References
Adams, G. E., & Wardman, P. (1977) in *Free Radicals in Biology* (Pryor, W. A., Ed.) Vol. 3, pp 53–95, Academic Press, New York.
Becvar, J. (1973) Ph.D. Thesis, University of Michigan.
Bisby, R. H., Cundall, R. B., Redpath, J. L., & Adams, G. E. (1976) *J. Chem. Soc., Faraday Trans. 1*, *72*, 51–63.
Faraggi, M., & Klapper, M. H. (1979) *J. Biol. Chem. 254*, 8139–8142.
Faraggi, M., Redpath, J. L., & Tal, Y. (1975) *Radiat. Res. 64*, 452–456.
Faraggi, M., Klapper, M. H., & Dorfman, L. M. (1978) *J. Phys. Chem. 82*, 508–512.
Hoffman, M. Z., & Hayon, E. (1972) *J. Am. Chem. Soc. 94*, 7950–7957.
Klapper, M. H., & Faraggi, M. (1979) *Q. Rev. Biophys. 12*, 465–519.
Land, E. J., & Swallow, A. J. (1969) *Biochemistry 8*, 2117–2125.
Massey, V., Stankovich, M., & Hemmerich, P. (1978) *Biochemistry 17*, 1–8.
Matheson, M. S., & Dorfman, L. M. (1969) *Pulse Radiolysis*, MIT Press, Cambridge, MA.
Mayhew, S. G., & Ludwig, M. L. (1975) *Enzymes, 3rd Ed. 12*, 57–118.

Characterization of the *Escherichia coli* X-ray Endonuclease, Endonuclease III†

Harold L. Katcher‡ and Susan S. Wallace*

ABSTRACT: The X-ray endonuclease endonuclease III of *Escherichia coli* has been purified to apparent homogeneity by using the criterion of sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The most purified fraction shows endonucleolytic activity against apurinic and apyrimidinic (AP) sites and a dose-dependent response to DNA that has been X irradiated, UV irradiated, or treated with OsO_4. The endonuclease also nicks OsO_4-treated DNA that has been subsequently treated with alkali to produce fragmented thymine residues and DNA treated with potassium permanganate. The enzyme does not incise the alkali-labile sites present in DNA X irradiated in vitro in the presence of hydroxyl radical scavengers. The most purified fractions exhibit two distinct activities, an AP endonuclease that cleaves on the 3′ side of the damage leaving a 3′-OH and a 5′-PO_4 and a DNA *N*-glycosylase that recognizes at least two substrates, thymine glycol residues and urea residues. The glycosylase activity is sensitive to *N*-ethylmaleimide while the AP endonuclease is not.

Ionizing radiation produces a variety of damages in DNA including single- and double-strand breaks, alkali-labile damages, and both DNA–DNA and DNA–protein cross-links [see Ward (1975) for review]. The primary criterion for establishing these categories is the particular measurement used to quantitate the damage. Base damages are the preponderant lesion produced (Cerutti, 1975a,b) and are included in all of these categories. However, the quantitation and characterization of base damages are difficult because small numbers of each particular type of damage are present in irradiated DNA, and the instability of some of these damages precludes their measurement by chemical means.

At present, one class of base lesions can be quantitated (Hariharan, 1980), the common radiolysis product of thymine (Scholes & Weiss, 1960), moieties of the 5,6-dihydroxydihydrothymine type (thymine glycols). Thymine glycols have been shown to be lethal lesions in both single-stranded (Hariharan et al., 1977) and double-stranded (Moran et al., 1980) phage transfecting DNAs. Further, *Micrococcus radiodurans* have been shown to specifically release this product into the medium following γ irradiation (Hariharan & Cerutti, 1972). Also, crude extracts of *Escherichia coli* remove thymine glycols from γ-irradiated or OsO_4-treated DNA (Hariharan & Cerutti, 1974). Thus, it is likely that an excision type repair mechanism might function in cells to remove X-ray-induced base damage from DNA.

A number of years ago this laboratory reported that partially purified extracts of *E. coli* were capable of specifically nicking X-irradiated (Strniste & Wallace, 1975) and OsO_4-treated (Armel et al., 1977) DNA; this activity was called the X-ray endonuclease. Subsequently, Radman (1976) reported the purification of an endonuclease from *E. coli*, endonuclease III, that recognized minor photoproducts of UV irradiation, and a similar activity was reported by Gates & Linn (1977b) to nick DNA that was heavily UV irradiated, X irradiated, OsO_4 treated, or depurinated by heat/acid treatment. This latter activity, believed to be endonuclease III, was a byproduct of the purification of endonuclease V (Gates & Linn, 1977a). It has been reported recently (Demple & Linn, 1980) that the enzyme isolated by Gates and Linn has an associated DNA glycosylase specific for thymine glycol residues. Another DNA glycosylase, specific for urea residues in DNA, has been found in 10-fold purified extracts of *E. coli* by Breimer & Lindahl (1980); such urea residues are known products of the radiolysis of thymine residues in DNA (Têoule et al., 1977).

In this paper we report purification of the X-ray endonuclease and show these highly purified preparations to contain

† From the Department of Microbiology, New York Medical College, Valhalla, New York 10595. *Received March 15, 1983.* This work was supported by Grants CA21342 and CA33657 awarded by the National Cancer Institute, U.S. Department of Health and Human Services, and a contract from the U.S. Department of Energy.
‡ Present address: Department of Mathematics, University of Southern California, Los Angeles, CA 90007.

0006-2960/83/0422-4071$01.50/0

all the activities ascribed to endonuclease III and the urea *N*-glycosylase. The X-ray endonuclease appears to be a dimer that has both an AP endonuclease activity and a DNA *N*-glycosylase that recognizes at least two different damaged DNA substrates.

Experimental Procedures

Bacteria and Bacteriophage. *Escherichia coli* AB3027, *thr-1*, *leuB6*, *thi-1*, *proA2*, *argE3*, *his-4*, *xthA14*, *polA20*, *lacY1*, *galK2*, *mtl-1*, *xyl-5*, *ara-14*, *rha-6*, *strA31*, *tsx-33*, λ–, *supE44*, obtained from the *E. coli* Genetic Stock Center, Yale, was used for all enzyme preparations. The bacteria were grown in a fermentor in modified Zubay's medium (Zubay et al., 1970), supplemented with casamino acids and streptomycin sulfate (50 μg/mL), to late log phase, collected by centrifugation, and stored at −20 °C. PM2 bacteriophage and host *Alteromonas espejiana* were grown as previously described (Wallace et al., 1981).

Enzymes. DNA polymerase I (*E. coli*) was purified by the method of Jovins et al. (1969) through fraction VII. Exonuclease III (*E. coli*) was purchased from Miles Laboratories; phosphodiesterase II (bovine spleen) and alkaline phosphatase (*E. coli*) were purchased from P-L Biochemicals. Uracil–DNA *N*-glycosylase was generously supplied by E. Friedberg.

Nucleic Acids and Derivatives. ^{3}H-Labeled PM2 DNA was prepared as previously described (Wallace et al., 1981); the specific activity was 30 000–120 000 cpm/μg. [2-^{14}C]Poly[dT(Na)], (2.75 Ci/mmol), was purchased from P-L Biochemicals as was poly(dA). [*methyl*-^{3}H]dTTP (58 Ci/mmol) was purchased from Schwarz/Mann. [*methyl*-^{3}H]dT (63 Ci/mmol) was bought from ICN Pharmaceuticals. *E. coli* tRNA was obtained from Boehringer Mannheim. Thymine glycol and thymidine glycol monophosphate, prepared by the method of Baudisch & Davidson (1925), were gifts of B. Erlanger, and *cis*-thymine glycol was a gift of J. Cadet.

Other Reagents. Agarose, type A, and streptomycin sulfate, grade b, were purchased from Calbiochem-Behring. Acrylamide and *N,N'*-bis(acrylamide), TEMED,[1] and 2-mercaptoethanol were purchased from Sigma Chemical Co. Dithiothreitol and *N*-ethylmaleimide are products of Eastman Kodak Co.; phosphocellulose (P-11), DEAE-cellulose (DE52), and 3MM chromatography paper are produced by Whatman Ltd. Polygram Cel 300 PEI/UV_{254} and Polygram Cell 300/UV_{254} MN were purchased from Brinkmann Instruments, Inc. Sephadex G-75 and G-100, CM-Sephadex, and DEAE-Sephadex were bought from Pharmacia Fine Chemicals. AG 50W-X8 cation-exchange resin and AG 1-X8 anion-exchange resin as well as hydroxylapatite (Bio-Gel HTP) and the protein molecular weight standards (Low Molecular Weight Gel Filtration Kit) were purchased from Bio-Rad.

Preparation of Substrates. For X-irradiated DNA, small volumes (33–83 μL) of ^{3}H-labeled PM2 DNA (approximately 10 μg/mL) in 10 mM Tris, pH 8.0, and 50 mM KI were irradiated by a Philips X-ray source with a Machlett tube (beryllium window) at 60 kVp and 2.5 mA. The dose rate was 27.5 krd/min as determined by the inactivation of coliphage T4.

For OsO_4-treated DNA, a solution of [*methyl*-^{3}H]thymidine-labeled PM2 DNA (5–50 μg/mL, 50 000 cpm/μg) was preheated to 70 °C for 2 min, brought to 0.02% OsO_4 from a 4% stock solution, and incubated for 5 min. Following incubation the tube was cooled to room temperature and extracted with 2 volumes of diethyl ether 3 times. The solution was then extensively dialyzed against 10 mM Tris and 1 mM EDTA. This treatment results in one to three enzyme sensitive sites per molecule. For determination of the DNA glycosylase activity, ^{3}H-labeled PM2 DNA (10 μg/mL, 500 000 cpm/μg) was preheated to 82 °C for 2 min and enough 4% OsO_4 added to bring the OsO_4 concentration to 0.2%. Incubation was continued for about 15 min or until the solution developed a light amber color. This procedure results in the oxidation of 5–30% of the thymine residues present.

For ultraviolet-irradiated DNA, a solution of ^{3}H-labeled PM2 DNA (100 μL at 10 μg/mL) was placed in a watch glass about 15 cm from a General Electric germicidal lamp, with a UV flux of about 4 J m^{-2} and irradiated from 0 to 10 min. The UV-irradiated DNA was used immediately.

Apurinic DNA was prepared according to the procedure of Lindahl & Andersson (1972). ^{3}H-Labeled PM2 DNA was dialyzed against a solution of 0.1 M NaCl, 10 mM Tris-HCl, and 10 mM sodium citrate, pH 5.2, and then heated at 70 °C for 10 min to produce one to two apurinic sites per molecule. The reaction was stopped by placing the solution on ice and adding enough 1 M Tris, pH 7.5, to bring the final Tris concentration to 50 mM. AP sites were quantified by comparing the number of strand breaks present in a preparation of DNA analyzed by neutral sucrose gradient sedimentation to the number present when analyzed by alkaline sucrose gradient sedimentation following incubation of the samples with an equal volume of 1 M glycine–KOH, pH 13.0, for at least 4 h.

For uracil-containing DNA, 1 volume of 2.0 M $NaHSO_3$, 1.0 M $Na_2S_2O_5$, and 0.5 M sodium acetate, adjusted to pH 5.9 with NaOH, was added to an equal volume of PM2 DNA (16 μg/mL) and incubated at 37 °C for 9 h. The DNA was dialyzed overnight against 50 mM Hepes, pH 8.0, and 1 mM EDTA. This preparation was assayed for the number of uracil residues present by treatment with a saturating amount of uracil–DNA glycosylase and assaying for AP sites.

For apyrimidinic DNA, uracil-containing DNA (above) was treated with saturating amounts of uracil–DNA glycosylase for 1 h at 37 °C. This DNA was then assayed for AP sites. All substrates, with the exception of UV-irradiated DNA, were quick frozen in dry ice–acetone and stored at −70 °C up to several months without breakdown.

OsO_4-treated poly(dT) was prepared by bringing a solution of 1 μCi/mL [2-^{14}C]poly(dT) (2.75 mCi/mmol) to 0.4% OsO_4, incubating it at 55 °C for 20 min, and ether extracting 4 times with 2 volumes of diethyl ether.

Poly(dT) containing fragmented thymine residues was prepared by taking OsO_4-treated poly(dT), bringing it to 0.3 M sodium acetate, and then adding 3 volumes of punctilious alcohol. The reaction tube was left on ice for 30 min and then spun for 10 min at 10 000 rpm in an SS34 rotor. The supernatant was discarded and the centrifuge tube washed once with cold ethanol and dried under vacuum. The precipitate was resuspended in 0.2 M KOH (0.5 mL), left at room temperature for 2 h, and neutralized with 1 M HCl. This DNA was then precipitated with 3 volumes of alcohol and dried under vacuum.

For "treated" poly(dT)·poly(dA), precipitated OsO_4-treated or OsO_4-treated, alkali-treated [2-^{14}C]poly(dT) was redissolved in 0.5 mL of reaction buffer containing an equimolar concentration of poly(dA). The solution was placed in a 55 °C

[1] Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; AP, apurinic/apyrimidinic; NEM, *N*-ethylmaleimide; PEI, poly(ethylenimine); TEMED, *N,N,N',N'*-tetramethylethylenediamine; TLC, thin-layer chromatography; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTT, dithiothreitol; EtSH, 2-mercaptoethanol; Cl_3CCOOH, trichloroacetic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

water bath for 1 min and then slow cooled.

Endonuclease Assay. Endonuclease assay reaction mixes contained 100–500 ng of ^{3}H-labeled PM2 DNA (50 000–100 000 cpm/μg) in 10 mM Tris, pH 7.5, and 1 mM each of EDTA and EtSH in a total volume of 45–95 μL. To this was added 5 μL of enzyme, and the solution was adjusted to 0.1 M KCl. The reaction was incubated for 10 min at 37 °C and stopped by one of three methods depending on the means of subsequent analysis. For analysis by velocity sedimentation using neutral sucrose gradients, 33 μL of 4 M KCl was added to the 100 μL total reaction mix and layered on 5-mL gradients of 5–20% sucrose. For analysis on agarose gels, 15 μL of stopping solution containing 5% sodium dodecyl sulfate, 30% glycerol, 0.1 M EDTA, and 0.05% bromophenol blue was added to a 50-μL reaction. The reaction mixes may be quick frozen in dry ice–acetone either before or after addition of the stopping solution and stored for several weeks at −70 °C before analysis without breakdown of the DNA. The unit of activity is defined as the number of femtomoles of substrate converted to strand breaks in 10 min under these reaction conditions.

DNA Glycosylase/Endonuclease Assay. In order to detect DNA glycosylase activity independent of endonuclease activity, the following assay was performed. Reaction conditions were identical with those of the endonuclease assay except that reactions were stopped by the addition of equal volumes of 1 M glycine–NaOH buffer, pH 12.8. These were then incubated at room temperature for 4 h, loaded onto cold 5-mL alkaline sucrose gradients (5–20% sucrose, 0.1 M NaOH, 1.0 M KCl, and 1 mM EDTA), and centrifuged at 45 000 rpm for 100 min. Incubation in alkali under these conditions was sufficient to convert all AP sites to strand breaks. The number of strand breaks revealed by this analysis should be independent of the presence of an AP endonuclease.

Electrophoresis. For the determination of type I and II PM2 DNA, 1.0 g of agarose was brought to 100 mL with 2 × TEB buffer (20 × TEB has 108 g of Tris base, 55.65 g of boric acid, and 4.65 g of Na_2EDTA brought to 500 mL with distilled water) and heated until the agarose dissolved. A slab gel, 2.5 mm thick, was formed with a 20-tooth well comb. The gel was loaded and run on a vertical slab gel apparatus (Aquabogue) in 1 × TEB buffer at a constant current of 35 mA for 3 h. The gel was then stained with ethidium bromide [1 drop of EtBr (10 mg/mL)/400 mL of TEB buffer), and after 30 min the bands corresponding to type I and type II DNA were cut out, acidified, melted, and placed in liquid scintillation fluid for counting.

For the analysis of [2-^{14}C]poly(dT)·(dA), a variation of the procedure of Maniatis et al. (1975) was followed. Slab gels (2.5 mm thick), containing 5% acrylamide [acrylamide–*N*,*N*′-bis(acrylamide), 30:1], were polymerized in TEB buffer containing 7 M urea and run in a vertical gel electrophoresis apparatus, the reservoirs of which were filled with 20 mM sodium phosphate, pH 7.5. The samples containing oligonucleotide were ethanol precipitated, dried under vacuum, redissolved in 98% deionized formamide, boiled for 2 min, and then brought to 0.025% in bromophenol blue. The gel was run at a constant voltage of 10 V/cm (90 V). After the dye marker reached the bottom of the gel, electrophoresis was stopped, and the gel was cut into eight equal pieces and counted directly in scintillation fluid (Liquiscint).

For analysis of proteins, a modification of the procedure of Laemmli (1970) was used. Proteins were concentrated to dryness by dialysis against dry Sephadex G-200 and redissolved in running buffer. The protein-containing solutions were brought to 10% glycerol, 1% EtSH, 1% $NaDodSO_4$, and 0.025% bromophenol blue and placed in a boiling water bath for 3 min. The protein was loaded onto a discontinuous slab gel consisting of an upper (stacking) gel [3% acrylamide (30:1), 0.125 M Tris-HCl, pH 6.8, and 1% $NaDodSO_4$] and a lower sizing gel [12.5% acrylamide (30:1), 0.375 M Tris-HCl, pH 8.8, and 1% $NaDodSO_4$]. The 2.5 mm thick gel was run in an Aquabogue vertical gel apparatus at a constant current of 50 mA until the dye marker reached the bottom of the gel. The Bio-Rad $NaDodSO_4$–polyacrylamide gel electrophoresis low molecular weight marker proteins were used as standards. Gels were stained with Comassie Brilliant Blue.

Analysis of the Acid/Alcohol-Soluble Products of the Digestion of DNA. For paper chromatography, Whatman 3MM paper was used in the descending mode for 9–10 h. The solvent consisted of the upper phase of a mixture of ethyl acetate–*n*-propyl alcohol–water (4:1:2). For thin-layer chromatography, a plastic-backed thin-layer plate was run in a tank preequilibrated with solvent. For the cellulose TLC the solvent used was propanol–H_2O, 3:1. For the PEI–cellulose TLC water was used as the solvent.

For the analysis of thymidine, thymine, and dTMP markers, visualization under UV light was used. Urea was visualized by spraying the chromatograms with Warner's reagent, a solution of 10% *p*-(dimethylamino)benzaldehyde in concentrated HCl which is mixed with 4 volumes of acetone immediately prior to use (Ekert, 1962). Thymine glycol derivatives, thymine glycol, thymidine glycol, and thymine glycol monophosphate, were visualized by a modification of Fink's technique (Fink et al., 1956). The chromatograms were first sprayed with 1 M methanolic NaOH, dried, and then sprayed with Warner's reagent. The thymine glycol derivatives were characterized by a yellow spot which over time becomes pink and then eventually blue.

DNA Polymerase I Binding Site Assay. DNA (375 ng/reaction), untreated, OsO_4 treated with three enzyme-susceptible sites per molecule, or heat/acid treated to produce about two AP sites per molecule, was incubated for 20 min at 37 °C in 25 mM Tris, pH 7.5, 1 mM EDTA, and 0.1 M KCl with or without a 10-fold excess of X-ray endonuclease (fraction V). After a 30-min incubation, reactions were stopped by heating at 70 °C for 3 min. The reactions were brought to 10 mM $MgCl_2$, and 1 unit of exo III/endo VI was added to test reactions. After a 10-min incubation at 37 °C, all reactions were heated for 10 min at 70 °C. For each DNA substrate (untreated, OsO_4 treated, or heat/acid treated), four samples were analyzed for susceptibility to DNA polymerase I: (1) no enzyme treatment, (2) X-ray endonuclease treated (3) exonuclease III/endonuclease VI treated, and (4) both X-ray endonuclease and exonuclease III/endonuclease VI treated. These reactions were placed on ice, and to each reaction was added 20 nmol each of dATP, dGTP, and dCTP as well as 0.8 nmol of cold dTTP together with 2.5 μCi of [^{3}H]dTTP (58 Ci/nmol). The reaction volume was brought to 200 μL with 25 mM Tris buffer, pH 7.5, and $MgCl_2$ was added to bring the final concentration to 8 mM $MgCl_2$. To this was added 0.86 unit of DNA polymerase I [fraction VIII of Jovins et al. (1969)]. After the samples were mixed, 25-μL aliquots were removed from each reaction and placed on glass fiber filters (GF/A), washed with ice-cold Cl_3CCOOH and then with ice-cold 95% ethanol. The reactions were then incubated at 37 °C, and 25-μL aliquots were withdrawn at 20-min intervals and treated as above. All filters were dried and assayed for radioactivity.

Glycerol Gradients. The X-ray endonuclease (fraction V) was diluted 1:4 with 10 mM Tris, pH 8.0, 1.0 M KCl, 1 mM

EDTA, and 1 mM DTT, and 100 μL of this solution was layered onto a 5-mL gradient of 20–40% glycerol in the same buffer. The gradients were centrifuged in a Beckman SW50.1 rotor for 48 h at 2 °C. The gradients were collected in 0.18-mL fractions and assayed for activity. The sedimentation coefficient was determined by comparison to three known proteins run simultaneously in separate gradient tubes (ribonuclease A, bovine chymotrypsinogen, and bovine serum albumin).

Enzyme Purification. Purification up to and including single-stranded DNA–agarose chromatography is described in detail elsewhere (Wallace et al., 1981). Frozen cells were thawed and mixed with one-third their volume with 10 mM Tris, pH 8.0, 1 mM EDTA, 1 mM EtSH, and 10% glycerol (buffer A) made 4 M with respect to KCl. To the resultant suspension was added an equal weight of glass beads, and the mixture was then ground in a Braun MSK homogenizer for 2 min with CO_2 cooling. Glass beads and cell debris were removed by centrifugation, and the pellet was discarded. To the clear supernatant, fraction I, was added half of its volume of a 30% solution of PEG 6000 in buffer A containing 1 M KCl followed by stirring for 20 min. The turbid suspension was centrifuged at 12000*g* for 15 min, and the pellet, containing the nucleic acids, was discarded. The supernatant, fraction II, was diluted with three parts of buffer A to a final concentration of 0.25 M KCl and then applied to a single-stranded DNA–agarose column at 2 column volumes/h. The column was washed with 3 column volumes of 0.3 M KCl in buffer A, drained, and eluted with 2 column volumes of 1.0 M KCl in buffer A. The eluate, fraction III, was then mixed with an equal volume of precooled glycerol and stored at −20 °C. At this point the enzyme can be stored for up to 1 year at −20 °C without loss of activity. Fraction III is approximately 50–100-fold purified and has no endonucleolytic activity against untreated PM2 DNA in the presence or absence of magnesium.

Hydroxylapatite was suspended in buffer A plus 0.5 M KCl, and 1.0 mL bed volume of hydroxylapatite was used for each 0.5 mg of protein fraction III. The ratio of column diameter to length was approximately 1:3. Fraction III was applied directly to the column which was then washed with 3 column volumes of 0.1 M potassium phosphate buffer, pH 8.0, 10% glycerol, 1 mM EDTA, and 1 mM DTT (0.1 M buffer B) and the activity, fraction IV, was eluted with 0.3 M buffer B. Fraction IV was concentrated 10–20-fold by dialysis against a 30% solution (w/v) of PEG 20000 in buffer A plus 1.0 M KCl. The concentrate was immediately applied to a Sephadex column.

A column with a bed volume of 500 mL (90 cm × 5.3 cm^2) of either Sephadex G-75 or Sephadex G-100 was equilibrated with buffer A plus 1.0 M KCl at 4 °C. Fraction IV, in a volume of about 5% of the Sephadex bed volume, was applied to the column and eluted with the equilibration buffer. Samples equal to half the applied volume were collected and assayed for endonuclease activity. The activity elutes as would a spherical protein of molecular weight 25000 (±10%). The active fractions were concentrated 10-fold by dialysis against PEG 20000 as above (fraction V).

Defined phosphocellulose was loaded as a thin slurry into a 1-mL column and equilibrated overnight with buffer A containing 0.25 M KCl. Fraction V was diluted with buffer A to 0.25 M KCl and applied to the column. The column was then washed with 2 mL of buffer A plus 0.25 M KCl, and the enzyme was eluted with a linear gradient of 0.3–0.6 M KCl (10 mL); 0.5-mL fractions were collected. The activity (fraction VI) elutes between 0.4 and 0.5 M KCl. The protein concentration of the active fraction was typically less than 5 μg/mL, and the enzyme activity is unstable at this point.

Table I: Purification of the *Escherichia coli* X-ray Endonuclease

fraction no.	description	protein concn (mg/mL)	sp act. (units/mg of protein)	yield (%)
I	crude extract PEG 6000	19	7000	100
II	supernatant	8	13000	100
III	DNA-agarose, 1 M KCl eluate	0.3	320000	25
IV	hydroxylapatite, 0.3 M eluate after PEG 20000 concn	0.9	780000	23
V	Sephadex G-75, M_r 25000	0.06	4.6×10^6	19
VI	phosphocellulose, 0.4–0.5 M eluate	<0.01	*a*	>0.3

[a] Single band on $NaDodSO_4$ gel.

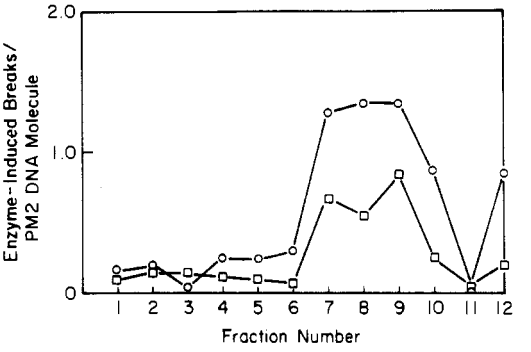

FIGURE 1: Coelution from phosphocellulose of endonuclease activities against AP and OsO_4-treated DNA. X-ray endonuclease (fraction V) was applied to a 0.5 × 3 cm column and eluted with a 10 column volume gradient of 0.25–0.6 M KCl in buffer A. Fractions (1 mL) were simultaneously assayed for endonuclease activity on AP DNA (□) and for DNA glycolsylase/endonuclease activity on OsO_4-treated DNA (○) as described under Experimental Procedures. Both activities coelute between 0.4 and 0.5 M.

The purification scheme shown in Table I is based on the nicking of X-irradiated DNA. Similar results were obtained when AP DNA, OsO_4-treated DNA, or UV-irradiated DNA was used. When the enzyme was assayed simultaneously with several substrates, all activities copurified at all stages (see Figure 1 for phosphocellulose chromatography using OsO_4-treated and AP DNA substrates). Further attempts to separate these activities by ion-exchange chromatography on CM-Sephadex and DEAE-cellulose or by sedimentation on glycerol gradients failed. The phosphocellulose fraction VI revealed a single band on $NaDodSO_4$–polyacrylamide gel electrophoresis.

The X-ray endonuclease was stable up to the concentrated Sephadex eluate step, fraction V, providing that the proper conditions of ionic strength, temperature, and reducing potential were maintained. Enzyme stored at ionic strengths below 0.1 M KCl showed a loss of activity toward both AP DNA and OsO_4-treated DNA substrates. Storage without the presence of a reducing environment (1 mM EtSH or 1 mM DTT) caused a loss in activity toward X-irradiated DNA and OsO_4-treated DNA but not toward AP DNA. At low ionic strength the enzyme formed aggregates that eluted in the void volume of a Sephadex G-100 column with the concomitant loss of 95% of the activity. The use of ion-exchange resins at any point in the purification led to a significant loss of activity.

Table II: X-ray Endonuclease Substrate Specificity

type of DNA treatment	probable lesion(s)	alkali-stable sites susceptible to enzyme	alkali-labile sites susceptible to enzyme	inhibition of nicking by NEM	DNA glycosylase activity
acid/heat	apurinic site	no	yes	no	
bisulfite	uracil residues	no	no		
bisulfite, uracil-DNA glycosylase	apyrimidinic sites	no	yes	NT[a]	
OsO_4 treatment	thymine glycol residues	yes	no	yes	yes
OsO_4 treatment followed by alkali	urea residues	yes	no	yes	yes
X-irradiated DNA (50 mM KI)	thymine glycols, peroxides, hydrates, and base fragment residues	yes	no	yes	yes
permanganate	thymine glycols and base fragment residues	yes[b]		NT	NT
ultraviolet	minor UV photoproducts and apyrimidinic sites	yes[b]		yes	NT

[a] NT = not tested. [b] Alkali stability of endonuclease-sensitive sites could not be tested as alkali produced complete conversion to type II DNA with or without enzyme added.

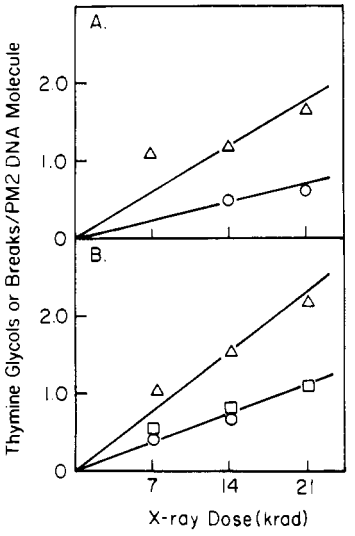

FIGURE 2: Relationship between X-ray endonuclease sites, strand breaks, and alkali-labile lesions in X-irradiated PM2 DNA. (A) ^{3}H-Labeled PM2 was irradiated in the presence of 50 mM KI at the indicated times, and strand breaks were measured immediately by neutral sucrose gradient centrifugation (○) or after incubation with saturating amounts of X-ray endonuclease (fraction VI) (△). (B) ^{3}H-Labeled PM2 DNA was X irradiated as above, incubated with alkali (as described under Experimental Procedures) and analyzed by alkaline sucrose gradient sedimentation either without enzyme (○), after incubation with saturating amounts of X-ray endonuclease (fraction VI) (△), or after incubation with X-ray endonuclease in the presence of 10 mM NEM (□).

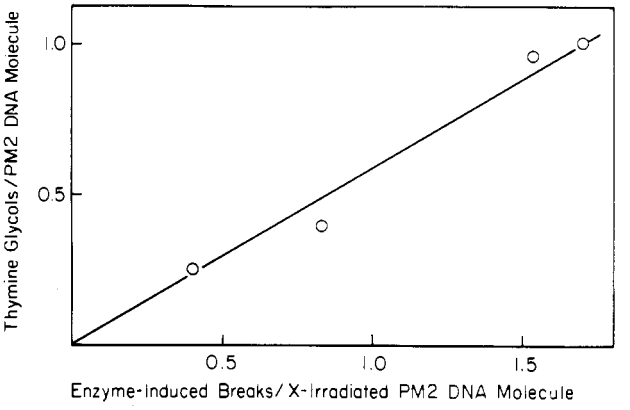

FIGURE 3: Relationship between X-ray-induced endonuclease-susceptible sites and X-ray-induced thymine glycols. ^{3}H-Labeled PM2 DNA (500 000 cpm/μg of DNA) was X irradiated in 10 mM Tris, pH 7.5, and 50 mM KI, and 200-ng aliquots were assayed for X-ray-induced/X-ray endonuclease susceptible sites by incubating with and without saturating quantities of X-ray endonuclease. Simultaneously, aliquots (4 μg) of the same DNA preparations were assayed for thymine glycol content by the alkali-degradative procedure of Hariharan (1980).

Results

Substrate Specificity. The X-ray endonuclease shows a dose-dependent response to DNA irradiated under several conditions (Strniste & Wallace, 1975). Figure 2 shows the activity against PM2 DNA X irradiated in the presence of the hydroxyl radical scavenger KI which effectively increases the ratio of enzyme-sensitive sites to frank strand breaks (Armel et al., 1977). There are approximately two enzyme-induced sites per strand break (Figure 2A), and this ratio remains constant from fraction III to fraction VI. The sites recognized by the X-ray endonuclease are primarily alkali stable as evidenced by the fact that the same number of enzyme-susceptible sites were seen whether the endonuclease assay or the DNA glycosylase/endonuclease assay was used.

Figure 3 depicts the relationship between production of thymine glycol type products as determined by the alkali degradative procedure of Hariharan (1980) and the production of X-ray endonuclease susceptible sites by X irradiation in the presence of 50 mM KI. The number of glycols produced is about 50% of the number of enzyme-susceptible sites. If, as seen below with OsO_4-treated DNA (Figure 4) there are two glycols (as determined by the alkali-degradative procedure) for every enzyme-susceptible site, the glycol type damages in X-irradiated DNA would account for about 25% of the total sites recognized by the X-ray endonuclease. The implication is that in an X-irradiated DNA substrate, the enzyme is recognizing alkali-stable base damages in addition to thymine glycols.

The enzyme does incise at apurinic sites generated by heat/acid treatment (Table II). The incised sites are about 85% of the total sites convertible to breaks by alkali (Lindahl & Andersson, 1972), and no increase in the number of breaks is observed if the apurinic DNA is incubated with the enzyme prior to alkali treatment. Apyrimidinic DNA produced by the bisulfite deamination of cytosine and subsequent incubation with uracil–DNA glycosylase is also a substrate for the enzyme as shown in Table II.

Treatment of DNA with OsO_4 at elevated temperatures or at high pH, which produces thymine glycol residues (Beer et al., 1966; Burton & Riley, 1966), also produces enzyme-susceptible sites. Highly labeled, freshly prepared PM2 DNA

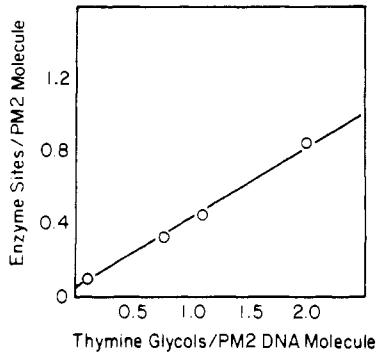

FIGURE 4: Relationship between thymine glycols and X-ray endonuclease sensitive sites in OsO_4-treated PM2 DNA. ^3H-Labeled PM2 DNA (500000 cpm/μg) was preheated to 70 °C, and a 4% solution of OsO_4 was added to 0.02% (w/v). Aliquots were removed at various times, incubated with saturating amounts of X-ray endonuclease (fraction VI), and analyzed by agarose gel electrophoresis, or incubated with 0.2 M KOH and assayed for thymine glycol type damages by the alkali-degradative procedure (Hariharan, 1980).

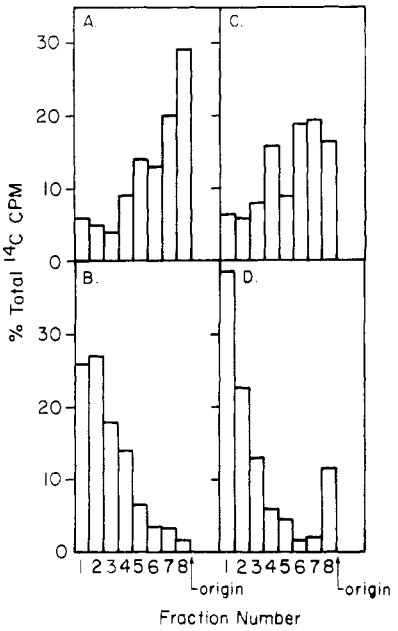

FIGURE 5: Polyacrylamide gel electrophoresis of [2-^{14}C]poly(dT)·(dA) containing either thymine glycol residues or thymine fragment (urea) residues. (A and B) [2-^{14}C]Poly(dT) was incubated with 0.4% OsO_4, extracted with diethyl ether, precipitated, and annealed with poly(dA). The duplex was either directly precipitated and redissolved in 98% formamide (A) or first digested with the X-ray endonuclease (B). The denatured polynucleotides were then analyzed by electrophoresis on 5% polyacrylamide gels containing 7 M urea as described under Experimental Procedures. (C and D) OsO_4-treated [2-^{14}C]poly(dT) (as above) was incubated in 0.2 M KOH for 2 h, neutralized, precipitated with alcohol, and annealed to poly(dA). The polynucleotide was then either directly precipitated and redissolved in 98% formamide (C) or first reacted with saturating quantities of the X-ray endonuclease (D). The polynucleotides were analyzed as above.

was analyzed for both endonuclease-sensitive sites by enzyme treatment followed by agarose gel electrophoresis and for thymine glycols by the procedure of Hariharan (1980). Although the data indicate (Figure 4) that there are approximately two thymine glycols present for every enzyme-sensitive site, the chemical analysis for the production of thymine glycols at these low levels is uncertain enough that a one to one relationship cannot be precluded.

If DNA, treated with OsO_4, is further treated with alkali under conditions that should result in the cleavage of thymine glycols into urea and N-substituted urea residues (Iida & Hayatsu, 1970), the same number of alkali-stable, enzyme-sensitive sites as were present in the original nonalkali treated OsO_4-treated DNA persists.

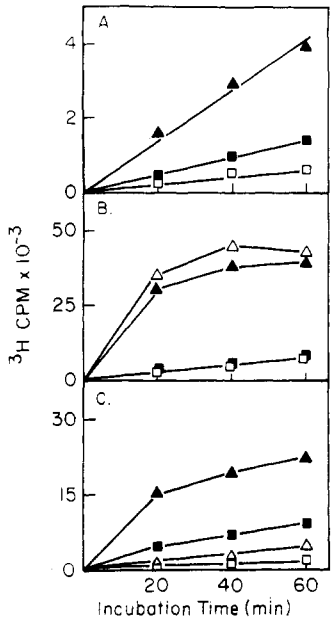

FIGURE 6: X-ray endonuclease induced nicks as substrates for *E. coli* DNA polymerase I. Unlabeled PM2 DNA either untreated (A), containing apurinic sites (B), or treated with OsO_4 (C) was incubated without enzyme (□), with exo III/endo VI (△), with X-ray endonuclease (fraction VI) (■), or with X-ray endonuclease followed by exo III/endo VI (▲) and added to a reaction mixture containing DNA polymerase I, Mg^{2+}, and all four dNTPs ([^{3}H]dTTP) as described under Experimental Procedures. Aliquots were removed initially and at 20-min intervals, and the acid-insoluble radioactivity was determined.

Figure 5 shows the results of an X-ray endonuclease digestion of [2-^{14}C]poly(dT) treated with OsO_4 and then annealed with poly(dA) and analyzed on denaturing polyacrylamide gels. Here (Figure 5B) it can clearly be seen that the enzyme has endonucleolytic action on poly(dT) containing thymine glycols and that it nicks the strand containing the damage. Figure 5D shows a similar experiment where the thymine glycols in the poly(dT) are fragmented by alkali before annealing and the resulting substrate is then incubated with X-ray endonuclease before analysis. This experiment corroborates the results shown above (Table II) with alkali-incubated OsO_4-treated DNA, and it can be concluded that the enzyme is capable of recognizing fragmented thymine glycol residues.

The X-ray endonuclease also nicks DNA treated with $KMnO_4$ (Table II) which produces a variety of lesions some of which correspond to those produced by OsO_4 (Howgate et al., 1968). Also, irradiation of DNA with UV light results in the dose-dependent generation of sites that are recognized by purified enzyme preparations; these represent about one site per 70 pyrimidine dimers. Thus the X-ray endonuclease recognizes a variety of damaged DNA substrates including AP sites, alkali-stable X-ray damages, and several degradation products of thymine.

Nature of the Nick. PM2 DNA either untreated or treated with OsO_4 to produce about three enzyme-sensitive sites per molecule or with heat/acid to produce about one AP site per molecule was incubated with the X-ray endonuclease followed by incubation with *E. coli* DNA polymerase I as described under Experimental Procedures. As was observed with endonuclease III preparations (Warner et al., 1980), the enzyme-induced nicks are not effective substrates for DNA polymerase I unless *E. coli* endonuclease VI is added prior to

Table III: Determination of the Presence of 5′-Phosphate Groups at X-ray Endonuclease Induced Nicks

reaction conditions	% acid soluble (cpm)
OsO_4-treated PM2 DNA + X-ray endonuclease	13
OsO_4-treated PM2 DNA + X-ray endonuclease + BALP	11
OsO_4-treated PM2 DNA + X-ray endonuclease + spleen phosphodiesterase	13
OsO_4-treated PM2 DNA + X-ray endonuclease + BALP + spleen phosphodiesterase	67

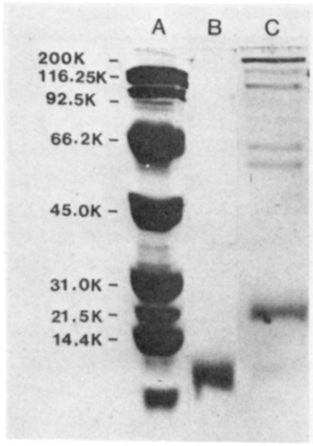

FIGURE 7: $NaDodSO_4$–polyacrylamide gel of X-ray endonuclease fractions. Samples were prepared and run according to the procedure of Laemmli (1970) as described under Experimental Procedures. (Lane A) Molecular weight standard proteins (Bio-Rad Laboratories low molecular weight standards for $NaDodSO_4$ gel electrophoresis); (lane B) phosphocellulose fraction (VI); (lane C) DNA–agarose fraction III.

the polymerase (Figure 6). This experiment indicates that the X-ray endonuclease nicks on the 3′ side of the damage which can be effectively cleaved on the 5′ side by endonuclease VI (Gossard & Verly, 1978). An alternative possibility, however, is that the X-ray endonuclease cleaves at the 5′ side of the damage leaving a 3′-PO_4 which can subsequently be removed by the phosphatase activity of endonuclease VI (Richardson & Kornberg, 1964), thus generating a polymerase-binding site.

To obviate this second possibility, DNA treated with OsO_4 and then with X-ray endonuclease was further incubated with or without bacterial alkaline phosphatase (BALP) and then incubated with phosphodiesterase II, and solubilization of DNA was measured. The results, shown in Table III, indicate that the PO_4 group is present on the 5′ side of the nick (requires BALP action before DNA can be digested by phosphodiesterase II). Taken together these data show that the X-ray endonuclease nicks on the 3′ side of AP sites and OsO_4-induced sites leaving a 3′-OH and a 5′-PO_4.

Molecular Properties. Sephadex chromatography of the X-ray endonuclease at 1 M KCl in buffer A yielded a molecular weight of 25 000 (±10%). Preliminary analysis by glycerol gradient sedimentation showed two peaks of activity against both AP and OsO_4-treated DNA, one corresponding to 25 000 (±20%) and another corresponding to half the native molecular weight [12 000 (±20%)]. This indication of possible subunit structure was further supported by the finding that $NaDodSO_4$–polyacrylamide gel analysis of the most purified fraction (VI) showed a single band corresponding to a molecular weight of 12 000–14 000 (Figure 7). Taken together

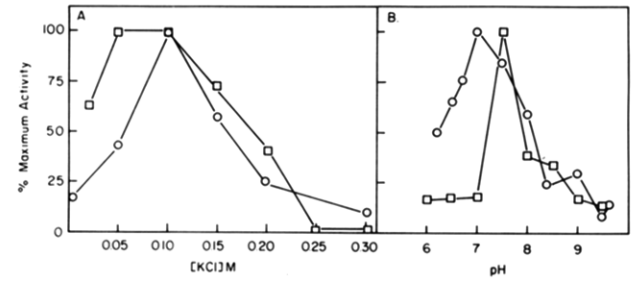

FIGURE 8: Effect of KCl and pH on the activities of the X-ray endonuclease. (A) ^3H-Labeled PM2 DNA treated to produce either AP sites (□) or OsO_4 damages (○) was incubated with subsaturating amounts of the X-ray endonuclease (fraction VI) at the KCl concentrations indicated. (B) ^3H-Labeled PM2 DNA containing either AP sites (□) or OsO_4 damages (○) was incubated with subsaturating amounts of the X-ray endonuclease (fraction VI) at the indicated pH. Samples (A and B) were assayed by either agarose gel electrophoresis or neutral sucrose gradient centrifugation.

these results indicate that the native enzyme might be a dimer either of two enzymatically identical subunits or of two distinct enzymatic subunits of nearly identical molecular weight. Studies are currently in progress to further elucidate the structure of the X-ray endonuclease.

Through all stages of purification the DNA glycosylase/endonuclease activity against X-ray- and OsO_4-induced damages copurified with the endonuclease activity against AP and UV sites. The DNA glycosylase/endonuclease assay relies on the fact that incubation with 1 M glycine–NaOH buffer quantitatively converts AP sites to single-stranded breaks. A DNA glycosylase assayed by this method will show the same activity whether or not an AP endonuclease is present so that DNA glycosylase activity may be assayed independently of AP endonuclease activity. Further analysis of purified enzyme by chromatography on DEAE-cellulose and CM-Sephadex using salt gradient elution showed no further resolution of these activities. The activity toward the complex substrate, X-irradiated DNA, was the same for all fractions from fraction III onward, with 0.04 (±10%) endonuclease-susceptible sites produced for each kilorad of X-rays.

Salt optimum studies showed some differences in the response of the X-ray endonuclease activity toward OsO_4-treated and AP DNA. As can be seen from Figure 8A the activity against OsO_4 sites shows optimum activity at 0.10 M KCl with 50% inhibition at 0.05 and 0.15 M KCl while the salt optimum for AP sites is between 0.05 and 0.10 M KCl with 50% inhibition at 0.02 and 0.12 M KCl. Similarly, Figure 8B shows the activity against AP DNA to have a sharp pH optimum at pH 7.5 with 50% inhibition at 0.3 pH unit on either side of this and the endonuclease against OsO_4-treated DNA to have a broader optimum at pH 7.0 with 50% inhibition at pH 6.2 and pH 8.1. Further, heat lability studies of the enzyme on X-irradiated and AP substrates showed the activity against X-irradiated DNA to be more labile (50% maximum at 5 min at 45 °C) than the activity against AP DNA (50% maximum at 10 min at 45 °C) (Figure 9A). Lastly, the activity against OsO_4-treated DNA is very sensitive to NEM, being about 30% maximal at 10 mM, whereas at this concentration almost complete activity remains toward AP DNA (Figure 9B). The activity against X-irradiated and UV-irradiated DNA is also inhibited by NEM. These data can be explained by postulating that the X-ray endonuclease contains two distinct activities: one an endonuclease specific for apurinic lesions and the other a DNA glycosylase specific for base damages.

Glycosylase Activity. Both TLC and paper chromatographic analyses (Figure 10A,B) show the acid-soluble prod-

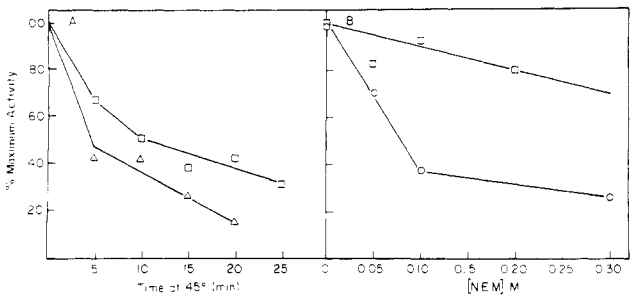

FIGURE 9: (A) Heat inactivation of the activities of the X-ray endonuclease. Samples (100 μL) of the X-ray endonuclease (fraction VI) were heated at 45 °C. Aliquots were removed immediately and, at 5-min intervals, diluted and simultaneously assayed for activity on AP DNA (□) or X-irradiated DNA (△) by neutral sucrose gradient centrifugation. (B) Differential effects of NEM on the activities of the X-ray endonuclease. ^{3}H-Labeled PM2 DNA containing either AP sites (□) or OsO_4-induced damages (○) was treated with subsaturating amounts of X-ray endonuclease (fraction VI) at the concentrations of NEM indicated and assayed by neutral sucrose gradient centrifugation.

ucts of exhaustively digested heavily labeled OsO_4-treated ^{3}H-labeled PM2 DNA (1–10% thymine glycol) to be thymine glycol as revealed by comigration with *cis*-thymine glycol and a mixture of *cis*- and *trans*-thymine glycol. The glycosylase activity, in addition to cleaving thymine glycols, can also remove urea fragments as shown in Figure 10C,D. Here, OsO_4-treated [2-^{14}C]poly(dT) annealed to poly(dA) was treated with alkali to fragment the thymine glycols and then incubated with the X-ray endonuclease. As can be seen with both paper chromatographic and TLC systems, a labeled fragment is released that comigrates with urea.

Further experiments where thymine glycol containing DNA is incubated with X-ray endonuclease in the presence or absence of 10 mM NEM clearly demonstrate that NEM inhibits the thymine glycol–DNA glycosylase activity (Figure 11). Lastly, analysis by PEI–cellulose TLC of a Cl_3CCOOH supernatant from a digest of heavily labeled, X-irradiated DNA by X-ray endonuclease showed an enzyme-dependent release of nonnucleotide radioactivity which was inhibited by the presence of 10 mM NEM (data not shown).

Discussion

Comparison to Other E. coli Repair Enzymes. Because of its broad specificity, the X-ray endonuclease is easily differentiated from the AP-specific endonucleases IV (Ljungquist, 1977) and VII (Friedberg et al., 1981) as well as from the AP endonuclease associated with exonuclease III and endonuclease VI (Verly & Rassart, 1975). Separation from the AP-specific endonucleases during the purification procedure was ensured by using *xth*$^-$ mutants (exo III$^-$) (Weiss, 1976) for the starting material and an assay procedure in which the activity of AP-specific endonucleases would not be observed; that is, the activity against X-irradiated DNA substrates was measured under conditions which convert the alkali-labile lesions to strand breaks. The fact that the X-ray endonuclease does not incise unirradiated DNA distinguishes it from endonuclease I (Lehman et al., 1962). This leaves two endonucleases which have broad substrate specificities, endonucleases V and III. Endonuclease V, although active on heavily UV-irradiated DNA and X-irradiated DNA, is unlike the X-ray endonuclease in that it prefers a single-stranded substrate, nicks untreated single-stranded DNA, requires Mg^{2+}, has a higher pH optimum, and acts on uracil-containing DNA (Gates & Linn, 1977a).

The X-ray endonuclease appears to be identical with endonuclease III in both its molecular and catalytic properties. Endonuclease III, originally purified by its activity toward heavily UV-irradiated DNA, has nearly the same salt optimum, pH optimum, and sedimentation coefficient as the X-ray endonuclease and is also sensitive to inhibitors such as single-stranded DNA and 1 M NaCl (Radman, 1976). An enzyme found as a byproduct of the purification of endonuclease V and purified with a heavily UV-irradiated DNA substrate was assumed to be endonuclease III (Gates & Linn, 1977b). The latter activity was shown to nick AP DNA, X-irradiated DNA, and OsO_4-treated DNA. However, it was not determined whether the sites nicked in X-irradiated DNA were stable to alkali. Endonuclease III was recently shown to be associated with a DNA glycosylase specific for 5,6-hydrated thymine moieties (Demple & Linn, 1980). Although it was not acknowledged, this enzyme may also act at urea residues since the method used to produce the thymine glycols (Gates & Linn, 1977b), OsO_4 oxidation of alkali-denatured DNA, employed a pH at which the produced glycols are unstable; the half-life of thymine glycols is less than 20 min at pH 12 (Iida & Hayatsu, 1970). Thus, a significant number of glycols should have been fragmented during their procedure.

Of the seven DNA glycosylases reported to occur in *E. coli*, only the uracil–DNA glycosylase may be definitively ruled out as being an activity of the X-ray endonuclease, as the latter does not nick uracil-containing DNA (Table II). Both the 5,6-hydrated thymine–DNA glycosylase activity possessed by endonuclease III and the urea–DNA glycosylase activity, reported in a 10-fold purified preparation (Breimer & Lindahl, 1980), appear to be components of the X-ray endonuclease. The urea–DNA glycosylase, like the X-ray endonuclease, has a molecular weight on the order of 25 000, does *not* require Mg^{2+}, and has a pH optimum (for the release of urea) of between 7.4 and 7.8. These properties are consistent with both activities residing on the same enzyme. If the urea–DNA glycosylase and the X-ray endonuclease represent identical activities, then the separation of the urea–DNA glycosylase from both the hypoxanthine–DNA glycosylase and the formamidopyrimidine–DNA glycosylase during its purification (Breimer & Lindahl, 1980) implies that these activities are not components of the X-ray endonuclease. Similarly, the inability of highly purified preparations of the 3-methyladenine–DNA glycosylase to release free urea from the substrate used to demonstrate the urea–DNA glycosylase activity (Breimer & Lindahl, 1980) indicates that this activity is also distinct from the X-ray endonuclease. The relationship between the X-ray endonuclease and the 7-methylguanine–DNA glycosylase remains unknown.

In summary it would appear that the X-ray endonuclease is a multifunctional enzyme comprising a bifunctional (at least) DNA glycosylase and a class I AP endonuclease and appears to be identical with the activities reported both as endonuclease III and as the urea–DNA glycosylase.

Substrate Specificity. The damages produced by X-irradiating DNA in dilute aqueous solution include frank strand breaks, base damages, and alkali-labile sites, the latter depending on whether incubation in alkali converts them to strand breaks. The X-ray endonuclease recognizes the alkali-stable base damages, presumably the ring saturation and fragmentation products of the bases, but does not act at the majority of alkali-labile damages. This is demonstrated in part by the observation that the number of X-ray endonuclease susceptible sites remains the same whether X-irradiated DNA is analyzed by the endonuclease assay or by the DNA glycosylase/endonuclease assay (Figure 2). Further, the number of nicks produced in X-irradiated DNA by the combined

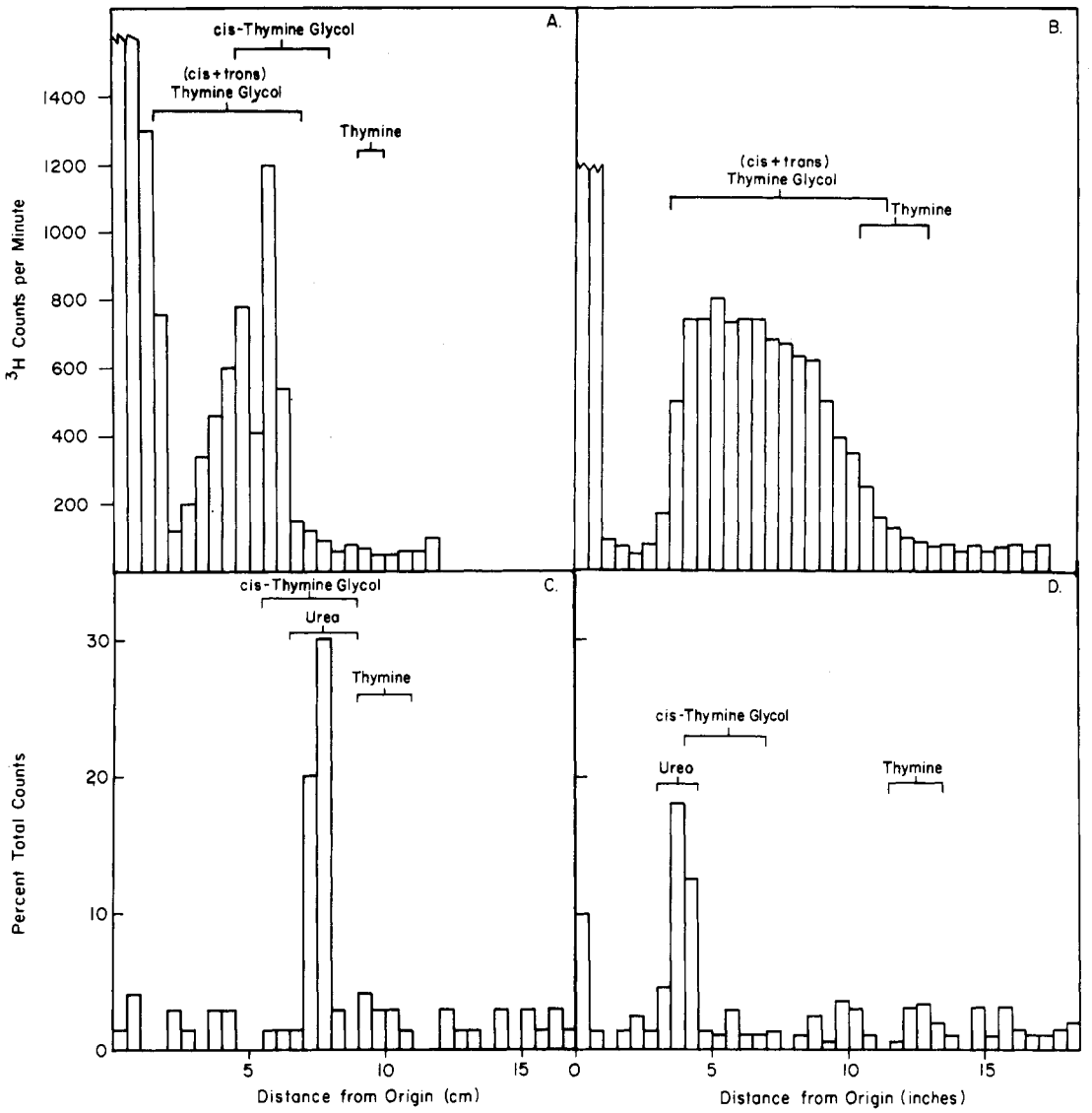

FIGURE 10: (A and B) Analysis of products released after incubation of OsO_4-treated DNA with X-ray endonuclease. ^{3}H-Labeled PM2 DNA ($\sim 10^6$ cpm/μg of DNA) was extensively treated with OsO_4 (see Experimental Procedures) and then incubated with saturating quantities of enzyme. The Cl_3CCOOH-soluble fraction was taken to dryness, and samples were spotted onto (A) Polygram Cel 300/UV_{254} MN thin-layer plates and developed in propanol–H_2O, 3:1. The developed plates were dried, cut into 1-cm strips, and counted. Samples were also spotted onto sheets of Whatman 3MM chromatography paper (B). Descending paper chromatography where the upper phase was a mixture of ethyl acetate–*n*-propyl alcohol–H_2O (4:1:2) (Breimer & Lindahl, 1980) was used. Counts were eluted by water from 1-in. strips. TMP, thymine glycol monophosphate, and polynucleotides remained at the origin during both chromatographic procedures. (C and D) Analysis of products released after X-ray endonuclease incubation with [2-^{14}C]poly(dT)·(dA) treated with OsO_4 followed by alkali. As described under Experimental Procedures, the alcohol-soluble material was (C) spotted onto Polygram Cel 300/UV_{254} MN thin-layer plates, developed in propanol–H_2O, 3:1, cut into 1-cm strips, and counted or (D) spotted on Whatman 3MM chromatography paper, developed with the upper phase of a mixture of ethyl acetate–*n*-propyl alcohol–H_2O (4:1:2), cut into 1-in. strips, eluted with water, and counted. Authentic markers were run simultaneously in different lanes.

action of *Saccharomyces cerevisiae* AP endonuclease E, a class II AP endonuclease from the yeast (Armel & Wallace, 1978), and the X-ray endonuclease is equal to the sum of the nicks introduced by each enzyme acting alone (unpublished results). This result appears paradoxical, as the X-ray endonuclease has been shown to nick both the apurinic sites generated by heating at low pH and the apyrimidinic sites generated by uracil–DNA glycosylase acting on uracil-containing DNA (Table II). This observation can be explained, however, by postulating that alkali-labile sites generated by X-rays are not "clean" AP sites but AP sites containing fragmented deoxyribose rings. Both types of alkali-labile lesions have been found attached to the DNA backbone in DNA X irradiated in vitro (Isildar et al., 1981). AP sites containing fragmented sugars appear not to be substrates for the AP endonuclease activity of the X-ray endonuclease although they are apparently substrates for other AP endonucleases (Armel & Wallace, 1978). Evidence that X-ray-induced alkali-labile lesions are different from heat/acid generated apurinic sites comes from observation that the rate constant for the conversion of X-ray-induced alkali-labile sites to strand breaks is considerably higher than that of apurinic sites (Katcher & Wallace, 1978; Lafleur et al., 1981).

The X-ray endonuclease was also shown to nick at or near the site of the thymine glycol (Figure 4) specifically produced in duplex DNA or poly(dT)·(dA) by OsO_4 treatment, at or near minor photoproducts produced by ultraviolet irradiation (Table II), and at or near urea residues (Figure 5) produced by the alkali-induced degradation of thymine glycols. Furthermore, on a complex substrate such as that produced by the X irradiation of DNA, there are twice as many enzyme-susceptible sites than 5,6-dihydroxydihydrothymine type res-

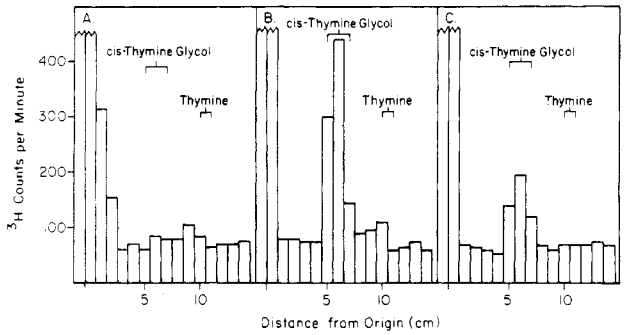

FIGURE 11: Effect of NEM on the thymine glycol–DNA glycosylase activity of the X-ray endonuclease. OsO_4-treated PM2 DNA either unincubated (A), incubated with X-ray endonuclease (fraction V) (B), or incubated with X-ray endonuclease in the presence of 10 mM NEM (C) was precipitated, and the supernatants were taken to dryness under vacuum, redissolved in ethanol, spotted onto Cel 300/UV_{254} MN plates, and developed in 1-propanol–H_2O, 3:1.

idues (Figure 3) as measured by the alkali-degradative method (Hariharan, 1980). As the lesions known to be recognized by the X-ray endonuclease are members of the class of lesions that cause minor distortions of the DNA helix (Cerutti, 1975b), it will be interesting to see if other members of this class, ring contraction and other ring fragmentation products, are also substrates.

Mechanism of Action of the X-ray Endonuclease. While the AP endonuclease activity is virtually uninhibited by the presence of NEM, a sulfhydryl blocking reagent (Figure 9), the endonuclease activity on both thymine glycol containing DNA and urea residue containing DNA are substantially inhibited (Table II) as is the thymine glycol–DNA glycosylase activity (Figure 11).

These data, taken together with data showing cochromatography of the AP endonuclease activity and the DNA glycosylase/endonuclease activity against OsO_4-treated DNA, indicate that this enzyme has at least two partly independent activities: a base-damage-specific DNA glycosylase and an AP endonuclease. The endonuclease activity against DNA containing base lesions may therefore result from the concerted action of both these activities. If the glycosylase against thymine glycol type damages and the glycosylase acting on urea fragments share a common active center, this multisubstrate DNA glycosylase might also recognize base damages that share some characteristics of both the glycol and urea residues such as lack of aromaticity or improper base stacking or base pairing. Alternatively, the two glycosylase activities might involve separate or overlapping active centers with unique specificities. Since both DNA glycosylase activities are NEM inhibitable, these alternatives remain unresolved.

Whether or not the multiple activities attributed to the X-ray endonuclease belong to the same enzyme has not been unequivocally determined, but several lines of evidence support this hypothesis. The AP endonuclease and OsO_4-damage DNA glycosylase/endonuclease activity cochromatograph at all stages in the purification procedure, and these activities have also been shown to coelute from CM-Sephadex and DEAE-cellulose. Glycerol gradient sedimentation analysis of fraction V shows two peaks of activity, one corresponding to the molecular weight of the native enzyme (about 25 000) and the other to a molecular weight of about half that molecular weight. These peaks appeared in the same positions whether the AP endonuclease or OsO_4-treated DNA glycosylase activity was assayed, providing preliminary evidence that the native enzyme might be a dimer of two subunits. Further support for this hypothesis is given by $NaDodSO_4$–polyacrylamide gel electrophoresis analysis of the most purified fraction (fraction VI) which shows a single band corresponding to a molecular weight of about 12 500. Although it cannot be unequivocally stated that this band corresponds to the X-ray endonuclease, a similar analysis on fraction V showed only three bands, with the molecular weight of the heaviest protein corresponding to ca. 16 000. It appears then that the X-ray endonuclease is a multisubstrate DNA glycosylase/AP endonuclease, possibly composed of two subunits of either identical or similar molecular weights.

The AP endonuclease activity of the X-ray endonuclease is a class I AP endonuclease, and we assume that it acts after the DNA glycosylase removes the damaged base. On an AP substrate, it gives rise to a nick which is not a primer terminus for DNA polymerase I (Figure 8). In conjunction with either a class II AP endonuclease or a 3′–5′-exonuclease it produces a gap that must be filled before ligation. It is possible, however, that a multifunctional endonuclease acts prior to the action of the DNA glycosylase. Analyses of the X-ray endonuclease digestion products of X-irradiated DNA and other complex substrates, as well as quantitative and kinetic studies, are being undertaken in an attempt to distinguish among these possibilities.

An Endonuclease Involved in Ionizing Radiation Repair? The damages introduced into DNA by X-rays can result from the production of high-energy species such as free radicals, hydrated electrons, and peroxides. These species are also produced by a variety of other cellular reactions. Interaction of DNA with the high-energy photons of UV light produces base damages similar to those produced by ionizing radiation (Hariharan & Cerutti, 1977), and metabolic processes are known to produce hydroxyl radicals, hydrogen peroxide, and superoxide radicals (Fridovich, 1978). Additional radiation arriving from space as well as from the natural decay of terrestrial atoms gives sufficient reason for an organism whose genetic integrity must be maintained to have enzymes to repair the resultant base damages. The elaboration of an enzyme such as the X-ray endonuclease able to recognize a class of damages such as those causing minor distortions of the DNA helix would be economical. The in vivo importance of the X-ray endonuclease in the repair of DNA base damage and the repair of AP sites awaits the isolation of mutants defective in this activity.

Registry No. Endonuclease III, 60184-90-9; *Escherichia coli* X-ray endonuclease, 71567-53-8; urea–DNA glycosylase, 85976-58-5; thymine glycol–DNA glycosylase, 85976-57-4; AP endonuclease, 61811-29-8.

References

Armel, P. R., & Wallace, S. S. (1978) *Nucleic Acids Res. 5*, 3347–3356.

Armel, P. R., Strniste, G. F., & Wallace, S. S. (1977) *Radiat. Res. 69*, 328–338.

Baudisch, O., & Davidson, D. (1925) *J. Biol. Chem. 64*, 233–239.

Beer, M., Stern, S., Carmalt, D., & Mohlenrich, K. H. (1966) *Biochemistry 5*, 2283–2288.

Breimer, L., & Lindahl, T. (1980) *Nucleic Acids Res. 8*, 6199–6211.

Burton, K., & Riley, W. T. (1966) *Biochem. J. 98*, 70–77.

Cerutti, P. A. (1975a) in *Photochemistry and Photobiology of Nucleic Acids* (Wang, S. Y., Ed.) Vol. 2, pp 181–239, Academic Press, New York.

Cerutti, P. A. (1975b) in *Molecular Mechanisms for the Repair of DNA* (Hanawalt, P. C., & Setlow, R. B., Eds.)

pp 3–12, Plenum Press, New York.
Demple, B., & Linn, S. (1980) *Nature (London) 287*, 203–208.
Ekert, B. (1962) *Nature (London) 194*, 278–279.
Fink, R. M., Cline, R. E., MacGhaughey, C., & Fink, F. (1956) *Anal. Chem. 28*, 4–6.
Fridovich, I. (1978) *Science (Washington, D.C.) 201*, 875–880.
Friedberg, E. C., Bonura, T., Love, J. D., McMillan, S., Radany, E. H., & Schultz, R. A. (1981) *J. Supramol. Struct. Cell. Biochem. 16*, 91–103.
Gates, F. T., III, & Linn, S. (1977a) *J. Biol. Chem. 252*, 1647–1653.
Gates, F. T., III, & Linn, S. (1977b) *J. Biol. Chem. 252*, 2802–2807.
Gossard, F., & Verly, W. G. (1978) *Eur. J. Biochem. 82*, 321–332.
Hariharan, P. V. (1980) *Radiat. Res. 81*, 496–498.
Hariharan, P. V., & Cerutti, P. A. (1972) *J. Mol. Biol. 66*, 65–81.
Hariharan, P. V., & Cerutti, P. A. (1974) *Biochem. Biophys. Res. Commun. 61*, 375–379.
Hariharan, P. V., & Cerutti, P. A. (1977) *Biochemistry 16*, 2791–2795.
Hariharan, P. V., Achey, P. M., & Cerutti, P. A. (1977) *Radiat. Res. 69*, 375–378.
Howgate, P., Jones, A. S., & Titensor, J. R. (1968) *J. Chem. Soc. C*, 275–279.
Iida, S., & Hayatsu, H. (1970) *Biochim. Biophys. Acta 213*, 1–13.
Isildar, M., Schuchmann, M. N., Schulte-Frohlinde, D., & Von Sonntag, C. (1981) *Int. J. Radiat. Biol. Relat. Stud. Phys., Chem. Med. 40*, 347–354.
Jovins, T. M., Englund, P. T., & Bertsch, L. L. (1969) *J. Biol. Chem. 244*, 2996–3008.
Katcher, H. L., & Wallace, S. S. (1978) *Int. J. Radiat. Biol. Relat. Stud. Phys., Chem. Med. 34*, 497–500.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Lafleur, M. V. M., Woldhuis, J., & Loman, H. (1981) *Int. J. Radiat. Biol. Relat. Stud. Phys., Chem. Med. 39*, 113–118.
Lehman, I. R., Rousos, G. G., & Pratt, E. A. (1962) *J. Biol. Chem. 237*, 819–828.
Lindahl, T., & Andersson, A. (1972) *Biochemistry 11*, 3618–3623.
Ljungquist, S. (1977) *J. Biol. Chem. 252*, 2808–2814.
Maniatis, T., Jeffrey, A., & van de Sande, H. (1975) *Biochemistry 14*, 3787–3793.
Moran, E., Zerler, B. R., & Wallace, S. S. (1980) *Radiat. Res. 83*, 41.
Radman, M. (1976) *J. Biol. Chem. 254*, 1438–1445.
Richardson, C. C., & Kornberg, A. (1964) *J. Biol. Chem. 239*, 242–250.
Scholes, G., & Weiss, J. (1960) *Nature (London) 185*, 305–306.
Strniste, G. F., & Wallace, S. S. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1997–2001.
Têoule, R., Bert, C., & Bonicel, A. (1977) *Radiat. Res. 72*, 190–200.
Verly, W. G., & Rassart, E. (1975) *J. Biol. Chem. 250*, 8214–8219.
Wallace, S. S., Katcher, H. L., & Armel, P. R. (1981) in *DNA Repair, A Laboratory Manual of Research Procedures* (Friedberg, E. C., & Hanawalt, P. C., Eds.) 1A, pp 113–125, Marcel Dekker, New York and Basel.
Ward, J. F. (1975) *Adv. Radiat. Biol. 5*, 181–239.
Warner, H. R., Demple, B. F., Deutsch, W. A., Kane, C. M., & Linn, S. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 4602–4606.
Weiss, B. (1976) *J. Biol. Chem. 251*, 1896–1901.
Zubay, G., Chamber, D. A., & Cheong, L. C. (1970) in *The Lactose Operon* (Beckwith, J. R., & Zipser, D., Eds.) Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.