight, the expression of Eq. 2 is still valid, but the terms in the sum are related to macromolecules of different weight. In this case the coefficient γ in Eq. 3 is $\gamma = L_i\tau_i^2/A$, where L_i is the molar concentration of corresponding macromolecules, A is a constant ($A = \eta_s k \cdot T \cdot \omega/\eta_{rel}$), η_s is the viscosity of the solvent, η_{rel} the relative viscosity of the solution, ω is the angular velocity of the rotor before switching off the field; k is Boltzmann's constant; T is absolute temperature.

We must now make a summation for all kinds of macromolecules present in the solution:

$$\theta(t) - \theta(\infty) = \sum_i \frac{L_i \tau_i^2}{A} \cdot e^{-t/\tau_i}. \tag{5}$$

In our case we have a continuous *MWD*, $f(M/M_r)$, where M_r is the number average molecular weight. By definition

$$f(M/M_r) = dW/d(M/M_r)$$

where dW is the probability of finding macromolecules in the intervale M, $M + dM$. Of course $\int_0^\infty f(M/M_r)d(M/M_r) = 1$.

Now we can introduce instead of Eq. 3 an equivalent expression describing the behavior of the whole statistical distribution of chain molecules

$$\theta(t) - \theta(\infty) = \beta \int_0^\infty \tau^2 \cdot e^{-t/\tau} f(M/M_r)d(M/M_r) \tag{6}$$

where β is a universal constant.

So we have a continuous spectrum of relaxation times. But we can apply Dirichlet's theorem of averages to our integral. This means that we interpolate our experimental curve for $\theta(t)$, as an exponential function

$$\theta(t) - \theta(\infty) \simeq \beta \tau_m^2 \cdot e^{-t/\tau_m} \tag{7}$$

where τ_m is an average retardation time for the entire distribution. Experiment shows that the differences between experimental points and interpolated straight lines are <5% (Fig. 1). Comparing Eqs. 5 and 6, we see that τ_m is the square average of retardation times of the whole statistical distribution

$$\tau_m = [\int_0^\infty \tau^2 f(M/M_r)d(M/M_r)]^{1/2}. \tag{8}$$

In reality, the retardation time depends mainly on the effective length of the macromolecules and increases by a factor of 3.35 going from circular to open structures of the same length (3). But if the chain is supercoiled, the dimensions of the molecular coil are strongly decreased. Our data strongly suggest that at low doses of γ irradiation DNA released from the lysed cells or nucleoids already contains sufficient single-strand breaks (SSBs), at least one, to release the supercoiling. This is the only sensible way to interpret the $\tau_i^\circ/\tau_o^\circ$ increase by a factor of 3.1–3.4 (Fig. 3) to be consistent with the interpretation, necessarily true for other reasons, that $\tau_i^\circ/\tau_o^\circ$ is the first DSB (see below). If SSBs that relax the supercoiled nucleoid do not exist, the entire interpretation of our data must be reexamined.

In our case, we cannot extrapolate the $\theta(t)$ curve to infinite time as do Zimm and co-workers (3), becuase after moderate γ-damage some open molecules always exist, and we would obtain by extrapolation the same τ°, referring to the open initial chains with the biggest length. On the other hand, interpolation yields an average value of τ°, but the error introduced in this case is small, as experiment reveals. The deviations of experimental points from the interpolated straight line are within 5%. In the following we shall use only one point of the dose curve, its extremum which gives the maximum of τ° because it coincides with the first DSB. The only feasible reason for the reduction of τ° is the diminution of the molecular chain length because of a second nonrepaired DSB.

RESULTS

The Maxima on Dose-Response Curves as Indicators of the First DSB

We have plotted relative values of τ_i° for irradiated cells (after a given ith dose) to initial values for undamaged nonirradiated cells τ_o°. In all experiments τ_o° is equal to 90 ± 10 s. Fig. 3 shows a typical dependence of $\tau_i^\circ/\tau_o^\circ$ on γ-ray dose. At first it increases with dose, attains a maximum and then drops. It is well known that SSBs induced by γ-radiation exceed DSBs by as much as a factor of 30–50, and that they lead to the relaxation of supercoiled DNA (13). Supercoiled DNA in solution forms a coil of smaller dimensions that does a partly or entirely relaxed chain (14). Therefore, we expect an increase of τ_i° with relaxation of supercoils. If one DSB is introduced into the relaxed circular molecule we obtain a statistical coil of maximal dimensions and accordingly a

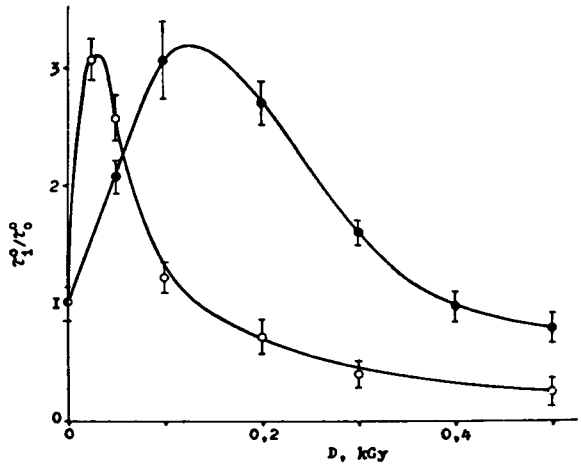

FIGURE 3 Dependence of the relative retardation time $\tau_i^\circ/\tau_o^\circ$ on the dose (D) of γ-irradiation for the wild-type strain CR 34/45 (τ_i°, the retardation time at an ith dose, extrapolated to zero DNA concentration; τ_o°, the retardation time of unirradiated DNA, concentration extrapolated to zero): ○, irradiated in M9 buffer; ●, irradiated in BA buffer.

maximal value of τ_i^o ; any additional DSBs can only decrease the chain length, and hence the diameter of the coil, thereby diminishing τ_i^o . The maximal value of τ_i^o/τ_o^o is ~3.5, due to the opening of the relaxed circle. The transition from supercoiled nucleoid to relaxed circle for *E. coli* yields a far larger τ_i^o/τ_o^o ratio than is seen in our data. Presumably, the pronase treatment relaxed the nucleoid domains and in combination with one or more SSBs induced by the isolation procedure yielded a relaxed circle. Therefore the increasing part of the curve demonstrates the effect of first DSB induction.

Generation and Repair of Double-Strand Breaks

Now we must consider a difficulty inherent in all experiments dealing with the measurement of DSB *in vivo*. It is difficult to fix the number of DSB in the chromosome at a particular moment. During γ-irradiation many SSB and other DNA lesions are formed before the first DSB is detected. The DNA damage can be a target for the action of cellular nucleases; single-strand gaps could be formed by exonucleolytic chain cleavage and when they are on opposite DNA strands or relatively close, DSB could occur (15). Therefore, following γ-irradiation and preparation of cells for lysis, which takes 10–15 min, DSB could be enzymatically generated. To distinguish primary from nuclease-generated secondary breaks, we used the BA-buffer as a stabilizing medium (16). Fig. 3 shows the comparative results of irradiation of cells in growth medium and BA medium which stops incision of γ-damages. We see that the production of SSB requires larger doses and that in this buffer the DSB begins to appear at a dose of 160 Gy based on the decrease in τ_i^o/τ_o^o.

As a result of enzymatic modifications, it is difficult to identify the production of DSBs and their repair. For example, in Fig. 4 are presented the results of irradiation in M9 buffer followed by up to 3 h of incubation in growth medium. Apparently there has been considerable repair, based on τ_i^o/τ_o^o maxima of 25 Gy and 170 Gy before and after incubation, respectively. However, as shown in Fig. 3, most DSBs appear to be due to enzymatic processing events. Further evidence for this is provided from experiments with JC5547 (*recA*13 *recB*2 *recC*22), in which nucleases are genetically inhibited. We see in Fig. 5 *b* that the decrease in τ_i^o/τ_o^o occurs at 100 Gy and that this is comparable to results with BA buffer, or if cells are incubated for 3 h following irradiation. Contrary to these results we find that in a *recA* mutant, which has excessive nuclease action following irradiation, only extremely low doses are required to observe DSBs in M9 buffer; protection is provided by the BA buffer. We thus conclude that DSBs detected at doses lower than 160 Gy originate through secondary processes.

In the above experiments, γ-irradiation took place at 4°C; at this temperature the activity of cellular enzymes is expected to be reduced considerably and therefore the induction of DSBs would depend on the balance between the action of endo- and exonucleases and polymerases. At 4°C the activity of nucleases decreased much less than that of DNA polymerases. As the temperature was raised, the

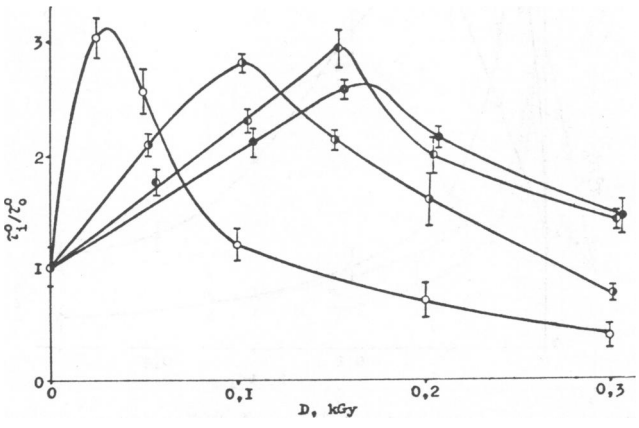

FIGURE 4 Dependence of the relative retardation time τ_i^o/τ_o^o on the dose (D) of γ-irradiation and the time of post-irradiation incubation at 42°C for the mutant LC 173: ○, immediately after irradiation; ◑, ◐, ●, after post-irradiation incubation for 1, 2 and 3 h, respectively.

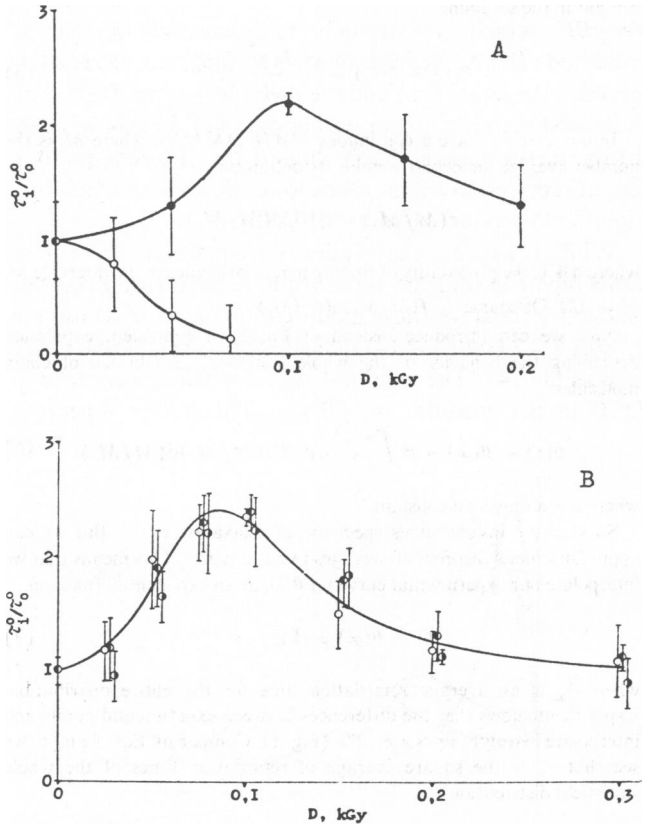

FIGURE 5 Dependence of the relative retardation time τ_i^o/τ_o^o on the dose (D) of γ-irradiation. (A) for the mutant AB 2463 *recA*13: ○, irradiated in M9 buffer; ●, irradiated in BA buffer. (B) for the mutant JC 5547 *recA*13 *recB*21 *recC*22: ○, irradiated in M9 buffer; ◐, irradiated in BA buffer; ◑, after irradiation in M9 buffer and 3 h of post-irradiation incubation.

enzymatic induction of DSBs might be expected to decrease depending on the balance of the two categories of enzymes. Cells were, therefore, irradiated in M9 medium at 4°, 37°, 45°, and 50°C without subsequent incubation. As seen in Fig. 6, the increase of temperature up to 45°C considerably reduces the formation of DSBs. At higher temperatures, more DSBs were observed, possibly because of thermally induced damages, such as apurinic sites. Optimal conditions (with a minimal yield of DSBs) are found near 45°C, at the temperature optimal for the activity of DNA polymerase I (17).

Correlation of the Lethal Dose with DSB Formation in Wild-Type and Resistant Strains

We have found that the average lethal dose (D_{37}) for LC173 and AB1157 was ~160 Gy. This corresponds roughly to the dose for the appearance of DSBs in these assays when secondary events are prevented (1, 18). We therefore conclude that the primary DSBs generated in DNA during γ-irradiation of these strains are not repaired and that they are the major cause of lethality. These results correlate with the data of other authors obtained with different biological systems (15, 19). For the mutant *recABC,* which is extremely radiosensitive in spite of inhibited DNA degradation, we find no correlation between the lethal dose and the dose for the first DSB. This is in good accord with our earlier data (1). In this deficient strain, death is presumably caused for different reasons, i.e., unrepaired lesions at much lower doses than needed for significant production of direct DSBs.

While it appears that under our conditions there is no DSB repair in the wild-type strain, results with a recently isolated γ-resistant (dose modification factor of 8) mutant *E. coli* K-12 Gam^{r}444 (4) indicate that considerable repair is possible (4). The minimal dose for the appearance of DSB in BA medium is 300 Gy. The higher dose may indicate that in wild-type strains irradiated in the presence of BA nucleases may still be present or that DSBs may be repaired in the resistant mutant. Even so we find that it takes doses of the order of 1,000 Gy before DSBs begin to appear when post-irradiation incubation in growth medium is allowed (Fig. 7). This corresponds to the lethal dose. We therefore conclude that DSBs produced in this strain are capable of being repaired and that the unrepaired breaks at the high doses are responsible for the observed lethality. The reason for the repair of DSBs induced directly by ionizing irradiation in this mutant is unclear. However, it is possible that the opportunity for repair mechanisms involving recombination may exist in this mutant. For example, there may be more duplicated chromosomes at the time of irradiation, thus allowing more opportunity for this type of repair (19, 20).

DISCUSSION

The results indicate that a significant amount of damage repair that can lead to DSBs can occur simultaneously with γ-irradiation that lasts 10–15 min. Our data suggest that, if special precautions are not taken to avoid enzymatic processing of γ-damage during irradiation and lysis, a large number of DSBs of secondary enzymatic origin are generated. It appears that when SSBs are efficiently repaired, as when there is incubation in nutrient medium, there are very few resulting DSBs. However, a quantitative contradiction is also present in our data. At 45°C, where polymerase can rapidly repair SSBs, the enzymatic DSB did not appear until 250 Gy, not the 130–160 Gy of the primary DSB of other experiments. This suggests that primary radiochemical DSBs may also be repaired, but much more efficiently at 45°C than at 37°C.

Nevertheless, by means of enzyme inhibitors, or by genetic damage of exonuclease loci, or by a temperature shift favoring DNA polymerase activity in comparison with that of nucleases, we can avoid most of the enzymati-

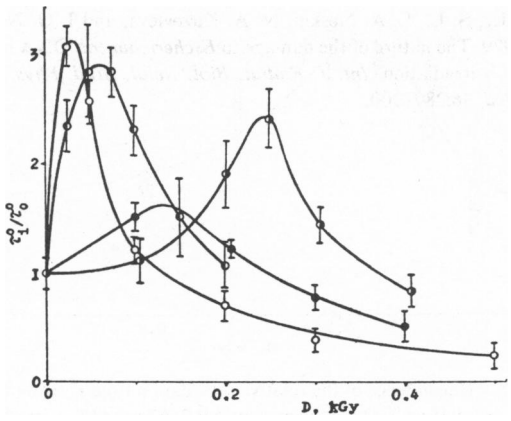

FIGURE 6 Dependence of the relative retardation time τ_i^o/τ_o^o on the dose (D) of γ-irradiation for the wild-type strain CR 34/45 for different temperatures of irradiation of cells: ○, for 4°C; ◐, for 37°C; ◑, for 45°C; ●, for 50°C.

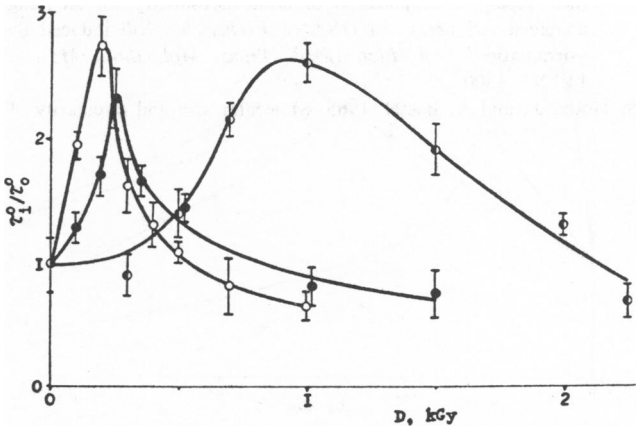

FIGURE 7 Dependence of the relative retardation time τ_i^o/τ_o^o on the dose (D) of γ-irradiation for the radioresistant mutant Gam^{r}444: ○, after irradiation in M9 buffer; ●, after irradiation in BA buffer; ◐, after irradiation in M9 buffer and 3 h of post-irradiation incubation.

cally generated DSBs. The enzymatic origin of most DSBs in γ-irradiated cells has been discussed elsewhere (15, 21). In some special cases (22), the DSBs were probably of radiochemical origin because the yield of DSBs in relation to the number of fissions was close to 1. But in the case of γ-irradiation, the number of DSBs formed is 30–50 times less than the yield of damages that can be recognized by incision enzymes and transformed into SSBs (23). The possibility of DSB repair by a recombinational mechanism such as proposed by Resnick (20) remains. Recombinational repair of DSBs was indicated by the experiments of Krasin and Hutchinson (19), wherein *E. coli* chromosomes were partially duplicated at the time of irradiation. It may be that the resistance of the Gam'444 strain used in this study is due to enhanced recombinational activity (V. L. Kalinin, personal communication).

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