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Ionizing Radiation Damage to the Folded Chromosome of *Escherichia coli* K-12: Sedimentation Properties of Irradiated Nucleoids and Chromosomal Deoxyribonucleic Acid

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The structures of the membrane-free nucleoid of *Escherichia coli* K-12 and of unfolded chromosomal deoxyribonucleic acid (DNA) were investigated by low-speed sedimentation on neutral sucrose gradients after irradiation with ^{60}Co gamma rays. Irradiation both in vivo and in vitro was used as a molecular probe of the constraints on DNA packaging in the bacterial chromosome. The number of domains of supercoiling was estimated to be approximately 180 per genome equivalent of DNA, based on measurements of relaxation caused by single-strand break formation in folded chromosomes gamma irradiated in vivo and in vitro. Similar estimates based on the target size of ribonucleic acid molecules responsible for maintaining the compact packaging of the nucleoid predicted negligible unfolding due to the formation of ribonucleic acid single-strand breaks at doses of up to 10 krad; this was born out by experimental measurements. Unfolding of the nucleoid in vitro by limit digestion with ribonuclease or by heating at 70°C resulted in DNA complexes with sedimentation coefficients of 1,030 ± 59S and 625 ± 15S, respectively. The difference in these rates was apparently due to more complete deproteinization and thus less mass in the heated material. These structures are believed to represent intact, replicating genomes in the form of complex-theta structures containing two to three genome equivalents of DNA. The rate of formation of double-strand breaks was determined from molecular weight measurements of thermally unfolded chromosomal DNA gamma irradiated in vitro. Break formation was linear with doses up to 10 krad and occurred at a rate of 0.27 double-strand break per krad per genome equivalent of DNA (1,080 eV/double-strand break). The influence of possible nonlinear DNA conformations on these values is discussed.

The major features of the structural organization of the folded chromosome of *Escherichia coli* are now fairly well understood and have been reviewed recently by Pettijohn (31). Much of the early work on nucleoids focused on the membrane-associated form, largely due to difficulties in obtaining quantitative yields of the membrane-free chromosomes. An observation by Worcel and Burgi (37) that Sarkosyl could release the nucleoid from the membrane in vitro has led to the development of isolation procedures specific for membrane-free chromosomes (19-21). We have devised a similar protocol (K. M. Ulmer, Ph.D. thesis, Massachusetts Institute of Technology, Cambridge, 1978) for isolation of membrane-free nucleoids from *E. coli* strains AB2497 (wild type) and AB2487 (*recA13*) in order to examine the effects of ^{60}Co gamma radiation on the nucleoid and on chromosonal DNA.

When membrane-free nucleoids are unfolded by RNase digestion or by thermal treatment, the resulting structures are believed to represent the intact, replicating form of bacterial DNA (21). Drlica and Worcel (16) proposed that the difference in sedimentation coefficients observed following these two methods of unfolding is positive evidence for protein-mediated stabilization of the nucleoid structure. After consideration of the rotor speed dependence of these unfolded structures, however, Hecht et al. (21) concluded that these differences in sedimentation rates are simply the result of differences in mass and not

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of residual protein-stabilized folding in the RNase-digested nucleoids. Similar treatment of nucleoids obtained by our isolation method resulted in unfolded chromosomes with sedimentation coefficients significantly different from those obtained by other investigators. Although part of this difference may be due to the slightly different strains and procedures involved, the bulk of the discrepancy can be attributed to apparent inconsistencies associated with the gradient-calibration procedure used by Hecht et al. (21).

In the present paper, theoretical consideration is given to the possible conformations of DNA from unfolded nucleoids, particularly as they relate to calculations of ionizing radiation-induced double-strand breaks (27). Our results for gamma irradiation of nucleoids and unfolded chromosomal DNA generally agree with those of Lyderson and Pettijohn (29), but the number of domains of supercoiling calculated by our method is somewhat higher.

MATERIALS AND METHODS

Chemicals and enzymes. Sarkosyl NL-97 (sodium *N*-lauryl-sarcosinate) was a gift of Ciba-Geigy, Dyestuffs and Chemicals Div. Flourinert FC-48 was a gift of the 3M Co. Angio-Conray (80% sodium iothalamate) was purchased from the Pharmaceutical Div., Mallinckrodt, Inc. Sucrose was density-gradient grade (RNase-free) and was obtained from Schwarz/Mann. All other chemicals were purchased from Sigma Chemical Co. [*methyl*-^{3}H]thymidine (52.5 Ci/mmol), [^{14}C]leucine (>270 mCi/mmol), Aquasol, and Mini-LSC-Vials were purchased from New England Nuclear Corp.

Egg white lysozyme (EC 3.2.1.17; muramidase; grade I; 3× crystallized; 25,000 U/mg) was obtained from Sigma. RNase (EC 2.7.7.16; bovine pancreas; grade A; 5× crystallized; 64 Kunitz units per mg) was purchased from Calbiochem.

Organisms. *E. coli* strains AB2497 (*rec*$^+$) and AB2487 (*recA13*) were kindly provided by David Botstein, Department of Biology, Massachusetts Institute of Technology (17, 22, 23). These are low-thymine-requiring strains which grow at 2 μg of thymine per ml.

Media and buffer solutions. M9 buffer contained the following ingredients (in grams per liter): Na_2HPO_4, 7; KH_2PO_4, 3; NaCl, 0.5; NH_4Cl, 1; and $MgSO_4 \cdot 7H_2O$, 0.25. Phosphate buffer (0.067 M at pH 7.0) contained 3.56 g of $NaH_2PO_4 \cdot H_2O$ and 5.79 g of Na_2HPO_4 per liter.

Culture conditions. M9 minimal medium was made by combining stock solutions immediately before use. A standard 20-ml culture was made by combining 10 ml of double-strength M9-buffered salts, 10 ml of an amino acid solution containing 2×10^{-3} M L-arginine, L-histidine, L-leucine, L-proline, and L-threonine, 0.15 ml of 40% glucose, 0.1 ml of thiamine (0.1 mg/ml; filter sterilized), and thymine (1.0 mg/ml; 0.2 ml for overnight cultures or 40 μl for radioactive labeling of cells).

Cultures were grown in sidearm flasks at 37°C with constant shaking (about 200 rpm) to provide aeration.

Radioactive labeling of cultures. DNA was labeled by growing cells in M9 minimal medium with a low concentration of thymine (2 μg/ml). When nucleoids were to be sedimented directly, 10 μCi of [^{3}H]thymidine was added to the 20-ml culture at the time of inoculation. When a preparative gradient was to be run to obtain purified nucleoids for in vitro experiments, this amount of label was usually doubled (20 μCi).

Gamma irradiation. A Gammacell 220, manufactured by Atomic Energy Ltd. of Canada, was used for all gamma irradiations. This ^{60}Co source was calibrated by Fricke dosimetry (15) and used with a contoured lead attenuator to give a dose rate of 55 rads/s. The standard procedure for gamma irradiation was to place the cell or nucleoid suspension in a glass test tube (12 by 75 mm) held in an ice water slurry inside a Dewar flask. The Dewar flask was then fitted inside the lead attenuator of the Gammacell. The suspensions remained aerobic during irradiation, as determined by oxygen electrode measurements.

Isolation of folded chromosomes. Cultures were grown to $\simeq 10^8$ cells per ml, rapidly chilled, and centrifuged at 7,500 rpm for 5 min. The pellet was resuspended in solution A, unless the experiment called for prior washing of the cells or some other procedure. Solution A contained 20% (wt/vol) sucrose, 0.1 M NaCl, and 0.1 M Tris-hydrochloride buffer (pH 8.1) at 0°C. The cell suspension was diluted with solution A so that the cell concentration did not exceed 4×10^8 cells per 0.2 ml. A 0.2-ml portion of the diluted cell suspension was placed in a glass test tube (12 by 75 mm) and held on ice. A 50-μl amount of solution B was added to the test tube at 0°C and blended slightly in a Vortex mixer; solution B contained 0.12 M Tris-hydrochloride buffer (pH 8.1), 50 mM ethylenedinitrilotetraacetic acid, disodium salt (EDTA), and 4 mg of egg white lysozyme per ml. The test tube was then transferred to a water bath at 25°C for 5 min with occasional swirling and returned to the ice water bath for 1 min before the addition of 0.25 ml of solution C, which contained 1% (wt/vol) Brij-58 (polyoxyethylene 20-cetyl ether), 0.4% (wt/vol) sodium deoxycholate, 2% (wt/vol) Sarkosyl NL-97, 2.0 M NaCl, and 10 mM EDTA. This detergent mixture at 0°C was added to the test tube, which was swirled gently to mix it with the spheroplasting suspension. The turbid solution cleared completely within several seconds, but was held on ice for 15 min before the lysate was gently layered onto sucrose gradients with a Clay-Adams pipette fitted with a wide-bore plastic tip. A 0.2-ml portion of the lysate was usually counted to determine the amount of radioactivity put on the gradient, as a check on recovery during fractionation.

Sucrose gradient centrifugation. All gradients were made up in untreated cellulose nitrate tubes (0.5 by 2 inches [ca. 1.3 by 5.1 cm]), using a gradient base containing 1.0 M NaCl, 0.01 M Tris-hydrochloride buffer (pH 8.1), and 1 mM EDTA. The high-density shelf was composed of 50% (wt/vol) sucrose dissolved in Angio-Conray solution. Gradients were centrifuged in an SW50.1 Ti rotor in a Beckman L5-75 ultracentrifuge at 4°C. For preparative gradients, 20,000 rpm was used, for a final integrated centrifugal force ($\omega^2 t$)

of 6.00×10^9 rads^2/s in the preset mode on the built-in ω^2t integrator. For analytical runs, the rotor speed was adjusted to about 3,000 rpm to minimize any rotor speed effects on nucleoids or unfolded DNA (21).

Gradients were fractionated with an ISCO model 640 density gradient fractionator set at a 0.5-ml/min flow rate and 0.2 ml/fraction; Fluorinert FC-48 was used as a dense chase solution. Fractions were collected directly in Beckman Mini-LSC-Vials unless the nucleoids were being purified for in vitro experiments, in which case a duplicate preparative gradient was fractionated first to determine the position of the nucleoid peak. The peak fraction, or that portion of the gradient between the half-maximum peak height points, was then diluted as necessary with gradient base. For scintillation counting, 3 ml of Aquasol plus 10% (vol/vol) water was added to the vial, shaken, and then counted in a Beckman LS-230 ambient-temperature scintillation counter. In all experiments, recovery from the gradients was >90%.

In vitro unfolding of nucleoids. Pooled peak fractions from a 10 to 30% preparative gradient were diluted with gradient base and mixed by gently rocking the test tube. Before the sample was layered on the gradient, a 0.5-ml cap containing 3.5% sucrose and 20 μg of RNase per ml in gradient base was layered on top of the 5 to 20% (wt/vol) neutral sucrose gradient.

Alternatively, nucleoids were prepared and diluted as described above and then applied to 5 to 20% neutral sucrose gradients without caps. Instead, 0.2 ml of hexadecane was floated over the sample, and the entire gradient was incubated in an oven at 72°C for about 45 min (21). The isokinetic behavior of the gradient was unaffected by this treatment. The gradients were then cooled to 4°C before centrifugation.

Calibration curves for neutral sucrose gradient analysis. Rate zonal density gradient centrifugation was the principle analytical method used in the present study. Considerable effort was made to insure the reproducibility and linearity of the various types of gradients required, and these were periodically checked by measuring the refractive index of collected fractions. Accurate calibration was also considered essential to achieve maximum resolution of small changes in the gradient profiles.

For most experiments, sedimentation markers were included in the gradients to serve as internal standards that would reveal any abnormality during sedimentation. Because we questioned the reliability and accuracy of the ratio method for determining sedimentation rates of experimental materials, we adopted a calibration similar to that used by Hecht et al. (21). A calibration curve that could be used directly in calculations of sedimentation coefficients or molecular weights was made for each type of gradient. To produce the curves, matched gradients were made, and one or more sedimentation markers were added to each. These included T4 bacteriophage (1,025S) (10, 11, 14) and lambda bacteriophage (410S) (35). All markers were labeled with [^{14}C]thymidine. The gradients were then centrifuged at 20,000 rpm for the phage markers and at 30,000 rpm for the T4 DNA, at the same temperature used for the experimental work (4°C). Zimm (39) has indicated that a rotor speed of 30,000 rpm is slow enough to avoid speed-dependent effects on DNA with a molecular weight similar to that of T4 (110×10^6; reference 18). At predetermined increments of ω^2t, gradients were removed from the centrifuge and fractionated while others were subjected to additional cycles of centrifugation. The first moments of the marker ^{14}C label distributions were determined, and these were plotted against the dimensionless quantity S × ω^2t, yielding calibration curves for any value of ω^2t (Fig. 1).

The 10 to 50% and 10 to 30% gradients depart from isokinetic behavior in the bottom portions of the gradients, as has been noted previously (10, 25). The two types of 5 to 20% gradients demonstrated true isokinetic behavior within experimental limits of detection (Fig. 1).

Calculations of average sedimentation coefficients and molecular weights. The rate of sedimentation of a particle in a sucrose gradient may be represented by the Burgi-Hershey relation (6): $s^0_{20,w} = \beta D/\omega^2 t$, where $s^0_{20,w}$ is the sedimentation coefficient at zero concentration corrected to water at 20°C, D is the distance sedimented, and β is a proportionality constant. Values of this constant for the various gradients used in the present study were determined by using calibration gradients (Fig. 1). The sedimentation coefficient corresponding to any position in the gradient can thus be calculated from the following equation: $s_i = (a_0 + a_1 v_i)/\omega^2 t$, where s_i is the sedimentation coefficient of material in the ith fraction, a_0 and a_1 are the regression coefficients from Fig. 1 for the appropriate type of gradient, and v_i is the cumulative volume fractionated to the midpoint of the ith fraction.

Since $n_i = w_i/M_i$ and w_i is proportional to cpm$_i$, where n_i is the number of molecules in the ith fraction, w_i is the mass or weight of molecules in the ith fraction, M_i is the molecular weight of molecules in the ith fraction, and cpm$_i$ is the counts per minute in the ith fraction, weight average (M_w) and number-average (M_n) molecular weights may be calculated from the following equations: $M_w = \Sigma \mathrm{cpm}_i M_i / \Sigma \mathrm{cpm}_i$ and $M_n = \Sigma \mathrm{cpm}_i / (\Sigma \mathrm{cpm}_i / M_i)$.

It is possible to calculate M_i with the relation derived by Studier (34), using the constants published by Clark and Lange (10), which have been corrected for changes in the apparent specific volume of the DNA due to the presence of 1 M NaCl in the gradients (10). The final equations for calculating average molecular weights are:

$$M_w = \frac{\Sigma\left[\mathrm{cpm}_i\left(\frac{a_1 v_i + a_0}{0.027\,\omega^2 t}\right)^{2.390}\right]}{\Sigma \mathrm{cpm}_i} \text{ and}$$

$$M_n = \frac{\Sigma \mathrm{cpm}_i}{\Sigma\left[\mathrm{cpm}_i \Big/ \left(\frac{a_1 v_i + a_0}{0.027\,\omega^2 t}\right)^{2.390}\right]}.$$

RESULTS

Isolation of membrane-free folded chromosomes. The published procedures for isolating folded chromosomes from *E. coli* differ greatly in their details, but they basically involve the spheroplasting of a cell suspension with lysozyme–EDTA and subsequent lysis by a mix-

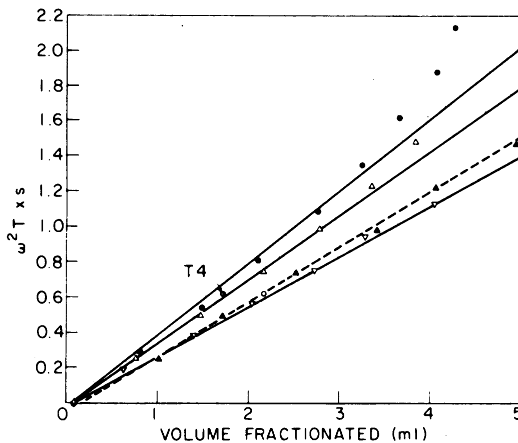

FIG. 1. *Calibration curves for neutral sucrose gradients. Symbols:* ●, *T4 phage in 10 to 50% gradient;* △, *lambda phage in 10 to 30% gradient;* ◑, *T4 phage in 10 to 30% gradient (single point);* ▲, *lambda phage in 5 to 20% gradient;* ○, *T4 phage in 5 to 20% gradient (single point);* ▽, *T4 DNA in 5 to 20% gradient.*

ture of mild, nonionic detergents in the presence of suitable counterions. With the original methods of Stonington and Pettijohn (33) and of Worcel and Burgi (36), a mixed population of nucleoids is obtained in which the proportion of membrane-free chromosomes can be increased by raising the temperature of the lysis. Although claims have been made that selective isolation of the desired form of the chromosome could be accomplished by simple temperature regulation, Ryder and Smith (32) and Korch et al. (25) have clearly demonstrated that this is largely due to an artifact caused by the low-speed centrifugation of the crude lysate before sucrose gradient analysis.

An early paper by Worcel and Burgi (36) reported that chromosomes could be released from the membrane in vitro by incubation with 1% Sarkosyl at 0°C for 15 min. Thus, 2% (wt/vol) Sarkosyl NL-97 was incorporated into solution C with the other detergents, so that the final concentration in the lysate was 1%.

When lysis occurred at 25°C, a very slowly sedimenting, often viscous peak of material was obtained. At 10°C this did not occur, but unlysed cells and membrane-associated chromosomes were present, even for lysozyme incubation times of 300 s at 0°C. Lysis at 0°C was similar, except that a rapidly sedimenting peak of viscous material was often obtained. Increasing the spheroplasting temperature to 25°C with lysis at 0°C finally gave the desired result (Ulmer, Ph.D. thesis). Lysozyme incubation at 25°C for 180 to 300 s followed by lysis at 0°C for 15 min lysed all of the cells and yielded a single, nonviscous peak sedimenting at about 1,600S relative to a T4 phage marker (1,025S). When cells were examined after spheroplasting, they were rounded in appearance. The turbid cell suspension cleared within seconds after the addition of the detergent mixture, and the membrane was apparently completely solubilized, since no debris was seen under the microscope. When the cells were labeled with [^{14}C]leucine, less than 1% of the recovered, acid-insoluble label cosedimented with the [^{3}H]thymidine peak (Fig. 2).

The incorporation of Sarkosyl into the detergent mixture was similar to other recently published methods (19-21), in which spheroplasting was performed without sucrose, but at higher NaCl and lysozyme concentrations. The incubation was for only 45 to 60 s at 4°C, but lysis was then at 24°C with a final concentration of 0.5% Sarkosyl. In the present study, however, this procedure was not satisfactory, at least for the strain of *E. coli* K-12 used; incomplete lysis was observed, and the yield of membrane-free chromosomes was greatly reduced.

In vitro unfolding of chromosomes with RNase. Several techniques were compared for unfolding the bacterial chromosome in vitro with RNase under conditions that would cause

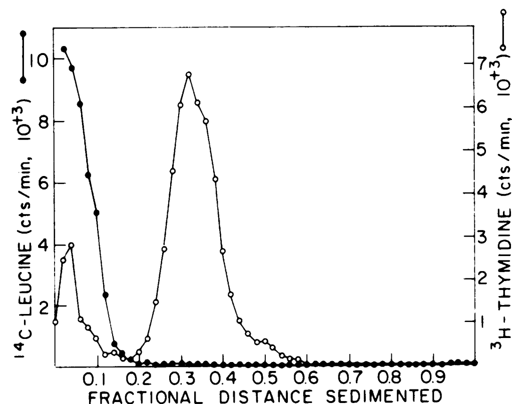

FIG. 2. *Dual-labeled profiles for an improved lysis procedure for the isolation of membrane-free nucleoids. An exponential-phase culture of E. coli strain AB2497 (wild type) was uniformly labeled with 5 μCi of [^{14}C]leucine and 10 μCi of [^{3}H]thymidine. Cells were harvested by centrifugation, washed in M9 buffer, and lysed according to the procedure described in the text. A 0.2-ml sample of the lysate was layered onto a 4.8-ml 10 to 50% neutral sucrose gradient with a 0.3-ml high-density shelf at the bottom and centrifuged in an SW50.1 rotor at 17,000 rpm at 4°C for 15 min. The gradient was fractionated onto Whatman 3MM filter disks (2 drops/filter) and dried under a heat lamp. The filters were washed at 0°C in bulk for 15 min in two changes of 5% trichloroacetic acid and once in acetone for 5 min, dried, and counted.*

a minimal amount of shearing. The first two methods (methods 1 and 2) involved the premixing of a diluted sample of purified nucleoids obtained from a preparative sucrose gradient with various concentrations of pancreatic RNase. The mixture of RNase and nucleoids was then carefully layered on top of 5 to 20% neutral sucrose gradients. In method 1, these gradients were incubated at room temperature for 1 h and then cooled to 4°C for 0.5 h before centrifugation at that temperature. In method 2 the gradients were centrifuged at low speed at 4°C immediately after the samples were applied. Methods 3 and 4 involved loading the gradient with either purified nucleoids or a diluted portion of a standard lysate, after first applying a 0.5-ml cap containing various concentrations of RNase to the gradient. In method 3 the gradients were loaded into the rotor immediately after the sample was applied, and the run was started at 4°C. In method 4 the gradients were spun at 5,000 rpm for 20 min to introduce the nucleoids into the RNase cap. At that point, the gradients were incubated at room temperature for 1 h and then cooled to 4°C for 0.5 h before they were returned to the centrifuge for the final run at that temperature. The calculated sedimentation coefficients for these unfolded chromosomes (method 4) were corrected for the small amount of sedimentation that occurred during the initial prespin.

No systematic difference was seen among the different methods, and the mean value of the sedimentation coefficient of RNase-digested chromosomes from all methods of unfolding was 1,030 ± 59S. Due to its convenience, method 3 was employed for all subsequent experiments requiring RNase unfolding. Method 3 eliminates both the need to premix the chromosomes with the RNase (thus minimizing handling and possible shearing, which is particularly important for irradiated DNA) and the need to incubate the gradients at room temperature; it is also equally suitable for direct application of lysates or for use with purified nucleoids. Finally, it is not necessary to make corrections for any precentrifugation, as with method 4.

Gamma irradiation of folded chromosomes. Gamma irradiation of purified folded chromosomes in vitro at 0°C in gradient base under aerobic conditions resulted in a gradual shift of the chromosome profile toward a slower rate of sedimentation (Fig. 3). The decrease was linear and amounted to a reduction of approximately 20% at a dose of 10 krads. The shapes of the profiles were not changed as a result of the irradiation, and no accumulation of low-sedimentation-coefficient material was seen at the top of the gradients.

The behavior of folded chromosomes obtained by lysing cells immediately after in vivo gamma irradiation under similar conditions was essentially the same as the behavior of chromosomes obtained by in vitro irradiation at doses above 2 krads (Fig. 3). Once again the shapes of the profiles were unchanged, and only a linear decrease in sedimentation rate was observed. The magnitude and rate of the change were essentially identical to those of the nucleoids irradiated in vitro. However, below 2 krads the chromosomes irradiated in vivo appeared to show a slight increase in sedimentation rate, which amounted to a 4% increase at 1 krad. The relative rates of change in sedimentation coefficients for these different conditions of irradiation are compared in Fig. 4.

RNase unfolding of gamma-irradiated nucleoids. When chromosomes were gently unfolded on the gradients by digestion with RNase after gamma irradiation in vitro, the changes in sedimentation rate became more pronounced (Fig. 3). For the same range of gamma irradiation doses, the observed changes in the sedimentation rate were much greater. At 10 krads the rate of sedimentation decreased by approximately 40%. Once again, no major changes were observed in the shapes of the profiles, and no material accumulated at either the light or the heavy ends of the gradients.

The slight increase in sedimentation rate observed for folded chromosomes gamma irradiated in vivo was abolished when the nucleoids were digested with RNase after irradiation (Fig. 3). The changes in the gradient profiles were identical to those for in vitro irradiation. The peaks for chromosomes irradiated in vivo appear to be wider than those for nucleoids irradiated in vitro because a broader range of sedimentation coefficients is present when a lysate is run directly. The chromosomes irradiated in vitro were obtained from the peak fraction of a preparative gradient. Relative changes in the sedimentation rates for these two methods of irradiation are compared in Fig. 4. The rates of change in both cases were identical, as was observed for folded chromosomes, but the rate of decrease was not linear. A 20% reduction in the relative sedimentation rate occurred with 2 krads, and an additional 20 to 25% decrease was then observed for doses up to 10 krads.

Thermal unfolding of gamma-irradiated nucleoids. In Fig. 5, a peak for unirradiated, thermally unfolded nucleoids is shown; it was sedimented almost to the bottom of the gradient and had a calculated average sedimentation coefficient of 610S. The changes that occurred in this profile at successively higher doses of gamma rays were qualitatively and quantita-

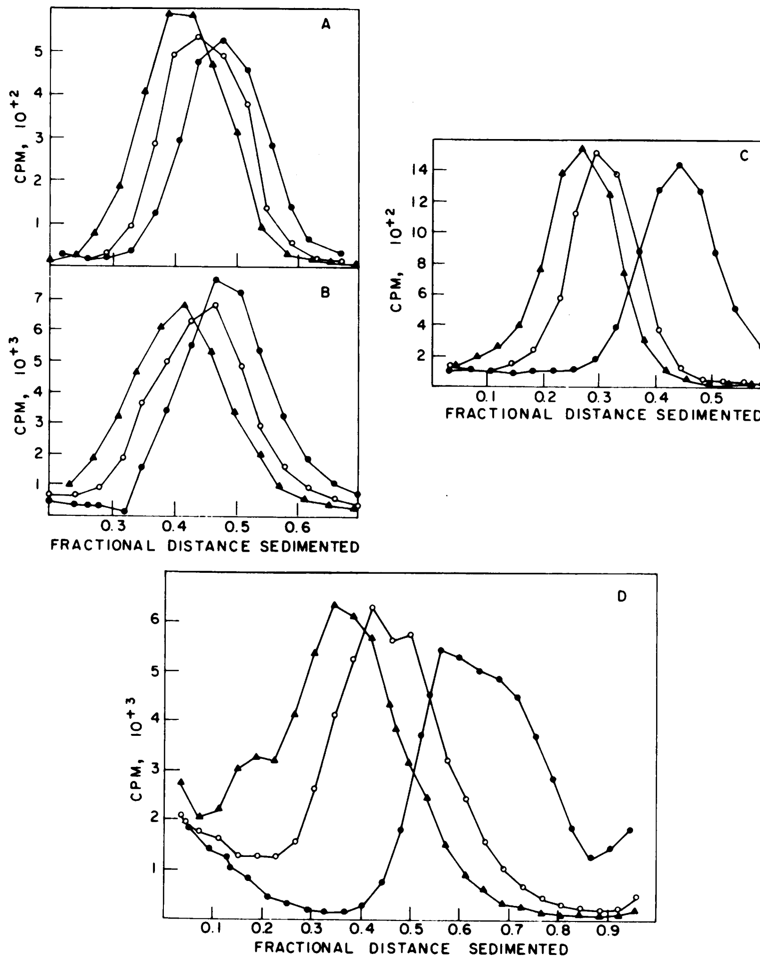

FIG. 3. *Gradient profiles for gamma-irradiated folded chromosomes and chromosomal DNA. Gradients were centrifuged at an average rotor speed of 2,936 rpm for a total ω^2t of 6.00 × 10^9 rads^2/s. Both ends of the gradients have been omitted for clarity, but there were no significant counts in those portions. (A) In vitro irradiation, folded chromosomes. (B) In vivo irradiated, folded chromosomes. (C) In vitro irradiated, RNase-unfolded chromosomes. (D) in vivo irradiated, RNase-unfolded chromosomes. Symbols: ●, 0 krad; ○, 5.0 krads; ▲, 10.0 krads.*

tively very different from the patterns observed for both the folded and the RNase-digested chromosomes. For the latter two structures, increasing doses of gamma rays caused a simple translation of the profile toward the top of the gradients, with little or no change in the shape or width of the peaks. The profiles for gamma-irradiated folded chromosomes presented by Lyderson and Pettijohn (29) are similar. In the case of thermally unfolded chromosomes, however, the initially sharp main peak of material became significantly broadened at 1 to 2 krads and at the same time underwent a large decrease in the average rate of sedimentation of the material (350S at 2 krads). At still higher doses, the profiles once again sharpened until a new peak was formed near the top of the gradient. Only a very slight additional shift in this peak was observed between 8 and 10 krads (118 to 107S). This pattern appears to be similar to that published by Crine and Verly (13) for the breakage of an initially homogeneous population of T7 DNA molecules. It also appears to be similar to the behavior observed by Lyderson and Pettijohn (29) for maximally unfolded nucleoids gamma irradiated either in vivo or in vitro.

Molecular weight calculations. If we assume for the moment that the thermally unfolded nucleoid is composed of native, linear DNA molecules, we can calculate the average molecular weights for the distributions in Fig. 5 (Table 1). Since direct calculation of number average molecular weights is highly sensitive to low-molecular-weight material near the top of

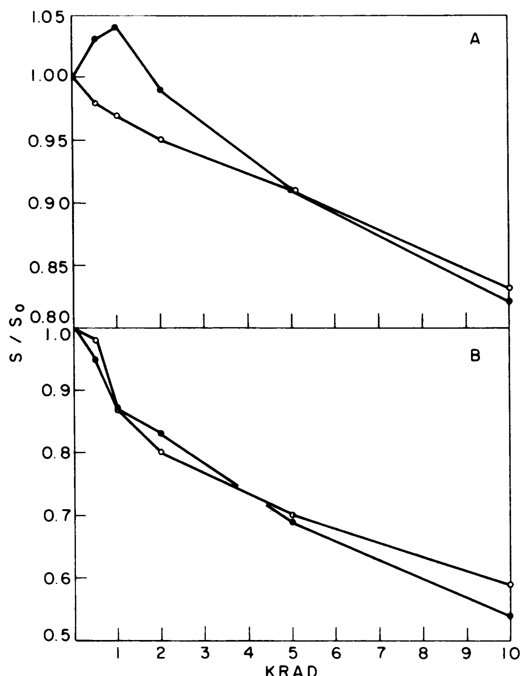

FIG. 4. *Changes in the sedimentation rates of gamma-irradiated folded chromosomes (A) and RNase-unfolded chromosomal DNA (B). Gradients were centrifuged at an average rotor speed of about 3,000 rpm for a total* $\omega^2 t$ *of* 6.00×10^9 *rads*^{2}*/s. Symbols: ●, in vivo; ○, in vitro.* S/S_0*, Ratio of the average sedimentation coefficients of irradiated and unirradiated DNA.*

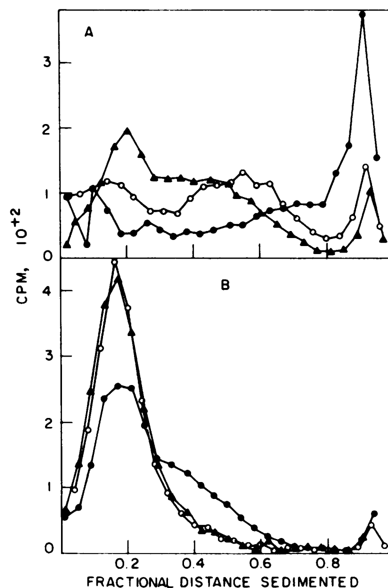

FIG. 5. *Gradient profiles for in vitro gamma-irradiated, thermally unfolded chromosomes. The gradients were centrifuged at an average rotor speed of about 3,000 rpm for a total* $\omega^2 t$ *of* 2.10×10^{10} *rads*^{2}*/s. (A) Symbols: ●, 0 krad; ○, 2.0 krads; ▲, 4.0 krads. (B) Symbols: ●, 6.0 krads; ○, 8.0 krads; ▲, 10.0 krads.*

the gradient, the five or six fractions at the very top of the gradients were excluded from the molecular weight calculations, as recommended by Bonura et al. (4, 5). For a homogeneous population of molecules, number average molecular weight equals weight average molecular weight (30). As the population becomes more random, however, number average molecular weight becomes less than or equal to weight average molecular weight, and for a completely random population, number average molecular weight equals weight average molecular weight/2 (24). In all cases, number average molecular weight is less than weight average molecular weight, indicating that the distributions are somewhat random. Indeed, the values of weight average molecular weight/2 are in fairly good agreement with the directly calculated values of number average molecular weight. In many studies of DNA repair, the number average molecular weight of a distribution is estimated as weight average molecular weight/2 because this value is less sensitive to low-molecular-weight material that accumulates at the top of the gradients. This procedure works well for major peaks located in the middle portion of the gradient. In Fig. 5, however, the peaks of interest at the higher doses are themselves at the top of the gradients. As Table 1 shows, the weight average molecular weight does not appear to accurately represent the observed behavior in this dose range. For these reasons, the directly calculated number average molecular weights were used for all further calculations.

TABLE 1. *Molecular weight calculations for thermally unfolded nucleoids gamma irradiated in vitro*

Dose (krads)	M_n (10^9)	$1/M_n$ (10^{-10})	M_w (10^9)	$M_w/2$ (10^9)	$2/M_w$ (10^{-10})
0	6.40	1.56	15.5	7.75	1.29
2	3.81	2.62	8.73	4.37	2.29
4	1.97	5.09	6.44	3.22	3.10
6	1.53	6.51	3.90	1.95	5.13
8	1.09	9.14	2.52	1.26	7.93
10	0.90	11.1	2.78	1.39	7.20

It should be noted that the number-average molecular weight for the unirradiated DNA in Fig. 5 is 6.4×10^9 or approximately 2.3 genome equivalents of DNA, if a linear molecule is assumed. This assumption is considered in detail below, but, for comparison, the highest molecular weight that could be isolated by the procedures of Bonura et al. (3-5) was 4×10^8 to 5×10^8, 13 times lighter than the material obtained

from nucleoids; this represents about one-sixth of an intact genome.

Double-strand break formation. The rate of formation of double-strand breaks is proportional to the slope of the reciprocal of the number average molecular weight plotted against dose (Fig. 6). A least-squares linear regression was fitted to the data, indicating that the rate of double-strand break formation was strictly linear up to at least 10 krads. The energy required to produce one double-strand break (4, 5) and the number of double-strand breaks per krad per genome equivalent of DNA were calculated and are shown in Table 2, along with other estimates of double-strand breakage rates from the literature.

The agreement with the most recent figures of Bonura and Smith (3) is quite good, but the most interesting comparison is with the two sets of values obtained by Lyderson and Pettijohn (29), whose experimental procedure was very similar to that used in the present investigation. Cells were irradiated in growth medium in vivo, or crude lysates containing nucleoids were irradiated in vitro and then unfolded by heating on the gradients to measure the number of double-strand breaks produced. The breakage rates obtained by Lyderson and Pettijohn (29) for both methods were higher than those obtained in the present study.

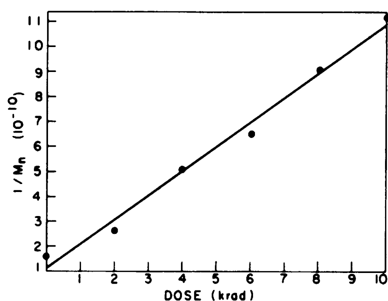

FIG. 6. *Reciprocal number average molecular weight (M_n) changes in thermally unfolded chromosomes gamma irradiated in vitro.*

Conformation of the thermally unfolded nucleoid. It was assumed above that the peak of material obtained after heating nucleoids on sucrose gradients represents native DNA free of proteins and RNA and existing as one continuous, linear strand. Calculating a molecular weight for this material by using the value of 610S, however, gives 2.5×10^{10}, or approximately 9 genome equivalents of DNA for *E. coli*. From the division rate of 50 min determined for the strain used here, it is possible to calculate an average of 2.0 genome equivalents of DNA per cell (12, 28). Hecht et al. (21) have determined that the nucleoids isolated from *E. coli* D-10 grown under similar conditions contain between 2.2 and 3.6 genome equivalents of DNA.

The discrepancy between these values and the 9 genome equivalents calculated from the sedimentation coefficient of the thermally unfolded nucleoids arises because the conformation of the *E. coli* genome is not linear, as was assumed for the purpose of that calculation. The replicating genome of *E. coli* is believed to exist in vivo as a theta structure (26) or complex-theta structure where multiple replication forks on a circular genome result in a covalently closed structure with multiple loops (26). When the chromosome is isolated in vitro, as with the extraction and unfolding of nucleoids, a number of breakage products of this complex-theta structure is possible. A single genome equivalent of DNA with a molecular weight of 2.8×10^9 would have a sedimentation coefficient of 242S if it existed as

TABLE 2. *Rates of double-strand break formation in bacterial genomes by ionizing radiation*

eV/double-strand break	Double-strand breaks/krad·genome[a]	Organism	Type of radiation	Atmosphere	Reference
1,088	0.27[b]	*E. coli*	Gamma	Air	This paper
786	0.37[c]		Gamma	Air	Lyderson and Pettijohn (29)
355	0.82[b]				
530	0.55		X	Air	Bonura et al. (5)
1,290	0.22		X	N_2	
1,211	0.24		Gamma	Air	Bonura and Smith (3)
1,000	0.28	*Salmonella*	Gamma	Air	Baraldi and Sinskey (unpublished data)
800	0.36	*Micrococcus radiodurans*	Gamma	Air	Kitayama and Matsuyama (24)
520	0.56		X	O_2	Burrell et al. (7)

[a] 2.8×10^9 daltons.
[b] In vitro nucleoid.
[c] In vivo nucleoid.

a simple linear molecule (10). If this same quantity of DNA were formed into a covalently closed circle, the resulting sedimentation coefficient would be increased by a factor of 1.18 (2). Another structure that might result from breakage of a theta structure is a Y-shaped structure containing a single replication fork. The sedimentation coefficients for Y structures that are either 50 or 100% replicated are greater than that of a simple linear equivalent by factors of 1.24 and 1.33, respectively (1). If a fully replicated theta structure were isolated intact, the expected sedimentation coefficient would be 1.517 times greater than that of a circular molecule or 1.79 times that of a linear equivalent (1). Such a structure containing two or three genome equivalents of DNA would then have a sedimentation rate between 579 and 686S. Although these are only simple approximations of the more complex structures that actually exist, they suggest that the 610S peak observed for thermally unfolded nucleoids consists of unbroken, replicating theta structures containing between two and three genome equivalents of DNA.

DISCUSSION

Relaxation of supercoiling and estimates of the number of domains. Single-strand breaks are one of the principle lesions formed in nucleic acids by ionizing radiation and are produced at a rate almost 100 times the rate of double-strand breaks (3, 38). Formation of single-strand breaks in the DNA of the folded chromosome would be expected to relax domains of supercoiling and lead to a gradual reduction of the sedimentation rate. Such behavior was observed in the present study for both in vivo- and in vitro-irradiated nucleoids. The shapes of the peaks were unaltered, and the changes in sedimentation rates were similar and of a magnitude indicative of relaxation in both cases.

By combining data for the rate of ionizing radiation-induced strand breakage (38), corrected for alkali-labile bonds, with the rate of relaxation of nucleoids, it is possible to make an improved estimate of 180 domains of supercoiling per genome. This is slightly higher than the value of 110 calculated by Lyderson and Pettijohn using the same procedure (29).

Gamma irradiation-induced unfolding of chromosomes. Current theories on the molecular organization of the nucleoid of *E. coli* propose that the highly compact folding of the chromosome is maintained largely by nascent RNA molecules attached to the DNA by their associated RNA polymerase molecules (21, 31). In addition, recent evidence indicates that many of the nascent RNA chains may have multiple sites of association with the DNA of the nucleoid (31). The possibility therefore exists that gamma irradiation of the nucleoid will introduce breaks in the RNA molecules responsible for maintaining the compact state of the nucleoid and result in unfolding.

If we assume an equal rate of breakage for RNA and DNA by gamma rays, we can compare the number of single-strand breaks per genome at a given dose with the estimated size of the RNA molecules involved in maintaining chromosome folding (32). At 10 krads, only 1 to 2 of the 74 ± 14 (31) RNA molecules contain a break. In light of the evidence for multiple sites of association of the RNA with the DNA, we would expect little or no unfolding of the chromosome due to radiation-induced strand breakage of RNA molecules at a dose of only 10 krads. This is supported by the present results obtained with nucleoids irradiated both in vivo and in vitro; at doses of gamma rays of up to 10 krads, no major dissociation or unfolding of the nucleoid was observed.

This contrasts somewhat with the findings of Lyderson and Pettijohn (29) for gamma-irradiated nucleoids. Based on determinations of the relative rotor speed dependence for nucleoids gamma irradiated both in vivo and in vitro, they concluded that in vitro irradiation caused little or no unfolding of the chromosome at doses up to 56 krads, whereas in vivo irradiation at similar doses caused a significant amount of unfolding. They further proposed that in vivo irradiation destabilizes or alters the RNA-DNA interactions in some manner, which results in the observed unfolding. Similar measurements of relative rotor speed dependence might have revealed unfolding in the in vivo-irradiated nucleoids in the present study as well, but the difference may also be attributable to the fact that Lyderson and Pettijohn (29) irradiated their cells in growth medium, whereas in our study the cells were washed and resuspended in buffer at 0°C before irradiation. It is possible that the unfolding they observed was the result of low-level physiological activity rather than of radiochemistry. It is not clear whether the observed unfolding involved breakage of the RNA molecules or some other reaction.

Sedimentation rates of RNase-digested and thermally unfolded nucleoids. Limit digestion of nucleoids with pancreatic RNase by several methods yielded material with an average sedimentation rate of 1,030 ± 60S at rotor speeds near 3,000 rpm. Hecht et al. (21), however, using similar techniques, obtained a sedi-

mentation rate of 450S for RNA-free nucleoid DNA. This difference appears to be due to differences in calibration of gradients. Their T4 DNA marker sedimented 25 ± 5% slower than expected relative to the intact T4 phage. Indeed, they state that "since the T4 DNA marker sedimented at an actual rate of 25S in the standard sucrose gradients at 4°C, the assumed values for $s^0_{20,w}$ ... were multiplied by 25/59 = 0.43 to estimate their real sedimentation velocities." Although the authors attempted to explain this discrepancy as due to density differences between the phage and the isolated DNA, it would perhaps appear more likely that their T4 DNA marker was somewhat degraded and did not in fact represent whole T4 DNA molecules. In the calibration gradients used for the present research, the T4 DNA sedimented exactly as predicted relative to lambda and T4 phage markers (Fig. 1). The value of the sedimentation coefficient of the T4 DNA used by Hecht et al. (59S) was also slightly lower than the more recent value (62.8S) of Clark and Lange (10), which was used in our analysis. If the correction of Hecht et al. (21) is eliminated, the sedimentation rate for RNase-digested nucleoids becomes 450S/0.43 = 1,046S, almost identical to the value obtained in the present work.

Using the same argument, the sedimentation rate for thermally unfolded nucleoids published by Hecht et al. (21) must also be corrected to 744S, which is somewhat higher than the value of 610 to 640S determined in the present study.

In another recent paper from the same laboratory, Lyderson and Pettijohn (29) report that the sedimentation rates of RNase-digested and thermally unfolded chromosomes differ by less than 10% and have sedimentation coefficients of about 250S, or about four times the rate of the T4 DNA marker. Whether this is again due to problems with the marker or actually represents a real difference in the conformations of the structures is unclear. The latter situation may in fact be the case, due to the slight differences in the strains, growth rates, and lysis procedures. Most significant may be the lysis procedure (21), in which the lysate is incubated with the detergent solution at 24°C rather than 0°C.

Effect of chromosome conformation on the calculations of the rate of formation of double-strand breaks by ionizing radiation. All of the values for the rate of formation of double-strand breaks by ionizing radiation listed in Table 2 were calculated assuming that the DNA was in the form of a single, continuous, linear polymer. The genome of *E. coli* is known to exist as a circle in vivo (26), and during replication the structure geometrically resembles a theta (8). In rapidly growing cells, the conformation of the DNA is even more complex, and multiple replication forks are present (12, 28). Several investigators have reported difficulty in reproducibly obtaining "free-sedimenting" DNA of intact, genome size from unirradiated cells (4, 5). For this reason, doses of ionizing radiation in excess of 10 krads were used to measure double-strand breakage rates. Extrapolation of reciprocal number average molecular weights into the biologically significant dose range of less than 10 krads suggested that break formation might be nonlinear below 10 krads. Bonura et al. (5) explained this observation as due to the formation of shear-sensitive sites in the DNA at these low doses. It appears more likely, however, that the nonlinearity reflects a changing conformation of the DNA.

For a linear polymer, one break will split the molecule in half on the average and thus produce a significant decrease in the observed rate of sedimentation. For a complex-theta structure, however, the formation of a single break is likely to open up only one loop, and the analytical form of the expression for the sedimentation coefficient will probably still be dominated by the presence of the several remaining loops. Thus, the sedimentation coefficient of such a structure might not be greatly different from that of a chromosome with all of the loops intact. The resulting change in the sedimentation coefficient would thus appear to be relatively less than in the case of the linear polymer. As the genome accumulates additional breaks, most of the loops will be broken, and the analytical form of the expression for the sedimentation rate will approach that of a chain with multiple branches. At still higher doses, after enough breaks have accumulated to reduce the size of the average piece of DNA to less than the average distance between replication forks, this will approach the condition for linear pieces of DNA. For a completely linear polymer, 5 to 10 breaks are required to convert an initially homogeneous population of molecules to a random distribution of sizes (9). The number of breaks required to produce a random distribution of pieces of DNA from an initial population of complex-theta structures with multiple replication forks is not as easy to predict.

In light of the above discussion, it is surprising that the plots of reciprocal number average molecular weight against radiation dose shown in Fig. 6 and also in the paper by Lyderson and Pettijohn (29) are linear. Furthermore, the rate of double-strand break formation determined in the present sudy is in close agreement with the most recent values of Bonura and Smith (3),

which were determined at doses in excess of 30 krads, where the assumption of linear DNA molecules is more plausible.

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