**Micrococcus luteus** Correndonuclceses

II. MECHANISM OF ACTION OF TWO ENDONUCLEASES SPECIFIC FOR DNA CONTAINING PYRIMIDINE DIMERS*

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Two endonucleases which act on ultraviolet-irradiated DNA have been isolated from *Micrococcus luteus* and in order of their elution from a phosphocellulose (Whatman P-11) column these have been designated as Py → Py correndonuclceses I and II. In the preceding paper (1), purification and general properties of these two enzymes have been described. Both the enzymes are highly specific for ultraviolet-damaged DNA with no significant specificity for native DNA modified by ionizing radiation and alklylation.

The major photoproduct formed at low levels of ultraviolet irradiation is a cyclobutane-type pyrimidine dimer (2, 3). This lesion is removed efficiently in ultraviolet-resistant cells but persists and leads to cell death of ultraviolet-sensitive strains (4–6). The experiments reported in this paper demonstrate that both enzymes are capable of an endonucleolytic incision close to and on the same strand as pyrimidine dimers, generating the 3'-hydroxyl and 5'-phosphoryl termini suitable for the priming required of DNA polymerases. In determining if their catalytic roles are unique, we have investigated and compared mechanisms of their action. The significance of similarity in photoproduct specificity is discussed, and data are presented for their selectivity of damage located in regions of the DNA with unique conformational structures. The involvement of these endonucleases in repair in *vivo* of ultraviolet radiation damage in *M. luteus* has been further studied and is the subject of the next paper (7).

A preliminary report of this work has been published (8).

**EXPERIMENTAL PROCEDURES**

**Materials**

Enzymes—Snake venom phosphodiesterase from *Crotalus adamanteus* venom, spleen phosphodiesterase, micrococcal nuclease, pancreatic deoxyribonuclease (DNase I), and bacterial alkaline phosphatase were purchased from Worthington Biochemicals. Venom phosphodiesterase was further purified on a concanavalin A-Sepharose 4B column to remove nonspecific endonuclease activities according to the method of Dolapchiev et al. (9). *Micrococcus luteus* DNA polymerase (10), *Escherichia coli* exonuclease VII (11), *E. coli* exonuclease VIII (12), yeast photoreactivating enzyme (13), phage T4 polynucleotide ligase (14), and *E. coli* binding protein (15) were kindly provided by Dr. Lester D. Hamilton, The Johns Hopkins University, Dr. Charles C. Richardson, Harvard University, Dr. Harold Werbin, University of Texas, Dr. Paul Modrich, Duke University, and Dr. Malcolm Gefter, MIT, respectively.

Nucleic Acids and Oligonucleotides—DNA unlabeled and radioactive labeled (1 to 10 × 10⁶ cpm/nmol) with [3H]thymidine or [3P]phosphoribonucleoside was prepared from *E. coli* by the method of Grossman (16). [3H]Thymidine triphosphate (56 cpm/nmol) was purchased from New England Nuclear Corp.

Poly(dA)poly(dT) and poly(dG)poly(dC) were purchased from Collaborative Research, Inc. (Waltham, Mass.), and renatured before use by warming to 40°C and slow cooling in a buffer of pH 7.4 containing 100 mM Tris-HCl and 160 mM sodium chloride.

Nucleic acid concentrations are expressed in nucleotide equivalents unless otherwise stated.

Other Materials—Norit was activated by extensive acid washing (17) until the absorbance at 260 nm of the supernatant fluid dropped to zero. The Norit carrier contained 20 mM sodium pyrophosphate, 25 mM potassium phosphate (pH 7.0), and 5 mg/ml of bovine serum albumin.

**Methods**

Ultraviolet Irradiation—*E. coli* DNA (100 nmol) in 1 ml of NaCl/citrate was ultraviolet-irradiated with a 254 nm lamp. An incident dose of 6 J/m²/s was employed for 2 min to introduce about 4.9 pmol of pyrimidine dimers/nmol of DNA.

1 300 mM NaCl and 30 mM sodium citrate.
DNA-Initial incision of the ultraviolet-irradiated DNA (5 pmol at pH 8.0 1 pmol of p-nitrophenol in 1 min at 25°C. were carried out in parallel experiments. Negligible single strand termini or the conversion of RF1 molecules to the relaxed form which were counted in Aquasol in a Tri-carb liquid scintillation counter.

It was convenient to define two different units for endonuclease activity depending upon the type of substrate, the amount of damage, and the kind of assay used. In a bacterial alkaline phosphatase assay in which 5 pmol of 32P-labeled DNA containing 20 pmol of pyrimidine dimer was used as a substrate, 1 unit of activity was defined as that which incised 10 pmol of pyrimidine dimer in 30 min at 37°C. This is referred to as a bacterial alkaline phosphatase unit.

In a 5x174 RFI DNA incision assay in which 25 pmol of the substrate containing 0.25 pmol of pyrimidine dimers/nmol of DNA was used, 1 unit was defined as that amount of enzyme which in 30 min at 37°C produced at least one break in 63% of 5X174 RF1 circles, each containing on the average five pyrimidine dimers.

Micrococcal nuclease and pancreatic DNase I were assayed as described in the previous paper (1) using a radioactive micrococcal nuclease mixture used for the micrococcal nuclease included 10 mM sodium borate (pH 8.8), 2 mM calcium chloride and that for pancreatic DNase I included 50 mM Tris/HCl (pH 6.5) and 5 mM magnesium chloride.

The polymerizing activity of the M. luteus DNA polymerase was assayed as described by Hamilton et al. (10), using activated calf thymus DNA (20).

Phoretectivation - The reaction mixture (0.1 ml) containing 10 mM Tris/HCl (pH 7.5), 10 mM 2-mercaptoethanol, 150 mM sodium chloride, 25 pmol of 5x174 RFI DNA (five pyrimidine dimers/RFI molecule) and 10 transforming units of yeast photolyase (phoretactivating enzyme). Samples were incubated at 37°C under two General Electric black light fluorescent bulbs at a distance of 20 cm. Light below 500 nm was excluded using a Pyrex glass filter. Controls were incubated under identical conditions but devoid of light. At various times during incubation, the reaction mixtures were diluted 3-fold with 10 mM Tris/HCl, pH 7.4, and further incubated at 37°C for 30 min in the presence of 1.0 unit of the endonuclease. The extent of incision in the photoretactivated DNA by Py -> Py correondonuclease I or II was determined by retention of the RFI molecules on B-6 membrane filters.

Action of DNA Polymerase on Ultraviolet Endonuclease-incised DNA - Initial incubation of the ultraviolet-irradiated DNA (5 pmol of pyrimidine dimers/nmol of DNA) was carried out by incubation for 60 min at 37°C of 2 to 5 pmol of E. coli DNA with 2.5 bacterial alkaline phosphatase units of Py -> Py correondonuclease I or II in 0.1 ml of the reaction mixture described above. The endonuclease was inactivated by heating at 65°C for 60 min and the mixture was adjusted to 50 mM Tris/HCl (pH 8.0), 5 mM magnesium chloride, and 0.05 mM 2-mercaptoethanol. The samples were then processed as described below.

Nucleotide Incorporation at Incised Sites - To determine if the sites generated could act as primers for polymerization, the incised unlabeled DNA was incubated at 37°C in the presence of 0.033 µmol of each of the four deoxynucleoside triphosphates, 0.06 µCi of [3H]thymidine triphosphate, and 2.5 units of M. luteus DNA polymerase. Incorporation of deoxynucleotides into the DNA was determined by precipitating with 6% trichloroacetic acid containing 10 mM sodium pyrophosphate, by filtering on a Whatman GF/C glass filter, and by counting the dried filters in Instafluor (Packard).

Pyrimidine Dimer Excision - Excision of pyrimidine dimers from 51P-labeled DNA, by the DNA polymerase, was carried out in the absence of synthesis (deoxynucleoside triphosphates withheld) at 30°C for 5 min. Precipitation of the DNA by the addition of 50 µl of 20% trichloroacetic acid in the presence of 15 µg of carrier DNA, resulted in a supernatant fraction containing the excised pyrimidine dimers, which were counted in Aquasol. The insoluble fraction was prepared by the method of Setlow and Carrier (22) to assess the levels of those pyrimidine dimers remaining in the DNA.

Action of Phospholipases on Incised DNA - E. coli 51P-labeled DNA (5 nmol) after ultraviolet irradiation was incised with 2.5 bacterial alkaline phosphatase units of Py -> Py correondonuclease I or II, precipitated with trichloroacetic acid, and redisolved in sodium hydroxide (as above). The identity of the termini produced by incision was determined either before or after bacterial alkaline phosphatase treatment, according to their susceptibility to the specific action of spleen phosphodiesterase in 83 mM sodium succinate buffer (pH 8.0), 3 mM magnesium chloride and 3 mM EDTA and that of venom phosphodiesterase in 80 mM Tris/acetate (pH 6.4) and 80 mM magnesium acetate.

Dephosphorylation of Incised Native DNA - Five nanomoles of 51P-labeled DNA was incubated with 2.5 units of E. coli DNA polymerase in the presence of 20 pmol of pyrimidine dimer as a substrate. Samples were incubated at 37°C for 5 min. The reaction mixture containing 50 mM Tris/HCl (pH 7.6), 8.6 mM MgCl2, 6 mM 2-mercaptoethanol, 20 mM Tris, and 1 unit of phage T7, ligase. The catalytic activity of polynucleotide ligase was measured by formation of closed circles which, after denaturation and renaturation, were not retained by B-6 membrane filters.

Action of E. coli Exonuclease VII on Incised DNA Duplexes - Three nanomoles of 51P-labeled and endonuclease-incised DNA was incubated for 15 min at 0°C with that amount of E. coli binding protein (15) which binds stoichiometrically to 3 nmol of thermally denatured DNA. The reaction mixture (0.2 ml) containing 67 mM potassium phosphate (pH 7.0), 8.3 mM EDTA, 10 mM 2-mercaptoethanol, and 10 units of E. coli exonuclease VII was then incubated at 37°C for various time intervals. The radioactivity released by the exonuclease was measured by precipitating the DNA with 50 µl of 20% trichloroacetic acid and counting an aliquot of the supernatant fluid in Aquasol.

**RESULTS**

**Specificity for Pyrimidine Dimers containing DNA** - The substrate specificity of each enzyme for DNA-containing pyrimidine dimers was demonstrated by the rapid loss in the ability of ultraviolet-irradiated 5x174 RFI DNA to act as a substrate for the incorporation of deoxynucleotides.
Mechanism of Action of Two Repair Endonucleases


Enzymatic Incision Generates 3'-OH Termini—When ultraviolet-irradiated DNA was used as a substrate for polymerase I, incorporation of nucleotides occurred with essentially equal facility (Fig. 3). Since DNA polymerase I requires an unesterified 3'-OH nucleophilic site the presence of a 3' phosphomonoester group would be refractory to polymerization (24). Both endonucleases must, therefore, generate such a site as a consequence of incision.

That a 3'-hydroxyl terminus at the site of incision is generated during incision was further investigated by examining the exonucleolytic hydrolysis of incised DNA by venom phosphodiesterase, Escherichia coli exonuclease I or the 3' → 5' exonuclease associated with DNA polymerase I. All three exonuclease I require for hydrolysis of single-stranded DNA a 3' unesterified terminus (11, 25, 26). When incised and subsequently denatured DNA was exposed to the action of venom phosphodiesterase before and after dephosphorylation, the rate of hydrolysis did not change significantly. This was comparable to substrates generated by pancreatic DNase I (Fig. 4, B and D). Similar results were obtained during hydrolysis with exonuclease I and the 3' → 5' exonuclease associated with DNA polymerase I. These experiments demonstrate that both exonucleases generate 3'-hydroxyl and by inference 5'-phosphoryl termini.

Incision Is Produced 5' to the Photoproduc—The next question was the position of the incision in relation to the pyrimidine dimer since this fixes the requirement for the direction of excision action for appropriate exonucleases. Spleen phosphodiesterase (5' → 3'), snake venom phosphodiesterase (3' → 5'), and E. coli exonuclease I (3' → 5') are all inhibited by pyrimidine dimers (27, 28); therefore, inhibition of hydrolysis is diagnostic for the presence of photoproducts at the termini required for exonuclease action. Calf spleen phosphodiesterase initiates exonucleolytic hydrolysis from a 5' nonesterified terminus and as a consequence it acts on DNase I hydrolyzed DNA (which has 5'-phosphoryl termini) only following bacterial alkaline phosphatase treatment (Fig. 4, A and C). However, DNA incised with Py → Py correndonuclease I or II was insensitive to the exonucleolytic action of spleen phosphodies-
Mechanism of Action of Two Repair Endonucleases

Excision of Pyrimidine Dimers from Incised DNA by DNA Polymerase I—In order to examine any differences in the specificity of the two endonucleases for the various pyrimidine dimers, incised DNA after treatment with the 5′→3′ exonuclease associated with DNA polymerase I or human exonuclease (29) was analyzed for the unexcised thymine-thymine, thymine-cytosine, and cytosine-cytosine dimers. In Table I are data showing that either endonuclease initiates the excision process by either DNA polymerase I or human exonuclease at all types of pyrimidine dimer sites. The loss from the incised ultraviolet-irradiated DNA, reaching a level of 50 to 75%, was the same regardless of the endonuclease catalyzing the incision. However, when the two endonucleases were used simultaneously to incise the ultraviolet-damaged DNA, removal of thymine-cytosine dimers decreased selectively.

Influence of DNA Structure on Endonuclease Action—To determine how nucleotide chain length, base composition, and conformation of the substrate influenced action of the two endonucleases, synthetic polymers and DNAs of different base ratio were used as competitors of irradiated φX174 RF1 DNA in an incision assay. The results presented in Table II show that, whereas E. coli DNA-containing pyrimidine dimers caused 94 to 96% inhibition, no such interference was observed when DNA was substituted by ultraviolet-irradiated decaomers of poly(dA).poly(dT), poly(dT).poly(dC), or free pyrimidine dimers. These data indicate that both enzymes required that the competing polymer must be of minimum chain length (>10 nucleotides). There was no observable preference by either enzyme for DNAs from Haemophilus influenzae (high AT), Micrococcus luteus (high GC), and E. coli (GC = AT), (Table III) whose proportions of thymine to cytosine dimers should reflect the differing base ratios (31).

The experiments involving synthetic polymers (Table II) suggest that the structure of the substrate might influence the activity of the two enzymes in a specific way. Thus, poly(dG).poly(dC) in 50 mM phosphate or in 10 mM Tris/Cl supplemented with 100 mM sodium chloride competes with equal facility for either endonuclease activity. However, poly(dA).poly(dT), under these conditions, has little affinity for Py Py endonuclease I (<30%) and none for Py Py endonuclease II. The affinity of the latter endonuclease for this polymer was observed in 10 mM Tris/Cl in which the enzyme was not optimally active. Since structure of synthetic polymers is very much dependent on the ionic strength of the environment (30, 31), these results indicate that these enzymes may require distinct conformations at the site of damage.

Availability of 5′-Phosphoryl Termini of Incised DNA for Dephosphorylation by Bacterial Alkaline Phosphatase—Irradiated DNA incised with the Py Py endonucleases serves as a substrate for E. coli exonuclease VII and human exonuclease, both of which prefer single-stranded DNAs. On the other hand, incised DNAs are also substrates for the 5′→3′ exonuclease of polymerase I, which prefers double-
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**Table I**

**Excision of pyrimidine dimers**

Thymine- or cytosine-labeled DNA (2 nmol) was incubated with 2.5 bacterial alkaline phosphatase units of each endonuclease, either individually or in combination. The incised DNA, after inactivation of the endonucleases at 70° for 5 min, was treated with DNA polymerase or human placental corexonuclease. The unexcised pyrimidine dimers remaining in the DNA were determined by its hydrolysis in 97% formic acid and paper chromatography of the hydrolysate in 1-butanol:acetic acid:water (80:12:30) as described by Setlow and Carrier (22).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pyrimidine dimer content of treated DNA</th>
<th>Per cent dimers excised</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T &lt;-&gt; T C &lt;-&gt; T C &lt;-&gt; C</td>
<td>T &lt;-&gt; T C &lt;-&gt; T C &lt;-&gt; C</td>
</tr>
<tr>
<td>Ultraviolet irradiation</td>
<td>8.50 8.05 3.15</td>
<td>54 51 55</td>
</tr>
<tr>
<td>Py &lt;-&gt; Py corexonuclease I + DNA polymerase I</td>
<td>3.91 3.94 1.42</td>
<td>55 35 58</td>
</tr>
<tr>
<td>Py &lt;-&gt; Py corexonuclease II + DNA polymerase I</td>
<td>3.49 3.22 1.10</td>
<td>59 60 65</td>
</tr>
<tr>
<td>Py &lt;-&gt; Py corexonuclease I and II + DNA polymerase I</td>
<td>3.83 5.23 1.21</td>
<td>55 35 58</td>
</tr>
<tr>
<td>Py &lt;-&gt; Py corexonuclease I + human corexonuclease</td>
<td>4.09 4.67 1.81</td>
<td>46 42 49</td>
</tr>
<tr>
<td>Py &lt;-&gt; Py corexonuclease II + human corexonuclease</td>
<td>2.13 2.17 1.13</td>
<td>75 73 64</td>
</tr>
<tr>
<td>Py &lt;-&gt; Py corexonuclease I and II + human corexonuclease</td>
<td>2.38 5.60 1.27</td>
<td>72 30 59</td>
</tr>
</tbody>
</table>

**Table II**

**Competition by ultraviolet-irradiated synthetic homopolymers in φX incision assay**

Synthetic homopolymers (0.005 A units) were ultraviolet-irradiated (100 J/m²) and mixed with 3H-labeled ultraviolet-irradiated φX174 RFI DNA at a nucleotide ratio of 10:1. The mixture was incubated at 0° for 5 min prior to incision with 1 unit of Py <-> Py corexonuclease I or II at 37° for 30 min. Percent competition was calculated from decrease in radioactivity retained on the B-6 membrane filters. Control experiments were run in parallel in which ultraviolet-irradiated polymer was substituted by the unirradiated polymer.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Reaction condition</th>
<th>Competition</th>
<th>I</th>
<th>II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(dI)-poly(dC)</td>
<td>Tris</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tris + 100 mM NaCl</td>
<td>63</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>Oligo(dI)·oligo(dC)ₙ</td>
<td>Tris</td>
<td>62</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50 mM phosphate</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Poly(dA)·poly(dT)</td>
<td>Tris</td>
<td>55</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tris + 100 mM NaCl</td>
<td>2</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Oligo(dA)·oligo(dT)ₙ</td>
<td>Tris</td>
<td>2</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50 mM phosphate</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Pyrimidine dimers in situ</td>
<td>Complete reaction</td>
<td>94</td>
<td>96</td>
<td></td>
</tr>
<tr>
<td>Pyrimidine dimers isolated from 2.5 nmol of E. coli DNA</td>
<td>Complete reaction</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

**Table III**

**Competition by ultraviolet-irradiated DNA in φX incision assay**

DNAs (500 pmol) of various base composition containing 2 pmol of pyrimidine dimers/nmol were used to compete for Py <-> Py corexonuclease I and II activities in the φX174 RFI DNA assay (see "Methods"). The nucleotide ratio of cold to labeled DNA and other reaction conditions were the same as described in the legend to Table II.

<table>
<thead>
<tr>
<th>DNA isolated from:</th>
<th>Treatment</th>
<th>Per cent competition for activities of Py &lt;-&gt; Py Corexonucleases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td><em>Haemophilus influenzae</em></td>
<td>Native</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Irradiated</td>
<td>59</td>
</tr>
<tr>
<td><em>Micrococcus luteus</em></td>
<td>Native</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Irradiated</td>
<td>30</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>Native</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Irradiated</td>
<td>46</td>
</tr>
</tbody>
</table>

stranded regions, and is capable of acting on DNase I-activated DNA with little or no affinity for denatured DNA (10, 26). It is possible that the photoproduc bearing termini present varying degrees of single-stranded character. The single strandedness of a terminus bearing a phosphomonoester group can be assessed by its sensitivity to bacterial alkaline phosphatase. Weiss et al. (32) demonstrated that those termini generated by the action of pancreatic DNase I on native DNA could not be dephosphorylated unless the substrate DNA was heated to near melting temperatures. These data suggested that internal phosphomonoesters were not accessible to bacterial alkaline phosphatase. In the present experiments 62% of Py <-> Py corexonuclease I-generated sites were susceptible to the action of bacterial alkaline phosphatase as opposed to only 12% of those produced by Py <-> Py corexonuclease II (Fig. 5). Complete dephosphorylation of sites generated by the latter endonuclease (Fig. 5) as those produced by pancreatic DNase I (32) was achieved only when the DNA was melted by
Fig. 5 (left). Temperature dependence of dephosphorylation by bacterial alkaline phosphatase (BAP) of native ultraviolet-irradiated (1440 J/m²)[^2] Escherichia coli DNA after incision with Py or Py correndonuclease I or II. After inactivating the endonuclease at 55°C for 60 min, the incised DNA was sealed at 30°C for 45 min with 2 units of phage T₄ polynucleotide ligase in the presence of 66 µM ATP. The ligase-treated DNA, after denaturation and renaturation, was filtered through B-6 membrane filters and the radioactivity going through was taken as an index of ligase activity.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Radioactivity retained on the B-6 filters (cpm)</th>
<th>Fraction retained: (A - B)/(A) x 100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreatic DNase I-incised</td>
<td>9,880</td>
<td>3,440</td>
</tr>
<tr>
<td>Py &lt;-&gt; Py correndonuclease I-treated DNA</td>
<td>8,270</td>
<td>7,870</td>
</tr>
<tr>
<td>Py &lt;-&gt; Py correndonuclease II-treated DNA</td>
<td>8,100</td>
<td>6,280</td>
</tr>
</tbody>
</table>

These data further support the suggestion that the former produced termini more single-stranded than those generated by the Py <-> Py II.

Fig. 6 (right). Kinetics of hydrolysis by exonuclease VII of native irradiated (1440 J/m²)[^2] DNA after incision with Py or Py correndonuclease I or II in the absence (open symbols) or presence (closed symbols) of saturating amounts of Escherichia coli binding protein. Three nanomoles of incised DNA was incubated for 15 min at 0°C with the binding protein prior to treatment with exonuclease VII at 37°C. The values are reported relative to the extent of hydrolysis of incised DNA by the exonuclease in the absence of binding protein.

**TABLE IV**

**Rejoining of endonuclease-incised DNA**

Twenty-five picomoles of φX174 RFI DNA containing five pyrimidine dimers/RFI molecule was incised at 37°C for 30 min with 1 unit of Py or Py correndonuclease I or II. After inactivating the endonuclease at 55°C for 60 min, the incised DNA was sealed at 30°C for 45 min with 2 units of phage T₄ polynucleotide ligase in the presence of 66 µM ATP. The ligase-treated DNA, after denaturation and renaturation, was filtered through B-6 membrane filters and the radioactivity going through was taken as an index of ligase activity.

<table>
<thead>
<tr>
<th>Temperature (°C) of Incubation with BAP</th>
<th>20</th>
<th>40</th>
<th>60</th>
<th>60</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radioactivity retained (cpm)</td>
<td>25</td>
<td>25</td>
<td>25</td>
<td>25</td>
<td>25</td>
</tr>
</tbody>
</table>

**DISCUSSION**

In the preceding paper (11), it was established that Py <-> Py correndonuclease I and II are discrete and separate proteins. The *in vitro* studies presented in this paper show that both these enzymes are pyrimidine dimer-specific and can initiate excision (Table I) and repair of the damage (Fig. 3) by polymerase I. Their dual role in cellular repair of ultraviolet-irradiated DNA will be supported in the succeeding paper (7) on biological data with mutants and transformants of *M. luteus* of differing sensitivities to ultraviolet irradiation.

Their separate and unique roles in the mechanism of repair may lie in the recognition of those conformationally distinct sites of DNA in which pyrimidine dimers are located. This was suggested by the results of the experiments involving synthetic polymers as competitors of the endonuclease assay. The
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substrate specificity of these polymers, however, was influenced as a result of changes of ionic strength in their environment which influence their physical properties (31). Ultraviolet-irradiated poly(dA)-poly(dT) effectively competed with ultraviolet-damaged φX RFI DNA for both activities in high salt, whereas poly(dA)-poly(dC) had a similar effect at low or negligible salt concentrations. The influence of substrate conformation was influenced by free pyrimidine dimers or dimers contained in decanucleotides. It should be mentioned that the correndonucleases act in the vicinity of pyrimidine dimers, suggesting that the physical configurations surrounding the damaged region may influence specificity.

That the region surrounding dimers determines the specificity for these enzymes is further suggested by the results of phosphomonoesterase-catalyzed dephosphorylation. Since the termini produced as a consequence of incision by Py correndonuclease I are more accessible to bacterial alkaline phosphatase and better protected by the binding protein against the hydrolytic action of exonuclease VII, it may be inferred that these regions yield termini which are more single-stranded in character. The results of the ligase experiment (1) and may have a role in recombinational repair.

The dose-response data shown in Fig. 2 suggest that approximately two Py correndonuclease II-sensitive sites are generated to one sensitive to Py correndonuclease I at low levels of irradiation. It should be emphasized, however, that DNA used in experiments in vitro does not represent the cellular in vivo configuration. Hence, this relationship may not hold true in cellular repair.

In E. coli, dark repair of ultraviolet damage can occur through two pathways involving excision system and the recombination system. The excision system is mediated through DNA polymerase I and produces a short patch, whereas the recombination system mediated through the recA gene product produces a long patch (35, 36). Both the systems are dependent on an endonucleolytic incision and are, therefore, blocked in incision-defective uvrA mutants (36, 37). It is difficult to ascertain if one of the correndonucleases is also involved in recombinational repair. Py correndonuclease I, however, can act on denatured ultraviolet-irradiated DNA (1) and may have a role in recombinational repair.

Acknowledgments—Some of the pyrimidine dimer excision experiments were carried out at the Brookhaven National Laboratories, for which we are thankful to Drs. R. B. Setlow and J. Doniger.

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