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RATE ZONAL DENSITY GRADIENT ULTRACENTRIFUGATION

ANALYSIS OF REPAIR OF RADIATION DAMAGE

TO THE FOLDED CHROMOSOME OF *ESCHERICHIA COLI*

by

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B.A., Williams College
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Institution

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2

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Submitted to the Joint Committee on Biological Oceanography of the Woods Hole Oceanographic Institution and the Massachusetts Institute of Technology, and the Department of Biology of the Massachusetts Institute of Technology on April 21, 1978, in partial fulfillment of the requirements for the degree of Doctor of Philosophy

ABSTRACT

The structure of the membrane-free nucleoid of *Escherichia coli* and of unfolded chromosomal DNA was investigated by sedimentation on neutral sucrose gradients after irradiation with ^{60}Co gamma-rays and ultraviolet light (254nm). Irradiation both *in vivo* and *in vitro* was used as a molecular probe of the constraints on DNA-packaging in the bacterial chromosome. The extremely gentle lysis and unfolding procedures which were developed yielded undamaged, replicating genomes, thus permitting direct measurement of the formation and repair of DNA double-strand breaks at biologically-significant doses of ionizing radiation.

In vitro UV-irradiation of nucleoids resulted in an increase in the observed rate of sedimentation due to the formation of an unknown photo-product. In contrast, UV-irradiation of wild-type cells *in vivo* showed evidence of the formation of incision breaks which resulted in the relaxation of supercoiling in the nucleoid. Strand breakage was also observed following *in vivo* UV-irradiation of a *wvrB-5* strain, but at a lower rate and also accompanied by considerable unfolding of the chromosome. Such lesions may have been the result of direct photochemical reactions in the nucleoid, or enzyme activity associated with a *wvr*-independent mode of repair.

The number of domains of supercoiling was estimated at 170 per genome equivalent of DNA based on measurements of relaxation caused by single-strand break formation in *in vivo*- and *in vitro*-gamma-irradiated folded chromosomes. Similar estimates based on the target size of RNA molecules responsible for maintaining the compact packaging of the nucleoid predicted negligible unfolding due to the formation of RNA single-strand breaks at doses up-to 10 Krad, and were borne out by experimental measurements.

Unfolding of the nucleoid *in vitro* by limit-digestion with RNase or by heating at 70°C resulted in DNA complexes with sedimentation coefficients of $1030 \pm 59\text{S}$ and $625 \pm 15\text{S}$ 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 2-3 genome equivalents of DNA.

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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 dose up to 10 Krad, resulting in 0.27 double-strand breaks per kilorad per genome equivalent of DNA and requiring 1080 eV/double-strand break. The influence of possible non-linear DNA conformations of these calculations is discussed.

Repair of ionizing radiation damage to folded chromosomes was observed within 2-3 hours of post-irradiation incubation in growth medium. A model based on recombinational repair is proposed to explain the formation of 2200-2300S material during early stages of incubation and subsequent changes in the gradient profiles. Such behavior is not observed for post-irradiation incubation of wild-type cells in buffer or for a *recA-13* strain incubated in growth medium. Association of unrepaired DNA with plasma membrane is proposed to explain the formation of a peak of rapidly sedimenting material (>>3100S) during the later stages of repair.

Direct evidence of repair of double-strand breaks during post-irradiation incubation in growth medium was obtained from gradient profiles of DNA from RNase-digested chromosomes. The sedimentation coefficient of broken molecules was restored to the value of unirradiated DNA after 2-3 hours of incubation, and the fraction of the DNA repaired in this fashion was equal to the fraction of cells which survived at the same dose. An average of 2.7 double-strand breaks per genome per lethal event was observed, suggesting that 1-2 double-strand breaks per genome are repairable in this strain of *E. coli*.

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Maintenance of the structural integrity of DNA is of primary importance for preservation of the functional integrity of the cell. Numerous chemical and physical agents are capable of inducing structural defects in DNA, and highly complex systems have evolved for the enzymatic repair of such lesions. The volume of literature on DNA damage and repair is enormous, and for a current and extensive review of the field the reader is referred to Hanawalt and Setlow (1975). Generally, damage to the heterocyclic bases of the DNA results in alteration of normal base-pairing and base-stacking in the double-helix, causing distortion of the higher-order structure. Cerutti (1975) has categorized such base damage according to the nature and degree of distortion introduced. When the damaging agent reacts with the sugar-phosphate backbone of the DNA, strand-breakage is usually the resulting lesion. Damage which results in major helix distortion or strand-breakage is amenable to analysis by sedimentation techniques, and has been studied extensively in a wide variety of biological systems ranging from bacteriophage to human cell lines, and employing an even broader spectrum of damaging agents including many types of radiation as well as chemical mutagens and carcinogens.

The folded chromosome of *Escherichia coli* is a compact structure in which the DNA is maintained in a highly-condensed, organized state by both RNA and protein (Pettijohn, 1977). Changes in the sedimentation properties of the nucleoid as a result of UV- or gamma-irradiation can provide new insight into the molecular organization of the bacterial chromosome. When nucleoids are gently unfolded and deproteinized, the chromosomal DNA is minimally damaged and of intact genome size. With careful consideration of rotor speed effects, the formation and repair of double-strand

breaks at biologically significant doses of gamma-rays can be measured.

II. LITERATURE SURVEY

A. Ionizing Radiation Damage and Repair

Measurement of strand-breakage is currently the most widely-used technique for the detection of ionizing radiation damage to DNA *in vivo* (Ward, 1975a). Most sedimentation methods for strand-break analysis are based on following the changes in the average molecular weight of the DNA, which can be related to the number of breaks introduced in the original distribution by using equations such as those developed by Charlesby (1954) or Montroll and Simha (1940). In the pioneering work of Freifelder (1965), T7 bacteriophage were irradiated with X-rays and the number of double-strand (DS) breaks and single-strand (SS) breaks was determined using an analytical ultracentrifuge technique in which the percentage of broken molecules was obtained from the shape of the sedimentation boundary for native and denatured DNA respectively. For irradiation in buffer, the number of inactivated phage equaled the number which had received a DS-break. Under radioprotective conditions (eg. 10^{-3} M histidine) only 40% of the dead phage contained DS-breaks. The number of SS-breaks/phage was much larger than the number of lethal hits/phage in both cases.

Problems associated with obtaining molecular weight distributions from boundary shapes, and limitations on the usable range of molecular weights greatly restricted the application of such analytical ultracentrifuge techniques (van der Schans *et al.*, 1969). McGrath and Williams (1966) introduced alkaline sucrose gradient centrifugation as a method which overcomes some of the problems associated with the quantitative

determination of SS-breaks. By lysing protoplasts directly on top of the gradients, handling and subsequent shearing of the DNA was minimized. Gradient profiles were converted to mean sedimentation coefficients ($\bar{S}_{20,w}$) by the relation derived by Burgi and Hershey (1963) and the equations of Studier (1965) were then used to obtain average SS-molecular weights. DNA from unirradiated *E. coli* had an average molecular weight of 2.2×10^8 daltons or about one-sixth of an intact SS-genome of 1.4×10^9 daltons. A 20 Krad dose of X-rays reduced the molecular weight to one-half its original value, indicating a breakage rate of 1 SS-break/3.3 Krad·SS-genome. For the radiation sensitive strain *E. coli* B_s-1 , this was equivalent to the LD_{37} value of about 3 Krad, and no increase in the molecular weight of broken strands was observed after post-irradiation incubation in growth medium. For the radiation resistant strain *E. coli* B/r , however, the LD_{37} was 15 Krad and incubation of the irradiated cells in growth medium resulted in almost complete restoration of the DNA to the unirradiated molecular weight. This was the first evidence of repair of this type, and the technique has now been used more extensively than any other.

Since DS-breaks had been shown to be lethal for X-irradiated T7 phage (Freifelder, 1965), the question of their repairability in *E. coli* was raised by Kaplan (1966). He modified the McGrath and Williams (1966) technique by using neutral sucrose gradients for determination of DS-breaks, thus necessitating the use of the detergent SDS to lyse the protoplasts on top of the gradients. Only the first moments of the gradient profiles, and not sedimentation coefficients or molecular weights were calculated, so the results were more qualitative in nature. Once

again however, extensive repair of SS-breaks was observed using alkaline gradients, but no change was noted in DS-break profiles at similar doses under Kaplan's conditions of post-irradiation incubation. It thus appeared that DS-breaks were lethal and not subject to repair, at least in this bacterial strain.

Van der Schans *et al.* (1969) conducted an extensive examination of sucrose gradient centrifugation in order to assess the requirements for true quantitative determination of molecular weight distributions of DNA. His recommendations included uniform unloading of the gradients from the top, testing of gradient linearity, use of corrections for wall-losses (Abelson and Thomas, 1966), and analytical procedures for conversion of gradient profiles to sedimentation coefficient or molecular weight distributions.

Further investigations with the analytical ultracentrifuge also had important implications for alkaline sucrose gradient work. Bopp and Hagan (1970) conducted enzymatic end-group determinations on gamma-irradiated calf-thymus DNA and discovered that treatment of irradiated DNA with alkali increased the number of SS-breaks by 50%. This finding was further supported by Kessler *et al.* (1971) for gamma-irradiated SV40 DNA. Measurement of SS-breaks could be conducted in this system by making use of the properties of supercoiled, closed covalent circular (CCC) DNA. Under neutral conditions, one SS-break converts a rapidly-sedimenting, supercoiled CCC Component I molecule to an open ring (Component II) which sediments more slowly, thus permitting separation in an analytical ultracentrifuge. Under alkaline conditions, Component I forms a rapidly-sedimenting coil, while Component II denatures to form a single-stranded

circle and a linear molecule which cannot be separated by sedimentation (Component III). The alkali -induced increase in SS-breaks was only observed for irradiation of the SV40 DNA in aqueous solution, however and not when the DNA was irradiated in the dry state. At about the same time Achey *et al.* (1971) used the supercoiled CCC ϕ X174 replicating-form DNA for velocity sedimentation studies in alkaline and neutral sucrose gradients. Gamma-irradiation at room temperature produced almost as many alkali-labile sites as true SS-breaks, but if irradiation occurred at -196°C both types of damage were greatly reduced.

The nature of the alkali -labile bond (ALB) has been the subject of extensive investigation by a number of workers. Ward (1975a,b) has used both model nucleotides and native DNA to study the kinetics of ionizing radiation-induced ALB's. Following irradiation, the number of ALB's and true SS-breaks increases for several hours, paralleling the release of damaged free bases. Similar lesions are also formed in mammalian cells by methylating agents, 4-nitroquinoline-N-oxide, or ultraviolet light (Walker and Sridhar, 1975). It now appears that the ALB is actually an apurinic or apyrimidinic site in the DNA resulting from cleavage of the N-glycosidic bond, and can be caused by any number of chemical and physical agents. Crine and Verly (1976) have developed a technique to distinguish between true SS-breaks and apurinic sites based on mild denaturation with formamide and sedimentation on neutral sucrose gradients. This subject has recently been reviewed by Lindahl and Ljungquist (1975). Depurination and depyrimidation proceed spontaneously at significant rates even at physiological pH and temperature, and may require repair for normal maintenance of DNA (Verly, 1975). Increasing temperature and pH

results in increasing rates of hydrolysis, as does alkylation of the DNA with chemical alkylating agents. The ALB's formed by ionizing radiation are the result either of direct attack of hydroxyl radicals on the deoxyribose C-1 or of base damage which weakens the N-glycosidic bond thus leading to base elimination. Loss of a purine or pyrimidine allows the furanose form of the deoxyribose to assume the open chain form with a free aldehyde group. Such lesions are still relatively stable under *in vivo* conditions, and only lead to breaks, by β -elimination, upon treatment with heat or alkali. This explains the differences seen on neutral and alkaline gradients. At low pH, however, the free aldehyde may cross-link to the complementary DNA strand forming a rapidly-sedimenting fraction. Such cross-linking of DNA has not been studied extensively, but can also be caused by a number of other agents, and may be repairable (Cole and Sinder, 1975). Repair of apurinic DNA appears to be highly efficient, proceeding by an excision pathway involving endonucleases specific for the apurinic sites (Verly, 1975; Lindahl and Ljungquist, 1975). No enzyme for direct replacement of the free purine has yet been reported.

The contribution of these various radiation-induced DNA lesions to biological inactivation has been elegantly studied by van der Schans *et al.* (1973). Supercoiled CCC DNA from ϕ X174 and PM2 were irradiated with ^{60}Co gamma-rays in oxygenated solution. From neutral sucrose gradient sedimentation measurements, the average number of SS-breaks and DS-breaks per molecule was first determined. Linearity of SS-break formation with dose was observed, but for DS-breaks, the curve was non-linear, becoming much steeper above 1 Krad. This was probably due to formation of DS-breaks from two nearly-adjacent SS-breaks in opposite strands. The

biological activity of the irradiated DNA and of mildly denatured and membrane-filtered fractions was then tested for transforming activity on bacterial spheroplasts. By combining this data, it was possible to calculate the percent contribution of SS-breaks, DS-breaks, and base damage (all other types) to biological inactivation. Surprisingly, DS-breaks, whose inactivation efficiency is 100%, only contributed 3-5% to biological inactivation. About 98% of the SS-breaks were non-lethal, and they therefore only accounted for about 8% of the inactivation. Apparently in this system, base damage was by far the major cause of biological inactivation.

The relative and total amounts of each type of damage formed are affected by a large number of factors, however. For example, Bonura *et al.* (1975c) have recently shown that 50 kVp X-rays were more efficient than ^{137}Cs gamma-rays for cell killing and for production of DS-breaks and unrepairable SS-breaks in *E. coli*. The nature and extent of the damage thus appear to be highly dependent on the linear energy transfer (LET) of the radiation involved, and must be considered whenever studies are compared. In addition, even in studies using the same form of radiation, there are differences of several orders of magnitude in the reported rates of formation of SS-breaks (Town *et al.*, 1972). The type of DNA (eg. phage, prokaryotic, or eukaryotic), its state during irradiation (eg. *in vivo*; *in vitro* - dry, frozen, or in solution), and the presence of radiosensitizers or radioprotective compounds must also be considered (Bonura and Smith, 1976).

The most thoroughly studied radiation modifier is oxygen. Most cells are two to three times more sensitive to killing by ionizing

radiation when irradiated in the presence of oxygen rather than under anaerobic conditions. Town *et al.* (1972) proposed that this effect was due to a qualitative difference in the type of DNA damage produced, rather than in the actual number of lesions. When *E. coli* was rapidly analyzed by alkaline sucrose gradient centrifugation, twice as many SS-breaks were observed after X-irradiation in the presence of oxygen as with irradiation under a nitrogen atmosphere. An ultrarapid repair pathway (Type I Repair) was proposed to explain this difference. According to this theory, breaks produced under nitrogen were rapidly repaired by direct action of DNA ligase, whereas irradiation in oxygen produced different end-groups at the SS-break which could not be used directly by polynucleotide ligase as a substrate. More recent work, however, has disproved this theory. Sapora *et al.* (1975) and Fox *et al.* (1976) have developed ultrarapid lysis techniques which permit SS-break determinations within 10msec after irradiation. No enzyme-dependent repair is observed between 10msec and 2 seconds, and the observed difference in the initial number of SS-breaks is therefore attributed to radiochemical processes.

At least two other pathways do exist for enzymatic repair of radiation-induced SS-breaks in *E. coli*. Town *et al.* (1973) were able to show that wild-type *E. coli* held in buffer after irradiation were able to repair 90% or more of their SS-breaks, while a *polA-1* mutant deficient in DNA polymerase I lacked this activity (Type II Repair). Similarly, they were able to show that transfer to growth medium after maximal repair in buffer resulted in repair of an additional 2-3 breaks per cell, and was dependent on *recA* and *recB* genes (Type III Repair). More recent work has further elaborated on the genetics and enzymology of excision repair,

showing the involvement of the *polC* gene product, DNA polymerase III, and the *exrA* gene, and overlap with the *uvr*-gene dependent pathway for repair of ultraviolet radiation damage (Youngs *et al.*, 1974). Very similar repair pathways have also been revealed in several other prokaryotic and eukaryotic systems (Hanawalt and Setlow, 1975).

The situation is not nearly as clear for repair of DS-breaks. The greater number of DS-breaks found after irradiation of *polA-1* cells appears to be caused by unsuccessful excision repair in this strain, rather than due to repair of DS-breaks in the *pol*⁺ strain (Bonura *et al.*, 1975a). Indeed, since Kaplan's (1966) initial report that *E. coli* could not repair DS-breaks, most workers have concentrated on more radiation-resistant bacteria. Kitayama and Matsuyama (1968,1971) demonstrated repair of DS-breaks by neutral sucrose gradient analysis of gamma-irradiated *Micrococcus radiodurans*. Precise quantitation of the breakage and repair rates was not possible, but repair was shown to be inhibitable by chloramphenicol, tetracycline, and mitomycin D. Working with the same *M. radiodurans*, Burrell *et al.* (1971) and Burrell and Dean (1975) also demonstrated repair of DS-breaks produced by X-irradiation. In their system, as many as 100 DS-breaks/genome could be repaired. This process seemed to involve binding of the broken DNA to the plasma membrane, forming a rapidly sedimenting complex which released DNA of normal molecular weight upon further incubation in growth medium. A similar involvement of membrane-bound DNA in DS-break repair was also observed by Baraldi and Sinskey (unpublished results) for a highly radiation-resistant strain of *Salmonella typhimurium* LT-2 developed by Davies (Davies and Sinskey, 1973; Davies *et al.*, 1973). All of these

studies shared common problems which interfered with more detailed analysis, however. First among these was the difficulty of reproducibly obtaining uncontaminated and unbroken DNA of genome-size molecular weight ($>10^9$ daltons) under neutral lysis conditions. Anomalous sedimentation patterns were also obtained in which the profiles were non-Gaussian, with sharpened leading edges.

B. Rotor Speed Effect

Kavenoff (1972) developed a very gentle lysis procedure for *Bacillus subtilis* which differed significantly from the normal sodium dodecyl sulfate (SDS) mediated lysis of lysozyme/EDTA derived spheroplasts. Instead, cells were treated overnight with a pronase/Brij-58/lysozyme solution before mixing with Sarkosyl NL-97 on top of neutral sucrose gradients. When the gradients were centrifuged, the calculated sedimentation coefficients showed a strong rotor speed dependence, being inversely proportional to ω^4 for low rotor speeds (<7000 rpm). The faster the gradients were centrifuged, the lower the S-value that was calculated. When S-values were extrapolated to zero rotor speed ($S^0 \approx 140$) and the molecular weight calculated ($1.8-2.5 \times 10^9$ daltons), they agreed closely with the value expected for the intact genome. It thus appeared that all previous workers on large DNA's had seriously underestimated the molecular weights since rotor speeds were generally greater than 2×10^4 rpm. Further work with gentle lysis procedures and speed dependence was conducted by Levin and Hutchinson (1973). *Bacillus subtilis* protoplasts were lysed by prolonged incubation (≈ 20 hours) in a rigid-walled dialysis cell with pronase and SDS at 50°C , and then analyzed on neutral sucrose gradients. Speed dependence was again observed and agreed well with a

theory proposed by Zimm (1974). At low rotor speeds, a broad band of material sedimented ahead of the main DNA peak. This rapidly sedimenting component formed a sharp peak at higher rotor speeds, and could be converted to the slower-sedimenting main peak DNA by low-level gamma-irradiation. Additional investigations of this phenomenon by Hariharan and Hutchinson (1973) indicated that a gamma-ray dose which produced 1 DS-break/genome was required to convert the rapidly sedimenting material to the main DNA peak. The rapidly-sedimenting peak reappeared after incubation in growth medium, and corresponded with the ability of the cells to form colonies. The authors proposed that the fast-sedimenting component was the circular, intact genome, and that its reappearance was an indication of repair of DS-breaks. Unfortunately, this study was conducted using high rotor speeds and speed dependence was not taken into account. Their conclusions were therefore questionable, and it was more likely that the fast-sedimenting component was an association with membrane, due to the heterogeneous nature of the material at low rotor speeds (Levin and Hutchinson, 1973), and because of the strong rotor speed dependence (Hecht *et al.*, 1977). Another recent study has highlighted the importance of using low centrifuge speeds by demonstrating that a low molecular weight DNA can actually be made to sediment faster than a higher molecular weight DNA at sufficiently high rotor speeds (Chia and Shumaker, 1974).

By adopting more gentle lysis procedures and allowing for speed dependence, several workers have recently re-investigated the formation and repair of both SS-breaks (Youngs and Smith, 1976b) and DS-breaks in *E. coli*. Bonura *et al.* (1975b) measured DS-breakage by X-rays under

aerobic and anaerobic conditions, finding 1.3-1.4 DS-breaks per lethal hit and therefore suggesting that DS-breaks still were not repairable. However, their number-average molecular weight for unirradiated DNA was $\approx 4 \times 10^8$ daltons or about one-seventh of an intact genome, indicating that their background level of DS-breaks in unirradiated DNA was six or more per genome. Furthermore the lowest X-ray dose used to measure any additional breakage was 20 Krad, which corresponds to $\approx 10^{-4}$ survival.

The only positive evidence for repair of DS-breaks in *E. coli* has come from Krisch *et al.* (1976). Cells were labeled with 5- $[^{125}\text{I}]$ iodo-deoxyuridine (a thymine analogue) and then stored frozen (-196°C) to allow for radioactive decay. Thawed cultures were analyzed for both DS-breaks, with consideration for rotor speed effects, and for the number of $[^{125}\text{I}]$ atoms incorporated into the DNA. The efficiency for DS-break formation due to $[^{125}\text{I}]$ decay was found to be 1.01 ± 0.05 in *rec+* cells, and 1.02 ± 0.23 in *recA* cells. Incubation of thawed cultures in growth medium for one hour permitted the repair of a maximum of 3-4 DS-breaks/genome in the *rec+* cells, with no repair in the *recA* cells. A 1:1 correlation was obtained from survival curves for both strains, between the number of lethal hits and the number of residual DS-breaks per cell.

C. Ultraviolet Radiation Damage and Repair

The major photoproduct resulting from UV (254nm) irradiation of DNA both *in vivo* and *in vitro* is the cyclobutane-type pyrimidine dimer (Hanawalt and Setlow, 1975). In *uvr+* cells, excision repair is highly efficient and is capable of removing hundreds of dimers/genome. The formation and repair of UV-induced incision breaks has been studied using alkaline sucrose gradients (Youngs *et al.*, 1974). Some repair occurred

when irradiated cells were incubated in buffer, and was dependent on DNA polymerase I, since *polA-1* strains showed greatly reduced levels of repair. Significant amounts of additional repair occurred when cells were subsequently incubated in growth medium, unless the cells contained *recA-13*, *recB-21*, or *exrA* mutations. Thus two branches for *uvr*-gene dependent excision repair exist (Youngs and Smith, 1973) which closely resemble Type II and III repair described earlier for ionizing radiation damage. Enzymatically induced DS-breaks, resulting from the overlap of excision gaps in opposite DNA strands, have been implicated as the ultimate lethal lesion in *uvr+* cells (Bonura and Smith, 1975a,b).

More recent investigations, however, have indicated that additional photoproducts are also involved. Youngs and Smith (1976a) have reported on the formation of SS-breaks in UV-irradiated *uvrA* and *uvrB* strains which lack the *uvrA-uvrB* endonuclease (Braun *et al.*, 1975). Some of these SS-breaks were produced by direct photochemical events, while the remainder appeared to result either from breakdown of a thermolabile photoproduct or as the result of *uvrA-uvrB*-independent incision. Such breaks were still formed after photoreactivation, indicating that they did not involve cyclobutane-type pyrimidine dimers. Repair of these SS-breaks required DNA polymerase I and polynucleotide ligase, but not the *recA*, *recB*, *recF*, *lexA*, or *uvrD* gene products.

Similar evidence for the involvement of other UV-photoproducts has come from irradiation of CCC phage DNA's. Denhardt and Kato (1973) reported an increase in the sedimentation rate of UV-irradiated ϕ X174 superhelical, replicative form I DNA. Photoreactivation appeared to reverse this effect, and the authors proposed that the formation of

cyclobutane-type pyrimidine dimers had altered the pitch of the helix, causing an increase in the superhelix content of the molecule, which resulted in an increased rate of sedimentation. Veldhuisen *et al.* (1976) have seriously questioned this conclusion, however. An endonucleolytic activity had been associated with the photoreactivating enzyme preparation used by Denhardt and Kato (1973) which resulted in the formation of a considerable amount of component II material. The component I peak did not completely return to the pre-irradiated position either. The experiment was repeated using CCC PM2 DNA. The increase in sedimentation coefficient was still observed, but photoreactivation of this preparation caused no further change. Complete photoreactivation was verified in these experiments by the use of *Micrococcus luteus* UV-endonuclease. It therefore appeared that a UV-photoproduct other than cyclobutane-type pyrimidine dimers was responsible for the observed increase in the sedimentation rate.

D. The Folded Chromosome of *Escherichia coli*

To date, all of the lysis techniques employed to isolate bacterial DNA in repair experiments have yielded the native, random-coil, linear form of the molecule. However, by lysing spheroplasts of *E. coli* with mild, non-ionic detergents in the presence of suitable counterions, it is possible to isolate the DNA as a condensed, non-viscous nucleoid, similar in appearance to the chromosome observed *in vivo* (Pettijohn, 1977). This was first accomplished by Stonington and Pettijohn (1971) who lysed spheroplasts of *E. coli* with Brij-58 (polyoxyethylene 20-cetyether) and deoxycholate in 1M NaCl. Neutral sucrose gradient analysis of the lysate revealed that the DNA sedimented as a highly-compact, 3200S

structure, whose composition was 80% DNA, 10% RNA, and 10% protein by weight. Polyacrylamide gel electrophoresis of the proteins indicated that they were primarily the α , β , β' subunits of RNA polymerase, while the RNA was half nascent messenger (mRNA) and half ribosomal (rRNA). The complex was stable to treatment with pronase or trypsin, but showed a marked increase in viscosity upon treatment with RNase, SDS, or heat. Measurement of the DNA molecular weight by unfolding the nucleoid with 0.5% SDS on neutral sucrose gradients revealed that it was of intact, genome size ($>10^9$ daltons). It was further shown that high ionic strength (1M NaCl) was critical for isolation of the complex, but could be reduced significantly (0.1M NaCl) after purification without viscosity increase. This rapidly sedimenting DNA complex was believed to represent the compact isolated form of the bacterial "nuclear body" which had been observed in electron micrographs. Due to the extreme sensitivity to RNase, it was further proposed that RNA played a role in maintaining this compact packaging of the DNA.

By using a procedure which differed mainly in the temperature of lysis, Worcel and Burgi (1972) were able to isolate a similar DNA complex with a somewhat lower sedimentation coefficient range of 1300-2000S. Amino acid starvation of the cells, to line-up the chromosomes at initiation, caused a shift in the profile toward the 1300S component. Readdition of the required amino acid brought about a return to the original distribution, prompting the authors to propose that the 1300S fraction represented single chromosomes lined-up at initiation, while the 2200S fraction was newly-replicated, double chromosomes. Their most interesting finding, however, was evidence of supercoiling in the DNA

complex, based on titration with ethidium bromide. When purified fractions of the DNA complex were rerun in gradients with ethidium bromide, the sedimentation coefficient first decreased to a minimum (900S) at a critical dye concentration of $2\mu\text{g/ml}$ ($5\mu\text{M}$), and then increased to slightly more than their original value. This behavior was identical to that observed for covalently-closed, circular (CCC) DNA's (Vinograd *et al.*, 1965, 1968). All CCC DNA's are believed to contain negative superhelical turns *in vitro*. Intercalation of ethidium bromide changes the pitch of the double helix (Wang, 1969), unwinding these negative superhelical turns and relaxing the molecule. This results in a decrease in the observed sedimentation coefficient, which reaches a minimum when all of the negative superhelical turns have been unwound. Further increase in the concentration of ethidium bromide then introduces positive superhelices and the sedimentation coefficient again increases. The presence of only one SS-break allows the DNA molecule to rotate and destroys this behavior. Such molecules remain relaxed, cannot have superhelical turns, and show only a slight decrease in sedimentation coefficient with increased binding of ethidium bromide due to increases in the frictional coefficient of the DNA. The *E. coli* DNA complex thus behaved as if there was not a single nick in the entire chromosome. Worcel and Burgi (1972) then deliberately nicked the DNA complex with DNase I in an attempt to relax the molecule and remove the supercoiling. Rather than requiring only one break per molecule, however, between 12 and 80 were needed to completely relax the structure based on measurements of SS-breaks from alkaline sucrose gradient profiles of the complex. A model for the chromosome of *E. coli* was developed based on all of these observations.

The chromosome was proposed to contain about fifty loops or folds, each of which was supercoiled. Nicking of one loop would only relax the supercoiling of that loop, and not others. The folded structure was maintained by a stabilizing "core" of RNA and protein which could be disrupted by RNase, SDS, or heat to yield a completely relaxed and unfolded DNA molecule *à la* Cairns (1963).

Further work by Pettijohn *et al.* (1973) revealed that the difference in sedimentation coefficients obtained by the two groups was due to differences in the lysing temperature. Lysis at 0-4°C results in 3200S nucleoids, whereas lysis at 20°C yields nucleoids with a mean sedimentation coefficient of 1600S. The more rapidly-sedimenting nucleoids were shown to be associated with a specific fraction of the cell membrane due to the presence of specific membrane proteins, lipids, and phospholipids (Pettijohn *et al.*, 1973; Worcel and Burgi, 1974). Direct examination of such nucleoids in the electron microscope also revealed membrane fragments associated with the DNA (Pettijohn *et al.*, 1973; Delius and Worcel, 1974). The 1600S nucleoids, however, were membrane-free and were shown to be a highly efficient template for *in vitro* RNA (Pettijohn *et al.*, 1973; Giorno *et al.*, 1975b) and DNA (Kornberg *et al.*, 1974) synthesis.

Worcel and Burgi (1974) extended their investigation of the folded chromosomes to include the membrane attached form. The free-sedimenting 1600S nucleoid could be released from the membrane-associated complex by *in vitro* incubation with 1% Sarkosyl for 15 minutes at 0°C. Release was also observed following amino acid starvation, with reattachment occurring only after readdition of the required amino acid, and resumption of protein and DNA synthesis. The authors suggested that DNA was released from the

membrane upon completion of a round of replication and required protein synthesis and reattachment to the membrane for initiation of a subsequent round of replication. These findings were questioned by Ryder and Smith (1974). By eliminating the 4000xg centrifugation of the crude lysate prior to sucrose gradient analysis, they were able to obtain quantitative recovery of membrane-associated folded chromosomes without any change in the sedimentation profile. Inhibition of protein synthesis by amino acid starvation or antibiotics resulted in formation of very rapidly sedimenting membrane associated nucleoids (5000-6000S). The release and reattachment pattern of Worcel and Burgi (1974) was not observed, and was believed to have been an artifact of the pre-centrifugation step which would have selectively removed this rapidly sedimenting form. Their original hypothesis was further questioned in light of a report that protein synthesis was required for replication of the terminal 0.5% of the chromosome (Marunouchi and Messer, 1973). This conflict was recently resolved by Korch *et al.* (1976). The 4000xg centrifugation was clearly shown to select for a small sub-fraction of the total DNA and the rapidly-sedimenting structure (6000S) obtained from amino acid starved cells was shown to contain cell wall components as well as membrane. This was due to poorer lysis of starved cells and could be corrected for by increased incubation with lysozyme. The resulting membrane-attached nucleoids still sedimented slightly faster than those from exponentially growing cells, which the authors proposed was due to blockage of terminal DNA replication and the accumulation of nearly-double chromosomes.

Isopycnic centrifugation in CsCl density gradients had been used by Giorno *et al.* (1975a) to measure the buoyant density of formaldehyde-

fixed nucleoids. Membrane-free nucleoids band at 1.69 ± 0.02 gm/cm³, while membrane-associated nucleoids are less dense, banding at 1.46 ± 0.02 gm/cm³. The ration of DNA:RNA:protein in folded chromosomes appears to be constant due to the nearly monodisperse appearance of the bands in CsCl, regardless of the position in the heterogeneous preparative sucrose gradient profile from which the nucleoids were obtained.

Several stringently controlled plasmid DNA's have been shown to co-sediment with the folded chromosome in both *E. coli* K-12 (Kline and Miller, 1975), and also in *Salmonella typhimurium* LT-2, from which membrane-free nucleoids have recently been isolated using Triton X-100 and deoxycholate to lyse spheroplasts (Manis *et al.*, 1976).

The factors responsible for stabilizing the folded chromosome have also been investigated. Flink and Pettijohn (1975) found that the polyamine spermidine could successfully substitute as a counterion to maintain the folded chromosome at reduced ionic strength, during storage, and during thermal treatment. Dworsky (1975a,b) developed a very gentle lysis procedure based on this finding which permitted isolation of nucleoids from cells treated with rifampicin to block RNA synthesis. The final detergent and NaCl concentrations were reduced (0.07% Brij-58, 0.03% deoxycholate, 0.2M NaCl) and 3mM spermidine was added along with a third detergent, cetyltrimethylammonium bromide (0.07%). The membrane-associated nucleoids obtained by this procedure were initially stable *in vitro*, but slowly unfolded during incubation in the lysing solution. A comparison of nucleoids obtained by this lysis procedure with those obtained using the original method of Stonington and Pettijohn (1971) has also been published (Dworsky, 1976), and indicated that membrane

could stabilize folded chromosomes at low salt concentrations or after treatment with rifampicin *in vivo* or RNase *in vitro*. By combining sedimentation and viscometry, Drlica and Worcel (1975) concluded that prolonged RNase treatment of nucleoids resulted in only partial unfolding of the chromosome yielding structures with sedimentation coefficients of 400-500S. Complete unfolding required treatment with trypsin, heat (60-70°C), or SDS to produce material which sedimented at 130S at 7000 rpm. This seemed to indicate that proteins were involved in stabilization of the folded chromosome.

Hecht *et al.* (1977) have recently questioned the involvement of proteins in the stabilization of RNase unfolded chromosomes after careful consideration of rotor speed effects. In the course of their investigation, these authors discovered that the sedimentation coefficients of both membrane-free and membrane associated chromosomes were inversely related to rotor speed, in a manner analagous to the effect observed for large, extended DNA molecules. Apparently, the loops of the folded chromosome were reversibly extended at high centrifugal fields, slowing the rate of sedimentation. The relative rotor speed effect was enhanced as the chromosomes were relaxed with ethidium bromide or unfolded by RNase, SDS, or heat. The maximal effect was observed for completely unfolded and deproteinized chromosomes ($S^0=320$), and was similar to the theoretical rotor speed effect predicted for a non-replicating, linear genome-equivalent of *E. coli* DNA ($S^0=150-200$). The value of the sedimentation coefficient for limit digestion of chromosomes with RNase was found to be $S^0=450$, which agreed well with the value published by Drlica and Worcel (1975) of 400-500S. However, the $S^0=320$ for deproteinized chromosomes

was significantly higher than the 130S value obtained by Drlica and Worcel (1975). Part of this difference was due to the fact that Hecht *et al.* (1977) had used different sedimentation markers for the two different types of unfolded chromosomes. The remainder of the difference may have been due to mass differences in the structures caused by the different lysis techniques (with and without Sarkosyl). Hecht *et al.* (1977) therefore also proposed that the slight difference between the sedimentation coefficients of the RNase digested and thermally unfolded chromosomes was also due to mass differences, and not due to a loss of protein-mediated folding which was resistant to RNase digestion. They finally proposed that the deproteinized nucleoid ($S^0=320$) represents the intact, unfolded, replicating *E. coli* DNA.

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A. Chemicals

1. General

Sarkosyl NL-97 (sodium N-laurylsarcosinate) was a gift of CIBA-GEIGY Dyestuffs & Chemicals Division, Greensboro, North Carolina. Fluorinert[®] FC-48 was a gift of 3M Company. Angio-CONRAY[®] (sodium iothalamate) was purchased from the Pharmaceutical Division of Mallinckrodt, Inc. Sucrose was density gradient grade (ribonuclease-free) obtained from Schwarz/Mann. All other general chemicals were purchased from Sigma Chemical Co.

2. Radiochemicals

Methyl- $[^3\text{H}]$ -thymidine (52.5 Ci/mmol), methyl- $[^{14}\text{C}]$ -thymine (52.5 Ci/mmol), $[^{14}\text{C}]$ -leucine (>270 mCi/mmol), Aquasol[®], and Mini-vials[®] were all purchased from New England Nuclear.

3. Enzymes

Egg white lysozyme (muramidase) Grade I, 3X crystalized, 25,000 U/mg was obtained from Sigma Chemical Co. Ribonuclease (bovine pancreas) A Grade, 5X crystalized, 64 Kunitz Units/mg was purchased from Calbiochem. Deoxyribonuclease I (bovine pancreas) RNase-free (<0.0005%), 2197 U/mg was a product of Worthington Biochemical Corp.

B. Organisms

All bacterial strains used in this study were derived from *Escherichia coli* K-12. Their designations and properties are listed in Table 1. Strains KU0100-KU0104 were kindly provided by Dr. David Botstein, Biology Department, M.I.T. as his strains D5608, D15, D16, D17, and D5407 respectively. The first four of these were originally from the collection of Dr. Paul Howard-Flanders (Howard-Flanders *et al.*, 1966; Howard-Flanders and Theriot, 1966; Emmerson, 1968) and were designated AB1157, AB1885,

TABLE 1: Strain characteristics for *Escherichia coli* K-12

Strain Number & Origin	uvrB-5				thy ⁺	arg						lac						T1			λ	T4	STREP	MITO C	SUP	SEX
	uvrB-5	uvrC-34	recA-13	polA-1		arg	his	leu	pro	thr	thi	lac	gal	ara	xyl	mtl	rha	T1	T6	T4						
KU0100 D5608 AB1157	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	+	S	S	S	S	S	R R	R R	Pm ⁺	F-
KU0101 D15 AB1885	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	+	S	S	S	S	S	R S	R S		F-
KU0102 D16 AB1884	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	+	S	S	S	S	S	R S	R S		F-
KU0103 D17 AB2463	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	+	S	S	S	S	S	R S	R S		F-
KU0104 D5407 JG112 W3110	+	+	+	-	-	+	+	+	+	+	+	-	+	+	+	+	-						R			F-
KU0105 +trim* KU0100	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	S	S	S	S	S	R R	R R	Pm ⁺	F-
KU0106 +trim KU0101	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	S	S	S	S	S	R S	R S		F-
KU0107 +trim KU0102	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	S	S	S	S	S	R S	R S		F-
KU0108 +trim KU0103	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	S	S	S	S	S	R S	R S		F-

* trim = trimethoprim selection

+thy⁻ strains will grow at 2 µg/ml thymine.

AB1884, and AB2463. Strain D5407 was a derivative of W3110 from the original collection of Dr. J. Lederberg (Bachmann, 1972). Strains KU0105-KU0108 were isolated after trimethoprim selection (Miller, 1972) by this author. Strains were selected which were capable of growing at a thymine concentration of 2 $\mu\text{g/ml}$ (low-level thymine requirement). Originally, all strains were maintained on LB-agar slants at 4°C. Recovery was found to be improved, however, if strains were stored at room temperature and transferred bi-weekly.

C. Media and Buffer Solutions

M9 buffer contained the following ingredients per liter: Na_2HPO_4 7gm, KH_2PO_4 3gm, NaCl 0.5gm, NH_4Cl 1gm, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.25gm. Phosphate buffer was 0.067 M at pH 7.0 and contained 3.56gm of $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ and 5.79 gm of Na_2HPO_4 per liter. LB broth contained 10gm Bacto-tryptone, 10gm NaCl , and 5gm Bacto-yeast extract per liter. All agars contained 15gm/l Bacto-agar. All media components were obtained from Difco.

D. Culture Conditions

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

Cultures were routinely grown in side-arm flasks at 37°C with constant shaking (≈ 200 rpm) to provide aeration.

E. Radioactive Labeling of Cultures

DNA was labeled by growing cells in M9 minimal medium with a low concentration of thymine (2 μ g/ml). If nucleoids were to be sedimented directly, 10 μ Ci (10 μ l) of [3 H]-thymidine was added to the culture when inoculated. If a preparative gradient was to be run to obtain purified nucleoids for *in vitro* experiments, this amount of label was usually doubled.

F. Ultraviolet Irradiation

A UV cabinet containing a single, GE germicidal lamp (G8T5) was used for all UV-irradiations. New lamps were aged for 20 hours and the lamp was always warmed-up prior to use. The dose delivered to the sample was measured by an International Light IL-600 research photometer equipped with a 254nm interference filter and a Model IL620 Integrator. Cell or nucleoid suspensions were irradiated with shaking in a watch glass or in a white porcelain spot-plate at the desired temperature. The dose rate was adjusted to 2.0 J/M²·sec.

G. Gamma-Irradiation

A Gammacell 220, manufactured by Atomic Energy of Canada Limited was used for all gamma-irradiations. This ^{60}Co source was calibrated at the factory using an ionization chamber. The central dose rate (CDR) on May 15, 1972 was 6.15×10^5 Roentgen/hour $\pm 10\%$. Using the relationship that 1 Roentgen = 0.869 rad in air (Etter, 1965), this converts to 11,795 rad/min. This value agrees well with the calibration obtained in this lab on November 3, 1973 using Fricke dosimetry (Davies and Sinskey, 1973). The CDR obtained in this calibration was 9657 rad/min and the calculated rate based on the amount of radioactive decay which had occurred since the first

calibration was 9680 rad/min. Using the contoured lead attenuator to reduce the dose rate resulted in a 44% reduction of the CDR. The standard procedure for gamma-irradiation was to place the cell or nucleoid suspension in a 12x75mm glass test tube which was held in an ice-water slurry inside a dewar flask. The dewar flask then fit inside the lead attenuator of the Gammacell. The suspensions remained aerobic as determined by oxygen electrode measurements.

H. Survival after UV-Irradiation

Strains KU0100-KU0104 were grown overnight at 37°C in M9 minimal medium. Fresh 20ml prewarmed cultures were inoculated with 0.4ml of the overnight cultures and grown to $\approx 10^8$ cells/ml (31-33 Klett). Cells were harvested by Millipore® filtration (0.22µ HA) and washed with 10ml of M9 salts. The cells were then resuspended in 20ml of M9 buffer and irradiated at 2.0J/M²·sec by shaking a 10ml aliquot at room temperature in a watch glass. Samples were withdrawn at intervals, diluted in phosphate buffer at 0°C and plated in duplicate (0.1ml/plate) on LB agar. All manipulations were carried out under dim light in order to minimize possible photoreactivation. Plates were incubated for 48 hours at 37°C and colonies were counted.

I. Survival after Gamma-Irradiation

Strains KU0104, KU0105, and KU0108 were grown overnight in M9 minimal medium. Fresh 20ml cultures were inoculated with 0.2ml of the overnight cultures and grown with shaking at 37°C for 4½-5 hours and then quickly cooled in ice-water and harvested by centrifugation at 5000xg for 5 minutes. The cells were washed with 20ml M9 salts (plus 10µg/ml thymine), centrifuged a second time, and finally resuspended in 12ml of M9 salts

plus thymine. Aliquots were held on ice in 12x75mm glass test tubes during irradiation. The dose rate with the lead attenuator was ≈ 3.3 Krad/min. Dilutions were made in phosphate buffer and the cells were plated in duplicate (0.1ml/plate) on M9-minimal medium plates. These were incubated at 37°C for 48 hours before colonies were counted.

J. Isolation of Folded Chromosomes

The development of this method is described in detail in Appendix A. Cultures were routinely grown into early exponential phase ($\approx 3-4$ hours), rapidly chilled in ice-water, and harvested by centrifugation at 5000xg for five minutes. The supernatant was decanted and the pellet was resuspended in Solution A unless the experiment called for washing of the cells or some other procedure first. Solution A contained 20%(w/v) ribonuclease-free sucrose, 0.1M NaCl, and 0.01M Tris-HCl 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/0.2ml. A 0.2ml aliquot of the diluted cell suspension was placed in a 12x75mm glass test tube and held on ice. Solution B contained 0.12M Tris-HCl buffer (pH 8.1), 50mM EDTA, and 4mg/ml egg white lysozyme. Fifty microliters of Solution B at 0°C was added to the test tube and vortexed slightly. The test tube was then transferred to a water bath at 25°C for 5 minutes with occasional swirling. The tube was then returned to the ice-water bath for 1 minute before the addition of 0.25ml of Solution C. This solution contained 1%(w/v) Brij-58 (polyoxyethylene 20-cetyl ether), 0.4%(w/v) sodium deoxycholate, 2%(w/v) Sarkosyl NL-97 (sodium N-laurylsarcosinate), 2.0M NaCl, and 10mM EDTA. The detergent mixture (at 0°C) was added to the test tube and swirled gently to mix with the spheroplasting suspension. The turbid solution

cleared completely within several seconds, but was held on ice for 15 minutes before gently layering the lysate onto sucrose gradients with a Clay-Adams pipette fitted with a wide-bore plastic tip. A 0.2ml portion of the lysate was usually counted to determine the amount of radioactivity put on the gradient as a check on recovery during fractionation.

K. Sucrose Gradient Centrifugation

All gradients were made-up with gradient base containing 1.0M NaCl, 0.01M Tris-HCl buffer (pH 8.1), 1mM EDTA, and density gradient grade (ribonuclease-free) sucrose. The 10-50% gradients used for analysis of membrane-associated folded chromosomes were made individually using a Buchler gradient mixing chamber and pump. The high-density shelf was composed of 50%(w/v) sucrose dissolved in Angio-CONRAY® (80% sodium iothalamate) solution. The 0.3ml shelf was just sufficient to fill the rounded portion of the cellulose nitrate centrifuge tube. A 4.8ml gradient was then formed on top of the shelf, and the gradients were cooled to 4°C before use. A Beckman Density Gradient Former was used to make 5-20%(w/v) and 10-30%(w/v) gradients three at a time. With this gradient maker, the solutions are combined in a mixing chamber by means of a cam-driven precision syringe drive. All gradients were made in untreated 1/2x2 inch cellulose nitrate centrifuge tubes. Gradients were centrifuged in an SW50.1Ti rotor in a Beckman L5-75 ultracentrifuge at 4°C. For preparative gradients, 20,000 rpm was used for a final $\omega^2 t$ of 6.00×10^9 rad²/sec using the Preset Mode on the built-in $\omega^2 t$ integrator. For analytical runs, the rotor speed was adjusted to ≈ 3000 rpm to minimize any rotor speed effects for nucleoids or unfolded DNA (see Appendix D).

Gradients were fractionated using an ISCO Model 640 Density Gradient

Fractionator set at 0.5ml/min flow rate and 0.2ml/fraction using Fluorinert[®] FC-48 (3M Company) as a dense chase solution. Fractions were collected directly into Beckman Mini-Vials[®] unless the nucleoids were being purified for *in vitro* experiments. In that 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, 3ml of Aquasol[®] (plus 10%v/v H₂O) was added to the vial, shaken, and then counted in a Beckman LS-230 ambient temperature scintillation counter.

L. In Vitro Unfolding of Nucleoids

1. RNase

Pooled peak fractions from a 10-30% preparative gradient were carefully diluted with gradient base by gently rocking the test tube to mix the solutions, and then treated as necessary for the experiment. Before gently adding the nucleoid sample to the gradient with a Clay-Adams pipette, a 0.5ml cap containing 3.5% sucrose and 20µg/ml RNase in gradient base was layered on top of the 5-20%(w/v) neutral sucrose gradient. After the sample was applied, the gradients were loaded into the rotor at 4°C and the low-speed centrifugation was started.

2. Thermal

Alternatively, nucleoids were prepared and diluted as above and then applied to 5-20% neutral sucrose gradients without caps. Instead 0.2ml of hexadecane was floated over the sample and the entire gradient was incubated in a 72°C oven for about 45 minutes. The gradients were then cooled to 4°C before loading in the rotor and beginning centrifugation.

IV. RESULTS

A. UV-Irradiation

1. Survival Curves

The survival curves for UV-irradiated wild-type, *uvrB-5*, *uvrC-34*, *polA-1*, and *recA-13* strains of *Escherichia coli* K-12 are shown in Figure 1. These results agree well with those of Youngs and Smith (1973) for related strains irradiated under similar conditions. The slightly higher survival of the wild-type strain used in this study may reflect a difference between the AB1157 background and that of the W3110 parent strain from which the *polA-1* mutant was derived (Youngs and Smith, 1976).

2. *In Vitro* UV-Irradiated Nucleoids

A fairly rapid increase in the sedimentation rate of *in vitro* UV-irradiated nucleoids is observed for fluences up-to about 100 J/M² (Figures 2 & 5). Little further increase is seen at higher doses, with a total change amounting to $\approx 12\%$ increase of the sedimentation coefficient at 1200 J/M². A comparison of these results with those obtained for *in vitro* UV-irradiation of supercoiled CCC DNA's from bacteriophages ϕ X174 RFI and PM2 is provided in Table 2. The sedimentation coefficients of the phages as well as their molecular weights are orders of magnitude different from the nucleoid, but the relative changes induced by UV-irradiation are quite similar and occur at similar UV doses.

3. *In Vivo* UV-Irradiated Nucleoids

When the wild-type strain KU0105 is irradiated with a similar dose of UV *in vivo*, a very large initial decrease in the average sedimentation coefficient of the nucleoid is observed (Figures 3 & 5). Between 40 and 360 J/M² the sedimentation coefficient is fairly constant, running between 80-85% of the unirradiated value. Above this dosage, the sedimentation

FIGURE 1. SURVIVAL CURVES FOR UV-IRRADIATION OF *ESCHERICHIA COLI* K-12.

Early exponential phase cultures in M9 medium were harvested by Millipore filtration, washed with M9 buffer, and resuspended in the same buffered salt solution for irradiation at room temperature. Cell suspensions were irradiated in a shallow layer (1-2 mm) in a watch glass with constant shaking. Aliquots were removed at intervals, diluted in cold phosphate buffer, and plated in duplicate on LB agar (0.1ml/plate). The plates were incubated at 37°C for 48 hours prior to counting colonies. The dose-rate was set at 2.0 J/M²·sec and the single G8T5 germicidal lamp was warmed-up for ½ hour prior to use.

FIGURE 2. GRADIENT PROFILES FOR *IN VITRO* UV-IRRADIATED FOLDED CHROMOSOMES.

Purified nucleoids from a preparative gradient were diluted in gradient base and irradiated at 0°C at a fluence rate of $2.0 \text{ J/M}^2 \cdot \text{sec}$. The UV-irradiated chromosomes were then run on 10-30% neutral sucrose gradients at an average rotor speed of 3371 rpm for a total $\omega^2 t$ of $6.00 \times 10^9 \text{ rad}^2/\text{sec}$.
 Upper Panel: ● — 0 J/M^2 ; ◇ — 40 J/M^2 ; ▲ — 80 J/M^2 .
 Lower Panel: ● — 120 J/M^2 ; ◇ — 600 J/M^2 ; ▲ — 1200 J/M^2 . The ends of the gradients have been omitted for clarity, but no significant counts were obtained from those portions.

TABLE 2

CHANGES IN THE SEDIMENTATION PROPERTIES OF *IN VITRO* UV-IRRADIATED SUPERCOILED DNA'S

DNA	S ₀	S _s (Δ%)	D _s (J/M ² x10 ⁻³)	S _m (Δ%)	D _m (J/M ² x10 ⁻³)	δ
ØX174 RF I	21	24(16)	4	25(19)	6-9	-0.036
PM2	28	33(19)	1	-----	---	-----
<i>E. coli</i> nucleoid	1800	1930(7)	0.08	2020(12)	1.2	-0.17

S₀ = initial sedimentation coefficient.S_s = sedimentation coefficient in shoulder region.S_m = maximum sedimentation coefficient.

Δ% = % increase of sedimentation coefficient.

D_s = UV dose in shoulder region.D_m = UV dose for maximum increase in S.δ^m = superhelix density (superhelical turns/10 base pairs).

FIGURE 3. GRADIENT PROFILES FOR *IN VIVO* UV-IRRADIATED FOLDED CHROMOSOMES FROM STRAIN KU0105 WT.

Strain KU0105 WT was harvested by centrifugation during exponential growth in M9 medium. The cells were washed and resuspended in M9 buffer at 0°C for irradiation at a UV-fluence rate of 2.0 J/M²·sec. Immediately after irradiation the cells were lysed and run on 10-30% neutral sucrose gradients at 4°C. The average rotor speed during centrifugation was 2940 rpm for a total $\omega^2 t$ of 6.00x10⁹ rad²/sec. Upper Panel: ●—● 0 J/M²; ◇—◇ 40 J/M². Lower Panel: ●—● 120 J/M²; ◇—◇ 600 J/M²; ▲—▲ 1200 J/M². The ends of the gradients have been omitted for clarity, but no significant counts were found in those portions.

coefficient once again increases back to within a few percent of the initial value, and then remains in this range between 600 and 1200 J/M².

In contrast, the *uvrB-5* strain irradiated *in vivo* under identical conditions shows a constant decrease in the sedimentation rate of the folded chromosomes within the same dose range (Figures 4 & 5). The initial rate of decrease is not as rapid as with the wild-type strain, but the sedimentation rate continues to decrease to almost one-half of the original value by 1200 J/M².

4. Ethidium Bromide Titration of Supercoiling in Folded Chromosomes Isolated from Strain KU0106 *uvrB-5* UV-Irradiated *In Vivo*

The nature of the decrease in the sedimentation coefficient of *in vivo* irradiated chromosomes from strain KU0106 *uvrB-5* was further investigated by assessing the amount of supercoiling remaining in the nucleoids by using gradients containing various concentrations of the intercalating dye ethidium bromide. The binding of an ethidium bromide molecule changes the pitch of the DNA helix. Closed covalent circular DNA is constrained by the topological relation that $\alpha = \tau + \beta$, where α is the topological linking number (invariant for a given CCC DNA), β is the duplex winding number, and τ is the superhelix winding number (Vinograd *et al.*, 1968). Any change in the winding of the helix (β) therefore results in a change of the opposite sense in the supercoiling (τ). All CCC DNA's observed to date, including bacterial nucleoids, possess negative superhelices *in vitro* (Dworsky, 1976). Thus with increased binding of ethidium bromide positive superhelical turns are introduced which are manifested by a change in the observed rate of sedimentation.

Nucleoids from unirradiated cultures show titration patterns like

FIGURE 4. GRADIENT PROFILES FOR *IN VIVO* UV-IRRADIATED FOLDED CHROMOSOMES FROM STRAIN KU0106 *uvrB-5*.

An exponential phase culture of strain KU0106 *uvrB-5* was harvested by centrifugation, washed, and resuspended in M9 buffer at 0°C. The cells were UV-irradiated at a fluence rate of 2.0 J/M²·sec and immediately lysed. The 10-30% neutral sucrose gradients were centrifuged at 4°C with an average rotor speed of 2626 rpm for a total $\omega^2 t$ of 6.00x10⁹ rad²/sec.

● — ● 0 J/M²; ◇ — ◇ 600 J/M²; ▲ — ▲ 1200 J/M². The bottom portions of the gradients have been omitted for clarity, but no significant counts were obtained from this region.

FIGURE 5. CHANGES IN THE AVERAGE SEDIMENTATION COEFFICIENT OF UV-IRRADIATED FOLDED CHROMOSOMES.

All UV-irradiated chromosomes were sedimented through 10-30% neutral sucrose gradients at 4°C using an average rotor speed of ≈ 3000 rpm for a total $\omega^2 t$ of 6.00×10^9 rad²/sec. ●—● Chromosomes isolated from strain KU0105 WT on a preparative gradient were diluted in gradient base and irradiated *in vitro* at 0°C. ▲—▲ The same strain was also UV-irradiated *in vivo* and the cells immediately lysed to obtain folded chromosomes. ◇—◇ Strain KU0106 *uvrB*-5 was irradiated *in vivo* and then immediately lysed to obtain nucleoids. The fluence rate in all cases was 2.0 J/M²·sec.

that of the upper-most curve in Figure 6. The sedimentation coefficient decreases to a minimum of about 75% of the initial value at a critical dye concentration of $2\mu\text{g/ml}$ ($5\mu\text{M}$). At higher dye concentrations, the nucleoids again return to their original rate of sedimentation due to the introduction of positive superhelices. After a dose of 40 J/M^2 is administered to the *uvrB-5* strain *in vivo*, the initial rate of sedimentation is slightly reduced. The sedimentation coefficient continues to decrease during titration, reaching a minimum at the same critical dye concentration ($2\mu\text{g/ml}$). This indicates that the superhelix density of the nucleoid has not changed. The superhelix density is the number of superhelical turns per 10 base pairs. At still higher dye concentrations, however, the sedimentation coefficient of the nucleoids does not increase beyond 80% of its initial value. With a still higher UV dose of 600 J/M^2 , the normal biphasic response during titration is even further reduced. The initial value of the sedimentation coefficient is only a little over 70% of that for unirradiated nucleoids, and declines to about 50% with little or no increase in sedimentation rate at higher concentrations of ethidium bromide.

5. Loss of *In Vivo* UV-Irradiated Folded Chromosomes During Centrifugation at High Rotor Speeds

An additional property of folded chromosomes from *in vivo* UV-irradiated strain *uvrB-5* was revealed when gradients were run at high rotor speeds (20,000 rpm). With increasing doses of UV, the chromosomes sedimented at progressively slower rates as has been observed above, but in addition, there is a decrease in the amount of DNA which was recovered from the gradients (Figure 7). All gradients initially contained the

FIGURE 6. RELAXATION OF SUPERCOILING IN *IN VIVO* UV-IRRADIATED FOLDED CHROMOSOMES FROM STRAIN KU0106 *uvrB-5*.

An exponential phase culture of strain KU0106 *uvrB-5* was harvested by centrifugation, washed, and resuspended in M9 buffer at 0°C for UV-irradiation at a fluence rate of 2.0 J/M²·sec. The cells were immediately lysed by the standard procedure for obtaining nucleoids and 0.2ml portions of the lysate were layered on top of 10-30% neutral sucrose gradients containing various concentrations of ethidium bromide. Gradients were centrifuged at ≈3000 rpm for a total $\omega^2 t$ of 6.00x10⁹ rad²/sec at a temperature of 4°C. UV Dose: ●————● 0 J/M²; ◇————◇ 40 J/M²; ▲————▲ 600 J/M².

FIGURE 7. GRADIENT PROFILES OF UV-IRRADIATED FOLDED CHROMOSOMES FROM STRAIN KU0106 *uvrB-5* AFTER HIGH-SPEED CENTRIFUGATION.

A culture of strain KU0106 *uvrB-5* in exponential growth was harvested by centrifugation, washed, and resuspended in M9 buffer at 0°C. The cells were irradiated *in vivo* in a spot plate at a UV-fluence rate of 2.0 J/M². The cells were immediately harvested by centrifugation and lysed by the standard technique. Equal numbers of counts from each lysate were then run on 10-30% neutral sucrose gradients at 4°C using a rotor speed of 20,000 rpm for a total $\omega^2 t$ of 6.00×10^9 rad²/sec. UV Dose: ● — ● 0 J/M²; ◇ — ◇ 120 J/M²; ▲ — ▲ 1200 J/M².

same amount of $[^3\text{H}]$ -labeled, irradiated DNA. Only about 20% of the original amount of material could be recovered from gradients where the cells had received the highest UV doses (600-1200 J/M²) as shown in Figure 8.

B. Gamma-Irradiation

1. Growth Curve

A growth curve for strain KU0105 wild-type is shown in Figure 9. The cells were grown with shaking at 37°C in M9 minimal medium supplemented exactly as in experiments where cells were radioactively labeled, except for substituting non-radioactive thymidine in place of the $[^3\text{H}]$ -compound. After this experiment was completed, it was found that pre-warming the medium prior to inoculation significantly reduced the lag period, and this procedure was then followed for all subsequent experiments. The growth rate under these conditions is exponential as can be seen by the least-squares regression which has been fitted to the data of Figure 9 after excluding the first few points at either end of the curve. From the coefficients of the regression, a doubling-time of 56 minutes has been determined. A similar experiment conducted by Richard Saunders (personal communication) using the same strain, medium, and growth conditions yielded a doubling-time of 50 minutes.

2. Survival Curves for Gamma-Irradiated *E. coli* K-12 Strains

Gamma-irradiation of exponential phase cells of strains KU0104 *polA-1*, KU0105 WT, and KU0108 *recA-13* at 0°C in M9 buffer under aerobic conditions resulted in survival on LB agar as indicated in Figure 10. The points shown in the Figure are the average of the results of three separate experiments and indicate that for the wild-type strain, there is an

FIGURE 8. LOSS OF DNA DURING HIGH-SPEED CENTRIFUGATION OF NUCLEOIDS FROM
IN VIVO UV-IRRADIATED STRAIN KU0106 *uvrB-5*.

A washed exponential phase culture of strain KU0106 *uvrB-5* was irradiated *in vivo* in M9 buffer at 0°C with a UV-fluence rate of 2.0 J/M². The cells were immediately lysed by the standard procedure for obtaining nucleoids and equal numbers of counts were placed on identical 10-30% neutral sucrose gradients. Centrifugation was at 4°C using a rotor speed of 20,000 rpm for a total $\omega^2 t$ of 6.00×10^9 rad²/sec.

FIGURE 9. GROWTH CURVE FOR STRAIN KU0105 WT AT 37°C IN M9 MINIMAL MEDIUM.

A side-arm flask containing 20ml of M9 minimal medium was inoculated with 0.2ml from an overnight culture of strain KU0105 WT and incubated with shaking at 37°C. Aliquots were withdrawn at intervals, diluted in phosphate buffer, and plated in duplicate (0.1ml/plate) on LB agar. The plates were incubated at 37°C for 48 hours and colonies were counted. The straight line is a least-squares linear fit to the data, excluding the first and last points. The doubling-time based on the coefficient of the regression is 56 minutes.

FIGURE 10. SURVIVAL OF STRAINS KU0104 *polA-1*, KU0105 WT, AND KU0108 *recA-13* AFTER GAMMA-IRRADIATION.

Exponential phase cultures of strains KU0104 *polA-1*, KU0105 WT, and KU0108 *recA-13* were harvested by centrifugation, washed in cold M9 buffer, and resuspended in the same buffered salt solution in 12x75mm glass test tubes. The test tubes were held in ice-water inside a dewar flask for irradiation with Co^{60} gamma-rays. At intervals, aliquots were withdrawn and diluted in cold phosphate buffer before plating in duplicate (0.1ml/plate) on LB agar. Plates were incubated at 37°C for 48 hours before counting colonies. The points shown are the average for three separate experiments.

exponential decrease in survival for doses up-to 100 Krad. The markedly enhanced sensitivity of strains *polA-1* and *recA-13* irradiated under identical conditions is also apparent in this Figure.

3. *In Vitro* Unfolding of Chromosomes

a. RNase

Several techniques were compared for unfolding the bacterial chromosome *in vitro* with RNase under conditions which would cause a minimal amount of shearing. These results are summarized in Table 3. The first two methods (I & II) involved 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 5-20% neutral sucrose gradients. In Method I, these gradients were incubated at room temperature for one hour and then cooled to 4°C for one-half hour before running in the centrifuge at that temperature. In the second method (II), the gradients were immediately loaded into the centrifuge after applying the samples, and the low-speed run was started at 4°C. The next two methods (III & IV) involved loading the gradient with either purified nucleoids or a diluted portion of a standard lysate, after first applying a 0.5ml cap to the gradient which contained various concentrations of RNase in 3.5% sucrose in gradient base. For the third technique (III), the gradients were loaded into the rotor immediately after applying the sample and the run was started at 4°C. In the fourth method (IV), these gradients were spun at 5000 rpm for 20 minutes in order to introduce the nucleoids into the RNase cap. At that point, the gradients were incubated at room temperature for one hour and then cooled to 4°C for one-half hour

TABLE 3

RNASE UNFOLDING OF NUCLEOIDS *IN VITRO*: COMPARISON OF METHODS

METHOD	NUCLEOIDS	RNase ($\mu\text{g/ml}$)	S	UNFOLDED CHROMOSOMES	MEAN $\pm$ SD
I. PREMIX & RUN	PURIFIED	20		1047	1047
II. PREMIX & INCUBATE	PURIFIED	2		1082*	
		10		975	
		20		1024	
		20		1094	
		40		1033	
		100		precipitated	1031 $\pm$ 49
III. CAP & RUN	LYSATE	2		1548*	
		2		1257*	
		20		986	
		20		910	
		20		1098	
	PURIFIED	20		1033	
		20		1061	1018 $\pm$ 73
IV. CAP, PRESPIN & INCUBATE	PURIFIED	20		1073	1073

*Excluded from the average.

before returning them to the centrifuge for the final run at that temperature. The calculated sedimentation coefficients for these unfolded chromosomes (Method IV) were corrected for the small amount of sedimentation which occurred during the initial pre-spin.

No systematic difference is seen among the different methods and the mean value of the sedimentation coefficient of RNase digested chromosomes from all methods of unfolding is $1030 \pm 59S$. Due to the convenience of Method III, it was employed for all subsequent experiments requiring RNase unfolding. This eliminated the need both to pre-mix 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. The method was also equally suitable for direct application of lysates or for use with purified nucleoids. Lastly, it was not necessary to make corrections for any pre-centrifugation as with Method IV. An example of the profiles obtained with this procedure is shown in Figure 11.

b. Thermal

Maximal unfolding and deproteinization of chromosomes can be achieved by incubation of the nucleoids at $70^{\circ}C$ (Drlica and Worcel, 1975; Hecht *et al.*, 1977). In order to minimize handling and thus possibilities for shearing the DNA, the unfolding was accomplished by incubating the entire gradient in a $72^{\circ}C$ oven for about 45-50 minutes. The first 15-20 minutes were required for the gradients to come up to temperature. A layer of hexadecane (0.2ml) was placed over the chromosome sample prior to heating in order to prevent evaporation and convective mixing, as suggested by Hecht *et al.* (1977). The isokinetic behavior of the

FIGURE 11. GRADIENT PROFILES FOR RNASE AND THERMAL UNFOLDING OF NUCLEOIDS
IN VITRO.

▲————▲ A portion of a standard lysate from strain KU0105 WT was diluted with gradient base and applied to a 5-20% neutral sucrose gradient with a 0.5ml cap containing 3.5% sucrose in gradient base. ◇————◇ This gradient was identical to the first except that the cap contained 20 μ g/ml of RNase. ●————● Purified nucleoids from a preparative gradient were diluted in gradient base and layered on top of a 5-20% neutral sucrose gradient. The nucleoid sample was then overlaid with 0.2ml of hexadecane. This gradient was held in a 72°C oven for 45 minutes and then cooled to 4°C before centrifuging with the other gradients at that same temperature. The average rotor speed was ≈ 3000 rpm for a total $\omega^2 t$ of 6.00×10^9 rad²/sec.

gradient was unaffected by this treatment, but the slope of the calibration gradient was slightly shallower than for the unheated RNase gradients (see Appendix B). This is probably due to increased diffusion during heating which would tend to flatten out the gradient.

A profile for chromosomes unfolded by this heating procedure is shown along with one for RNase unfolding in Figure 11. The sedimentation coefficient of thermally unfolded DNA is about 640S, and the peak is sharpened considerably even compared with the RNase-treated material. The tendency for the peaks to sharpen with increased unfolding is quite reproducible and appears to be characteristic of unfolded nucleoids. Quite similar behavior was seen by Hecht *et al.* (1977) and Drlica and Worcel (1975), but was not commented on.

4. Gamma-Irradiation of Folded Chromosomes

a. *In Vitro*

Gamma-irradiation of purified folded chromosomes *in vitro* at 0°C in gradient base under aerobic conditions results in a gradual shift of the chromosome profile toward a slower rate of sedimentation (Figure 12). The decrease is fairly linear, amounting to approximately a 20% reduction at a dose of 10 Krad. The shapes of the profiles are not appreciably changed as a result of the irradiation and no accumulation of low sedimentation coefficient material is seen at the top of the gradients.

b. *In Vivo*

The behavior of folded chromosomes obtained by lysing cells immediately after *in vivo* gamma-irradiation under similar conditions is essentially the same as for *in vitro* irradiation at doses above 2 Krad (Figure 13).

Once again the

FIGURE 12. GRADIENT PROFILES FOR *IN VITRO* GAMMA-IRRADIATED FOLDED CHROMOSOMES.

Purified nucleoids from a preparative gradient were diluted in gradient base and irradiated at 0°C in air. The irradiated chromosomes were then analyzed on 10-30% neutral sucrose gradients at 4°C using an average rotor speed of 2936 rpm for a total $\omega^2 t$ of 6.00×10^9 rad²/sec. Both ends of the gradients have been omitted for clarity, but there were no significant counts in those portions. Upper Panel: ● — ● 0 Krad; ◇ — ◇ 0.5 Krad; ▲ — ▲ 1.0 Krad. Lower Panel: ● — ● 2.0 Krad; ◇ — ◇ 5.0 Krad; ▲ — ▲ 10.0 Krad.

FIGURE 13. GRADIENT PROFILES FOR *IN VIVO* GAMMA-IRRADIATED FOLDED CHROMOSOMES.

An exponential phase culture of strain KU0105 WT was harvested by centrifugation, washed, and resuspended in M9 buffer for gamma-irradiation in air at 0°C. The cells were immediately lysed and run on 10-30% neutral sucrose gradients at 4°C with an average rotor speed of 2822 rpm for a total $\omega^2 t$ of 6.00×10^9 rad²/sec. The ends of the gradients have been omitted for clarity, but no significant counts were present in those portions.

Upper Panel: ● — ● 0 Krad; ◇ — ◇ 0.5 Krad; ▲ — ▲ 1.0 Krad.
 Lower Panel: ● — ● 2.0 Krad; ◇ — ◇ 5.0 Krad; ▲ — ▲ 10.0 Krad.

shapes of the profiles are unchanged, and only a linear decrease in sedimentation rate is observed. The magnitude and rate of the change are essentially identical to the *in vitro* irradiated nucleoids. However, below 2 Krad the *in vivo* irradiated chromosomes appear to show a slight increase in sedimentation rate. At 1 Krad this amounts to only a 4% increase for the wild-type strain and a 1% increase for the *polA-1* strain. The relative rates of change in sedimentation coefficients for these different conditions of irradiation are compared in Figure 14.

c. Speed Dependence

Lydersen and Pettijohn (1977) have performed quite similar experiments. Cells of *E. coli* D-10 were irradiated at 0°C in growth medium with ^{60}Co gamma-rays and then lysed by the procedure of Hecht *et al.* (1977) to obtain *in vivo* irradiated nucleoids. *In vitro* gamma-irradiation was provided by irradiating crude lysates directly at 0°C. When the *in vivo* and *in vitro* irradiated nucleoids (containing different radioactive labels) were sedimented together in neutral sucrose gradients at low rotor speeds, the sedimentation coefficients were decreased as a function of dose, but they differed from each other by less than 10% even after a dose as high as 56 Krad. At this dose, the sedimentation coefficients had decreased from 1800-1900S to 1100-1200S. This behavior is quite similar to that observed in this research (Figure 14).

When the same gradients were centrifuged at higher rotor speeds, however, significant differences were observed between *in vivo* and *in vitro* irradiated nucleoids. The relative rotor speed dependence for the *in vitro* irradiated nucleoids was indistinguishable from that of the unirradiated nucleoids, whereas the *in vivo* irradiated nucleoids exhibited

FIGURE 14. CHANGES IN THE SEDIMENTATION RATES OF GAMMA-IRRADIATED FOLDED CHROMOSOMES.

All gradients were 10-30% neutral sucrose run at 4°C with an average rotor speed of ≈ 3000 rpm for a total $\omega^2 t$ of 6.00×10^9 rad²/sec. *in vivo* gamma-irradiated nucleoids from strain KU0105 WT; *in vivo* gamma-irradiated nucleoids from strain KU0104 *polA-1*; *in vitro* gamma-irradiated nucleoids from strain KU0104 WT.

a strong rotor speed dependence characteristic of nucleoids which had been partially digested with RNase (Lydersen and Pettijohn, 1977).

5. RNase Unfolding of Gamma-Irradiated Nucleoids

a. *In Vitro* Irradiation

When chromosomes are gently unfolded on the gradients by digestion with RNase after gamma-irradiation *in vitro*, the changes in sedimentation rate become more pronounced (Figure 15). For the same range of gamma-radiation dose, the observed changes in the sedimentation rate are much greater. There is an approximately 40% decrease in the rate of sedimentation at 10 Krad. Once again, no major changes are observed in the shapes of the profiles and no material accumulates at either the light or the heavy ends of the gradients.

b. *In Vivo* Irradiation

The slight increase in sedimentation rate observed for *in vivo* gamma-irradiation of folded chromosomes is abolished when the nucleoids are digested with RNase after irradiation (Figure 16). The changes seen in the gradient profiles are identical to those for *in vitro* irradiation, with the exception of a small shoulder at the low-S side of the 10 Krad profile. The peaks for the *in vivo* irradiated chromosomes appear to be wider than those for *in vitro* irradiated nucleoids because a broader range of sedimentation coefficients is present when a lysate is run directly. The chromosomes which are irradiated *in vitro* are obtained from the peak fraction of a preparative gradient. Relative changes in the sedimentation rates for these two methods of irradiation are compared in Figure 17. The rates of change in both cases are identical as was observed for folded chromosomes, but in this Figure the rate of decrease is not linear. A

FIGURE 15. GRADIENT PROFILES FOR *IN VITRO* GAMMA-IRRADIATED CHROMOSOMES UNFOLDED WITH RNASE.

Purified nucleoids from a preparative gradient were diluted in gradient base and irradiated at 0°C in air. The irradiated chromosomes were then layered on 5-20% neutral sucrose gradients with 0.5ml caps containing 20 µg/ml RNase in 3.5% sucrose in gradient base. The gradients were centrifuged at 4°C with an average rotor speed of 2937 rpm for a total $\omega^2 t$ of $6.00 \times 10^9 \text{ rad}^2/\text{sec}$. The bottoms of the gradients have been omitted for clarity, but no significant counts were present in those portions. Upper Panel: ● — 0 Krad; ◇ — 0.5 Krad; ▲ — 1.0 Krad. Lower Panel: ● — 2.0 Krad; ◇ — 5.0 Krad; ▲ — 10.0 Krad.

FIGURE 16. GRADIENT PROFILES FOR *IN VIVO* GAMMA-IRRADIATED CHROMOSOMES UNFOLDED WITH RNASE.

An exponential phase culture of strain KU0105 WT was harvested by centrifugation, washed, and resuspended in M9 buffer at 0°C and irradiated in air. The cells were immediately lysed and run on 5-20% neutral sucrose gradients with 0.5ml caps containing 20µg/ml RNase in 3.5% sucrose in gradient base. The gradients were centrifuged at 4°C with an average rotor speed of 3271 rpm for a total $\omega^2 t$ of 9.00×10^9 rad²/sec. ●.....● 0 Krad; ◇-----◇ 0.5 Krad; ▲-----▲ 1.0 Krad; ●-----● 2.0 Krad; ◇.....◇ 5.0 Krad; ▲-----▲ 10.0 Krad.

FIGURE 17. CHANGES IN THE SEDIMENTATION RATE OF GAMMA-IRRADIATED, RNASE-UNFOLDED CHROMOSOMES.

Chromosomes were irradiated *in vitro* — , or *in vivo* — and then run at 4°C on 5-20% neutral sucrose gradients with 0.5ml caps which contained 20µg/ml RNase in 3.5% sucrose in gradient base.

20% reduction in the relative sedimentation rate occurs within 2 Krad and an additional 20-25% decrease is then observed for doses up-to 10 Krad.

c. Speed Dependence

Lydersen and Pettijohn (1977) have also examined gamma-irradiated nucleoids after digestion with RNase. The behavior of *in vitro*- and *in vivo*-irradiated nucleoids are qualitatively very similar to those reported here. However, the absolute magnitude of the sedimentation coefficients of the peaks are significantly different. This may be attributable to differences in the calibration procedures used as examined in the Discussion, but may also reflect differences in the irradiation procedures or the actual conformation of the RNase digested material. Lydersen and Pettijohn (1977) found that in order to achieve the same decrease in the sedimentation rate, *in vitro* irradiated nucleoids required only one-half the dose administered *in vivo*. The speed dependence of nucleoids irradiated *in vitro* and *in vivo* with doses which caused an equal decrease in the sedimentation rate at low speeds was identical.

6. Thermal Unfolding of Gamma-Irradiated Nucleoids

As indicated in Figure 11, thermal unfolding of nucleoids results in a greatly sharpened distribution of material in the gradient. In Figure 18 a similar peak for unirradiated, thermally-unfolded nucleoids has been sedimented almost to the bottom of the gradient, and has a calculated average sedimentation coefficient of 610S. The changes which occur in this profile at successively higher doses of gamma-rays are qualitatively and quantitatively very different from the patterns observed

FIGURE 18. GRADIENT PROFILES FOR *IN VITRO* GAMMA-IRRADIATED, THERMALLY-UNFOLDED CHROMOSOMES.

Purified chromosomes from a preparative gradient were diluted in gradient base and irradiated at 0°C in air. The irradiated chromosomes were gently layered onto 5-20% neutral sucrose gradients and then overlaid with 0.2ml of hexadecane. The gradients were heated for 45 minutes in a 72°C oven and then cooled to 4°C before centrifuging at that same temperature with an average rotor speed of ≈ 3000 rpm for a total $\omega^2 t$ of 2.10×10^{10} rad²/sec.

Upper Panel: ● — ● 0 Krad; ◇ — ◇ 2.0 Krad; ▲ — ▲ 4.0 Krad.
 Lower Panel: ● — ● 6.0 Krad; ◇ — ◇ 8.0 Krad; ▲ — ▲ 10.0 Krad.

for both the folded chromosomes and the RNase digested chromosomes. For these latter two types of 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 Lydersen and Pettijohn (1977) behaved in a similar manner. In the case of thermally-unfolded chromosomes, however, the initially sharp peak of material becomes significantly broadened at 1-2 Krad while at the same time undergoing a large decrease in the average rate of sedimentation of the material (350S @ 2 Krad). At still higher doses, the profiles once again sharpen until a new peak is formed near the top of the gradient. Only a very slight additional shift in this peak is observed between 8 and 10 Krad (118S $\rightarrow$ 107S). This pattern appears to be similar to that published by Crine and Verly (1976) for the breakage of an initially homogeneous population of T7 DNA molecules. It also appears to be similar to the behavior observed by Lydersen and Pettijohn (1977) for maximally unfolded nucleoids which had been gamma-irradiated either *in vivo* or *in vitro*.

7. Rate of Double-Strand Break Formation

a. Molecular Weight Calculations

If we assume for the moment that the thermally unfolded nucleoid is essentially native, linear DNA molecules, we can calculate average molecular weights for the distributions in Figure 18 by the procedures outlined in Appendix C. Direct calculation of number-average molecular weights is highly sensitive to low molecular weight near the top of the gradient. As recommended by Bonura *et al.* (1975b) the five or six fractions at the

very top of the gradients were excluded from the molecular weight calculations in order to minimize this source of error. The calculated average molecular weights are compiled in Table 4. For a homogeneous population of molecules, $M_n = M_w$ (Montroll and Simha, 1940). As the population becomes more random, however, $M_n < M_w$ and for a completely random population, $M_n = M_w/2$ (Kitayama and Matsuyama, 1971). All of these values have been listed in the Table for comparison. In all cases, $M_n < M_w$ indicating that the distributions are somewhat random. Indeed the values of $M_w/2$ are in fairly good agreement with the directly calculated values of M_n . In many repair studies, the number-average molecular weight of a distribution is estimated as $M_w/2$ because this value is less sensitive to low molecular weight material which accumulates at the top of the gradients. This procedure works well for major peaks which are located in the middle portion of the gradient. In Figure 18 however, the peaks of interest at the higher doses are themselves at the top of the gradients. As can be seen from Table 4, 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 will be used for all further calculations.

Before continuing we should note that the number-average molecular weight for the unirradiated DNA in Figure 18 is 6.4×10^9 daltons or approximately 2.3 genome equivalents of DNA assuming a linear molecule. This assumption will be considered in detail in a later section, but for comparison the highest molecular weight which could be isolated by the procedures of Bonura *et al.* (1975b) and Bonura and Smith (1976) was

TABLE 4

MOLECULAR WEIGHT CALCULATIONS FOR THERMALLY UNFOLDED, *IN VITRO* GAMMA-IRRADIATED NUCLEOIDS

DOSE (Krad)	M_n ($\times 10^{-9}$)	$1/M_n$ ($\times 10^{10}$)	M_w ($\times 10^{-9}$)	$M_w/2$ ($\times 10^{-9}$)	$2/M_w$ ($\times 10^{10}$)
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

$4-5 \times 10^8$ daltons. This is thirteen times lighter than the material obtained from nucleoids and represents about one-sixth of an intact genome.

b. Rate of Breakage

The rate of formation of double-strand breaks is proportional to the slope of the reciprocal number-average molecular weight plotted against dose. This relation is shown in Figure 19. A least-squares linear regression has been fitted to the data, indicating that the rate of double-strand break formation is strictly linear up to at least 10 Krad. The number of additional breaks introduced per initial number-average piece of DNA is given by:

$$N = \left(\frac{M_{n1}}{M_{n2}} \right) - 1 \quad (1)$$

where M_{n1} and M_{n2} are the number-average molecular weights after doses of D_1 and D_2 (in rads) respectively (Bonura *et al.*, 1975b). The number of breaks per gram of DNA is then given by the expression:

$$B = N(6.02 \times 10^{23} / M_{n1}) \quad (2)$$

and the energy required to produce one double-strand break is calculated as:

$$\text{eV/DS-break} = 6.25 \times 10^{13} (D_2 - D_1) / B \quad (3)$$

where the energy is in electron volts (Bonura *et al.*, 1975b). This value may also be expressed in terms of DS-breaks per kilorad per genome equivalent of DNA using the formula:

$$\text{DS-breaks/Krad} \cdot \text{genome} = N(2.8 \times 10^9) / [(D_2 - D_1) \times 10^{-3} M_{n1}] \quad (4)$$

A value of 2.8×10^9 daltons was used as the molecular weight of the *E. coli*

FIGURE 19. RECIPROCAL NUMBER-AVERAGE MOLECULAR WEIGHT CHANGES IN *IN VITRO* GAMMA-IRRADIATED, THERMALLY UNFOLDED CHROMOSOMES.

The number-average molecular weights for the profiles shown in Figure 18 were calculated according to the procedure outlined in Appendix C. The reciprocal number-average molecular weights were then plotted against the gamma-ray dose in kilorads. The line is a least-squares linear regression fitted to the data.

TABLE 5

RATES OF DOUBLE-STRAND BREAK FORMATION IN BACTERIAL GENOMES BY IONIZING RADIATION

eV/DS-BREAK	DS-BREAKS/KRAD·GENOME ¹	STRAIN	RADIATION	ATMOSPHERE	REFERENCE
1088	0.27 ²	<i>Escherichia coli</i>	γ	AIR	THIS WORK
786	0.37 ³		γ	AIR	Lydersen & Pettijohn (1977)
355	0.82 ²				
530	0.55		X	AIR	Bonura <i>et al.</i> (1975b)
1290	0.22		X	N ₂	
1211	0.24		γ	AIR	Bonura & Smith (1976)
1000	0.28	<i>Salmonella typhimurium</i>	γ	AIR	Baraldi & Sinskey (unpublished data)
800	0.36	<i>Micrococcus radiodurans</i>	γ	AIR	Kitayama & Matsuyama (1971)
520	0.56		X	O ₂	Burrell <i>et al.</i> (1971)

¹ 2.8x10⁹ daltons.² *in vitro* nucleoid.³ *in vivo* nucleoid.

genome (Cairns, 1963). Values calculated by Eqs. 3 and 4 are included in Table 5 along with other estimates of DS-breakage rates from the literature.

The agreement with the most recent figures of Bonura and Smith (1976) is quite good, but the most interesting comparison is with the two sets of values obtained by Lydersen and Pettijohn (1977). Their 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 in order to measure the number of double-strand breaks produced. For both methods of irradiation, the breakage rates obtained by Lydersen and Pettijohn (1977) were higher than those obtained here. These differences will be examined in detail in the Discussion, but may be related to the slight differences in the procedures.

8. Conformation of the Thermally-Unfolded Nucleoid

In the previous section it was assumed that the peak of material obtained after heating nucleoids on sucrose gradients represented native DNA free of proteins and RNA and existing as one continuous, linear strand. Calculating a molecular weight for this material using the value of 610S, however, gives 2.5×10^{10} daltons or approximately nine genome equivalents of DNA for *E. coli*. From the division rate of 50 minutes which has been determined for the strain used here (Figure 9) it is possible to calculate the expected average number of genome equivalents of DNA. Using the formula of Cooper and Helmstetter (1967), the average number of genome equivalents of DNA per cell is given by:

$$\bar{G} = (\tau/C \ln 2) 2^{(C+D)/\tau} - 2^{D/\tau}$$

where τ is the doubling-time (50 minutes), C is the time for a replication point to proceed from the origin to the terminus of the chromosome (47 minutes), and D is the time interval between replication of the terminus and cell division (25 minutes). The values for C and D are those of Kubitschek and Freedman (1971). This yields an average DNA content per cell of 2.0 genome equivalents. Hecht *et al.* (1975) 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 nine 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 (Kornberg, 1974) or complex-theta structure where multiple replication forks on a circular genome result in a covalently-closed structure with multiple loops. 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 are possible. Five of the simpler forms which have been treated analytically are listed in Table 6. The expected sedimentation coefficient for each type of structure is calculated for multiple genome equivalents of DNA using the formulae in the first column. Thus a single genome equivalent of DNA with a molecular weight of 2.8×10^9 daltons would have a sedimentation coefficient of 242 if it existed as a simple linear molecule (Clark and Lange, 1977). If this same quantity of DNA was formed into a covalently closed circle, the resulting sedimenta-

TABLE 6

THEORETICAL SEDIMENTATION COEFFICIENTS AT LOW ROTOR SPEEDS FOR VARIOUS
CHROMOSOME CONFORMATIONS WITH MULTIPLE GENOME EQUIVALENTS OF DNA

GENOME EQUIVALENTS (2.8×10^9 daltons)				
FORMULA	REFERENCE	1	2	3
$S_{\text{linear}} = 0.0270 M^{0.4184}$	1	242	324	383
$S_{\text{circle}} = 1.18 S_{\text{linear}}$	2	286	382	452
$S_{Y,50\%} = 1.24 S_{\text{linear}}$	3	300	402	475
$S_{Y,100\%} = 1.33 S_{\text{linear}}$	3	322	431	509
$S_{O,100\%} = 1.517 S_{\text{circle}}$	3	434	579	686

- 1 Clark & Lange (1976)
2 Bloomfield & Zimm (1966)
3 Bloomfield (1968)

tion coefficient would be increased by a factor of 1.18 (Bloomfield and Zimm, 1966). Another possible structure which might result from breakage of a theta structure is a Y containing a single replication fork. The sedimentation coefficients for Y-structures which are either 50% or 100% replicated are greater than that of a simple linear equivalent by factors of 1.24 and 1.33 respectively (Bloomfield, 1968). If a fully replicated theta structure was isolated intact, the expected sedimentation coefficient would be 1.517 times greater than a circular molecule or 1.79 times a linear equivalent (Bloomfield, 1968). Such a structure containing two or three genome equivalents of DNA would then have a sedimentation rate between 579S and 686S. Although these are only simple approximations of the more complex structures which actually exist, they suggest that the 610S peak observed for thermally-unfolded nucleoids consists of unbroken, fully-replicated theta structures containing between two and three genome equivalents of DNA. The implications of the existence of such structures for the strand-breakage calculations presented earlier are considered in the Discussion.

9. Repair of Gamma-Radiation Damage

a. Folded Chromosomes

When the wild-type strain KU0105 is incubated at 37°C in complete M9 medium after gamma-irradiation in M9 buffer at 0°C, major changes occur in the gradient profiles of folded chromosomes. Figures 20 and 21 demonstrate the effects of post-irradiation incubation after doses of 10 and 5 Krad respectively. Immediately after irradiation, the nucleoids are shifted toward the lighter end of the gradients by an amount consistent with the results described in Figure 14. After one-half hour in growth

FIGURE 20. GRADIENT PROFILES FOR REPAIR OF GAMMA-IRRADIATED (10KRAD) FOLDED CHROMOSOMES.

An exponential phase culture of strain KU0105 WT was harvested by centrifugation, washed, and resuspended in M9 buffer. The cells were irradiated at 0°C in air with a dose of 10 Krad and then immediately added to a side-arm flask containing 20ml of prewarmed M9 medium with non-radioactive thymidine. The flask was incubated with shaking at 37°C and aliquots were withdrawn at intervals and harvested again by centrifugation. The cells were resuspended at 0°C in lysing solution A until all of the samples had been taken, at which time they were all lysed by the standard method. The lysates were applied to 10-30% neutral sucrose gradients and centrifuged at 4°C with an average rotor speed of 2949 rpm for a total $\omega^2 t$ of 6.00×10^9 rad²/sec. Upper Panel: ● — ● pre-irradiation; ◇ — ◇ immediate post-irradiation; ▲ — ▲ ½ hr incubation. Lower Panel: ● — ● 1 hr incubation; ◇ — ◇ 2 hr incubation; ▲ — ▲ 3 hr incubation.

FIGURE 21. GRADIENT PROFILES FOR REPAIR OF GAMMA-IRRADIATED (5KRAD) FOLDED CHROMOSOMES.

An exponential phase culture of strain KU0105 WT was harvested by centrifugation, washed, and resuspended in M9 buffer. The cells were irradiated at 0°C in air with a dose of 5 Krad and then immediately added to a side-arm flask containing 20ml of prewarmed M9 complete medium with non-radioactive thymidine. The flask was incubated with shaking at 37°C and aliquots were withdrawn after various periods of incubation. The cells were again harvested by centrifugation and resuspended in lysing solution A at 0°C until all samples had been taken. The cells were then lysed at the same time by the standard technique and the lysates were applied to 10-30% neutral sucrose gradients. These were centrifuged at an average rotor speed of 3261 rpm for a total $\omega^2 t$ of $6.00 \times 10^9 \text{ rad}^2/\text{sec}$. Upper Panel: ● —● pre-irradiation; ◇ —◇ immediate post-irradiation; ▲ —▲ ½ hr incubation. Lower Panel: ● —● 1 hr incubation; ▲ —▲ 2 hr incubation; ◇ —◇ 3 hr incubation.

medium, the entire peak shifts to the heavy side of the original, unirradiated profile. The material is more heterogeneous in nature and the peaks are broadened accordingly. At one hour of incubation, the peaks have sharpened more and the material has moved back toward the light end of the gradient to a position at the low-S side of the unirradiated distribution. Finally after three hours, the nucleoids once again shift slightly toward heavier material. With increasing incubation, increasing amounts of rapidly-sedimenting material appear in the last few fractions at the bottom of the gradients as well.

This complex behavior is presented in a more quantitative fashion in Table 7. The average sedimentation coefficients for the unirradiated nucleoids are nearly identical for both the 5 Krad and 10 Krad experiments at 1751S and 1762S respectively. Immediately after irradiation, the sedimentation coefficients are reduced to 1624S and 1370S. The percent change from the unirradiated sedimentation coefficients are in good agreement with the data of Figures 12-14. After one-half hour, the chromosomes from cells irradiated with 5 Krad have an increased sedimentation rate of 2236S which then progressively decreases to 1939S at two hours of incubation. By three hours, however, the value has again increased to 2045S. The situation is slightly different for the 10 Krad exposure. In this case, the sedimentation rate at one-half hour of incubation is only increased to 2050S. This is still higher than the rate for unirradiated nucleoids, and the coefficient continues to increase further reaching a maximum of 2318S at one hour of incubation. At two hours, the rate has again decreased to a value of 1686S and by three hours the sedimentation coefficient has increased to 1793S which is

TABLE 7
REPAIR OF FOLDED CHROMOSOMES AFTER GAMMA-IRRADIATION

AVERAGE SEDIMENTATION COEFFICIENTS						
HOURS OF INCUBATION						
DOSE (Krad)	UNIRRADIATED	0	$\frac{1}{2}$	1	2	3
5	1751	1624	2236	2194	1939	2045
10	1762	1370	2050	2318	1686	1793
						72
						61

essentially the same as the unirradiated nucleoids. The percentage of the material present in the unirradiated peak which returns to that same initial position in the gradient after three hours of incubation is 72% and 61% and 5 and 10 Krad respectively. The remainder of the material appears at either the very top or the very bottom of the gradients. Any material in the last fraction at the bottom of the gradient has a sedimentation coefficient $>>3100S$.

The chromosomes with sedimentation coefficients of 2200-2300S which are observed within $\frac{1}{2}$ -1 hours after irradiation are in the range of the most rapidly sedimenting nucleoids which are present in the distribution of unirradiated chromosomes, whereas the very rapidly sedimenting material at the bottom of the gradients is not found in the range of sedimentation coefficients of unirradiated nucleoids. The nature of this material will be considered further in the Discussion, but is in the range of sedimentation coefficients for membrane-associated nucleoids.

b. Folded Chromosomes from KU0108 *recA-13*

When strain KU0108 *recA-13* is incubated at 37°C in complete M9 medium after receiving a dose of 5 Krad in M9 buffer at 0°C, the gradient profiles for nucleoids are significantly different from those observed with the wild-type strain (Figure 22). Immediately after irradiation the peak is shifted toward the light end of the gradient as has been observed previously, but upon incubation the sedimentation coefficients of the material continue to decrease further until most of the material is at the top of the gradient. No heavy material is formed during incubation as revealed by the total absence of radioactivity below the position of the unirradiated peak in the 10-50% gradients used for this experiment.

FIGURE 22. GRADIENT PROFILES FOR REPAIR OF GAMMA-IRRADIATED (5KRAD)
FOLDED CHROMOSOMES FROM STRAIN KU0108 *recA-13*.

An exponential phase culture of strain KU0108 *recA-13* was harvested by centrifugation, washed, and resuspended in M9 buffer. The cells were irradiated at 0°C in air with a dose of 5 Krad and then immediately added to a side-arm flask containing 20ml of prewarmed M9 medium with non-radioactive thymidine. The flask was incubated with shaking at 37°C and aliquots were removed at intervals and again harvested by centrifugation. These cells were resuspended in lysing solution A at 0°C until all samples had been taken, at which time they were all lysed by the standard method. The lysates were applied to 10-50% neutral sucrose gradients and then centrifuged at 4°C at an average rotor speed of 2781 rpm for a total $\omega^2 t$ of $6.00 \times 10^9 \text{ rad}^2/\text{sec}$. ● — ● pre-irradiation; ◇ — ◇ immediate post-irradiation; ▲ — ▲ ½ hr incubation; ● — ● 3 hr incubation.

A high-density shelf was included in these gradients as well in order to detect any very rapidly sedimenting material which might be lost to the bottom of the tube. The only indication of any type of possible repair is the presence of a small shoulder on the 3 hour incubation profile at the heavy end of the initial, unirradiated peak.

c. Two-Minute Post-Irradiation Incubation

Within one-half hour after the start of post-irradiation incubation in growth medium, strain KU0105 WT shows the formation of material with an average sedimentation coefficient in the range of 2200-2300S. In order to determine whether this peak was formed immediately after returning the cells to growth medium, a two-minute incubation experiment was conducted. An exponential phase culture was harvested by centrifugation, washed, and resuspended in M9 buffer at 0°C. A portion of this culture was then gamma-irradiated at 0°C with a dose of 10 Krad while the unirradiated portion was simply held on ice. Immediately after irradiation, flasks containing 20ml of prewarmed M9 buffer (plus thymidine 40µg/ml) or M9 complete medium were inoculated with either irradiated or unirradiated cells. The flasks were incubated at 37°C with shaking for only 2 minutes and then rapidly chilled and harvested by centrifugation. The cells were then lysed by the standard method and run on 10-50% neutral sucrose gradients at 4°C. The resulting profiles are shown in Figure 23.

Only a slight decrease in sedimentation rate is observed for nucleoids obtained immediately after irradiation, and after 2 minutes of incubation in complete M9 medium there is no apparent change in the profile. Irradiated cells incubated in M9 buffer for 2 minutes, however, show a large decrease in sedimentation rate with a major portion of the

FIGURE 23. GRADIENT PROFILES FOR TWO-MINUTE REPAIR OF GAMMA-IRRADIATED FOLDED CHROMOSOMES.

An exponential phase culture of strain KU0105 WT was harvested by centrifugation, washed, and resuspended in M9 buffer. Half of the cells were irradiated with a dose of 10 Krad in air at 0°C while the others were simply held on ice. These two batches of cells were then split and a portion of each was added to side-arm flasks containing 20ml of either prewarmed M9 buffer or prewarmed M9 complete medium. The flasks were incubated with shaking at 37°C for only two minutes at which time the cells were harvested again by centrifugation and lysed by the standard method. The lysates were applied to 10-50% neutral sucrose gradients and centrifuged at 4°C at an average rotor speed of ≈ 3000 rpm for a total $\omega^2 t$ of 6.00×10^9 rad²/sec. Upper Panel: ●—● pre-irradiation; ◆—◆ immediate post-irradiation; ▲—▲ irradiated cells, incubated in M9 complete medium for 2 minutes. Lower Panel: ●—● unirradiated cells incubated in M9 complete medium for 2 minutes; ◆—◆ irradiated cells incubated in M9 buffer for 2 minutes; ▲—▲ unirradiated cells incubated in M9 buffer for 2 minutes.

TABLE 8

TWO-MINUTE REPAIR OF GAMMA-IRRADIATED FOLDED CHROMOSOMES

INCUBATION	AVERAGE SEDIMENTATION COEFFICIENTS
PRE-IRRADIATION	1725
IMMEDIATE POST- IRRADIATION	1595
IRRADIATED CELLS 2 MIN M9 MEDIUM	1590
IRRADIATED CELLS 2 MIN M9 BUFFER	832
UNIRRADIATED CELLS 2 MIN M9 MEDIUM	1873
UNIRRADIATED CELLS 2 MIN M9 BUFFER	1433

material remaining near the top of the gradient. Unirradiated cells incubated in buffer under the same conditions show only a slight decrease in sedimentation rate compared with the pre-irradiation profile. A slight increase in sedimentation rate is observed for unirradiated cells after return to growth medium for the 2 minute incubation period. No rapidly sedimenting material is observed in any of these gradients and no significant counts are found on top of the high density shelves. The average sedimentation coefficients for each condition of incubation are tabulated in Table 8.

d. RNase Unfolded Chromosomes

When nucleoids are unfolded by digestion with RNase after post-irradiation incubation in M9 complete medium, gradient profiles like those in Figure 24 are obtained. A large decrease in the sedimentation rate is observed immediately after irradiation. The sedimentation coefficient for the unirradiated nucleoids is 1098S which is reduced to only 786S after 5 Krad (Table 9). Return of this material to the position of unirradiated material in the gradients is quite rapid as indicated by the half-hour incubation profile. This material is fairly heterogeneous, including a range of sedimentation coefficients on either side of the unirradiated nucleoid peak. With further incubation, the profile sharpens until by three hours the width is the same as the unirradiated profile. Some fine-structure in the shape of the profiles is also present at 2 and 3 hours of incubation. Two separate peaks are clearly resolved at the high and low ends of the range of sedimentation coefficients which comprise the unirradiated distribution. The mean sedimentation coefficient after 3 hours of incubation is slightly increased over the unirradiated

FIGURE 24. GRADIENT PROFILES FOR REPAIR OF RNASE UNFOLDED CHROMOSOMES AFTER 5 KRAD GAMMA-IRRADIATION.

An exponential phase culture of strain KU0105 WT was harvested by centrifugation, washed, and resuspended in M9 buffer. The cells were irradiated at 0°C in air with a dose of 5 Krad and immediately added to a side-arm flask containing 20ml of prewarmed M9 medium with non-radioactive thymidine. The flask was incubated with shaking at 37°C and aliquots were withdrawn at intervals and the cells harvested again by centrifugation. These were resuspended in lysing solution A at 0°C and held until all of the samples had been taken at which time they were all lysed by the standard method. The lysates were applied to 5-20% neutral sucrose gradients with 0.5ml caps containing 20µg/ml RNase in 3.5% sucrose in gradient base. The gradients were centrifuged at 4°C at an average rotor speed of ≈ 3000 rpm for a total $\omega^2 t$ of 9.00×10^9 rad²/sec. Upper Panel: ●—● unirradiated; ◇—◇ immediate post-irradiation; ▲—▲ ½ hr incubation. Lower Panel: ●—● 1 hr incubation; ◇—◇ 2 hr incubation; ▲—▲ 3 hr incubation.

TABLE 9

REPAIR OF RNASE-UNFOLDED CHROMOSOMES AFTER 5 KRAD GAMMA-IRRADIATION

INCUBATION (HOURS)	AVERAGE SEDIMENTATION COEFFICIENT
UNIRRADIATED	1098
IMMEDIATE POST- IRRADIATION	786
$\frac{1}{2}$	1042
1	1071
2	1059
3	1133

value (Table 9).

The extent of repair after two hours of post-irradiation incubation was determined in a final experiment for several doses of gamma-rays as indicated in Figure 25. Complete recovery occurs after doses of 0.5-2.0 Krad within this two hour incubation period, while some material with lower sedimentation coefficients is still present in the profiles for 5.0 and 10.0 Krad. This is consistent with the results of Figure 24 where 3 hours of incubation was required before all of the low-S material had been repaired. Fine-structure similar to that described above is also present in several of the profiles, including that for unirradiated nucleoids. That fraction of the material which does not return to the position for unirradiated chromosomes appears to sediment to the very bottom of the gradient, although some small amount of material also appears at the top of the 5 and 10 Krad profiles. The percentage of material which does return to the position of unirradiated chromosomes is indicated in Table 10 along with the percent survival at the same doses from Figure 10. The agreement between the two sets of numbers is quite good, indicating that the percentage of RNase unfolded chromosomes which is repaired is equivalent to the percentage of cells which survive.

FIGURE 25. GRADIENT PROFILES FOR TWO-HOUR REPAIR OF RNASE UNFOLDED CHROMOSOMES AFTER SEVERAL DOSES OF GAMMA-RAYS.

An exponential phase culture of strain KU0105 WT was harvested by centrifugation, washed, and resuspended in M9 buffer. The cells were irradiated at 0°C in air and then immediately added to flasks containing 20ml of M9 medium with non-radioactive thymidine. The prewarmed flasks were incubated at 37°C with shaking and then harvested again by centrifugation. The cells were lysed according to the standard method and the lysates were applied to 5-20% neutral sucrose gradients with 0.5ml caps containing 20µg/ml RNase in 3.5% sucrose and gradient base. Centrifugation was carried out at 4°C at an average rotor speed of 3584 rpm for a total $\omega^2 t$ of 9.00×10^9 rad²/sec.

Upper Panel: ● — ● unirradiated; ◇ — ◇ 0.5 Krad; ▲ — ▲ 1.0 Krad; ● — ● 2.0 Krad; ◇ — ◇ 5.0 Krad; ▲ — ▲ 10.0 Krad.

TABLE 10
RECOVERY OF RNASE DIGESTED NUCLEOIDS AFTER 2 HOUR
POST-IRRADIATION INCUBATION, AND CELL SURVIVAL

DOSE (Krad)	%RECOVERY OF NUCLEOIDS ¹	%SURVIVAL ²
0.5	98	95
1.0	93	91
2.0	81	83
5.0	79	63
10.0	41	40

¹ From Figure 25.

² From Figure 10.

A. UV-Irradiation1. *In Vitro*

The increase seen in the sedimentation rate of *in vitro* UV-irradiated folded chromosomes is similar to that observed following *in vitro* UV-irradiation of small covalently-closed, circular DNA's isolated from bacteriophage. Denhardt and Kato (1973) reported that the sedimentation coefficient of superhelical ϕ X174 RFI DNA increased from 21S to 25S at UV doses up to 10^4 J/M². Similar though smaller increases were also observed for non-superhelical RFI DNA synthesized *in vitro*, and even for the RFI component. The authors proposed that the observed increases were due to the formation of cyclobutane-type pyrimidine dimers which altered the pitch of the helix and thereby changed the superhelix density of the molecules in a manner analogous to the effect of ethidium bromide. Simple photoreactivation experiments designed to remove the dimers without nicking the DNA seemed to indicate that the sedimentation rate returned to near-normal following such treatment. Considerable endonucleolytic activity was associated with their photoreactivation, however, and the gradient profiles actually showed only partial recovery.

More recently, Veldhuisen *et al.* (1976) have attempted to repeat this earlier work using DNA from the phage PM2. Once again, an increase in the sedimentation rate was observed which was proportional to the UV dose and resulted in a 19% increase at 10^3 J/M². No significant change in the sedimentation rates of either Components II or III were reported, however. In addition, careful photoreactivation experiments whose effectiveness was checked by subsequent incubation with UV-endo-nuclease from *Micrococcus luteus* were performed. Any dimers remaining

after photoreactivation were nicked by the *Micrococcal* enzyme thus converting the molecule to a more slowly sedimenting Component II molecule. Maximal photoreactivation left only 0.7 dimers per molecule, but the increased sedimentation coefficient remained unchanged and the rate did not return to the original value. The cyclobutane-type pyrimidine dimer thus does not appear to be the photoproduct which is responsible for this observed increase in the sedimentation rate.

The differences observed in the relative magnitudes of the changes in sedimentation rates for phage and nucleoid DNA's, as well as the doses at which such changes were manifested, may be due to several factors. Whereas the sedimentation properties of the bacteriophage DNA's are due largely to the conformation of the naked DNA molecules, the bacterial chromosome is a more complex structure which contains significant amounts of RNA and protein. The superhelix density of the folded chromosome is also significantly different from that of the phage DNA's (Dworsky, 1976), as is the molecular weight. Thus the degree of distortion introduced by the formation of a given photoproduct is likely to affect the conformation and superhelical content of these different genomes to different degrees.

2. *In Vivo*

a. Strain KU0105 wild-type

Markedly different results were obtained when *E. coli* was UV-irradiated *in vivo* and nucleoids were immediately isolated and analyzed. In the wild-type strain KU0105, a 15-20% decrease in the sedimentation rate was observed, even for the lowest dose used in this study (40 J/M^2). A broad plateau was then observed up to about 360 J/M^2 where little additional change was noted. Above this dose, however, the sedimentation

rate once again increased and indeed returned to almost normal by 1200 J/M². Although the cells were irradiated on ice and lysed immediately thereafter, there is a period of five minutes during the lysozyme incubation where the temperature is raised to 20°C. This may have been sufficient for the UV-endonuclease to incise the irradiated DNA near cyclobutane-type pyrimidine dimers and to begin excision repair. The presence of only one such nick would relax the supercoiling of one loop in the folded chromosome, resulting in a decrease in the sedimentation rate. If all loops in the chromosome were so nicked, the structure would be completely relaxed and additional incisions would cause little further change in the sedimentation coefficient. The observed plateau may have been an indication of such complete relaxation of the nucleoid. The sedimentation rate of chromosomes completely relaxed by ethidium bromide is only about 75% ($\approx 1350S$) of the unrelaxed rate, which is comparable to the value observed at the plateau for *in vivo* UV-irradiated nucleoids ($\approx 1400S$). Using the figure from Veldhuisen *et al.* (1976) for the rate of formation of dimers ($0.7/10 \text{ J/M}^2 \cdot 6 \times 10^6$ daltons), we can calculate the number expected at 40 J/M² for one genome equivalent of *E. coli* DNA (2.8×10^9 daltons) to be on the order of ≈ 1200 . Since it is estimated by Worcel and Burgi (1972) that the folded chromosome contains about 50 loops, this would have been more than a sufficient dose to provide at least one dimer per loop as possible substrate for the UV-endonuclease.

The subsequent increase in the sedimentation rate at higher doses is more difficult to explain. Inactivation of the UV-endonuclease or failure of the incision step may occur at these super-lethal doses (compare Figures 1 and 5). This may have been combined with the formation

of photoproducts which caused an increase in the sedimentation rate, as was observed for *in vitro* UV-irradiated chromosomes. Under these conditions the return to near-normal sedimentation rate at very high UV doses would make sense.

b. Strain KU0106 *uvrB-5*

If this was indeed the case, then *in vivo* UV-irradiation of a strain which lacked the UV-endonuclease should have resulted in behavior more like that observed for *in vitro* irradiation, and should not show a decrease and plateau in the sedimentation rate. When strain KU0106 *uvrB-5* was UV-irradiated *in vivo*, however, a major reduction in the sedimentation rate was observed which was roughly proportional to the dose. At 1200 J/M² the sedimentation rate was almost one-half of the unirradiated rate. Relaxation of the supercoiling was again suspected, and the irradiated chromosomes were subjected to ethidium bromide titration in order to test this hypothesis. With increased UV dose, the initial rate of sedimentation at zero dye concentration decreased as had been observed above. Upon titration with ethidium bromide, the nucleoids showed a decrease in sedimentation rate to a minimum at a critical dye concentration of 2µg/ml. Above this concentration the sedimentation coefficient once again increased to near normal values. Such a minimum is still present in the titration of chromosomes UV-irradiated with 40 J/M², but only partial recovery of the sedimentation rate occurs at higher dye concentrations. At the highest UV dose of 1200 J/M² the minimum occurs between 2-3µg/ml with no increase apparent at higher dye concentrations. This behavior is very similar to that observed by Worcel and Burgi (1972) for chromosomes which had been nicked by treatment with DNase I under

conditions which cause only single-strand breaks. It thus appears that even in the *uvrB-5* strain, breaks appear following *in vivo* UV-irradiation which result in relaxation of the supercoiling in the chromosome.

The degree to which the sedimentation coefficient is reduced in this strain, however, suggests additional loss of chromosome structure and possible unfolding of the nucleoid. As cited earlier, the completely relaxed chromosome which contains no supercoiling (either at the critical dye concentration of 2 μ g/ml or after extensive nicking by DNase I) has a sedimentation coefficient which is only 70-80% (1200-1400S) of the non-relaxed structure. In the case of the *in vivo* irradiated *uvrB-5* strain, however, the sedimentation rate continues to decrease to almost 50% (900-1000S) of the unirradiated value. This degree of change is characteristic of chromosomes which have been partially digested with RNase and are partially unfolded (Drlica and Worcel, 1975).

Additional evidence that UV-irradiated *uvrB-5* chromosomes contain damage more severe than maximum relaxation was obtained somewhat unexpectedly from those experiments in which *in vivo* irradiated folded chromosomes were centrifuged at high rotor speeds (20,000 rpm). Prior to the discovery that nucleoids exhibited a rotor speed dependent rate of sedimentation 20,000 rpm was the standard speed used in all experiments. The shape of ethidium bromide titration curves for unirradiated nucleoids was unaffected by the higher speed, and recovery of material from the gradients was uniform (>90%) at all dye concentrations. When UV-irradiated nucleoids were run at these rotor speeds, however, the amount of material recovered from the gradients was inversely related to the UV dose, leveling off at about 25% recovery at the highest doses. Similar losses of high molecular

weight DNA from gradients run at high rotor speeds has been reported by Schumaker and Zimm (1973a,b) indicating that the UV-irradiated chromosomes have probably undergone extensive unfolding in addition to relaxation of supercoiling. Pettijohn and Hecht (1973) have shown that partial unfolding of the nucleoid is possible without loss of supercoiling under conditions of limited digestion with RNase.

c. UV-Induced Strand-Breaks in *uvr*⁻ Strains

Youngs and Smith (1976a) have recently published their findings concerning the formation of single-strand breaks in UV-irradiated strains of *E. coli* K-12 which lack the endonuclease coded for by the structural genes *uvrA* and *uvrB*. Their investigation was prompted by a report of Radman (1976) on the isolation of a second UV-endonuclease. Youngs and Smith found that indeed strand scission does occur at significant rates in *uvr*⁻ strains, and appears to be associated with a photoproduct other than the cyclobutane-type pyrimidine dimer. A portion of the breaks they observed appeared to result from a direct photochemical process. Cells UV-irradiated at 0°C and immediately lysed showed a dose-dependent increase in the reciprocal number-average molecular weight of the single-stranded DNA. Single-strand breaks were formed at the rate of ≈ 0.05 per half-genome per J/M². A slightly lower rate can be calculated from the data of Denhardt and Kato (1973) for *in vitro* UV-irradiation of ϕ X174, giving 0.025-0.030 per half-genome per J/M².

Post-irradiation incubation of these strains resulted in considerable additional strand-breakage, even after photoreactivation to eliminate cyclobutane-type pyrimidine dimers (Youngs and Smith, 1976a). The formation of these additional breaks was temperature dependent and may have

been the result of enzyme activity or of the break-down of a thermo-labile photoproduct. Repair of many of these breaks was demonstrated, showing a requirement for DNA polymerase I and polynucleotide ligase.

Applying these rates of strand breakage to the folded chromosomes, we calculate that 30-60 breaks will occur per genome equivalent at a dose of 600 J/M^2 . For chromosomes UV-irradiated *in vitro* this dose corresponds to the point of maximum increase in the sedimentation rate. The formation of such single-strand breaks which would tend to relax the structure and decrease the sedimentation rate may cancel the effect of the photoproduct which is responsible for causing the initial increase. Since break formation was observed for *in vitro* UV-irradiation of phage DNA, this would appear more likely than the possibility that strand-breaks do not occur for UV-irradiation of the nucleoid *in vitro*.

For the wild-type strain irradiated *in vivo*, the number of dimers far exceeds the number of possible direct strand breaks, and the more rapid decrease in sedimentation rate occurs at a lower UV dose due to the action of the *uvrA-uvrB* endonuclease. The return to near normal sedimentation again is best explained by the inactivation of the enzyme at super-lethal doses. In the *uvrB* strain which lacks this endonuclease, the rate of decrease in the sedimentation rate following *in vivo* irradiation is much slower. At 600 J/M^2 enough direct photochemical strand breaks would have occurred (30-60 per genome equivalent) to cause a significant amount of relaxation of the chromosome. In addition to such relaxation, however, we have also noted a significant amount of unfolding at this dose. The presence of such unfolded regions in the chromosome could account for the failure of the sedimentation rate to return to

higher values at increased UV doses. The cause of the unfolding is subject to speculation. The possibility exists that the *wvrB-5* strain contains mutant characteristics in addition to the lack of a functional *wvrA-wvrB* endonuclease which may result in the formation of a different class of UV lesions which are responsible for the observed unfolding. More likely, perhaps, is the possibility that in the absence of the *wvrA-wvrB* endonuclease, a lesion which is normally repaired via the *UVR*-gene dependent excision repair pathway is now subject to a different mode of repair which results in unfolding of the chromosome. The nuclease isolated by Radman (1976) may perform the incision step in such a pathway. In addition, the non-dimer lesion which is responsible for the increase in sedimentation rate seen following *in vitro* irradiation, may serve as the substrate for such an enzyme.

B. Gamma-Irradiation

1. Relaxation of Supercoiling and Estimates of the Number of Domains

Single-strand breaks are one of the principal lesions formed in nucleic acids by ionizing radiation, and are produced at a rate almost one hundred times that for double-strand breaks (Youngs and Smith, 1976b; Bonura and Smith, 1976). 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 for both *in vivo*- and *in vitro*-irradiated nucleoids. The shapes of the peaks were unaltered, and the changes in sedimentation rates were similar in both cases and of a magnitude which is indicative of relaxation.

Early estimates of the number of domains of supercoiling present in

the nucleoid were based on alkaline gradient sedimentation of DNase I digested chromosomes. By calculating the number of single-strand breaks which were produced by an incubation with DNase which was sufficient to cause complete relaxation of the supercoiling in the nucleoid, Worcel and Burgi (1972) estimated that 12-80 domains of supercoiling were present per genome equivalent of DNA. This broad range is indicative of the difficulties encountered in obtaining high molecular weight single-stranded DNA from nucleoids by denaturation under alkaline conditions. Similar problems were encountered by this author in attempting to repeat these experiments. The reliability of these figures is also in doubt due to the fact that Worcel and Burgi (1972) did not take into account possible rotor speed effects, and used a rotor speed of 40,000 rpm. In addition, the action of DNase I did not appear to produce breaks at random. Some portions of the DNA in the nucleoids may have been inaccessible to DNase action.

By combining data for the rate of ionizing radiation-induced strand breakage with the rate of relaxation of nucleoids, it is possible to make an improved estimate of the number of domains of supercoiling. Several assumptions are required in order to proceed:

1. All domains of supercoiling are of the same size.
2. Domains of supercoiling are distributed at random in the nucleoid.
3. The contribution of double-strand breaks to relaxation is insignificant compared with single-strand breaks.
4. The decrease in the sedimentation rate observed for gamma-irradiated nucleoids is directly proportional to the fraction of domains which have been relaxed.

From Figure 14 we can estimate that nucleoids irradiated with a dose of

10 Krad have undergone an 18% reduction in sedimentation rate. Complete relaxation of all domains at 2 μ g/ml of ethidium bromide results in a 28% decrease in the sedimentation rate (Figure 6). If assumption four above is valid, then the nucleoids irradiated with a dose of 10 Krad have undergone only 65% of maximal relaxation. This also implies that 35% of the domains of supercoiling do not contain a single-strand break, while the remaining 65% must contain at least one. By using Poisson statistics, it is then possible to calculate the average number of single-strand breaks per domain. The Poisson distribution is given by:

$$P(m) = \frac{a^m e^{-a}}{m!} \quad (1)$$

where $P(m)$ is the fraction of domains containing m breaks and a is the average number of breaks per domain. If 35% of the domains contain no breaks:

$$P(0) = \frac{a^0 e^{-a}}{0!} = e^{-a} = 0.35 \quad (2)$$

$$a = 1.05 \quad (3)$$

We can also estimate that at 10 Krad, about 325 single-strand breaks occur per genome equivalent of DNA (32.4 SS-breaks/Krad $\cdot$ 2.8×10^9 daltons; Youngs and Smith, 1976b). Thus

$$\frac{1.05 \text{ breaks}}{\text{DOMAIN}} = \frac{325 \text{ breaks}}{2.8 \times 10^9 \text{ daltons}} \quad (4)$$

$$\text{DOMAIN} = 9.0 \times 10^6 \text{ daltons} \quad (5)$$

$$\text{DOMAINS/GENOME} = 310 \quad (6)$$

Lydersen and Pettijohn (1977) have used a similar analysis to obtain an estimate of the number of domains of supercoiling in the nucleoid.

They measured the number of alkalai-labile sites created in the nucleoid following *in vitro* gamma-irradiation using alkaline sucrose gradient sedimentation, and at the same time measured the amount of relaxation which had occurred using neutral sucrose gradients. The number of true single-strand breaks was calculated as 0.55 times the total number of breaks measured under alkaline conditions. This correction was based on measurements with CCC PM2 DNA which indicated that 45% of the breaks seen after *in vitro* irradiation were due to the cleavage of alkalai-labile bonds. Nucleoids irradiated *in vitro* with a dose of 22.4 Krad therefore contained 250 true single-strand breaks per genome equivalent of DNA and all but 10% appeared to be completely relaxed based on neutral gradient sedimentation. Using Poisson statistics again, the average number of single-strand breaks per domain is given as:

$$P(0) = e^{-a} = 0.10 \quad (7)$$

$$a = 2.30 \quad (8)$$

$$\frac{2.30 \text{ breaks}}{\text{DOMAIN}} = \frac{250 \text{ breaks}}{2.8 \times 10^9 \text{ daltons}} \quad (9)$$

$$\text{DOMAIN} = 2.6 \times 10^7 \text{ daltons} \quad (10)$$

$$\text{DOMAINS/GENOME} = 110$$

The figures used in expression (4) for the number of single-strand breaks/genome at 10 Krad has not been corrected for alkalai-labile bonds which should not contribute to relaxation. If the correction of Lydersen and Pettijohn (1977) is applied, the estimate is revised as follows:

$$\frac{1.05 \text{ breaks}}{\text{DOMAIN}} = \frac{179 \text{ breaks}}{2.8 \times 10^9 \text{ daltons}} \quad (4b)$$

$$\text{DOMAIN} = 1.6 \times 10^7 \text{ daltons} \quad (5b)$$

Both of these new estimates are considerably higher than the range published by Worcel and Burgi (1972). From electron microscopic observations, it has been estimated that each nucleoid contains 144 ± 48 loops of DNA which can be resolved upon spreading. Since these nucleoids contain between 2 and 3 genome equivalents of DNA, this would indicate that each visually-observable loop may contain several discrete domains of supercoiling (Pettijohn, 1977).

2. Gamma-Radiation 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 largely maintained by nascent RNA molecules attached to the DNA by their associated RNA-polymerase molecules (Hecht *et al.*, 1977; Pettijohn, 1977). In addition, recent evidence indicates that many of the nascent RNA chains may have multiple sites of association with the DNA of the nucleoid (Pettijohn, 1977). This view is supported by the observation that digestion with RNase which is sufficient to cause about 4 breaks per RNA molecule results in a gradual unfolding of the nucleoid, rather than an all-or-none dissociation. These same RNA molecules are dissociated from the DNA of the chromosome by heating to 70°C , thus suggesting that hydrogen-bonding is involved in the DNA-RNA association. Kornberg (1974) has presented a model in which a short, single-stranded RNA molecule is hybridized to two different regions of the DNA which are separated by a length which is equivalent to the size of one domain of supercoiling. 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. Pettijohn (1977) has indicated that these RNA molecules average 1200 bases in length. Using an average value of 330 daltons/base (Kornberg, 1974) this gives a molecular weight for the RNA of only about 4×10^5 daltons. From the previous section, the number of true single-strand breaks per genome at 10 Krad is 179. Thus

$$\frac{\# \text{ RNA breaks}}{4 \times 10^5 \text{ daltons}} = \frac{179 \text{ DNA breaks}}{2.8 \times 10^9 \text{ daltons}} \quad (1)$$

$$\# \text{ RNA breaks} = 0.025 = a \quad (2)$$

$$P(0) = e^{-a} = 0.97 \quad (3)$$

After a dose of 10 Krad, therefore, only 3% of the RNA molecules will contain one or more breaks. The actual number of RNA molecules involved in maintaining the folding has been estimated at 74 ± 14 (Pettijohn, 1977) and thus only 1 or 2 would contain any breaks. Due to the small target size and 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 Krad. This is supported by the results obtained with nucleoids irradiated both *in vivo* and *in vitro*. At doses of gamma-rays up to 10 Krad, no major dissociation or unfolding of the nucleoid was observed.

This is somewhat in contrast to the findings of Lydersen and Pettijohn (1977) for gamma-irradiated nucleoids. Based on determinations

of the relative rotor speed dependence for both *in vivo* and *in vitro* gamma-irradiated nucleoids, these authors concluded that *in vitro*-irradiation caused little or no unfolding of the chromosome at doses up to 56 Krad, whereas *in vivo*-irradiation at similar doses caused a significant amount of unfolding. They further proposed that *in vivo*-irradiation destabilized or altered the RNA-DNA interactions in some manner, which resulted in the observed unfolding. Similar measurements of relative rotor speed dependence might have revealed unfolding in the *in vivo*-irradiated nucleoids in this study as well, but the difference may also be attributable to the fact that Lydersen and Pettijohn (1977) irradiated their cells in growth medium whereas in this study the cells were washed and resuspended in buffer at 0°C prior to irradiation. It is possible the unfolding they observed was the result of low-level physiological activity, rather than radiochemistry. It is not clear whether the observed unfolding involved breakage of the RNA molecules, or some other reaction.

3. Sedimentation Rates of the RNase-Digested and Thermally-Unfolded Nucleoids

Limit digestion of nucleoids with pancreatic RNase by several methods yielded material with an average sedimentation rate of $1030 \pm 60S$ at rotor speeds near 3000 rpm (Table 3). The most recent publication of Hecht *et al.*, (1977) however, gives a sedimentation rate for RNA-free nucleoid DNA obtained by similar techniques of 450S. This is similar to Drlica and Worcel (1975) who had found sedimentation rates of 400-500S at 7000 rpm. This rotor speed is in the range where rotor speed effects become significant, however, and these values would appear to be under-

estimated considerably (Hecht *et al.*, 1977). The value of 450S obtained by Hecht *et al.* (1977) is also subject to question in light of the problems which these authors reported with their sedimentation markers. In their calibration gradients, the T4 DNA marker gave a sedimentation rate which was $25 \pm 5\%$ lower than expected relative to the intact T4 phage marker. 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 $S_{20,w}^0$ were multiplied by $25/59=0.43$ to estimate their real sedimentation velocities." Although the authors attempted to explain this discrepancy in the sedimentation rates of their markers 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 this research, the T4 DNA sedimented exactly as predicted relative to lambda and T4 phage markers (Appendix B). The value of the sedimentation coefficient of T4 DNA was also slightly lower (59S) in the Hecht *et al.* (1977) report compared with the more recent value of Clark and Lange (1976) which was used in this work (62.8S). If the correction of Hecht *et al.* (1977) is eliminated, the sedimentation rate for RNase digested nucleoids becomes $450\text{S}/0.43 = 1046\text{S}$ which is almost identical to the value obtained in this work.

Using the same argument, the value of thermally-unfolded nucleoids published by Hecht *et al.* (1977) must also be corrected in this same fashion. Thus the 320S sedimentation coefficient becomes $320/0.43 = 744\text{S}$. This is somewhat higher than the value of 610-640S determined in this study.

In another recent paper from the same laboratory, Lydersen and Pettijohn (1977) 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 fact that in the Pettijohn lysis procedure (Hecht *et al.*, 1977), the lysate is incubated with the detergent solution at 24°C rather than at 0°C (Appendix A). This may give rise to changes in the nucleoid structure which result in the observed differences in sedimentation rates after unfolding of the chromosomes. As Pettijohn (1977) has pointed out, "At present the methods for nucleoid isolation have elements of empiricism and are very much an art."

4. The Effect of Chromosome Conformation on 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 5 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* (Kornberg, 1974) and during replication, the structure geometrically resembles a theta (Cairns, 1963). In rapidly growing cells, the conformation of the DNA is even more complex and multiple replication forks are present (Cooper and Helmstetter, 1968; Kubitschek and Freedman, 1971). Several investigators have reported difficulty in reproducibly obtaining "free-sedimenting" DNA of intact,

genome size from unirradiated cells (Bonura *et al.*, 1975a,b). For this reason, these investigators were required to use doses of ionizing radiation in excess of 10 Krad to measure double-strand breakage rates. When the plots of reciprocal number-average molecular weight were extrapolated back into the biologically significant dose range, the extrapolation did not intersect the points for the few values of the molecular weight of DNA from unirradiated cells. This suggested that break formation might be non-linear below 10 Krad. Bonura *et al.* (1975b) attempted to explain this observation in terms of problems associated with the formation of shear-sensitive sites in the DNA at these low doses. It appears more likely, however, that the apparent lower rate of formation of breaks at low doses may reflect 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 for 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 complex, replicating 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, this will approach the condition for linear

pieces of DNA. This will occur after enough breaks have accumulated to reduce the size of the average piece of DNA to less than the average distance between replication forks in the intact, replicating genome. For a completely linear polymer, 5-10 breaks are required to convert an initially homogeneous population of molecules to a random distribution of sizes (Charlesby, 1954). 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.

A Monte Carlo calculation similar to that used by Crine and Verly (1976) would probably be required in order to make such a prediction. A computer would start with an initial population of complex-theta structures. As breaks were introduced at random by the computer, new sedimentation coefficients would be calculated based upon the new conformation of the molecule. This would depend upon where in the original structure the break had occurred. The conformation of the resulting structure would determine the analytical form of the expression used to calculate the new sedimentation rate. The computer would continue to redistribute broken molecules in this manner, using a large initial population in order to simulate the breakage caused by the radiation. Unfortunately, the analytical expressions for most of the intermediate structures which result upon breakage of a complex theta structure have not been derived. Such a proposed Monte Carlo calculation will thus have to await further work by researchers like Bloomfield and Zimm (Bloomfield and Zimm, 1966; Bloomfield, 1968).

In light of the above discussion, it is surprising that the plots

of reciprocal number-average molecular weight against radiation dose shown in Figure 19 and also in the paper by Lydersen and Pettijohn (1977) are linear. That the relation should be of this form is somewhat surprising. Furthermore the rate of double-strand break formation determined in this study is in close agreement with the most recent values of Bonura *et al.* (1976) which were determined at doses in excess of 30 Krad. The assumption of linear DNA molecules above this dose is more plausible. Perhaps the similarity of the values is just favorable coincidence.

5. Repair of Gamma-Radiation Damage to Folded Chromosomes

When wild-type cells are incubated for 2 to 3 hours in complete growth medium at 37°C following gamma-irradiation in buffer at 0°C under aerobic conditions, significant amounts of material return to the position of unirradiated nucleoids in the gradients. This same behavior is not observed following irradiation of a *recA-13* strain, suggesting that repair of folded chromosomes may require recombinational repair. This possibility is further supported by the formation of peaks of material with sedimentation coefficients in the range of 2200-2300S. This is essentially the range of the most rapidly-sedimenting nucleoids which are present in the distribution obtained from normally growing cells. Nucleoids within this range of sedimentation coefficients are believed to represent almost completely replicated, double-chromosomes (Korch *et al.*, 1976). In recombinational repair, we would expect to see the formation of a large pool of nearly-double chromosomes. The first shift in the profiles during incubation may thus represent the formation of a recombinational structure in the DNA. Since the profile for folded

chromosomes is shifted to lighter sedimentation coefficients immediately after irradiation, it is possible to rule out direct radiation-induced cross-linking of the nucleoid DNA to either proteins or membrane fragments as responsible for producing this more rapidly-sedimenting peak of material.

Recombinational repair is also growth medium-dependent (Town *et al.*, 1973). When gamma-irradiated cells were incubated in M9 buffer at 37°C for two minutes, the sedimentation rate decreased drastically. Under these conditions of incubation, excision repair is possible and the resulting decrease in sedimentation rate may reflect the activity of enzymes involved in the incision and excision steps of Type II repair (Youngs and Smith, 1976b). Irradiated cells which were incubated in complete growth medium for this period showed no change in their sedimentation rate, further supporting the idea that the formation of 2200-2300S material is not the result of a rapid-binding or cross-linking of the nucleoid to some other component of the cell which is thus responsible for the observed increase in the sedimentation rate. When recombination is complete and the daughter chromosomes have separated, we would expect to see the distribution of sedimentation coefficients for the nucleoids shift toward the lower end of the distribution. Irradiation would thus tend to synchronize the cell population. The cells would then resume normal replication starting with essentially single chromosomes. As normal replication proceeded, the distribution of the nucleoids would return to the profile observed for unirradiated cells. The fine-structure of the changes in the sedimentation patterns during post-irradiation incubation very nicely fit this proposed model of recombinational repair.

Several researchers have reported on the formation of very-heavy, membrane associated material during repair of ionizing radiation damage (Burrell *et al.*, 1971; Burrell and Dean, 1975; Baraldi and Sinskey, unpublished observations). During the latter stages of post-irradiation incubation observed in the present experiments, large amounts of material with sedimentation coefficients in excess of 3100S are formed. This is within the range of sedimentation rates for membrane-associated nucleoids (Pettijohn, 1977). The peak of very rapidly sedimenting material appears to arise from a portion of the nucleoids which are present in the 2200-2300S range. The replicon model of control of DNA replication in bacteria (Jacob *et al.*, 1963) predicts that binding of DNA to the cell membrane is required in the process of segregation of the daughter chromosomes. Following recombinational repair, the portion of the nucleoids which still contain the original radiation damage sites may remain bound to the membrane to form this rapidly sedimenting peak, while the repaired chromosomes would be released from the membrane under the lysis conditions used here, and return to the position of unirradiated chromosomes in the gradient.

6. Repair of Double-Strand Breaks

The problem which has confronted all previous investigators of repair of double-strand breaks in *E. coli* has been to measure viability and the rate of formation of double-strand breaks in the same biologically-significant dose range. Unfortunately this has generally not been possible. The background level of double-strand breaks accumulated during cell lysis set an upper limit on the number of breaks which might be repaired, but precluded measurement of repair of less than six or seven

double-strand breaks per genome. In an attempt to circumvent this shortcoming, the number of breaks expected in the biologically significant dose range was estimated by extrapolation from measurements of breakage at higher doses and compared with the number of lethal hits which was determined from survival curves at the lower doses. The most recent calculations of this type indicate that 1.3-1.4 double-strand breaks occur per lethal event (Bonura *et al.*, 1975b). This figure has been increasing slightly in the past few years as lysis and sedimentation procedures have improved.

The procedure for isolating nucleoids yields minimally-damaged DNA. Few, if any, single-strand breaks occur during isolation as evidenced by the presence of supercoiling and when nucleoids are examined in the electron microscope, no free ends are observed indicating that no double-strand breaks are formed either. The gentle unfolding techniques which have been developed (RNase or heat) yield DNA of very high molecular weight, essentially eliminating the problem of background breaks, and permitting direct measurement of double-strand break formation by gamma-rays in the biologically significant dose range less than 10 Krad. A similar calculation of the number of double-strand breaks per lethal event can now be made based on the data obtained with nucleoid DNA. An average of one lethal event per cell corresponds to a survival of 37% ($a=1$, $P(0)=e^{-1}=0.37$) which occurs at a dose of 10 Krad for this strain (Figure 10). The average number of double-strand breaks per genome at this dose can be determined from the data in Table 5, giving a value of 2.7 DS-breaks/genome based on data from these experiments, or 3.7 DS-breaks/genome using the breakage rate of Lydersen and Pettijohn (1977)

for *in vivo* gamma-irradiation of nucleoids. The absolute value of these figures is still subject to question, however, due to uncertainties surrounding the initial conformation of the DNA and these conclusions can only be considered suggestive of double-strand break repair.

The more conclusive evidence of double-strand break repair comes from the experiments where post-irradiation incubation of the wild-type cells results in the return of initially broken, RNase-unfolded, chromosomal DNA to the same position in the gradient as unirradiated DNA. The fraction of the DNA which is repaired in this fashion at different doses agrees closely with the fraction of cells which survive gamma-irradiation with the same doses.

The only other evidence of this type which directly demonstrates the repair of broken DNA on neutral sucrose gradients was obtained using the same strain of *E. coli* K-12 (AB2497), but using decay of $[^{125}\text{I}]$ incorporated into the DNA to produce the double-strand breaks (Krisch *et al.*, 1976). These results indicated that the wild-type strain could repair up-to 3-4 double-strand breaks per genome, with no repair observed for the *recA-13* strain.

It thus appears that *Escherichia coli* K-12 is indeed capable of repairing several double-strand breaks per genome by a pathway involving genetic recombination.

1. An improved lysis procedure was developed for quantitative isolation of membrane-free folded chromosomes from *E. coli* K-12. The new modifications included the addition of Sarkosyl to the detergent mixture, as well as changes in the cell concentration and in the times and temperatures for spheroplasting and lysis.
2. Nucleoids obtained by this procedure were found to exhibit a rotor speed dependent rate of sedimentation similar to that which has recently been reported by another investigator.
3. UV-irradiation of supercoiled, folded chromosomes *in vitro* resulted in a relative increase in the rate of sedimentation similar to that observed following *in vitro* UV-irradiation of supercoiled, closed, covalent circular DNA's from bacteriophage.
4. Relaxation of supercoiling occurred following *in vivo* UV-irradiation of nucleoids in wild-type cells, apparently due to the formation of *uvr* gene-dependent incision breaks at cyclobutane-type pyrimidine dimers. In *uvrB-5* cells UV-irradiated *in vivo*, a reduced rate of incision and relaxation was observed. These breaks may have been the result of direct or indirect photochemical lesions and/or incision of the DNA by an endonuclease distinct from the *uvrA-uvrB* enzyme, and acting on photoproducts other than cyclobutane-type pyrimidine dimers.
5. In addition, unfolding of *in vivo* UV-irradiated chromosomes occurred in the *uvrB-5* strain as evidenced by the magnitude of the change in sedimentation rate which was observed at higher UV doses, as well as rotor speed dependent loss of such nucleoids from the gradients. The cause of this unfolding is unknown, but may have been the result of processes associated with alternative repair pathways to *uvr*-dependent excision

6. Gamma-irradiation of the folded chromosome resulted in a gradual, linear decrease in the sedimentation coefficient. The rate was the same for nucleoids irradiated *in vitro* or *in vivo*, and appeared to reflect a relaxation of domains of supercoiling due to the formation of single-strand breaks. Estimates based on this assumption indicate that there are about 170 domains of supercoiling per genome. Similar calculations for the RNA molecules which maintain the compact folding of the nucleoid suggested that they were probably too small and too few in number to accumulate enough strand breaks to result in unfolding at these doses. No evidence of radiation-induced unfolding was in fact observed for doses up to 10 Krad.

7. Limit digestion of nucleoids with RNase, or thermal unfolding of chromosomes by heating at 70°C resulted in structures with average sedimentation coefficients of 1030±59S and 625±15S respectively. These sedimentation rates are higher than values reported in the literature, which may be due to considerable differences in calibration methods, or to other variables such as strains used, growth rate differences, and differences in lysis procedures. The somewhat lower rate obtained after heating is probably caused by mass differences which result from more extensive deproteinization of the complexes.

8. Both structures are believed to represent the intact, replicating form of the genome of *E. coli*. When the sedimentation rates of these structures were considered in light of the expected DNA content of these cells, determined from growth rate measurements, it appeared most likely that the DNA from maximally unfolded and deproteinized chromosomes was in the form of a completely replicated theta structure containing 2-3 genome

9. The sedimentation rate of DNA obtained from RNase digested nucleoids after gamma-irradiation decreased in a non-linear manner. The steeper initial rate of decrease may have reflected a change in the conformation of the DNA involving the breakage of loops in the replicating genome. The responses were similar for chromosomes irradiated *in vitro* or *in vivo*.

10. The rate of double-strand break formation was determined from calculations of the reciprocal number-average molecular weight of thermally unfolded chromosomes gamma-irradiated *in vitro*. The rate was linear for doses up-to 10 Krad, and the energy required to produce a double-strand break was 1080 eV giving 0.27 double-strand breaks per kilorad per genome equivalent of DNA.

11. A Monte Carlo calculation was proposed to simulate random breakage in a population of complex theta structures. Such calculations will be required in order to make more accurate measurements of double-strand break formation at these very low doses.

12. Repair of radiation damage to folded chromosomes was observed during post-irradiation incubation in growth medium at 37°C. Structures in the sedimentation range of double-chromosomes were observed within one hour after the start of incubation. No such increase in the sedimentation rate was observed during post-irradiation incubation in growth medium of a *recA-13* strain, or after 2 minute incubation of the wild-type strain in buffer at 37°C. In both cases the sedimentation rates continued to decrease from the irradiated value. This evidence suggested that growth-medium dependent, recombination repair was responsible for the restoration of the profile of folded chromosomes observed after 2-3 hours of incubation. At the point where repaired daughter chromo-

some appeared to segregate from the double-chromosome/recombination complex, a rapidly-sedimenting fraction was observed to form in the range of sedimentation coefficients for membrane associated nucleoids. This may represent the binding of unrepairable portions of the DNA to sites on the membrane which would be associated with dead cells.

13. Direct evidence for the repair of double-strand breaks was obtained from similar experiments where irradiated nucleoids were unfolded by RNase digestion before gradient analysis. Incubation of the irradiated cells in growth medium at 37°C for 2-3 hours restored the broken DNA profile to the same shape and position in the gradient as unirradiated DNA. The fraction of the DNA which was repaired in this manner at several different doses agreed closely with values for cell survival. From consideration of the average number of double-strand breaks per lethal event, it appeared that repair of 1-2 double-strand breaks per genome was possible in this strain, in agreement with a similar value for possible double-strand break repair in this same strain obtained by other investigators using a very different procedure.

1. Further investigation of the UV-induced increase in sedimentation rate for *in vitro* UV-irradiated nucleoids might be attempted using photoreactivation *in vivo* or *in vitro* to eliminate cyclobutane-type pyrimidine dimers in an effort to discern the photoproduct(s) responsible for the effect.
2. The effects of metabolic inhibitors on repair of gamma-radiation damage to folded chromosomes, as well as more extensive work with other repair deficient strains, may provide further insight into the details of the repair mechanism. Particular attention should be paid to other *rec* mutations involved in the regulation of genetic recombination.
3. Determination of the actual conformation of the RNase- and thermally-unfolded chromosome also presents a challenge. Extremely gently protein-monolayer spreading techniques might permit direct visualization of the intact structure in the electron microscope, or autoradiography could be performed similar to the procedure of Cairns (1963).
4. On the theoretical level, it would be useful to attempt the proposed Monte Carlo calculation for breakage of theta structures. This would entail some analytical work to derive the necessary expressions for possible intermediate structures, but the subsequent simulation might prove very useful in establishing the kinetics of double-strand break formation at very low doses.
5. It will also be necessary to resolve the discrepancies in sedimentation coefficients for unfolded chromosomal DNA. Direct comparison of the lysis procedures might be helpful in this regard.

6. Differences in irradiation conditions should also be examined, particularly for *in vitro* gamma-irradiation of nucleoids in crude lysates or as purified chromosomes in simple buffer, and *in vivo* gamma-irradiation of cells in growth medium or after washing and resuspending in buffer.

7. Determinations of rotor speed effects appears to be a powerful tool for discerning differences in the conformational constraints of these DNA-complexes, and probably should be rigorously applied to the material obtained at different stages of repair in order to further elucidate their structure.

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APPENDIX A

An Improved Method for the Isolation of Membrane-Free Folded Chromosomes from *Escherichia coli* K-12.

The published procedures for isolating folded chromosomes differ greatly in their details (Table 11), but basically involve spheroplasting of a cell suspension with lysozyme/EDTA and subsequent lysis by a mixture of mild, non-ionic detergents in the presence of suitable counterions. Before beginning repair studies, it was desirable to develop an isolation procedure which was highly reproducible, quantitative, and yielded only membrane-free chromosomes. With the original methods of Stonington and Pettijohn (1971) and Worcel and Burgi (1972), a mixed population of nucleoids was obtained in which the proportion of membrane-free chromosomes could be increased by raising the temperature of the lysis. Though claims were made that selective isolation of the desired form of the chromosome could be accomplished by simple temperature regulation, Ryder and Smith (1974) and Korch *et al.* (1976) 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. Elimination of this step permits quantitative isolation of chromosomes, and by using 10-50% gradients with a high-density shelf at the bottom instead of the 10-30% gradients used by the earlier workers, it is possible to clearly resolve membrane-free chromosomes, membrane-associated chromosomes, and any unlysed cells (Korch *et al.*, 1976). By making these improvements, it was possible to analyze the details of the isolation procedure in an attempt to obtain only membrane-free nucleoids.

By reducing the cell concentration in the lysate from 3×10^9 to

TABLE 11 Published methods for the isolation of folded chromosomes from *Escherichia coli* K-12†

REFERENCE	1,2	3	4	5	6	7	8	9	10	11
STRAIN	D10	CR34 D10	DG75	15 TAU-bar	DG75	H560 DG75	D10	D10	D10	AB1157 DERIV.
CELL CONC. $\times 10^{-9}$	4	1-3	1-3	1	2.6	1-3	1-4	1-4	0.6-1	<0.4
NaN ₃ (mM)	10	10	10	10	100	10	0	0	0	0
SUCROSE (%w/v)	20	20	20	20	20	20	10	0	0	20
TRIS BUFFER (M)	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.1	0.01	0.01
pH	8.1	8.2	8.2	8.0	8.2	8.2	8.1	8.1	7.6	8.1
NaCl (M)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	1.0	1.0	0.1
TEMP. (°C.)	0	0	0	0	0	0	0	0	0	0
EDTA (mM)	50	50	50	50	50	50	50	50	50	50
TRIS BUFFER (M)	0.12	0.12	0.12	0.12	0.12	0.12	0	0.1	0.02	0.12
pH	8.1	8.2	8.2	8.0	8.2	8.2	8.1	8.1	7.6	8.1
LYSOZYME (mg/mL)	4	4	4	4	4	4	10	10	10	4
TEMP. (°C.)	0	0	0	0/22	0	0	4	4	4	25
TIME (sec.)	30	≈30	30	180	30	30	45	45	45-60	300
BRIJ-58 (%w/v)	1	1	1	1	1	1	1	1	1	1
DEOXYCHOLATE (%w/v)	0.4	0.4	0.4	0.4	0.4	0.4	0.5	0.5	0.4	0.4
SARKOSYL NL-97 (%w/v)	0	0	0	0	0	0	1	1	1	2
EDTA (mM)	10	10	10	10	10	10	0	10	10	10
NaCl (M)	2	2	2	2	2	2	2	1	1	2
TEMP. (°C.)	0	RT	10-25	4/22	10-25	22	20	4/24	24	0
TIME (min.)	≈5	10-30	<10	20-30	<3	<10	5	30/6	5-6	15
4000 x g	+	+	+	±	±	+	-	-	-	-

¹Pettijohn *et al.*, 1973²Stonington & Pettijohn, 1971³Worcel & Burgi, 1972⁴Worcel & Burgi, 1974⁵Ryder & Smith, 1974⁶Korch *et al.*, 1976⁷Drlica & Worcel, 1975⁸Giorno *et al.*, 1975a⁹Giorno *et al.*, 1975b¹⁰Hecht *et al.*, 1977¹¹This work

†see also: Dworsky, 1976

1×10^8 , it was possible to lyse essentially all of the cells using the standard method at 25°C . However, a large amount of membrane-associated chromosomes was still present. In an effort to eliminate these, the lysozyme incubation time was extended to 180 seconds. Unfortunately lysis at 25°C now produced only a very sharp peak of viscous material. The lysis temperature was therefore reduced to 0°C , resulting mainly in membrane-associated nucleoids. Extending the detergent incubation time up to 40 minutes caused no improvement, but increasing the lysozyme incubation time to as much as 300 seconds increased the yield of membrane-free chromosomes while also reducing the sedimentation coefficient of the membrane-associated form. Raising the lysozyme incubation temperature to 25°C for 180 seconds further improved the yield of membrane-free chromosomes, but still did not eliminate all of the membrane-associated material. It appeared that simple manipulation of the cell concentration or the times and temperatures for spheroplasting and lysis was not sufficient to obtain only membrane-free chromosomes.

An early paper by Worcel and Burgi (1972) reported that chromosomes could be released from the membrane *in vitro* by incubation with 1% Sarkosyl at 0°C for 15 minutes. This concentration of Sarkosyl was incorporated into the sucrose gradients in an effort to release the chromosome during sedimentation. Although a peak for the membrane-free nucleoids was obtained, a much larger amount of material sedimented to the shelf and was very viscous. Next, 2% (w/v) Sarkosyl NL-97 was incorporated into Solution C with the other detergents, so that the final concentration in the lysate would be 1%. The above parameters were then re-evaluated using this new detergent mixture.

If lysis occurred at 25°C, a very slow-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 seconds 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. Lysozyme incubation at 25°C for 180-300 seconds followed by lysis at 0°C for 15 minutes lysed all of the cells and yielded a single, non-viscous peak sedimenting at $\approx 1600S$ relative to a T4 phage marker (1000S). 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 due to the total absence of any debris under the microscope. When the cells were labeled with [^{14}C]-leucine, less than 1% of the recovered, acid-insoluble label co-sedimented with the [3H]-thymidine peak (Figure 26). The limit of cell concentration in the 0.5ml lysate was found to be 4×10^8 cells, above which a rapidly sedimenting, viscous peak was obtained. During this analysis it was also observed that the lysozyme Solution B was quite stable as reported by Korch *et al.* (1976) and could be made-up fresh monthly. The new detergent mixture (Solution C*) which contained Sarkosyl was found to deteriorate during prolonged storage, and was therefore also prepared monthly.

The incorporation of Sarkosyl into the detergent mixture is similar to a procedure now used in David Pettijohn's lab (Giorno *et al.*, 1975a,b; Hecht *et al.*, 1976). Spheroplasting is performed without sucrose, but at

higher NaCl and lysozyme concentrations. The incubation is only for 45-60 seconds at 4°C, but lysis is then at 24°C with a 0.5% final concentration of Sarkosyl. This procedure, however, was not satisfactory, at least for the KU strain series of *E. coli* K-12 used here. Incomplete lysis was observed with this method, and the yield of membrane-free chromosomes was greatly reduced.

FIGURE 26. DUAL-LABELED PROFILES FOR AN IMPROVED LYSIS PROCEDURE FOR THE ISOLATION OF MEMBRANE-FREE NUCLEOIDS.

An exponential phase culture of strain KU0105 WT was uniformly labeled with 5 μ Ci of [14 C]-leucine and 10 μ Ci of [3 H]-thymidine. Cells were harvested by centrifugation, washed in M9 buffer and lysed according to the procedure described in Materials and Methods. A 0.2ml sample of the lysate was layered onto a 4.8ml 10-50% neutral sucrose gradient with a 0.3ml high-density shelf at the bottom and centrifuged in an SW50.1 rotor at 17,000 rpm at 4°C for 15 minutes. The gradient was fractionated onto Whatman 3MM filter discs (2 drops/filter) and dried under a heat lamp. The filters were washed in bulk for 15 minutes in two changes of 5% trichloroacetic acid and once in acetone for 5 minutes. The washing was conducted at 0°C and then the filters were dried again under the heat lamp. Filters were counted in 10ml of scintillation fluid and corrected for spill-over.

Calibration Curves for Neutral Sucrose Gradient Analysis.

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

For most experiments, sedimentation markers were included in the gradients to serve as an internal standard which would reveal any abnormality during sedimentation. The reliability and accuracy of the ratio-method for determining the sedimentation rate of the experimental material was questioned, and a calibration procedure similar to that used by Hecht *et al.* (1977) was adopted instead. A calibration curve was made for each type of gradient, which could be used directly in calculations of sedimentation coefficients or molecular weights. To produce the curves, matched gradients were made and one or more sedimentation markers were added to each. These included T4 bacteriophage (1025S Cummings, 1964; Conley and Wood, 1975), T4 DNA (62.8S Clark and Lange, 1976), and lambda bacteriophage (410S Weigle, 1966). All markers were labeled with $[^{14}\text{C}]$ -thymine. The gradients were then centrifuged at 20,000 rpm for the phage markers and 30,000 rpm for the T4 DNA at the same temperature used for experimental work (4°C). Zimm (1974) has indicated that a rotor speed of 30,000 rpm is slow enough to avoid speed-dependent effects for DNA with a molecular weight similar to that of T4 (110×10^6 daltons, Freifelder, 1970). At predetermined increments of $\omega^2 t$, gradients were removed from

the centrifuge and fractionated while the others were subjected to additional cycles of centrifugation. The first moments of the positions of the marker $[^{14}\text{C}]$ label distributions were determined, and this was plotted against the product of the sedimentation rate of the marker (in units of seconds and not Svedbergs) and the total $\omega^2 t$ to which that gradient had been subjected. The product $s \times \omega^2 t$ is a dimensionless quantity which permits the calibration curves to be used for experiments conducted at any given value of $\omega^2 t$. These curves are shown in Figures 27-29.

From Figure 27 it is easily seen that the 10-50% gradient departs from isokinetic behavior in the bottom portion of the gradient. This quality has been noted previously (Korch *et al.*, 1976; Clark and Lange, 1976). A least-squares linear regression was fitted to the first six points at the top of the gradient to represent the isokinetic portion.

The 10-30% gradient calibration also displays a slight departure from isokinetic behavior towards the bottom (Figure 28). Again a least-squares linear regression has been fitted to the data, in this case after excluding the very last point. Also note that the single data point for the T4 phage marker at about 2ml fractionated volume falls on this line. The fact that this gradient is not perfectly isokinetic did not appreciably affect the experimental results, however, since in most cases, the nucleoids were not sedimented much past the midpoint of the gradient which is still well within the isokinetic portion.

The two types of 5-20% gradients demonstrated true isokinetic behavior within experimental limits of detection (Figure 29). In the gradients used for RNase unfolding of the nucleoids, both the lambda and

FIGURE 27. CALIBRATION CURVE FOR 10-50% NEUTRAL SUCROSE GRADIENTS.

Eight identical 10-50% neutral sucrose gradients were made with a Buchler gradient maker on top of 0.3ml high-density shelves (50% sucrose in 80% iothalamate). The gradients were cooled at 4°C for two hours before use. A 0.2ml portion of [¹⁴C]-labeled T4 bacteriophage in gradient base was layered onto each gradient and centrifugation was carried out at 4°C at a rotor speed of 20,000 rpm for an $\omega^2 t$ of 6.00×10^9 rad²/sec per cycle. At the end of each cycle, one of the gradients was removed and fractionated while the rest of the gradients were subjected to additional cycles of centrifugation. The line in the figure is a least-squares linear regression fitted to the first six points of the curve, and represents the isokinetic portion of the gradient.

FIGURE 28. CALIBRATION CURVE FOR 10-30% NEUTRAL SUCROSE GRADIENTS.

Six identical 10-30% neutral sucrose gradients were made with a Beckman Density Gradient Former and cooled to 4°C for two hours before use. Bacteriophage markers labeled with $[^{14}\text{C}]$ were mixed with gradient base and 0.2ml portions were applied to each gradient. These were centrifuged at 4°C at 20,000 rpm for an $\omega^2 t$ of 6.00×10^9 rad²/sec per cycle. At the end of each cycle, one of the gradients was removed from the rotor and fractionated while the rest were subjected to additional cycles of centrifugation. The solid line in the figure is fitted to the data points after excluding the last one on the right, and represents the isokinetic portion of the gradient. ● λ phage (410S); ◆ T4 phage (1025S).

FIGURE 29. CALIBRATION CURVES FOR 5-20% NEUTRAL SUCROSE GRADIENTS.

RNASE GRADIENTS: Six identical 5-20% neutral sucrose gradients were made with a Beckman Density Gradient Former and cooled to 4°C for two hours before use. Bacteriophage markers labeled with $[^3\text{H}]$ were mixed with gradient base and 0.2ml portions were applied on top of 0.5ml caps which contained 3.5% sucrose in gradient base. The gradients were centrifuged at 4°C at 20,000 rpm for an $\omega^2 t$ of $6.00 \times 10^9 \text{ rad}^2/\text{sec}$ per cycle. At the end of each cycle, a gradient was removed from the rotor and fractionated while the rest were subjected to additional cycles of centrifugation. A least-squares linear regression has been fitted to the data, indicating isokinetic behavior throughout the gradient. — — — — —; $\diamond$ λ phage (410S); $\blacktriangle$ T4 phage (1025S).

HEATED GRADIENTS: Gradients were prepared as above except for the caps. A portion of $[^3\text{H}]$ -labeled T4 phage in nucleoid lysing mixture (0.2ml) was layered on top of the gradients and then overlaid with 0.2ml of hexadecane. The gradients were heated for 50 minutes in a 72°C oven and then cooled to 4°C before centrifuging at 30,000 rpm at this temperature. After each cycle of $3.00 \times 10^{10} \text{ rad}^2/\text{sec}$, a gradient was removed from the rotor and fractionated while the rest were subjected to additional cycles of centrifugation. The dashed line in the figure is a least-squares linear regression fitted to the points for T4 DNA (62.8S) $\bullet$, and indicates that the gradients are isokinetic throughout. — — — — —

T4 phage markers fell on the same line (dashed line). The slope of the regression for the heated gradients containing T4 DNA is slightly lower, which probably reflects the increased diffusion and flattening which is to be expected after heating. The coefficients from the least-squares linear regressions of all the gradients are presented in Table 12 along with the coefficients of determination (r^2) and the standard errors of the estimates. These values thus take into account all errors present in making gradients, handling and loading of samples, conditions of centrifugation, fractionation, and scintillation counting. These are the same coefficients used to calculate average sedimentation coefficients and molecular weights as detailed in Appendix C.

TABLE 12

COEFFICIENTS OF LEAST-SQUARES LINEAR REGRESSIONS FOR CALIBRATION GRADIENTS

GRADIENT	SLOPE (a_1)	INTERCEPT (a_0)	r^2	STANDARD ERROR
10-50% with shelf	0.417	-0.066	0.997	0.0138
10-30%	0.365	-0.036	0.999	0.0073
5-20% RNase	0.308	-0.045	0.999	0.0015
5-20% Heated	0.291	-0.023	0.999	0.0013

Calculations of Average Sedimentation Coefficients and Molecular Weights.

The rate of sedimentation of a particle in a sucrose gradient is a complex function of the physical properties of the particle, the composition of the gradient, and the conditions of centrifugation (Clark, 1976). In an isokinetic gradient, values for these variables are selected such that the sedimentation rate is constant and the sedimentation coefficient of the particle is thus a linear function of the distance sedimented. This may be represented by the Burgi-Hershey relation (Burgi and Hershey, 1963):

$$S_{20,w}^0 = \frac{\beta D}{\omega^2 t} \quad (1)$$

where $S_{20,w}^0$ is the sedimentation coefficient at zero concentration adjusted to 20°C in water, D is the distance sedimented, $\omega^2 t$ is the integrated centrifugal force, and β is a proportionality constant. Values of this constant for the various gradients used in this study were determined experimentally as detailed in Appendix B. Sedimentation markers with known sedimentation coefficients were centrifuged for known values of $\omega^2 t$. The product $S \times \omega^2 t$ was then plotted against the distance sedimented by the marker or, in this case, the volume fractionated (from the indicator on the ISCO fraction collector). Least-squares linear regressions fitted to the isokinetic portions of the gradients yielded the coefficients listed in Table 12. The sedimentation coefficient corresponding to any position in the gradient can thus be calculated as:

$$S_i = \frac{a_0 + a_1 v_i}{\omega^2 t} \quad (2)$$

where S_i is the sedimentation coefficient of material in the i th fraction,

a_0 and a_1 are the regression coefficients from Table 12 for the appropriate type of gradient, and v_i is the cumulative volume fractionated to the midpoint of the i th fraction. The weight-average sedimentation coefficient for a gradient profile is then calculated as:

$$\bar{S}_w = \frac{\sum (CPM_i (a_0 + a_1 v_i) / \omega^2 t)}{\sum CPM_i} \quad (3)$$

where CPM_i is the counts per minute in the i th fraction.

Weight-average and number-average molecular weights are defined as:

$$M_n = \frac{\sum n_i M_i}{\sum n_i} \quad (4)$$

$$M_w = \frac{\sum w_i M_i}{\sum w_i} \quad (5)$$

where n_i is the number of molecules in the i th fraction, w_i is the mass or weight of molecules in the i th fraction, and M_i is the molecular weight of molecules in the i th fraction. Since $n_i = w_i / M_i$, we may substitute in Eq. 4 to yield:

$$M_n = \frac{\sum w_i}{\sum w_i / M_i} \quad (6)$$

The mass of molecules in a given fraction is proportional to the amount of radioactivity associated with the fraction $w_i \propto CPM_i$. Substituting into Eqs. 5 & 6 gives:

$$M_w = \frac{\sum CPM_i M_i}{\sum CPM_i} \quad (7)$$

$$M_n = \frac{\sum CPM_i}{\sum CPM_i / M_i} \quad (8)$$

It is possible to calculate M_i using the relation derived by Studier (1965):

$$S = KM^\alpha \quad (9)$$

where S is the sedimentation coefficient, M is the molecular weight, and K and α are constants. The values chosen for these constants are those published by Clark and Lange (1976). They were carefully determined using isokinetic gradients to obtain sedimentation coefficients for several bacteriophage DNA's of known molecular weight. The sedimentation coefficients were calculated from the nature of the gradients and the conditions of sedimentation, rather than by comparison with some other sedimentation standard. Studier's equation thus takes the form:

$$S_{20,w}^* = 0.0270 M^{0.4184} \quad (10)$$

where $S_{20,w}^*$ indicates that the sedimentation coefficient has been corrected for changes in the apparent specific volume of the DNA due to the presence of 1M NaCl in the gradients (Clark and Lange, 1976). If we substitute Eq. 2 for $S_{20,w}^*$ in Eq. 10 and rearrange, the molecular weight of DNA in fraction i is given by:

$$M_i = \left(\frac{a_1 v_i + a_0}{0.027 \omega^2 t} \right)^{2.390} \quad (11)$$

This expression for M_i may now be substituted in Eqs. 7 & 8 to give the final equations for calculating average molecular weights:

$$M_w = \frac{\sum \left(CPM_i \left(\frac{a_1 v_i + a_0}{0.027 \omega^2 t} \right)^{2.390} \right)}{\sum CPM_i} \quad (12)$$

$$M_n = \frac{\sum CPM_i}{\sum \left[CPM_i / \left(\frac{a_1 v + a_o}{0.027 \omega^2 t} \right)^{2.390} \right]} \quad (13)$$

Rotor Speed Dependence of the Sedimentation Rate of Folded Chromosomes.

The dependence of the sedimentation rate of high molecular weight DNA on rotor speed has been recognized for several years now (Rubenstein and Leighton, 1971). Early workers with nucleoids assumed that these effects would not be present for the highly compact folded chromosome, and gradients were routinely spun at 17,000 rpm. Recently, however, Hecht *et al.* (1976) have reported that speed dependence is also observed for both the membrane-associated and the membrane-free forms of the nucleoid. Just prior to the publication of their report, the same effect was observed for the membrane-free nucleoids used in this study. Figure 30 shows the gradient profiles obtained after centrifuging lysates at average rotor speeds between 3148 and 38,761 rpm. At the higher rotor speeds, the profiles are sharpened as has been observed for extended DNA fibers and the rate of sedimentation is greatly reduced. The profiles broaden appreciably below 10,000 rpm and the sedimentation rate at the lowest rotor speed used is twice that obtained at the highest speed. This relationship is more clearly shown in Figure 31. Hecht *et al.* (1976) have presented similar findings, but their more extensive experiments seemed to indicate that the speed dependence was more sigmoid in nature. The sedimentation rate reached a maximum plateau at about 1900S for speeds below 10,000 rpm with a steeper transition to slower rates which then levels off above 30,000 rpm for a minimum sedimentation coefficient of about 1000S. For membrane-associated nucleoids, the relationship is even steeper, but is essentially linear from 5000-25,000 rpm. In order to avoid problems in the interpretation of gradient profiles, all subsequent experiments were performed at rotor speeds near 3000 rpm.

FIGURE 30. GRADIENT PROFILES FOR SEDIMENTATION OF FOLDED CHROMOSOMES AT VARIOUS ROTOR SPEEDS.

A standard lysate of strain KU0105 WT was split and applied to identical 10-30% neutral sucrose gradients and centrifuged at various average rotor speeds at a temperature of 4°C. The total $\omega^2 t$ in each case was 6.00×10^9 rad²/sec. $\diamond$ ----- $\diamond$ 3,148 rpm; $\blacktriangle$ ----- $\blacktriangle$ 9,748 rpm; $\bullet$ ----- $\bullet$ 19,685 rpm; $\diamond$ ----- $\diamond$ 27,486 rpm; $\bullet$ ----- $\bullet$ 34,577 rpm.

FIGURE 31. ROTOR SPEED DEPENDENCE OF THE SEDIMENTATION COEFFICIENT OF FOLDED CHROMOSOMES.

Six identical 10-30% neutral sucrose gradients were prepared and cooled to 4°C for two hours before use. Nucleoids were prepared from strain KU0105 WT and 0.2ml of the lysate was layered on each gradient along with ¹⁴C -labeled T4 phage (1025S). Gradients were centrifuged at different average rotor speeds but the total $\omega^2 t$ in each case was 6.00×10^9 rad²/sec. The final position of the T4 phage markers was the same for each gradient. A least-squares linear regression has been fitted to the data points.

Kevin Michael Ulmer, born January 12, 1951, graduated Magna Cum Laude in three years from Williams College in Williamstown, Massachusetts. In June of 1972 he was awarded a Bachelor of Arts degree with Highest Honors in Biology and Highest Honors in Physics, and was elected to Phi Beta Kappa. His undergraduate thesis entitled "A Tunable Organic Dye Laser Microbeam" received Sigma Xi Recognition for Undergraduate Research. That same June, Kevin entered the Joint Program in Biological Oceanography of the Woods Hole Oceanographic Institution and the Massachusetts Institute of Technology, and has been a Research Fellow of the Joint Program since that time. His continuing interest in the biological effects of radiation at the cellular and molecular levels led to the research presented in this thesis. He is currently a member of the American Society for Photobiology, the American Association for the Advancement of Science, and Sigma Xi.