Micrococcus luteus Correndonuclease

I. RESOLUTION AND PURIFICATION OF TWO ENDONUCLEASES SPECIFIC FOR DNA CONTAINING PYRIMIDINE DIMERS*

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Five peaks of endonuclease activity showing a preference for ultraviolet-damaged DNA have been chromatographically identified from extracts of Micrococcus luteus. They are numerically designated as I to V in order of their elution from phosphocellulose (Whatman P-11) columns. The first two of these peaks have been highly purified by a combination of gel filtration and affinity chromatography and are catalytically homogeneous judging from their effect on transforming DNAs. Peak I, which has an isoelectric point of 4.7, is heat-stable, requires high ionic strength for optimal activity, acts with equal facility on ultraviolet-irradiated native and denatured DNA, and has been designated as Py $\leftrightarrow$ Py correndonuclease I. Peak II which has a pH value of 8.7, is heat-labile, is inhibited by high ionic strength, acts on ultraviolet irradiated native but not denatured DNA, and has been designated as Py $\leftrightarrow$ Py correndonuclease II. Both enzymes are inhibited by Ca$^{2+}$ and Zn$^{2+}$, do not show any cofactor or sulfhydryl requirement, act optimally between pH 7.0 and 7.4, and have molecular weights between 11,000 and 15,000. Py $\leftrightarrow$ Py correndonuclease I requires a dose about 1.6 times that for Py $\leftrightarrow$ Py correndonuclease II for incision saturation of irradiated φX174 RF1 DNA.

The ability of living cells to survive the lethal effects of ultraviolet radiation damage has been ascribed in part to efficient removal of the damage through an excision repair pathway (1–3). Carrier and Setlow (4) and Boyce and Howard-Flanders (5) originally demonstrated excision of pyrimidine dimers in Escherichia coli cells, a mechanism which is now believed to be present in eukaryotic cells as well. This process of repair is initiated by an endonuclease-catalyzed incision in the vicinity of the damage subsequently removed exonucleolytically. The 5' → 3' double strand-specific exonuclease associated with DNA polymerase I has been shown to remove photochemical damage (6, 7). In addition, unassociated single strand-specific exonucleases which can excise pyrimidine dimers from ultraviolet-irradiated incised DNA have been isolated from Micrococcus luteus (8), E. coli (9), and human placenta (10). After removal of the damage, DNA polymerases completely reinsert complementary nucleotides, providing a substrate structure suitable for polynucleotide ligase restoring the integrity of duplex DNA (1, 7).

Endonucleases specifically requiring ultraviolet-damaged DNA as a substrate have been isolated from M. luteus (11–13), E. coli (14, 15), phage-infected cells (16, 17), rat liver (18), calf thymus (19), and slime molds (20). However, an endonuclease acting on ultraviolet-irradiated DNA in vitro does not necessarily involve it in DNA repair in vivo. Braun and Grossman (14) located an endonuclease (peak II) from wild type E. coli which demonstrated a dependence on ultraviolet-irradiated DNA and was present at normal wild type levels in the radiation-sensitive uvrA and uvrB mutants. This type of enzyme may have other roles such as the correction of mismatched base pairs in DNA (15). Furthermore, DNA damaged by heavy radiation can also serve as a substrate for single strand-specific endonucleases (21, 22). The action in this case is due to recognition of locally denatured regions produced in the DNA as a consequence of ultraviolet irradiation. The role of endonucleases in DNA repair mechanisms requires that they not only recognize and incise at the damage site but also produce termini suitable for removal of the damage by excision enzymes, reinsertion of nucleotides by polymerases, and final phosphodiester bond formation by ligase.

In this communication we have demonstrated the existence in M. luteus of five distinctly separable peaks of activity specific for ultraviolet-damaged DNA. These have been tentatively designated as peaks I to V (in order of their chromatographic elution from Whatman P-11) until their role in vivo is determined and correct nomenclature is established. We, furthermore, describe a chromatographic procedure capable of resolving these five activities and purification of peaks I and II. Studies on the mechanism of action of peaks I and II have demonstrated that they initiate in vitro excision and repair of pyrimidine dimers and are absent from an ultraviolet light-sensitive mutant of M. luteus (23). We propose that peak I and II enzymes be designated as Py $\leftrightarrow$ Py correndonuclease I and II in that they are endonucleases whose action on pyrimidine dimer-containing DNA eventuates in correctional repair.
mechanisms. Nomenclature for correctional endonucleases is tentative, pending identification of their structural genes and complete substrate specificity. The other three peaks, which also act on x-irradiated and alkylated DNA, are being further purified and their physiological significance is being investigated.

A preliminary report of this work has been published (23).

EXPERIMENTAL PROCEDURES

Materials

Cells, Enzymes, and Chemicals—Micrococcus luteus wild type strain was obtained from the American Type Culture Collection (ATCC 4698) and maintained on nutrient broth. Grade 1 lysosome from egg white was purchased from Sigma Chemical Company. Whatman DEAE-dextran (DE52) was used without further treatment. Phosphocellulose powder (Whatman P-11) was prewashed according to the manufacturer's instructions. Sephadex G-75 was obtained from Pharmacia Fine Chemicals and swollen for 24 h before use.

Nucleic Acids—Trinitium-labeled ϕX174 RF DNA prepared by the method of Marmur (25) and 1 mM ethylenediaminetetraacetic acid was irradiated in a 2-mm path length quartz cuvette. A low pressure mercury lamp (Sylvania) emitting primarily at 254 nm was employed without any filters. The incident dose determined by a Black Ray ultraviolet monitor (Ultra-Violet Products, Inc.) was 1.4 J/m²/s.

Ultraviolet Irradiation—Trinitium-labeled ϕX174 RF DNA at a concentration of 1 to 2 μg/ml in 10 mM tris(hydroxymethylamino) methane hydrochloride (pH 7.4) and 1 mM ethylenediaminetetraacetic acid was irradiated in a 2-mm path length quartz cuvette. A low pressure mercury lamp (Sylvania) emitting primarily at 254 nm was employed without any filters. The incident dose determined by a Black Ray ultraviolet monitor (Ultra-Violet Products, Inc.) was 1.4 J/m²/s.

Ultraviolet-irradiated DNA-cellulose—Cellulose (Munktell No. 410) was washed several times with boiling ethanol to remove pyridine, reduced at 60° for 60 min with 0.2% sodium borohydride, washed with water to neutrality, and lyophilized.

Treated cellulose (10 g) was mixed into a slurry with 20 ml of calf thymus DNA (2 mg/ml) which had been ultraviolet-irradiated for 1 h at an incident dose of 15 J/m²/s, spread in a thin layer on a glass surface, and dried overnight with a stream of hot air. The dried mass was resuspended in 20 ml of water and the drying procedure was repeated. Finally, it was resuspended in 100 ml of 5% ethanol, irradiated for 10 min with the same radiation dose, filtered, and washed exhaustively with 10 mM sodium chloride. The product was air-dried and stored at −20°.

Enzyme Assays—The standard assay mixture (0.3 ml) contained 10 mM Tris/HCl buffer (pH 7.4), 1 mM EDTA, 50 mM NaCl, 25 pmol of [3H] ϕX174 RF DNA (10,000 cpm) containing 6 pmol of pyrimidine dimers/nmol of DNA, and 1 to 10 units of endonuclease activity. One unit of enzyme is that amount which, from a Poisson distribution, produces, on the average, one break/174 RF1 DNA molecule in a total of 25 pmol of substrate in 30 min at 37°.

Binding Assay—The assay mixture incubated at 0° for 5 min was diluted with 2 ml of ice-cold 0.15 m NaCl/0.015 m citrate and immediately filtered through HAWP Millipore filters at a flow rate of 10 ml/min. The reaction tubes were washed once with 2 ml of cold NaCl/citrate and the filters were dried and counted in 2 ml of toluene-containing scintillation fluid, in a Packard scintillation counter. The radioactivity retained on the filters was taken as a measure of endonuclease binding activity.

Incision Assay—[3H] ϕX174 RF DNA was incubated at 37° for 30 min, denatured by adding 2 ml of a pH 12 buffer containing 100 mM sodium phosphate, 30 mM sodium chloroide, and 25 mM EDTA, and finally neutralized to pH 8.0 by adding 0.4 ml of 2 M Tris/HCl (pH 4.0) solution. [3H] ϕX174 RF DNA was conserved under these conditions whereas [3H] ϕ-X174 RF DNA was converted to single stranded species, which were retained when the mixture was filtered through B-6 (Schleicher and Schuell) membranes (26). The reaction tubes were washed twice with 4 ml of cold NaCl/citrate and 0.015 m citrate were dried and counted as mentioned above. The radioactivity retained on the membrane filters was taken as a measure of endonuclease incising activity. The ultraviolet damage specific activity was measured by the difference in activities against ultraviolet-irradiated and unirradiated DNA.

Endonuclease—Protein was determined by the method of Lowry et al. (27) using bovine serum albumin as a reference. Salt gradients were checked with a Radiometer conductivity meter. All pH measurements were made at a buffer concentration of 50 mM at room temperature. X-irradiation of the DNA at a concentration of 1 to 2 μg/ml was carried out under ultraviolet light (ϕ7.4) and 1 mM EDTA; DNA was alkylated with methyl methanesulfonate and ethyl methanesulfonate according to the method of Verly et al. (28). DNA was cross-linked using 4,5',8-trimethylpsoralen (Paul Elder and Co.) as described by Cole (29).

Isolation and Purification of Endonucleases

Unless otherwise indicated, all operations were performed at 4°.

Growth of Cells—M. luteus cells were grown at 34° under forced aeration in a Microferm laboratory fermenter (New Brunswick Scientific) in 12 liters of Columbia broth (Difco). Cells were harvested at a cell density of about 5 x 10⁹/ml by centrifugation in a Sharples continuous flow centrifuge, washed with 50 mM potassium phosphate buffer (pH 7.6), and stored at −20°.

Preparation of Extract—Frozen cells (75 g) were suspended by homogenization in a Waring Blender in 50 mM potassium phosphate (pH 7.6), 1 mM 2-mercaptoethanol, 1 mM EDTA, and 10% glycerol (Buffer X). The cell suspension (2 ml) was centrifuged at 100,000 g for 30 min at 37° for 10 min and cooled quickly by the addition of 200 ml of frozen Buffer X. Under these conditions, the cells lysed, yielding a dark brown viscous mass.

The cell lysate was broken by sonic irradiation for 7 min at full power with a sonifer sonifier. Cell debris was washed with Buffer X and stored at −20° for further purification (Fraction A).

DEAE-cellulose Chromatography—A column of Whatman DE52 (30.3 cm x 12 cm) was prepared and washed with 6 liters of Buffer X at pH 7.0. Fraction A was loaded onto both the binding and incision assays (Figs. 1 and 2). The first two peaks of Py −→ Py correndonuclease activity eluted between 125 and 175 mm potassium phosphate (peaks I and II in Fig. 2) and were pooled together for further purification (Fraction B), and peaks III, IV, and V were separately pooled at −20° for further investigation.

Sephadex G-75 Chromatography—A column of Sephadex G-75 (4.9 cm x 100 cm) was prepared and washed with 100 mM potassium phosphate (pH 7.6), 5 mM 2-mercaptoethanol, 5 mM EDTA, and 10% glycerol (Buffer Y). Fraction B after dialysis against 100 mM Tris/HCl (pH 7.6) and 1 mM EDTA was loaded onto the column at 2 ml/min and washed with 300 ml of the same buffer. The flowthrough and wash were combined to a final volume of about 900 ml (Fraction C).

Phosphocellulose Chromatography—A column of Whatman P-11 (4.9 cm x 23 cm) was equilibrated by washing with 4 liters of Buffer X at pH 7.0. Fraction C (0.6 g of protein) after dialysis against 10 volumes of Buffer X at pH 7.0 was applied to the column with an adjusted flow rate of 1 ml/min and washed with 200 ml of Buffer X. The protein was eluted in 15-ml fractions with a 1000-mm linear gradient of 50 to 300 mM potassium phosphate (pH 7.4) containing 1 mM 2-mercaptoethanol, 1 mM EDTA, and 10% glycerol. On completion of the gradient, the column was washed with 500 ml of 300 mM and finally 300 ml of 500 mM potassium phosphate (pH 7.4) containing 1 mM 2-mercaptoethanol, 1 mM EDTA, and 10% glycerol to elute peaks IV and V, respectively. The ultraviolet damage-dependent endonuclease activities were detected both by binding and incision assays (Figs. 1 and 2). The first two peaks of Py −→ Py correndonuclease activity eluted between 125 and 175 mm potassium phosphate (peaks I and II in Fig. 2) and were pooled together for further purification (Fraction C), and peaks III, IV, and V were separately pooled at −20° for further investigation.

Sephadex G-75 Chromatography—A column of Sephadex G-75 (4.9 cm x 100 cm) was prepared and washed with 100 mM potassium phosphate (pH 7.6), 5 mM 2-mercaptoethanol, 5 mM EDTA, and 10% glycerol (Buffer Y). Fraction C after concentration by precipitation with ammonium sulfate was layered onto the column at 2 ml/min and finally washed with a 3-ml aliquot of Fraction D.

A 3-ml aliquot of Fraction D was dialyzed against 4 liters of 100 mM NaCl in Buffer Z. Twenty 1.5-ml fractions were collected and assayed by the binding and incision methods for the presence of activities against ultraviolet-irradiated DNA (Fig. 3). The major activity eluting at a nonsymmetrical peak between Fractions 44 and 51 was pooled and concentrated to 5 ml by adsorption to a 2.5-ml column of phosphocellulose and eluting with 10 ml of 300 mM potassium phosphate at pH 7.6. The concentrated enzyme was dialyzed against 2 liters of Buffer Y for about 3 h and divided into small aliquots for storage in liquid nitrogen (Fraction D).

Phosphocellulose Chromatography—A column of phosphocellulose (0.28 cm x 8 cm) was prepared and washed with 100 ml of 10 mM Tris/HCl (pH 7.6), 1 mM EDTA, 1 mM 2-mercaptoethanol (Buffer Z). A 0.5 ml aliquot of Fraction D was dialyzed for 4 h against 4 liters of Buffer Z and applied to the column at a rate of 1 ml/20 to 30 min. The column was washed with 5 ml of the same buffer and eluted with a 50-ml gradient of 2 mM NaCl in Buffer Z. Finally 1.5-ml fractions were collected and tested for the presence of endonuclease activity (Fig. 4) by the incision assay using lightly ultraviolet-

1 300 mM NaCl and 30 mM sodium citrate.
irradiated DNA (20 J/m²). Fractions 3 to 5 were pooled separately from Fractions 10 to 14, dialyzed against 3 liters of Buffer Z, and stored in liquid nitrogen.

Isoelectric Focusing—Two milliliter aliquots of Fraction D were focused in a vertical isoelectric focusing apparatus (LKB, type 6101) containing a 104-ml linear gradient of 5 to 50% glycerol with 2% ampholine in the pH range 3 to 10. At 600 V, the separation was complete in 72 h and 62 fractions were collected and assayed for endonuclease activity. Two activities with isoelectric point 8.7 (Fractions 11 to 14) and 4.7 (Fractions 36 to 37) were separated (Fig. 5). The respective fractions were pooled and dialyzed against Buffer Z and stored in liquid nitrogen.

RESULTS

The results of a typical purification are summarized in Table I.

DEAE-cellulose Chromatography—This DEAE-cellulose column was used to remove the DNA and a major part of the exonuclease. About 30 to 50% of the total proteins were removed with a minimal loss of endonucleases. This step was necessary in separating peak III from the major protein peak on the next column.

Phosphocellulose Chromatography The result of a typical binding assay with fractions from a P-11 column is shown in Fig. 1. There are two peaks of activity which bind specifically to ultraviolet-irradiated DNA (I and II) and three others which bind to x-irradiated and alkylated DNAs (III, IV, and V). Peaks I and II are eluted ahead of the major protein peak at a concentration of 125 to 175 mM potassium phosphate (pH 7.4). Peak III is eluted immediately after the protein peak, whereas Peaks IV and V require elution concentrations of 300 and 500 mM potassium phosphate, respectively. From the results of the incision assay it can be seen (Fig. 2) that peaks III, IV, and V are active only against DNA heavily damaged by ultraviolet radiation (500 J/m²) in contrast to peaks I and II which act on DNA irradiated at low biological doses (10 J/m²). The first two peaks of endonuclease are not well separated from each other on this column; therefore, these are pooled in an attempt to achieve complete separation in subsequent chromatographic steps.

Sephadex Chromatography—This column as shown in Fig. 3 removed a high molecular weight nonspecific endonuclease from the damage-dependent ones which were not separated but eluted as a broad peak in the vicinity of the cytochrome c marker (M₀ = 13,000). On the basis of the weak binding of peak I to ultraviolet-irradiated DNA (compare Figs. 1 and 2), it was established that this enzyme eluted in the first half of the broad peak and, therefore, had a slightly higher molecular weight than peak II enzyme. The pooled peak (Fraction D) when used under conditions described under “Experimental Procedures” was over 97% specific for ultraviolet-irradiated DNA and had less than 3% contamination of endonuclease activities. At this stage, the enzymes are very stable and have been stored at −20° in Buffer Y containing 50% glycerol for 3 years without appreciable loss in activity. Fraction D used in experi-

![Fig. 1. Elution pattern of DNA-binding activities from phosphocellulose (P-11). The activities were determined by the "binding assay" using native (Δ—Δ), ultraviolet-irradiated (●—●), x-irradiated (○—○), and alkylated DNA (▲—▲). Absorbance was measured at 280 nm (□—□).](http://www.jbc.org/)

![Fig. 2. Elution pattern of endonucleolytic activities from a phosphocellulose (P-11). The 4X RFI incision assay was used to measure activity against native DNA (Δ—Δ), lightly ultraviolet-irradiated (10 J/m²) DNA (○—○), and heavily ultraviolet-damaged (500 J/m²) DNA (●—●). The concentration of the eluting phosphate buffer was obtained from a measurement of the conductivity (———).](http://www.jbc.org/)

TABLE I

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Peaks</th>
<th>Volume</th>
<th>Activity</th>
<th>Protein</th>
<th>Specific activity</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ml</td>
<td>UV-dependentać</td>
<td>Non-specific</td>
<td>mg/ml</td>
<td>µmol/mg</td>
</tr>
<tr>
<td>Extract</td>
<td>I → V</td>
<td>580</td>
<td>216</td>
<td>193.5</td>
<td>13.8</td>
<td>26.9</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>I → V</td>
<td>900</td>
<td>200</td>
<td>105.9</td>
<td>5.5</td>
<td>49.8</td>
</tr>
<tr>
<td>Phosphocellulose</td>
<td>I + II</td>
<td>120</td>
<td>104</td>
<td>4.9</td>
<td>0.6</td>
<td>1370</td>
</tr>
<tr>
<td>III</td>
<td>60</td>
<td>0.8</td>
<td>0.1</td>
<td>1.0</td>
<td>14.2</td>
<td>0.03</td>
</tr>
<tr>
<td>IV</td>
<td>45</td>
<td>0.5</td>
<td>0.04</td>
<td>0.1</td>
<td>118</td>
<td>0.02</td>
</tr>
<tr>
<td>V</td>
<td>45</td>
<td></td>
<td>1.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sephadex G-75</td>
<td>I + II</td>
<td>40</td>
<td>54.3</td>
<td>0.3</td>
<td>0.04</td>
<td>27,200</td>
</tr>
<tr>
<td>DNA-cellulose</td>
<td>I</td>
<td>4</td>
<td>1.7</td>
<td></td>
<td></td>
<td>0.6</td>
</tr>
<tr>
<td>II</td>
<td>7</td>
<td>11.5</td>
<td></td>
<td></td>
<td></td>
<td>4.6</td>
</tr>
<tr>
<td>Isoelectric focusing</td>
<td>I</td>
<td>3</td>
<td>0.9</td>
<td></td>
<td></td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>5</td>
<td>3.9</td>
<td></td>
<td></td>
<td>1.6</td>
</tr>
</tbody>
</table>

* Specific activity was defined as bacterial alkaline phosphatase units of ultraviolet dependent activity per mg of total proteins.

* UV-dependent activity was determined by the difference in total activities against ultraviolet-damaged DNA and native DNA.

* A BAP unit was defined as the amount of activity which incised 10 pmol of pyrimidine dimers in 30 min at 37°.
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DNA-cellulose Chromatography—This affinity column, shown in Fig. 4, resolved the two peaks of activity which eluted in the same order as obtained with the phosphocellulose column. Py $\rightarrow$ Py correndonuclease I (peak I) was eluted by 200 to 400 mM NaCl, whereas Py $\rightarrow$ Py correndonuclease II was more tenaciously bound to DNA-cellulose requiring 1.2 to 1.3 M salt for elution.

Isoelectric Focusing—Complete separation of Py $\rightarrow$ Py correndonuclease I and II was also achieved during isoelectric focusing of Fraction D in a pH 3 to 10 gradient (Fig. 5). However, this method of resolution has not been used for preparative purposes since the ampholines are inhibitory and difficult to remove completely from the isolated enzymes.

When the two enzymes separated on a DNA-cellulose column were individually subjected to isoelectric focusing, there was only one peak of activity recovered. These results confirm not only the purity of these two peaks but also their existence as distinctly separate enzymes. The activity which had a pI value of 4.7 (Fig. 5) corresponds with Py correndonuclease I on DNA-cellulose or phosphocellulose columns. The other enzyme which had a pI value of 8.7 is designated as Py $\rightarrow$ Py correndonuclease II.

Properties of Purified Py $\rightarrow$ Py Correndonuclease

Stability—The two correndonuclease after separation on a DNA-cellulose column were very unstable, undergoing complete inactivation in 3 to 4 weeks in 50% glycerol at -20°. The losses in activity, however, were considerably minimized when the enzymes were stored in liquid nitrogen.

Purity—The purified enzymes were not contaminated with endonucleolytic activities against native $\phi$X174RFI or single-stranded circular $\phi$X174 DNA. They further resulted in no loss to transforming activity of Bacillus subtilis DNA when incubated with the DNA either singly or together under the standard assay conditions. There was only one peak of activity recovered when either enzyme was individually subjected isoelectric focusing.

pH Optima and Buffer Requirements—Both the enzymes exhibit broad pH optima between 7.0 and 7.4 in either Tris/HCl or potassium phosphate buffer. No activity is observed below pH 4.0 or above pH 10.0. Activities in potassium phosphate are slightly higher than in Tris/HCl but the two buffers can be used interchangeably without any serious loss in activity.

Effect of Ionic Strength—High ionic strength was required for normal levels of Py $\rightarrow$ Py correndonuclease I activity. Thus this enzyme showed optimal activity in either 10 mM Tris/HCl supplemented with 50 mM NaCl or in 50 mM potassium phosphate. Py $\rightarrow$ Py correndonuclease II, however, required lower ionic strength and was fully active in only 10 mM Tris/HCl (Table II).

Requirement for Cations—There was no absolute requirement for divalent cations and the two enzymes were active even in the presence of 5 mM EDTA (Table II). Increasing the concentration of EDTA to 20 mM, however, decreased the Py $\rightarrow$ Py correndonuclease II activity. This effect of the chelating agent may be attributed to increased ionic strength. There was no requirement for monovalent cations and the stimulatory effects of NaCl or KCl on Py $\rightarrow$ Py correndonuclease (Fig. 6) may be due to increased ionic strength. Calcium and magnesium ions were not required.

TABLE II

Requirements for Py $\rightarrow$ Py correndonuclease I and II activity

<table>
<thead>
<tr>
<th>Condition</th>
<th>Py $\rightarrow$ Py correndonuclease I</th>
<th>Py $\rightarrow$ Py correndonuclease II</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>NaCl + 1.2 mM ATP</td>
<td>80</td>
<td>120</td>
</tr>
<tr>
<td>NaCl + 7 mM MgCl₂</td>
<td>100</td>
<td>60</td>
</tr>
<tr>
<td>NaCl + 7 mM MgCl₂ + 1.2 mM ATP</td>
<td>125</td>
<td>77</td>
</tr>
<tr>
<td>NaCl + 5 mM EDTA</td>
<td>65</td>
<td>78</td>
</tr>
<tr>
<td>NaCl + 20 mM EDTA</td>
<td>95</td>
<td>45</td>
</tr>
<tr>
<td>NaCl + 50 mM phosphate buffer, pH 7.4</td>
<td>110</td>
<td>93</td>
</tr>
<tr>
<td>+3 mM 2-mercaptoethanol</td>
<td>102</td>
<td>91</td>
</tr>
<tr>
<td>+25 mM N-ethylmaleimide</td>
<td>97</td>
<td>77</td>
</tr>
<tr>
<td>+0.1 to 1.2 mM cofactors</td>
<td>90-115</td>
<td>88-110</td>
</tr>
</tbody>
</table>
zinc inhibited both enzymes. Py correnendonuclease II was inhibited in a high ionic strength environment and, therefore, lost over 75% of its optimal activity in the presence of 100 mM NaCl or KCl or 15 mM MgCl₂ (Fig. 6).

Sulfhydryl Requirement—The enzymes neither required 2-mercaptoethanol in the standard assay mixture nor was the activity of the two enzymes significantly changed by the presence of 25 mM N-ethylmaleimide (Table II). This indicates, therefore, there was no sulfhydryl requirement for enzymic activity.

Co-factor Requirement—The two enzymes did not require any of the co-factors listed in Table II for activity. Although rATP increased Py correnendonuclease I activity, the stimulation was replaced by NaCl or MgCl₂ in the assay mixture (Table II). These results indicate that the stimulation could be an effect of ionic strength (Table II).

Dose Response—The incision activities of the two enzymes as a function of the extent of photochemical damage to the φX174RFI DNA is shown in Fig. 7A. Py correnendonuclease II produces 63% incision of the molecules at 7 J/m² as opposed to 11 J/m² required by the other endonuclease to generate a similar level of breaks. However, these values were extremely low when compared with those of peaks III, IV, and V which required 390, 150, and 250 J/m² respectively, to produce 63% incision (Fig. 7B).

Specificity—The two enzymes had no appreciable activity against x-irradiated or alkylated DNA (Fig. 1). Py correnendonuclease I acted on ultraviolet-irradiated native and denatured DNA, whereas with Py correnendonuclease II the specificity was limited to native DNA (Table III).

Activity against Cross-linked DNA—It was somewhat surprising that neither enzyme was active against psoralen cross-linked DNA (Fig. 8). It was anticipated from genetic experiments in Escherichia coli that resistance to ultraviolet light
and cross-linking agents may be controlled by the same structural genes (30).

Heat Inactivation — Stability of the two enzymes during incubation at 45° and 55° is shown in Fig. 8. In addition to the monophasic inactivation demonstrated by both enzymes, Py ←→ Py correndonuclease I was more stable than Py ←→ Py correndonuclease II at either temperature. During a 5-min incubation at 55°, the activity of the latter enzyme was reduced to less than 25%, whereas the former endonuclease I still retained 80% of its original activity.

**DISCUSSION**

Five different peaks of activity specific for DNA irradiated in vitro with a low pressure mercury lamp have been isolated from Micrococcus luteus and partially resolved by phosphocellulose chromatography.

Two of the five peaks that eluted between 125 and 175 mM potassium phosphate were not well resolved on phosphocellulose columns. When resolution of these enzymes was attempted on a DEAE-cellulose column, it was still not satisfactory even though Py ←→ Py correndonuclease I was bound more tightly to this positively charged resin than the second endonuclease. Complete separation was achieved by DNA-cellulose affinity chromatography. It is difficult to explain the poor resolution on the charged resins of two proteins with isoelectric values of 4.7 and 8.7.

These two enzymes have been highly purified when judged by isoelectric focusing and lack of interference in transformation assays. The purified enzymes have no requirements for co-factors, cations, or sulphhydryl compounds, have similar molecular weights, and show the same pH optima. Despite these similarities, the enzymes show marked differences in heat sensitivity, response to ultraviolet dose, and stimulation by salt. These differences, however, do not rule out the possibility that one of these enzymes is derived from the other in vivo.

Although the presence in a bacterium, of two seemingly different proteins with strikingly similar enzymatic properties is paradoxical, their involvement in repair in vivo is clear from a comparative study of the mutants and transformants of differing radiosensitivities (31).

The three other peaks which represent distinctly separate enzymes as judged from their dose response (Fig. 7B) are being further purified and characterized and will appear in later publications. It is clear, however, that they act either directly or indirectly on a site provided by severely damaged DNA. Peak III which has been studied the most extensively, requires extremely high ultraviolet doses and is active against x-irradiated DNA, alkylated and OsO₄-treated DNA, and depurinated DNA. It is possible that this enzyme can recognize common secondary effects in the DNA molecule arising from the diverse forms of damaging agents.

The presence of several endonucleasees specific for ultraviolet light-damaged DNA is not surprising. When DNA is ultraviolet-irradiated in vitro, several lesions, in addition to the well characterized pyrimidine dimers, are produced (32, 33). Hariharan and Cerutti (34) demonstrated that 5,6-dihy- droxydifumaral is formed when DNA is exposed to 254 nm irradiation. Feldberg and Grossman (24) recently discussed production of damage other than pyrimidine dimers during low levels of ultraviolet irradiation of DNA. The present data show that enzymes specific for such photoproducts exist in M. luteus cells. The levels of peaks III, IV, and V, in contrast to the Py ←→ Py correndonuclease I and II, were not changed even in the most ultraviolet-sensitive mutant strain (34). The physiological significance of the enzymes acting on heavily ultraviolet-damaged DNA is, therefore, not clear at this juncture. In any event, these studies suggest that in all enzymatic analyses a clear distinction should be made between a primary "repair endonuclease" and a "damage-dependent endonuclease" which may recognize the secondary effects of heavy irradiation or non-pyrimidine dimer damage which has yet to be correlated to the lethal effects of such irradiation. An ultraviolet repair endonuclease is defined as that enzyme which is primarily specific for regions of DNA containing pyrimidine dimers and is absent in ultraviolet-sensitive mutants. In contrast to it, an ultraviolet-dependent endonuclease may act on any modification introduced in this case by heavy irradiation of DNA. This would also include a single strand-specific endonuclease which can act on irradiated DNA because of its affinity for the locally denatured regions (19, 20) consequent to gross damage. Such enzymes are not necessarily involved in excision repair in vivo and, therefore, may be present in incision-defective mutants.

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**REFERENCES**

S Riazuddin and L Grossman

endonucleases specific for DNA containing pyrimidine dimers.

Micrococcus luteus correndonucleases. I. resolution and purification of two


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