Role of ATP in Removal of Psoralen Cross-links from DNA of Escherichia coli Permeabilized by Treatment with Toluene*

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The removal of interstrand cross-links from DNA was examined in Escherichia coli permeabilized by treatment with toluene. Under these conditions, the reaction requires ATP and Mg²⁺, and the mechanism appears to be similar to that occurring in whole cells. Under optimum conditions, the rate constant was 0.06 min⁻¹. Genetical, physical, and biochemical analysis of the repair process suggest the following mechanism. In an ATP-dependent reaction, the uvrA and uvrB gene products cleave a phosphodiester bond on the 5' side of one arm of the cross-link, producing a 3'-OH terminus. Subsequently, DNA polymerase I (5'-3' exonuclease activity) makes a second strand cut on the 3' side of the cross-link in the same DNA strand, completing removal of the covalent link between complementary strands. The second reaction did not occur in a uurD strain, which had normal levels of DNA polymerizing activity. The uurD gene may regulate the specificity or activity of the 5'-3' exonuclease of DNA polymerase I in vivo.

The repair of DNA containing interstrand cross-links in Escherichia coli occurs by a sequential process. This involves an initial removal of the cross-link followed by an obligatory recombination event which restores the intact base sequence to a cross-link-damaged region of the chromosome (1). Previous in vivo studies have shown that interstrand cross-links were removed from E. coli DNA, and that this reaction required the products of the uvrA, uvrB, uvrC, and polA (5'-3' exonuclease activity) genes (2).

It is also known that E. coli treated with toluene, or cells subjected to osmotic shock by treatment with high concentrations of sucrose become permeable to small molecules, such as inorganic ions or phosphorylated nucleotides, as well as small proteins such as DNase I or the UV endonuclease of bacteriophage T4 (3-11). Studies utilizing permeabilized cells thus represent an intermediate stage between reactions in whole cells and the corresponding reaction in vitro with purified enzymes. Such studies have been quite informative in elucidating the role of small molecules in metabolic processes of replication (3, 4) and repair (5-11) of DNA.

In this paper, we report the removal of interstrand cross-links from the DNA of E. coli permeabilized by treatment with toluene. The genetic control for this reaction and the role of small molecules were determined and compared to properties reported for the reaction in whole cells. DNA isolated at various stages of cross-link removal was characterized physically and biochemically. These results demonstrate a mechanism for the removal of interstrand cross-links in E. coli which is consistent with, and extends previous studies done with whole cells.

MATERIALS AND METHODS

Bacterial Strains—The following Escherichia coli strains were provided by Drs. A. Rorsch and I. Mattern (Medical Biological Laboratory, Rijswijk, ZH), The Netherlands: KMBL 1790 endA101 thyA301 bio87 pheA97 argA103, KMBL 1085 uurD101 endA101 thyA301 bio87, KMBL 1054 uurB endA101 thyA301, KMBL 1479 recB21 endA101 thyA301 bio87 pheA97 metE72, KMBL 1481 recC22 endA101 thyA301 bio87 pheA97, metE72, KMBL 1482 endA101 thyA301 bio87 pheA97 metE72, KMBL 1381 polA1 endA101 thyA301 bio87, KMBL 1794 polA107 endA101 thyA301 bio87 pheA97 argA103 metE72 srA, KMBL 1465 uurD101 endA101 thyA301 bio87. The presence of the endA101 mutation in each strain was confirmed by the methyl green staining procedure (12).

Media and Growth—Bacteria were grown in the medium described by Clark and Malee (13), supplemented with the following (per liter of distilled H₂O): 10.0 g of glucose, 10.0 g of casamino acids (vitamin-free), 10 mg of L-phenylalanine, 10 mg of L-methionine, 5.0 mg of biotin, 10 mg of thymidine. Cellular DNA was labeled during growth in this medium by addition of [methyl-¹⁴C]thymidine to a concentration of 10 to 20 μCi/ml. Cultures were grown to 2 X 10⁸ cells/ml, chilled in an ice bath for 15 min, and collected by centrifugation at 5,000 rpm for 5 min in a Sorvall SS-34 rotor at 0-2°C. Cells were washed once with, and then resuspended in phosphate buffer (50 mm KPO₄, pH 7.4; 10 mm MgCl₂) at a final concentration of approximately 4 X 10⁸ cells/ml, and placed on ice until treatment with toluene. Stationary phase cultures were prepared by overnight growth in Penassay broth at 37°C with aeration.

Toluene Treatment and Cross-linking of DNA—Cells were permeabilized by treatment with toluene as described by Moses and Richardson (3, 6). Cell concentrations used in DNA replication studies were not optimal for cross-link removal. The reaction mix-
tasures described here contain about 5 × 10⁶ cells/ml, and under these conditions DNA replication was not reduced appreciably.

After toluene treatment, cells were sedimented at 1930 × g and resuspended in 1 volume of phosphate buffer at approximately 2 × 10⁶/ml, then sedimented again and resuspended in buffer at approximately 5 × 10⁶/ml, pelleted, and resuspended until piarcescent mixtures at 35°C and incubated from 5 to 40 min. In some experiments, psoralen treatment preceded permeabilization. However, when cells were treated with toluene prior to cross-linking treatment, psoralen was added at the first wash step, and incubation at 0°C was continued for 5 min to equilibrate the drug with the cells. Toluen-treated cells were then exposed to light, washed once and resuspended for incubation as described above. The order of treatments did not affect eventual cross-link removal. However, the yield of cross-links in cellular DNA is increased 4 to 8-fold by a prior toluene treatment. Cross-linking was done at the following concentrations: 1.35 mM ATP, 1.5 mM GTP, 1.5 mM CTP, 36 μM dCTP, 36 μM dGTP, 36 μM dTTP, 2.5 mM NAD, and 5.0 mM MnMN. Toluene-treated cells (5 × 10⁶/ml) were placed in reaction mixtures consisting of various combinations of reagents listed above and incubated with gentle rotation (50 to 70 rpm) at 35°C. After various time periods, reactions were terminated by placing cells in solution at 0°C containing: 10 mM Tris (pH 8.0), 50 mM EDTA, and 100 mM sodium pyrophosphate.

Isolation of DNA Lysates were prepared by a modification of the procedure described by Rupp and Howard-Flanders (16). Cells were collected by centrifugation as described above and resuspended in 3.5% sucrose, 70 mM Tris (pH 8.1). The following reagents were added, lysozyme (0.09%), EDTA (0.4 mM), and the solution was incubated at 0°C before addition of Sarkosyl 97 to 0.65% final concentration. Some lysates were then treated with T1 (50 μg/ml) and pancreatic ribonuclease (50 μg/ml) (Worthington) for 30 min at 37°C. For experiments measuring cross-link removal in less than 10⁶ cells, DNA samples were prepared by extracting cell lysates with phenol, and in procedures which required larger numbers of cells, DNA was isolated by centrifugation to equilibrium in neutral CsCl gradients. In both procedures, samples were then dialyzed 18 h to 24 hr against three changes of 50 mM sodium phosphate buffer, pH 6.8, containing 0.25% NaCl. To reduce breakage of DNA during isolation, samples were not mixed on a Vortex mixer or agitated vigorously. DNA was collected with sodium chloride by sedimentation in alkaline sucrose gradients, using [3H]-labeled DNA from bacteriophage T7 as a single-stranded molecular weight standard of 12 × 10⁶ (17). The extent of DNA breakdown to acid-precipitable material was measured by separating the denaturation products by treatment of native or denatured DNA at severalfold higher DNA concentrations than used for experimental samples tested. Suitable conditions for enzymatic digestion were confirmed by treatment of native or denatured DNA at several fold higher DNA concentrations than used for experimental samples tested.

DNA Polymerase I Activity in Cell Extracts – Overnight cultures grown in Penassay broth were chilled in an ice bath for 15 min and pelleted by centrifugation at 500 × g (22). Cells were resuspended in 0.5 ml of 20% sucrose 10 mM Tris/HCl (pH 8.0), 0.2 ml of 35 mM EDTA containing 35 μg of lysozyme was added. After incubation for 30 min at ice, the solution was placed at room temperature and 0.2 ml of a Brij-80 (0.5%) and MgSO₄ (0.01 M) mixture was added. After 2 min at room temperature, the partially cleared mixture was centrifuged at 1500 × g for 30 min at 25°C. DNA Polymerase I activity was assayed by adding 0.2 ml of the Brij-80 extract to a solution containing: 50 mM phosphate buffer (pH 7.4); 5 mM MgCl₂; 1 mM β-mercaptoethanol; 23 μM GTP; 23 μM dGTP, and 3 μM TTP (specific activity, 18 μCi/μmol) (total volume, 0.5 ml). The reaction was started by adding "activated" calf thymus DNA (23) at a final concentration of 50 μg/ml. Samples were incubated at 37°C, and the reaction stopped by placing 66 μl in 4 ml of cold 7.5% trichloroacetic acid containing 100 mM sodium pyrophosphate. Calf thymus DNA was added to each tube to 50 μg/ml final concentration. Samples were mixed on a Vortex (24, 25) for 10 s. After the samples were chilled on ice for 10 min, the samples were added to a solution of 2% sodium dodecyl sulfate, 50 mM Tris (pH 8.0), and 0.1% sodium dodecyl sulfate (24, 25, 26) for 10 min. Samples were removed and then placed at 37°C. Radioactivity was counted as described previously (14).

DNA Sedimentation Analysis – The molecular weight distribution of DNA samples was measured by a modification of the procedure of McGrath and Williams (20). An aliquot of cells (~2 × 10⁶) or purified DNA was placed in a solution containing 0.05 M NaOH, 1 mM EDTA, and 0.5% Sarkosyl 97 which was then layered on top of 5 ml alkaline sucrose gradients grown in Penassay broth were chilled in an ice bath for 15 min and pelleted by centrifugation at 500 × g (22). Cells were resuspended in 0.1-ml underlayer of a CsCl 0.8 g/ml and sucrose (60%) solution. After 30 min at room temperature, DNA was sedimented by centrifugation in an SW 50.1 rotor (20%) at 40,000 rpm for 40 to 55 min. When cells were lysed directly on gradients, 27 to 30 fractions were collected on Whatman No. 17 filter paper strips and counted as described (21). When purified DNA was sedimented, fractions were collected in vials, neutralized with 1 N HCl and counted in 10 ml of Triton X-100 liquid scintillation fluid. The number average molecular weight was calculated using bacteriophage T7 DNA as a standard (22).

Treatment with SI Nuclease – DNA samples prepared from 50 ml reaction mixtures of toluene-treated cells was denatured as described above, and separated into permanently denatured and reversibly renaturing DNA by the batch elution method described by Britten et al. (22). Samples were dialyzed extensively against 1 mM Tris buffer (pH 8.0) with 0.1 mM EDTA, and a sample of each was counted for radioactivity. The specific activity of samples free of RNA was calculated from its optical density at 260 nm. SI nuclease from Aspergillus oryzae (23) was purified by the method of Vogt and Sheen (24, 25) from a nuclease preparation (Sigma) by R. R. Sinden in these laboratories. Conversion of acid-precipitable radioactive acid to soluble material was measured as follows. Reaction mixtures (0.5 ml) contained 50 mM NaOAc (pH 5.0), 0.1 mM NaCl, 1.0 mM ZnSO₄, 5% glycerol, 0.02 to 0.1 μM DNA, and 10 μl of SI nuclease. After 30 min at 37°C, reactions were terminated by addition of calf thymus DNA (0.5 μg/ml), and perchloric acid to 2.7% final concentration. A duplicate of each sample, and two control tubes with no enzyme were incubated for each experimental sample. Suitable conditions for enzymatic digestion were confirmed by treatment of native or denatured DNA at several fold higher DNA concentrations than used for experimental samples tested. DNA Concentrates generated from SI nuclease assay. Each enzyme digest was run in duplicate. In addition, control samples with single-stranded and native DNA of identical specific activities to experimental samples were prepared for use as standards to establish relative molecular weights.

DNA Cross-links – Removal of DNA Cross-links

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mixture and held on ice for 30 min, filtered onto Millipore GF filters, washed six times with 5 ml of cold 10% trichloroacetic acid and once with 15 ml of 95% ethanol. Filters were dried for 15 min at 60\(^\circ\)C and counted for radioactivity in 10 ml of scintillation fluid (in milliliters per liter) as follows: 600 ml of toluene, 40 ml of a permanganate and 20 ml of H\(_2\)O.

**RESULTS**

Cross-link Removal in Toluene-treated *Escherichia coli*—*E. coli* grown in medium containing [\(^{3}H\)]thymidine to label the DNA uniformly, and permeabilized by treatment with toluene as described under "Materials and Methods." After treatments producing 60 to 88 cross-links/*E. coli* chromosome (15), cells were incubated in different reaction mixtures as described. DNA isolated after various incubation times was denatured, and the number of interstrand cross-links remaining was measured by the hydroxylapatite chromatography technique (2). Results in Fig. 1 show that cross-links were removed selectively from DNA by a reaction that occurred with apparent first order kinetics. The rate constant of 6 \times 10^{-2} \text{ min}^{-1} determined here was nearly equal to that reported for the reaction in whole cells (2). Significantly, cross-link removal did not occur in permeabilized cells in the absence of ATP or Mg\(^{2+}\). Although ATP and Mg\(^{2+}\) appear to be the only small molecules required, results in Fig. 1 show that the reaction was slightly faster when the four deoxyribonucleotide triphosphates (dNTPs) were included at 36 \mu M. Under these conditions, 90 to 95% of the cross-links were removed.

A maximum rate constant for cross-link removal of 6 \times 10^{-2} \text{ min}^{-1} occurred for ATP concentrations between 1.2 and 2.0 mM (Fig. 2). As shown in Table I, no cross-links were removed when guanosine or cytosine triphosphate were substituted for ATP (similar data were obtained for KM11482, data not shown). ATP may be hydrolyzed during the strand-cutting reaction or it may regulate the activity of UV endonuclease, or both. To distinguish among these possibilities, methylene-substituted derivatives of ATP which are resistant to hydrolysis were tested. As shown in Table I, the reaction did not occur in the presence of either \(\alpha,\beta\)-methylene ATP or \(\beta,\gamma\)-methylene ATP at 1.35 mM. Additionally, neither of these analogues acted as inhibitors blocking the reaction when ATP was included.

In whole cells, the time required for total cross-link removal increased with the extent of psoralen and light exposures, suggesting that treatments producing more than 200 cross-links/chromosome somehow saturated the cell's capacity to repair these damages (2). As shown in Table II, a similar effect was noted for toluene-treated cells exposed to treatments producing between 80 and 190 cross-links/chromosome. During a given incubation time, the fraction of cross-links removed decreased with higher psoralen and light exposures. However, the initial rate constant for cross-link removal was similar to that determined for lower yields of cross-links (Fig. 2 and 3). One interpretation of these data is that toluene-treated *E. coli,* and perhaps whole cells, have less than 80 active repair enzymes available for each damaged chromosome.

It has been reported that DNA ligase of *E. coli* can rejoin DNA strands by UV endonuclease acting on DNA containing pyrimidine dimers (29, 30). The ligation reaction requires nicotinamide adenine dinucleotide (NAD) as a cofactor, and is inhibited by a reaction product, nicotinamide mononucleotide (NMN) (31). To determine whether ligation affected cross-link removal, the reaction was studied in the presence of 2.2 mM NAD or 5 mM NMN. Neither of these reagents affected cross-link removal (Table I), suggesting that ligation of cut DNA strands did not occur as a competing reaction under these conditions.

**Mutations Affecting Cross-link Removal**—In previous in *vivo* studies, cross-link removal was blocked in strains with mutations at *uvrA*, *uvrB*, *uvrC*, and *polA (5'-3' exonuclease)* (2). As shown in Table II, cross-link removal occurred normally in permeabilized cells containing recB21, recC22 (data not shown) or polA1 (data not shown) mutations. No reaction was detected in *uvrA6* or *uvrB5* (similar to *uvrA6*, data not shown) mutants and in *polA107* (similar to *uvrD101*, data not shown) or *uvrD101* cross-link removal was barely detectable or occurred at a greatly reduced rate. The apparent slow removal in whole cells with a *polA107* mutant was attributed to nonspecific DNA breakage (2). A similar explanation...
Removal of DNA Cross-links

Removal - It has been shown previously that cross-linked DNA in whole cells is cut, such that upon denaturation, two procedures. In some experiments, permeabilized cells were lysed directly on 5-ml alkaline sucrose gradients (21). Alternately, cells were lysed by addition of Sarkosyl 97 to spheroplasts prepared with lysozyme/EDTA. This lysate was incubated in the presence of ATP, Mg2+, and dNTPs. Sedimentation profile of DNA in alkaline sucrose from several Escherichia coli strains permeabilized with toluene, treated with psoralen and light, and incubated in the presence of Mg2+ and dNTPs, as described in the text. The panels show data from wild type cells (a), a uvrA6 strain (b), and a polA107 strain (c). Symbols refer to the following incubations and treatment conditions: C, control cells incubated 40 min in the presence of ATP, Mg2+, and dNTPs. This control is shown for wild type cells only, (as all other strains gave similar results, and 40-min incubations did not affect the sedimentation pattern significantly); O-O, cells exposed to psoralen and light, but not incubated; A-A, cells incubated in the presence of Mg2+ and dNTPs, but without ATP; D, cells incubated in the presence of ATP, Mg2+, and dNTPs. Sedimentation is from right to left, and DNA from bacteriophage T7, added as an internal molecular weight standard, sedimented as a peak at 0.22 the length of the gradient.

Fig. 3 (left). The number of cross-links removed per chromosome in Escherichia coli permeabilized by toluene treatment is shown after various incubation periods. Symbols refer to different psoralen and light exposures producing the following number of cross-links per chromosome: O, 80; D, 120; C, 170; and A, 100.

Fig. 4 (right). Shown here are typical sedimentation profiles of DNA in alkaline sucrose from several Escherichia coli strains permeabilized with toluene, treated with psoralen and light, and incubated in the presence of ATP, Mg2+, and dNTPs, as described in the text. The panels show data from wild type cells (a), a uvrA6 strain (b), and a polA107 strain (c). Symbols refer to the following incubations and treatment conditions: O, control cells incubated 40 min in the presence of ATP, Mg2+, and dNTPs. This control is shown for wild type cells only, (as all other strains gave similar results, and 40-min incubations did not affect the sedimentation pattern significantly); O-O, cells exposed to psoralen and light, but not incubated; A-A, cells incubated in the presence of Mg2+ and dNTPs, but without ATP; D, cells incubated in the presence of ATP, Mg2+, and dNTPs. Sedimentation is from right to left, and DNA from bacteriophage T7, added as an internal molecular weight standard, sedimented as a peak at 0.22 the length of the gradient.

Table I

| Incubation reagent mixture, 35°, 40 min | Average single-strand distance between cross-links | Fraction reversibly remating DNA | Cross-links removed |
|---------------------------------------|---------------------------------|--------------------------------|-------------------|
| Control untreated                     | 29 daltons x 10^6              | 0.47                           | 0                 |
| Cross-linked untreated                | 72 daltons x 10^6              | 0.56                           | 0                 |
| 1.35 mM ATP only                      | 29 daltons x 10^6              | 0.08                           | 89                |
| 1.35 mM ATP, 36 μM dNTPs, no Mg2+    | 60 daltons x 10^6              | 0.46                           | 2                 |
| 1.5 mM GTP, CTP 36 μM dNTPs          | 103 daltons x 10^6             | 0.75                           | 0                 |
| Control untreated                     | 72 daltons x 10^6              | 0.56                           | 0                 |
| Cross-linked untreated                | 20 daltons x 10^6              | 0.07                           | 94                |
| 1.35 mM ATP, 36 μM dNTPs, 5.0 mM NMN | 31 daltons x 10^6              | 0.80                           | 0                 |
| Control untreated                     | 19 daltons x 10^6              | 0.75                           | 7                 |
| Cross-linked untreated                | 1.35 μM α,β-Methylene-ATP, 36 μM dNTPs | 0.79                           | 1                 |
| 1.35 mM β,γ-Methylene-ATP, 36 μM dNTPs | 0.099                          | 90                             |
| 1.35 mM ATP, 36 μM dNTPs             | 0.089                          | 90                             |

may also apply to permeabilized cells here. Thus, gene products mediating the reaction in permeabilized cells appear to be the same as those for whole cells.

Sedimentation Properties of DNA Undergoing Cross-link Removal - It has been shown previously that cross-linked DNA in whole cells is cut, such that upon denaturation, strands dissociate into segments having a length equal to twice the average cross-link spacing (2). Strand cutting was investigated in permeabilized cells in order to determine the effects of ATP and to characterize intermediates which might be unstable in whole cells but accumulate in permeabilized E. coli. For sedimentation analysis, DNA was prepared by extraction with freshly distilled phenol and then dialyzed against 0.05 phosphate buffer, pH 6.8. Although both procedures gave the same results, the former method was superior for distinguishing differences between uvrA-, uvrB-, and polA107 mutants (Fig. 4). Psoralen and light exposures used for most of the sedimentation studies produced cross-links spaced an average of 90 x 10^6 daltons apart. Typical data obtained from permeabilized cells lysed directly on top of gradients is shown in Fig. 4. DNA from control cells incubated 40 min after toluene treatment, sedimented a distance corresponding to a molecular weight at the peak position of 160 x 10^6 (Fig. 4a). After the cross-linking treatment and before incubation, the DNA sedimented to the bottom of the tube, as shown. This increased sedimentation is due to the higher molecular weight of the cross-linked strands, as well as the unusually fast sedimentation property of DNA molecules containing many cross-links (32). After a 40-min incubation in the presence of 1.35 mM ATP and 13 mM Mg2+, the number average molecular weight of the denatured DNA was 38 x 10^6, or about twice the average cross-link spacing in single strands. In the absence of ATP, no strand cutting occurred during a 40-min incubation, and the DNA remained as fast sedimenting molecules, as shown. Sedimentation of DNA undergoing cross-link removal in permeabilized strains

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gene products make the first phosphodiester bond cleavage on the 5'-3' exonuclease activity) then makes a second cut on the 3' side of the cross-link in the same DNA strand, completing the limited strand cutting in the latter two mutants is considered further in the analysis of DNA, as described below.

Table II

| Pertinent genotype | 35° Incubation conditions | Time (min) | Reversibly renaturing DNA % | Cross-links removed % | Radiolabeled acid precipitable % |
|--------------------|--------------------------|-----------|----------------------------|----------------------|----------------------------------|
| Wild type          | 1.35 mM ATP 35 µM dNTP   | 0         | 69.0                       | 0                    | 100                              |
|                    |                          | 5         |                            |                      |                                  |
|                    |                          | 1         | 37                         | 46                   | 98                               |
|                    |                           | 20        | 19                         | 73                   | 97                               |
|                    |                          | 40        | 4.9                        | 93                   | 94                               |
| No ATP             | 35 µM dNTP               | 10        | 65                         | 5                    |                                  |
|                    |                          | 40        | 63                         | 9                    | 99                               |
| uvrA               | 1.35 mM ATP 35 µM dNTP   | 0         | 53                         | 0                    | 100                              |
|                    |                          | 10        | 52                         | 3                    | 98                               |
|                    |                           | 20        | 50                         | 5                    | 104                              |
|                    |                           | 40        | 46                         | 6                    | 96                               |
| recB               | 1.35 mM ATP 35 µM dNTP   | 0         | 75                         | 0                    | 100                              |
|                    |                          | 10        | 42                         | 43                   | 99                               |
|                    |                           | 20        | 23                         | 69                   | 102                              |
|                    |                           | 40        | 6.3                        | 92                   | 93                               |
| No ATP             | 35 µM dNTP               | 40        | 68                         | 7                    | 99                               |
| polA               | 1.35 mM ATP 35 µM dNTP   | 0         | 44                         | 0                    | 100                              |
|                    |                          | 5         | 51                         | 52                   | 99                               |
|                    |                           | 40        | 6.1                        | 98                   | 90                               |
| uvrD               | 1.35 mM ATP 35 µM dNTP   | 0         | 66                         | 0                    | 100                              |
|                    |                          | 10        | 62                         | 4.8                  | 98                               |
|                    |                           | 20        | 66                         | 0                    |                                   |
|                    |                           | 40        | 58                         | 12                   | 98                               |
| No ATP             | 35 µM dNTP               | 40        | 66.8                       | 0                    | 105                              |

with mutations at polA1, recB21, or recC22 gave results (not shown) similar to those obtained with wild type cells. Results with the uvrA6 mutant in Panel b demonstrate that strand cutting did not occur even in the presence of ATP (Fig. 4b). Similar results (not shown) were obtained with the uvrB5 strain. With the polA107 mutant, strand cutting was not observed in the absence of ATP. However, some strand cutting (but not cross-link removal) was observed in the presence of ATP, and the estimated number average molecular weight of the distribution was 150 to 170 x 10^6, or about 8 times the average cross-link spacing, as shown in Table III. A similar pattern of limited strand cutting was also observed for the uvrD101 mutant (33, 34) (data not shown). Thus, in polA107 and uvrD101, cross-link removal did not occur in the presence of ATP, and the apparent molecular weight of DNA after incubation in permeabilized cells was substantially larger than in wild type cells treated with psoralen and light. These results show that some strand cutting occurred, and that it depended upon the uvrA and uvrB gene products and ATP. However, complete reaction required in addition the 5'-3' exonuclease of DNA polymerase I and the uvrD+ gene. The significance of the limited strand cutting in the latter two mutants is considered further in the analysis of DNA, as described below.

Structure of DNA Undergoing Cross-link Removal—As described earlier, cross-link removal is thought to occur in two separate steps. By this mechanism, the uvrA and uvrB gene products make the first phosphodiester bond cleavage on the 5' side of one arm of the cross-link. DNA polymerase I (5'-3' exonuclease activity) then makes a second cut on the 3' side of the cross-link in the same DNA strand, completing removal of the covalent link between the strands. If this mechanism is correct, then DNA having the structures shown in Fig. 5 should accumulate under conditions blocking the second strand-cutting reaction. Following denaturation and rapid renaturation of DNA with one cut at each cross-link, the average molecular weight of this product should be about 8 times the average cross-link spacing, as was observed in the sedimentation analysis of polA107 and uvrD101 mutants. Additionally, products of rapid renaturation of such molecules should have single strand "tails" comprising 25% of the total mass.

To determine the structure of DNA undergoing cross-link removal and to characterize the step requiring ATP, DNA isolated at various stages of reaction was tested for its susceptibility to partial digestion by S1 nuclease and snake venom phosphodiesterase. DNA from permeabilized cells was isolated and denatured as described, and reversibly renaturing molecules were separated from permanently denatured DNA by hydroxyapatite chromatography. For these experiments, the average cross-link spacing was 6 to 7 x 10^6 daltons, and the molecular weight of DNA from control samples was 20 x 10^6. This closer spacing of cross-links was necessary in order to ensure that most of the end groups were produced by the strand-cutting reactions, rather than by shear breakage during isolation procedures.

S1 nuclease from Aspergillus oryzae acts as an endonuclease which specifically hydrolyzes single strand DNA as well as single-stranded regions attached to duplex molecules (23-25). As shown in Table III, less than 1% of reversibly renatured DNA from toluene-treated Escherichia coli was digested by S1 nuclease. As shown in Table IV, removal of the covalent link between the strands. If this mechanism is correct, then DNA having the structures shown in Fig. 5 should accumulate under conditions blocking the second strand-cutting reaction. Following denaturation and rapid renaturation of DNA with one cut at each cross-link, the average molecular weight of this product should be about 8 times the average cross-link spacing, as was observed in the sedimentation analysis of polA107 and uvrD101 mutants. Additionally, products of rapid renaturation of such molecules should have single strand "tails" comprising 25% of the total mass.

To determine the structure of DNA undergoing cross-link removal and to characterize the step requiring ATP, DNA isolated at various stages of reaction was tested for its susceptibility to partial digestion by S1 nuclease and snake venom phosphodiesterase. DNA from permeabilized cells was isolated and denatured as described, and reversibly renaturing molecules were separated from permanently denatured DNA by hydroxyapatite chromatography. For these experiments, the average cross-link spacing was 6 to 7 x 10^6 daltons, and the molecular weight of DNA from control samples was 20 x 10^6. This closer spacing of cross-links was necessary in order to ensure that most of the end groups were produced by the strand-cutting reactions, rather than by shear breakage during isolation procedures.

S1 nuclease from Aspergillus oryzae acts as an endonuclease which specifically hydrolyzes single strand DNA as well as single-stranded regions attached to duplex molecules (23-25). As shown in Table III, less than 1% of reversibly renatured DNA from toluene-treated Escherichia coli was digested by S1 nuclease. As shown in Table IV, removal of the covalent link between the strands. If this mechanism is correct, then DNA having the structures shown in Fig. 5 should accumulate under conditions blocking the second strand-cutting reaction. Following denaturation and rapid renaturation of DNA with one cut at each cross-link, the average molecular weight of this product should be about 8 times the average cross-link spacing, as was observed in the sedimentation analysis of polA107 and uvrD101 mutants. Additionally, products of rapid renaturation of such molecules should have single strand "tails" comprising 25% of the total mass.
ing DNA from unincubated cells treated with psoralen and light, was hydrolyzed after treatment with S1 nuclease. However, after a 5-min incubation of treated wild type cells, 13% of the reversibly renaturing radioactivity became susceptible to S1 nuclease. This value decreased to 2% after 40 min when cross-link removal ceased in permeabilized cells under these conditions. When DNA was isolated from wild type cells incubated 40 min in the absence of ATP, less than 1% of the reversibly renaturing material was digested by S1 nuclease. DNA isolated from the polA1 strain (data not shown) behaved similarly to that from wild type cells. Also shown in Table III are results from reversibly renaturing DNA of the uvrA6 mutant, in which no single strand regions were detected. Substantial amounts of S1-sensitive DNA accumulated and persisted for up to 40 min in both the uvrD101 and polA107 mutants (data not shown). The 12 to 15% of the reversibly renaturing material that was digested by S1 nuclease appears to be substantially less than the 25% predicted as shown in Fig. 5. This apparent discrepancy can be accounted for by the limited activity of the UV endonuclease during cross-link removal after more intense psoralen and light treatments. In order to be valid, the enzyme analysis of the 3' end required at least 200 cross-links/chromosome, or more than 4 cross-links/DNA fragment isolated. However, only 90 cross-links had been removed after 40 min. Data in Table III show that after 40 min, there are few strand cuts at the remaining 107 cross-links, which would produce single strand tails upon denaturation. Thus, strand cutting occurred to a maximum of 50% under these conditions, and a similar limiting value would be expected for polA107 and uvrD mutants. Strand cutting at only one-half of the cross-links would effectively double the size of segments released during denaturation, and would reduce the amount of material existing as single strand tails to 12.5%, similar to what was detected in these experiments.

To determine whether material sensitive to S1 nuclease was a tail attached to a double-stranded segment, and whether it terminated with a 3'-hydroxyl group, reversibly renaturing DNA was treated with snake venom phosphodiesterase. This enzyme is an exonuclease which initiates digestion specifically at 3'-hydroxyl termini of single-stranded DNA, releasing mononucleotides processively (26). Percentages of reversibly renaturing DNA susceptible to hydrolysis by snake venom phosphodiesterase or S1 nuclease are compared in Table III for several selected strains and incubation times or conditions. It was found that nearly all of the DNA susceptible to S1 was also hydrolyzed by snake venom phosphodiesterase. This indicates that the single-stranded DNA regions attached to rapidly renaturing DNA existed as tails terminating with 3'-hydroxyl groups, as shown in Fig. 5. These molecules were not formed in uvrA6 or uvrB5 mutants or in wild type cells in the absence of ATP. They accumulated and persisted in the uvrD101 and polA107 strains.

**DNA Polymerase I Activity of Brij Extracts** - As described above, partially cut DNA with similar structure accumulated in both polA107 and uvrD101 mutants, and DNA polymerase I catalyzed the second strand-cutting reaction completing cross-link removal. One possible interpretation might be that the uvrD gene product regulates the activity or stability of DNA polymerase I. To test whether these cells contained normal levels of DNA polymerase I, extracts were prepared from Brij lysates of wild type, uvrD101 and polA1 strains. "Activated" calf thymus DNA, the four dNTPs and 3H-labeled thymidine triphosphate were added to the extracts, and after various incubation times, incorporation of radioactivity into acid-insoluble material was determined (27, 28). These data

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**Table III**

Partial hydrolysis of reversibly renaturing DNA from toluene-treated Escherichia coli by S1 nuclease and snake venom phosphodiesterase

The number average molecular weight of DNA preparations from control cells was typically 20 x 10^6, as determined by sedimentation analysis in alkaline sucrose.

| Pertinent genotype | Incubation conditions | Time (min) | Fraction of DNA renaturing reversibly | Cross-links removed/2.5 x 10^6 daltons | Reversibly renaturing DNA hydrolysed by S1 nuclease | Snake venom phosphodiesterase |
|--------------------|-----------------------|------------|---------------------------------------|----------------------------------------|--------------------------------------------------|-------------------------------|
| wild type          | Control untreated     | 0          | 0                                     | 0.76                                   | 0.92                                             |                                |
|                    | Cross-linked unincubated | 0          | 0.96                                 | 0                                      | 12.5                                             | 11.0                           |
|                    | 1.35 mM ATP, 36 μM dNTPs | 5          | 0.92                                 | 35                                     | 9.3                                              |                                |
|                    |                       | 10         | 0.90                                 | 45                                     | 4.9                                              |                                |
|                    |                       | 20         | 0.85                                 | 64                                     | 4.9                                              |                                |
|                    |                       | 40         | 0.74                                 | 93                                     | 1.8                                              |                                |
| No ATP, 36 μM dNTPs | 10                    | 0.95                                 | 4                                      | 0.78                                              |                                |
|                    |                       | 20         | 0.94                                 | 13                                     | 0.95                                              |                                |
|                    |                       | 40         | 0.95                                 | 8                                      | 1.1                                              |                                |
| uvrA6              | Control untreated     | 0          | 0                                     | 0.92                                   | 1.0                                              |                                |
|                    | Cross-linked unincubated | 0          | 0.94                                 | 0                                      | 0.94                                             |                                |
|                    | 1.35 mM ATP, 36 μM dNTPs | 40        | 0.93                                 | 9                                      | 0.94                                             |                                |
|                    | No ATP, 36 μM dNTPs   | 40         | 0.94                                 | 9                                      | 0.94                                             |                                |
| polA107            | Control untreated     | 0          | 0                                     | 0.92                                   | 1.0                                              |                                |
|                    | Cross-linked unincubated | 0          | 0.95                                 | 0                                      | 0.92                                             |                                |
|                    | 1.35 mM ATP, 36 μM dNTPs | 5          | 0.85                                 | 4                                      | 8.7                                              |                                |
|                    |                       | 10         | 0.95                                 | 7                                      | 8.7                                              |                                |
|                    |                       | 20         | 0.95                                 | 3                                      | 16.9                                             |                                |
|                    |                       | 40         | 0.93                                 | 27                                     | 15.2                                             | 13.0                           |
| No ATP, 36 μM dNTPs | 40                    | 0.95                                 | 1                                      | 0.93                                              |                                |
RecB- or recC- strains. Since the methylene-substituted ATP in E. coli varies between 0.2 mM for stationary phase and 1.7 mM for exponentially dividing cells, it cannot be distinguished whether hydrolysis of ATP was required for reaction.

endocase appears to act in a processive fashion," and such a mechanism might require ATP in cells. ATP-dependent permeabilized cells. For example the Micrococcus luteus UV endonuclease does not appear to require ATP in vitro (35, 36), exonuclease V activity does not appear to be involved in cross-link removal, as the reaction proceeded normally in permeabilized cells. One interpretation of these results is that the complex repair sequence, described in the introduction (2, 30, 40) is not initiated at ATP concentrations which are insufficient for completion of the process.

Thus, the reaction rate varies markedly in the range of ATP concentrations normally found in whole cells. In agreement with this, psoralen cross-links were not removed in whole cells starved for a carbon source, or in stationary phase, where intracellular ATP concentrations are well below those required for efficient cross-link removal in permeabilized cells.

endocase coded for by the usrA and usrb genes makes a strand cut on the 5' side of each cross-link. This reaction requires ATP, and produces a hydroxyl group at 3' end of the strand cut. Next, DNA polymerase I (5'-3' exonuclease activity) makes a second cut on the 3' side of the cross-link, in the same DNA strand, and this reaction is blocked in uwrD- cells.

DISCUSSION

Psoralen interstrand cross-links were removed selectively from DNA in Escherichia coli permeabilized with toluene in a reaction similar to that previously studied in whole cells (2). Cross-link removal requires ATP in toluene-treated cells. Since permeabilized cells represent an intermediate stage between reactions in vitro and in whole cells, these results indicate that cross-link removal normally requires ATP.

The results obtained suggest a two-step mechanism for cross-link removal (Fig. 7). The UV endonuclease, coded for by the usrA and usrb genes (29, 35), in the presence of ATP makes a strand cut on the 5' side of one arm of the cross-link attached to one DNA strand, producing a 3'-OH terminus. Subsequently, DNA polymerase I (5'-3' exonuclease activity) makes a second strand cut on the 3' side of the cross-link. This reaction completes cross-link removal, as the complementary strands can now diffuse independently after denaturation.

ATP could interact directly with UV endonuclease, or regulate the reaction at a prior step. Although E. coli UV endonuclease does not appear to require ATP in vitro (35, 36), this does not necessarily reflect the situation in whole or permeabilized cells. For example the Micrococcus luteus UV endonuclease appears to act in a processive fashion, and such a mechanism might require ATP in cells. ATP-dependent exonuclease V activity does not appear to be involved in cross-link removal, as the reaction proceeded normally in recB- or recC- strains. Since the methylene-substituted analogues of ATP were inactive, it cannot be distinguished whether hydrolysis of ATP was required for reaction.

It may be significant that the intracellular concentration of ATP in E. coli varies between 0.2 mM for stationary phase and 1.7 mM for exponentially dividing cells, and that the break in the curve in Fig. 3, showing dependency of cross-link removal rate on ATP concentration, occurs at 1.1 mM ATP.

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