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Enzymatic Repair of DNA. III. Properties of the UV-Endonuclease and UV-Exonuclease*

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ABSTRACT: Two enzymes have been purified from *Micrococcus luteus* which together are responsible for the *in vitro* excision of photoproducts from UV-irradiated DNA. The first enzyme, UV-endonuclease, catalyzes the formation of single-strand incisions in irradiated duplex DNA. It is inactive on native, irradiated denatured, and unirradiated denatured DNA. The apparent K_m value for irradiated native DNA is equal to 1.25×10^{-5} M at 37° and the pH optimum is between pH 7.0 and 7.5. The UV-endonuclease is stimulated by but not dependent on Mg^{2+}. This enzyme is not inhibited by EDTA or caffeine. Acridine orange is inhibitory at high concentrations (1×10^{-2} M). Based on gel filtration, this enzyme has been assigned a molecular weight of 15,000. The DNA substrate-dose dependency relationship is correlated with UV-endonuclease activity. A direct correlation exists between the number of pyrimidine dimers formed and the number of single-strand breaks which in turn is dependent on the DNA source. The second enzyme, UV-exonuclease, degrades exposed regions of DNA resulting from incision by the UV-endonuclease. The UV-exonuclease hydrolyzes unirradiated and irradiated heat-denatured DNA at equivalent rates and to the same extent but does not attack native DNA or RNA. Unlike most known exonucleases, it is not inhibited by the presence of photoproducts in DNA. The apparent K_m value for irradiated denatured DNA is equal to 4.46×10^{-6} M at 37°. The pH optimum lies between pH 7.0 and 7.5 and the enzyme requires Mg^{2+} for activity. It is inhibited by Mn^{2+}, Ca^{2+}, Zn^{2+}, and EDTA. An unusual feature of this enzyme is the sigmoidal shape of its enzyme concentration curve. The initial lag in this curve can be abolished by certain monovalent cations: K^+, NH_4^+, Na^+, or Li^+. The UV-exonuclease is characterized with respect to its activity on denatured DNA. It degrades this substrate from *both* the 3′ and 5′ termini in an exonucleolytic manner, yielding as its final degradation product, 5′-monoucleotides. When irradiated denatured DNA is hydrolyzed to completion, the major products are mononucleotides although some tri- and tetranucleotides are also released. Single-stranded polydeoxythymidylic acid, poly-[d(pT)$_{200}$], is degraded entirely to mononucleotides but the digestion products of irradiated poly[d($\widehat{pT}$)$_{200}$] comprise a mixture of mono-, di-, and trinucleotides.

Many of the biological effects of ultraviolet irradiation have been attributed to the formation of pyrimidine dimers in DNA (Wacker, 1962; Setlow and Setlow, 1963). The recovery of organisms from UV-irradiation damage can occur by several mechanisms: photoreactivation (Kelner, 1949), postreplication repair (Rupp and Howard-Flanders, 1968), and dark repair (Haynes, 1966). The dark repair process which is a multistep enzymatic system involves the excision of pyrimidine dimers from UV-irradiated DNA (Setlow and Carrier, 1964; Boyce and Howard-Flanders, 1964). The removal *in vitro* of photoproducts from UV-irradiated DNA in *Micrococcus luteus* requires two enzymes: the UV-endonuclease and the UV-exonuclease. These two nucleases have been isolated and purified extensively (Grossman *et al.*, 1968; Kaplan *et al.*, 1969). Initially the UV-endonuclease recognizes a photoproduct-containing region, breaking a single phosphodiester bond in the vicinity of each dimer. The UV-exonuclease subsequently removes the photoproduct region leaving a cavity in the excised DNA. In this communication we describe the separate properties of these two enzymes and in the accompanying paper the mechanism of photoproduct excision.

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Experimental Section

Materials. ^{32}P-Labeled DNA was prepared from *E. coli* B or *M. luteus* by the method of Grossman (1967). UV-irradiated double-stranded and heat-denatured DNAs were prepared as previously described (Kaplan *et al.*, 1969). 3′-Hydroxyl terminally ^{14}C-labeled DNA was synthesized by established procedures using the DNA polymerase from *E. coli* (Adler *et al.*, 1958). Terminally 5′-^{32}P-labeled DNA was prepared by the method of Weiss *et al.* (1968). [^{3}H]Thymine-labeled polythymidylic acid ([^{3}H]poly[d(pT)$_{200}$] and [^{3}H]poly-d(pT)$_{200}$]) were kindly provided by Dr. Regis B. Kelley. Polycytidylic, polyadenylic, and polyuridylic acids were purchased from Miles Chemical Corp. while total yeast RNA was a gift of Dr. Raymond Adman. Oligonucleotide standards for chromatography were synthesized chemically according to the method of Khorana and Vivsolyi (1961).

The UV-endonuclease and UV-exonuclease from *M. luteus* were prepared as described in a previous publication (Kaplan *et al.*, 1969). *E. coli* DNA was purified according to the method of Richardson (1966) and 5′-nucleotidase from *Crotalus adamanteus* was prepared as described previously (Lehman *et al.*, 1962). Polynucleotide kinase was the generous gift of Dr. Charles C. Richardson and bacterial alkaline phosphatase was a product of the Worthington Biochemical Corp.

Bovine serum albumin was obtained from Nutritional Biochemical Corp., [γ-^{32}P]ATP from ICN, acridine orange from Fischer Scientific Corp., DE-81 chromatography paper from Reeve Angel Corp., and G-75 Sephadex from Pharmacia Corp. Caffeine was the gift of Dr. Inga Mahler.

Methods

Enzyme Assays. UV-ENDONUCLEASE ASSAY. The UV-endonuclease assay measures the formation of bacterial alkaline phosphatase sensitive phosphomonoesters produced by endonucleolytic hydrolysis. The enzyme is diluted in 0.01 M Tris-HCl buffer (pH 7.5). The incubation mixture (0.3 ml) contains 5.0 μmoles of Tris-HCl buffer (pH 7.5), 3 μmoles of magnesium chloride, 5 μmoles of 2-mercaptoethanol, 7.5 μmoles of UV-irradiated double-stranded [^{32}P]DNA [total incident dose = 1.3×10^{5} ergs/mm^{2} (precalibrated 15-W GE germicidal lamp); specific activity 10×10^{4} cpm/mμmole of nucleotide equivalent], and 2–4 units of enzyme. After incubation for 30 min at 37°, 0.2 ml of 2.5 mg/ml calf thymus DNA and 0.3 ml of cold 7% perchloric acid are added. The precipitate obtained from a 10-min centrifugation at 12,500*g* is redissolved in 0.5 ml of 1 N NaOH at room temperature. The pH is adjusted to 8.0 with 0.05 ml of 2 M Tris-HCl buffer (pH 6.45). Bacterial alkaline phosphatase (2 units) is added, and the solution is incubated at 45° for 30 min. The DNA is precipitated by adding 0.3 ml of cold 7% perchloric acid and centrifuged at 7500*g* for 10 min. The supernatant fraction is counted in Bray's–dioxane scintillation fluid. *One unit of activity is defined as that amount required to produce 10 μmoles of [^{32}P]P$_i$ released by bacterial alkaline phosphatase from UV-irradiated double-stranded DNA.* The enzyme assay is linear in the range between 0.5 and 6 units of enzyme.

UV-EXONUCLEASE ASSAY. The UV-exonuclease assay measures the conversion of ^{32}P-labeled irradiated denatured DNA into acid-soluble nucleotides. The enzyme is diluted in 0.01 M potassium phosphate buffer (pH 7.5). The incubation mixture (0.15 ml) contains 1 μmole of potassium phosphate buffer (pH 7.5), 1 μmole of $MgCl_2$, 2.5 nmoles of irradiated denatured [^{32}P]DNA (specific activity $1–5 \times 10^{4}$ cpm/nmole of nucleotide equivalent), and 0.1–0.5 unit of enzyme. When the protein concentration is less than 0.1 mg/ml, an equivalent amount of bovine serum albumin is included in the reaction mixture. After incubation at 37° for 30 min, 0.2 ml of calf thymus DNA (2.5 mg/ml) and 0.3 ml of cold 7% perchloric acid are added. The reaction mixture is centrifuged for 10 min at 12,500*g* and the supernatant fraction is counted in Bray's–dioxane scintillation fluid. The acid-soluble fraction from control incubations, with enzyme omitted, contains 0.2% of the radioactivity. *One unit of activity is defined as that amount required to produce 1 nmole of acid-soluble nucleotide in 30 min at 37°.* The assay is linear within the range 0.1 and 0.5 unit of enzyme providing the protein concentration is at least 100 μg/ml.

Additional Procedures. 5′-Nucleotidase is assayed according to the method of Heppel and Hilmoe (1955). One unit of activity corresponds to the liberation of 1 μmole of phosphorus in 60 min. Bacterial alkaline phosphatase activity is determined by the method of Garen and Levinthal (1960).

Norit Assay. The samples are acidified with 1 N HCl to pH 3 and passed over a Norit–Celite column composed of 0.5 ml of 20% activated Norit and 0.5 ml of 50% Celite in a sintered-glass filtered column. The column is washed with 30 ml of 0.01 N HCl. The sample is applied under vacuum and [^{32}P]P$_i$ is quantitatively washed through the column in 8 ml of 0.01 N HCl. All nucleotide-containing material remains adsorbed to the column. The amount of [^{32}P]P$_i$ is determined by counting in Bray's–dioxane scintillation fluid.

DEAE Paper Chromatography. DEAE paper chromatography is carried out using the solvent systems developed by Kelly *et al.* (1970). Nucleotide-containing spots are identified under ultraviolet light and their mobilities compared to known markers. Each chromatogram is cut into strips (1 × 2 cm) and counted in toluene–liquiflor scintillation fluid.

Molecular Weight Determination of the UV-Endonuclease. A solution of UV-endonuclease (0.3 ml) is layered onto a Sephadex G-75 column (1 × 100 cm) which has been pre-equilibrated with 0.2 M potassium phosphate buffer (pH 7.5) and 10% ethylene glycol (v/v). Elution is carried out with the same buffer. The UV-exonuclease and UV-endonuclease are assayed as described in the previous section and the standard (horse heart cytochrome *c*, mol wt 12,340) is located by its characteristic absorbance at 390 nm.

Dephosphorylation of Digestion Products Resulting from the Limited Hydrolysis of ^{32}P-Labeled Denatured DNA. Each reaction mixture (0.45 ml) contains 30 mμmoles of the ^{32}P-denatured DNA (specific activity 1×10^{5} cpm/mmole of nucleotide equivalent), 40 μmoles of Tris-HCl buffer (pH 8.0), 3 μmoles of $MgCl_2$, and 30 μg of bovine serum albumin and UV-exonuclease (0.1–0.4 unit). After incubation for 30 min at 37°, the tubes are chilled and one-half is processed as described for the UV-exonuclease assay. The other samples are held at 65° for 5 min and chilled in ice. Bacterial alkaline phosphatase (20 units) is added to the reaction mixture which subsequently is incubated for 30 min at 65°. The reaction is terminated by the addition of 1 N HCl (0.5 ml) and the samples treated with the Norit assay as described.

Distribution of Products after a Limited Hydrolysis of Denatured DNA by the UV-Exonuclease. Each reaction mixture (0.25 ml) contains 15 nmoles of denatured ^{3}H-labeled DNA (specific activity 2×10^{3} cpm/mmole of nucleotide equivalent), 2.0 μmoles of potassium phosphate buffer (pH 7.5), and 2 μmoles of $MgCl_2$ and UV-exonuclease (0.1–0.6 unit). After incubation for 30 min at 37°, the samples are held at 65° for 5 min and chilled in ice. The solutions are spotted directly

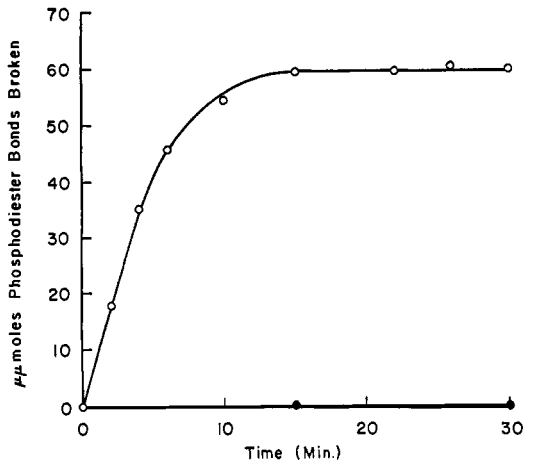

FIGURE 1: Kinetics of DNA incision at 37°. The enzyme was assayed as described in Methods. The incubation mixture (0.3 ml) contained 5 μmoles of Tris-HCl buffer (pH 7.5), 3 μmoles of $MgCl_2$, 5 μmoles of 2-mercaptoethanol, 6.3 nmoles of ^{32}P-labeled UV-irradiated double-stranded *E. coli* DNA (1.1×10^{-5} ergs/mm²), and 6.0 units of enzyme. At the indicated times, tubes were removed and the reaction terminated. The figure shows activity of UV-endonuclease on irradiated DNA (○) and native DNA (●).

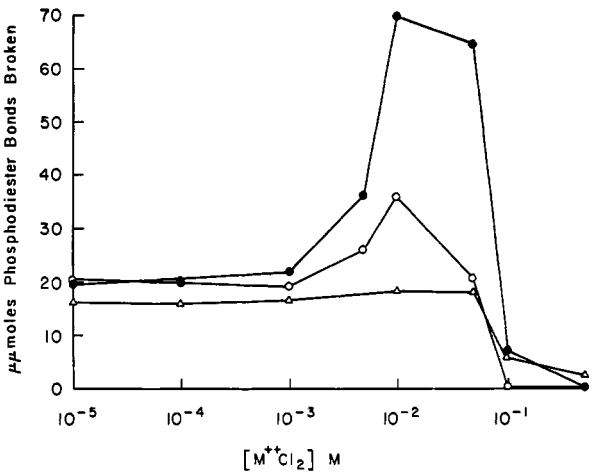

FIGURE 2: Effect of metal ions on UV-endonuclease activity. The enzyme was assayed as described in Methods at 37° after it had been dialyzed against 0.05 M potassium phosphate buffer (pH 7.5)–10% glycerol (v/v). The following salts were included in the incubation mixture at concentrations of 1.0×10^{-3}–5.0×10^{-1} M. $MgCl_2$ (●), $MnCl_2$ (○), and $CaCl_2$ (▲).

onto DEAE-chromatography paper and processed by the chromatographic procedures described in the Methods section.

Treatment of ^{32}P-Labeled Denatured DNA, Partially Digested by UV-Exonuclease, with 5′-Nucleotidase. Each reaction mixture (0.5 ml) contains 30 nmoles of ^{32}P-labeled denatured DNA (specific activity 1×10^5 cpm/nmole of nucleotide equivalent), 30 nmoles of glycine buffer (pH 8.5), 3 μmoles of $MgCl_2$, and 30 μg of bovine serum albumin and UV-exonuclease (0.1–0.4 unit). After incubating for 30 min at 37°, the tubes are chilled and one-half of them is processed as described for the UV-exonuclease assay. The other samples are held at 65° for 5 min and chilled in ice. The enzyme 5′-nucleotidase (2.0 units) is added to the reaction mixture which subsequently is incubated for 15 min at 37°. The reaction is terminated by the addition of 1 N HCl (0.5 ml) and the samples subjected to the Norit assay as described.

UV-Exonuclease Hydrolysis of Terminally Labeled Denatured DNA. The incubation mixture (1.8 ml) contains 2.34 μmoles of denatured DNA terminally labeled either at the 3′-hydroxy with [^{14}C]deoxyadenylic acid residues or at the 5′-phosphoryl terminus with [^{32}P]phosphomonoester groups, 12.1 μmoles of $MgCl_2$, 12.1 μmoles of potassium phosphate buffer (pH 7.5), bovine serum albumin (100 μg/ml), and 4 units of UV-exonuclease. The reaction mixture is incubated at 37° and at various time intervals, aliquots (0.3 ml) are removed and added to 0.2 ml of calf thymus DNA (2.5 mg/ml) and 0.5 ml of cold 1 N perchloric acid. After 5 min at 0°, the tubes are centrifuged for 10 min at 10,000*g*. The absorbance of the supernatant fluid is determined at 260 nm and the radioactivity measured in Bray's–dioxane scintillation fluid.

Results

Properties of the UV-Endonuclease. The UV-endonuclease has been shown to be specific for UV-irradiated native DNA and unable to act on unirradiated native denatured or irradiated denatured DNA (Kaplan *et al.*, 1969). It has also been demonstrated that the enzyme catalyzes the formation of single-strand breaks (Grossman *et al.*, 1968) in UV-irradiated double-stranded DNA.

Kinetics of DNA Incision. The appearance of phosphodiester-bond breaks in UV-irradiated *E. coli* DNA occurs rapidly, leveling off completely after 15 min (Figure 1). While the total number of phosphodiester-bond breaks is a function of dose, the appearance of such breaks reaches a final and steady state at all doses tested. In addition, phosphodiester-bond cleavage proceeds in a linear manner. The initial rates of such reactions are the same over a dose range from 2.58×10^4 to 2.3×10^5 ergs per mm² (Kushner, 1970).

Effect of Metal Ions on UV-Endonuclease Activity. The purified enzyme is stimulated between three- and fourfold by 1×10^{-2} M Mg^{2+} (Figure 2). Other divalent cations such as Mn^{2+} and Ca^{2+} produce little if any stimulation at comparable

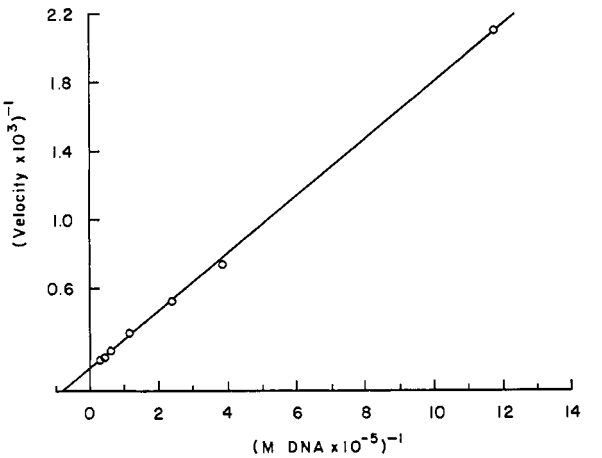

FIGURE 3: Substrate saturation of the UV-endonuclease. The UV-endonuclease was assayed as described in Methods against varying amounts of ^{32}P-labeled UV-irradiated double-stranded *E. coli* DNA (1.1×10^5 ergs/mm², 0.025–3.35 μg). The incubation mixture (0.3 ml) contained 5.0 μmoles of Tris-HCl (pH 7.5), 3.0 nmoles of $MgCl_2$, 5.0 μmoles of 2-mercaptoethanol, and enzyme (4.0 units). All points were run in duplicate and the molarity of DNA was expressed in terms of total nucleotide equivalents.

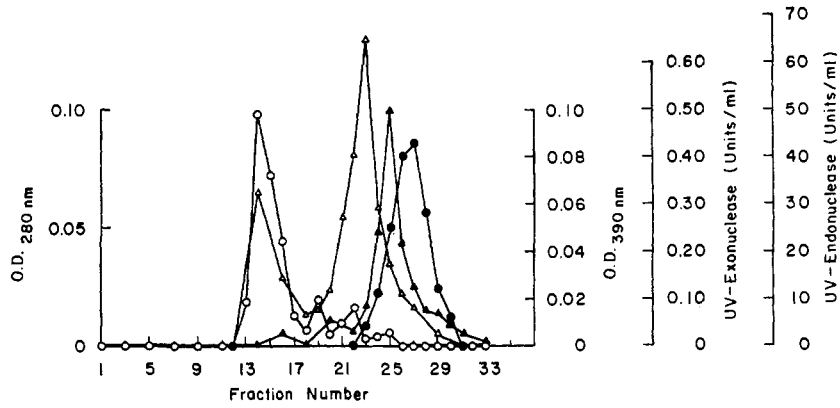

FIGURE 4: Molecular weight of the UV-endonuclease. The enzyme (0.3 ml) was layered onto a Sephadex G-75 column (1.0 × 34 cm) which had been preequilibrated with 0.01 M potassium phosphate buffer (pH 7.5)–10% ethylene glycol (v/v). The column was eluted with the same buffer. The figure illustrates protein, $OD_{280\ nm}$ (○), horse heart cytochrome *c* $OD_{390\ nm}$ (●), UV-exonuclease activity (△), and UV-endonuclease activity (▲).

concentrations. Although a similar UV-endonuclease has been purified by different means from this organism, it is not possible to evaluate its cation requirement since EDTA is included in the reaction mixtures to inhibit exonuclease contamination (Carrier and Setlow, 1970). UV-endonuclease which is independent of magnesium ions is not inhibited by EDTA at concentrations up to 1.0×10^{-2} M. At 5×10^{-1} M the residual activity decreases by only 50%. In the absence of magnesium ions, monovalent cations such as potassium, lithium, and sodium are not effective as activators.

Inhibition of UV-Endonuclease Activity. Caffeine and acridine orange were tested as possible inhibitory agents because of their known action as inhibitors of dark repair in *E. coli* (Witkin, 1958; Lieb, 1961). Caffeine has no effect in the concentration range tested (1×10^{-5} to 5×10^{-2} M). However, complete loss of enzymatic activity is obtained with acridine orange at 1×10^{-2} M when included in a reaction mixture protected from visible light. Since the dye is known to intercalate DNA, this effect is probably attributable to its action on the substrate rather than on the enzyme (Bradley and Wolf, 1959).

Dependence of the UV-Endonuclease Activity on pH. The UV-endonuclease is active between pH values 4.5 and 9 with a maximum between 7 and 7.5 in both potassium phosphate buffer and Tris-HCl buffer. The enzyme has slightly greater activity in potassium phosphate buffer.

Temperature Stability of the UV-Endonuclease. The highly purified enzyme is relatively stable in the presence of added exogenous DNA. In its absence, however, enzymatic activity decays rapidly at most temperatures tested. It has not been established that the enzyme is actually binding to the DNA.

Substrate Saturation of the UV-Endonuclease. The endonucleolytic activity of the UV-endonuclease, when plotted as a function of DNA concentration, results in a hyperbolic curve. The basic kinetic constants have been determined using the Lineweaver–Burk double-reciprocal plot. A straight line is generated, which on extrapolation, yields an apparent K_m value for irradiated native DNA of 1.25×10^{-5} M with the molarity of DNA expressed in terms of total nucleotide equivalents (Figure 3). The V_{max} is equal to 8700 nmoles of phosphodiester bonds broken per mg of protein per min.

Molecular Weight of the UV-Endonuclease. It has been reported previously that the UV-endonuclease is a low molecular weight protein (Kaplan *et al.*, 1969; Carrier and Setlow, 1970). Based on its elution pattern from Sephadex G-75, an approximate molecular weight of 15,000 has been assigned to the protein (Figure 4). Of interest is the appearance of a UV-exonuclease peak eluting at a molecular weight position slightly greater than that of the UV-endonuclease. This peak of activity is not observed when high concentrations of the UV-exonuclease protein are chromatographed and may represent a subunit of the enzyme which dissociates at low protein concentration.

Dose Dependence of the UV-Endonuclease. When enzymatic activity is followed as a function of UV dose, using constant DNA levels, a sigmoidal curve is obtained (Kaplan *et al.*, 1968). An identical phenomenon is observed when either *E. coli* or *M. luteus* DNA is used as a substrate (Figure 5). The low yield of single-strand breaks observed with the *M. luteus* DNA could be a reflection of the fewer thymine–thymine dimers formed in a high GC DNA. Since the quantum yield of cytosine–thymine and cytosine–cytosine dimers is lower than that for thymine–thymine dimers, the insensitivity of this DNA may reflect either fewer photoproducts formed at these doses or there may be greater conformational stability about a thymine dimer region. It has been reported by Setlow and Carrier (1966) that at 4×10^4 ergs/mm², a total yield of pyrimidine photoproducts in *M. luteus* DNA is half that obtained with *E. coli* DNA. Another factor contributing to this insensitivity may be related to the susceptibility of irradiated DNA, which is rich in GC, to the UV-endonuclease activity. It has not been established whether the UV-endonuclease recognizes CT or CC dimeric photoproducts.

Properties of the UV-Exonuclease Specificity. The UV-exonuclease is highly selective for denatured or single-stranded DNA and polydeoxynucleotides. When native and denatured *E. coli* DNAs are compared as substrates for the UV-exo-

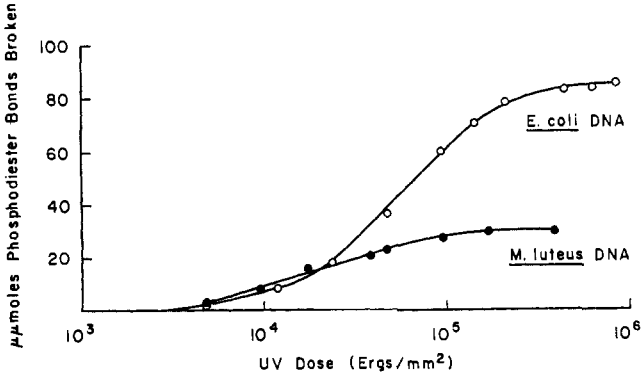

FIGURE 5: Dose dependence of the UV-endonuclease. The enzyme was assayed as described in Methods at 37°. Either ^{32}P-labeled native *E. coli* DNA (○) or native *M. luteus* DNA (●) was irradiated in a Bausch and Lomb monochromator at 280 nm. The incident radiation intensity was measured with an Eldorado photometer and was approximately 40 ergs/mm² per sec.

TABLE I: Hydrolysis of DNA, RNA, and Polynucleotides by UV-Exonuclease of *M. luteus*.[a]

Substrate	Amount of Substrate (nmoles)	Total Nucleotide Released (nmoles)	Extent of Hydrolysis (%)
1. Denatured DNA	2.4	2.26	94
2. Irradiated denatured DNA	2.4	2.4	100
3. Native DNA	2.4	0.030	1.2
4. Irradiated native DNA	2.4	0.025	1.0
5. Poly[d(pT)$_{200}$]	2.4	2.4	100
6. RNA (yeast)	2.4	0.010	0.4
6. Polycytidylic acid	2.4	0.005	0.2
8. Polyuridylic acid	2.4	0.006	0.25
9. Polyadenylic acid	2.4	0.005	0.2

[a] All substrates were incubated under standard assay conditions as described in Methods for a 2-hr period at 37°. The incubation mixture (0.15 ml) included 1.0 μmole of $MgCl_2$, 1.0 μmole of potassium phosphate buffer (pH 7.5), 2.4 nmoles of substrate, 10 μg of bovine serum albumin, and 0.5 unit of enzyme.

nuclease, under conditions of enzyme excess, the initial rate of hydrolysis of native DNA is less than 0.1% of the rate observed with denatured or irradiated denatured DNA (Figure 6). While the UV-exonuclease quantitatively degrades irradiated denatured DNA to acid-soluble products, only a negligible release of nucleotides occurs from irradiated native DNA. It has been shown previously that the UV-exonuclease, unlike certain other exonucleases, is not inhibited by the presence of photoproducts in DNA (Grossman *et al.*, 1968).

Data presented in Table I indicate that the enzyme requires a polydeoxynucleotide structure for its activity. The UV-exonuclease is unable to degrade yeast RNA, polycytydylic, polyuridylic, or polyadenylic acids at a detectable rate (less than 1% of the rate observed with denatured DNA) but does quantitatively hydrolyze poly[d(pT)$_{200}$] to acid-soluble products. It has been previously described that the UV-exonuclease also degrades oligodeoxynucleotides, including dinucleotides, to monomeric units (Grossman *et al.*, 1968). The length of a polynucleotide chain, therefore, apparently does not influence the specificity of the enzyme and its limit digestion product consists of 5′-deoxynucleoside monophosphate. In order to function as a repair enzyme the UV-exonuclease should be able to excise photoproduct regions from irradiated DNA. In fact, irradiated native DNA becomes a substrate for the UV-exonuclease only after it has first been incised with the UV-endonuclease (Kaplan *et al.*, 1969).

Substrate Saturation of the UV-Exonuclease. When the UV-exonuclease activity is examined under standard assay conditions, a hyperbolic DNA concentration curve is generated (Figure 7). The basic kinetic constants have been determined using the Lineweaver–Burk double-reciprocal plot. The apparent K_m value for irradiated denatured DNA is equal to 4.46×10^{-6} M. The molarity of the DNA is expressed in terms of total nucleotide equivalents. The V_{max} is equal to 154 nmoles of nucleotide released per mg of protein per min.

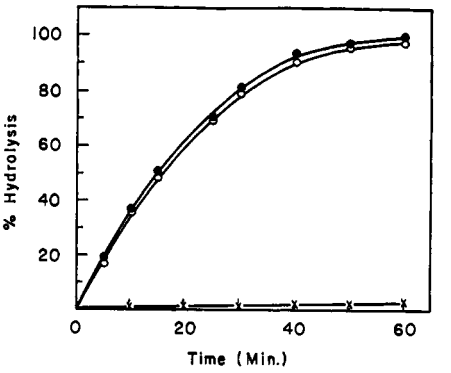

FIGURE 6: Kinetics of DNA hydrolysis at 37°. The enzyme was assayed as described in Methods. The incubation mixture (0.15 ml) included: 1 μmole of $MgCl_2$, 1.0 μmole of potassium phosphate buffer (pH 7.5), 2.4 nmoles of ^{32}P-labeled DNA, and 0.2 unit of enzyme. At the indicated times, tubes were removed and the reaction terminated. The hydrolysis of denatured DNA (●), irradiated denatured DNA (○), and native DNA (X) is shown. The DNA was irradiated at 254 nm for 10 min (dose = 1.0×10^5 ergs/mm^2).

Dependence of UV-Exonuclease Activity on pH. The rate of hydrolysis of irradiated denatured DNA has been determined over the pH range 5.5–10.5. The UV-exonuclease is active between pH 6 and 10 and the pH curve displays a maximum between 7 and 8. At the pH optimum the activity is twofold greater in potassium phosphate buffer than in Tris-HCl or sodium carbonate buffers. At pH values of 5.5, 6.5, 8.5, and 10.0 the rates are 10, 73, 82, and 24%, respectively, of the rate observed at pH 7.5.

Temperature Stability. The purified enzyme is unstable when incubated in the absence of DNA. Enzyme solutions are completely inactivated when heated at 45° for 2 hr. After 1 hr, only 5% of the original activity remains while at 37°, 57% of the enzyme activity is lost in the same time period.

Effect of Divalent Metal Ions. Purified UV-exonuclease is dependent on magnesium ion for activity. The optimal concentration is 6.7×10^{-3} M. At 10^{-3}, 10^{-2}, and 10^{-1} M the rates are 98, 37, and 11.2%, respectively, of that at 6.7×10^{-3} M. Magnesium cannot be replaced by either calcium, manganese, zinc, or copper. EDTA (5×10^{-3} M) produces 100% inhibition of activity.

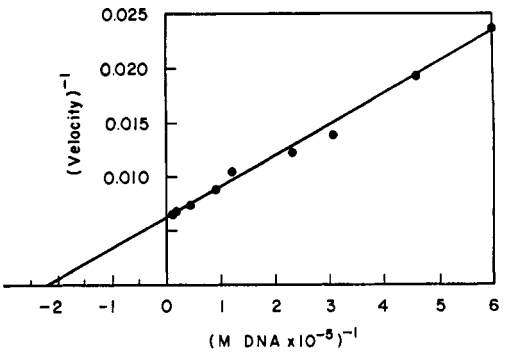

FIGURE 7: Substrate saturation of the UV-exonuclease. The enzyme was assayed as described in Methods against varying amounts of ^{32}P-labeled irradiated denatured DNA (0.025–2.5 μg). The incubation mixture (0.15 ml) contained 1.0 μ mole of $MgCl_2$, 1.0 μmole of potassium phosphate buffer (pH 7.5), 10 μg of bovine serum albumin, and 0.2 and 0.4 unit of enzyme. Two levels of enzyme were used in order to check proportionality of the assay. The molarity of DNA was expressed in terms of total nucleotide equivalents.

TABLE II: Proof of Exonucleolytic Attack on DNA Using Bacterial Alkaline Phosphatase.[a]

Expt	Enzyme Added: UV-Exonuclease (Units)	Enzyme Added: Alkaline Phosphatase (Units)	Acid-Soluble [^{32}P]Nucleotides: nmoles	Acid-Soluble [^{32}P]Nucleotides: %	^{32}P Released from Acid-Soluble Nucleotides: nmoles	^{32}P Released from Acid-Soluble Nucleotides: %
1	None	None	0.07	0.23	0.07	100
2	0.1	20	2.8	8.9	2.88	103
3	0.2	20	6.8	21.8	5.85	86
4	0.4	20	11.1	35.9	10.76	96
5	0.4	None	10.9	35.2	0.00	
6	None	20			0.00	

[a] The experimental procedure was carried out as described in Methods.

Dependence of UV-Exonuclease Activity on Enzyme Concentration. As shown in Figure 8 there is a sigmoidal dependence of acid-soluble nucleotide release as a function of protein concentration. The specific activity of the UV-exonuclease varies over a wide range of enzyme concentrations increasing fourfold when the protein is raised from 5 to 60 μg per ml. The initial lag in this curve can be abolished in the presence of crystalline bovine serum albumin (5–100 μg/ml) or certain monovalent cations.

Effect of Monovalent Cations. Certain monovalent ions enhance the UV-exonuclease reaction and abolish the lag in the enzyme concentration curve. Figure 9 compares the effect of increasing concentrations of potassium, sodium ammonium, and lithium sulfates on UV-exonuclease activity. Chloride salts also activate in the same maner but less efficiently. High concentrations of these cations strongly inhibit the enzymatic reaction. Since cation reactivation is most pronounced at low protein concentrations, it may be interpreted as an aggregation of the protein which upon dilution dissociates to monomeric species.

Products of Hydrolysis. The products of hydrolysis of denatured DNA, irradiated denatured DNA, and poly[d(pT)$_{200}$] were examined both chromatographically and in terms of their sensitivity to venom 5′-nucleotidase. In order to demonstrate that mononucleotides were produced at all stages of digestion, denatured DNA, labeled with [^{32}P]P_i, was hydrolyzed to varying extents and the reaction mixture analyzed for mononucleotide content by measuring its susceptibility to bacterial alkaline phosphatase (Table II). Since the extent of breakdown of denatured DNA was closely matched by the production of mononucleotides during the initial part of the reaction, it was concluded that the UV-exonuclease is attacking from an end of the DNA chain to produce mononucleotides in a stepwise manner. The products of limited DNA hydrolysis were also examined chromatographically to determine whether any of the acid-soluble material included oligonucleotides (Table III). The chromatographic system separated mononucleotides and oligonucleotides of chain length 2–8 from longer oligonucleotides that remained at the origin (Kelly *et al.*, 1970). At all stages of enzymatic hydroly-

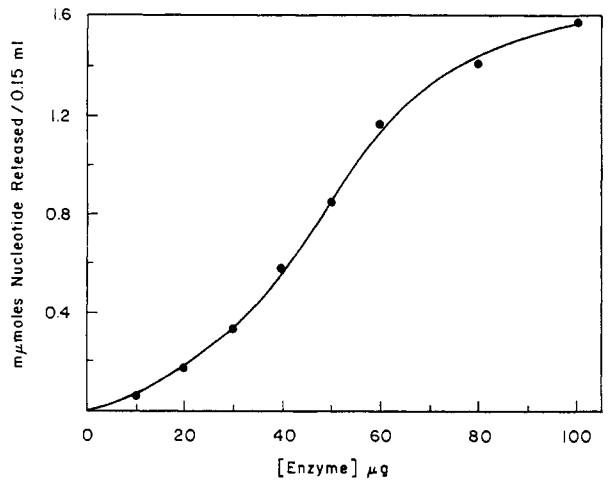

FIGURE 8: Dependence of UV-exonuclease activity on enzyme concentration. The enzyme was assayed as described in Methods. The incubation mixture (0.15 ml) contained 1.0 μmole of $MgCl_2$, 1.0 μmole of potassium phosphate buffer (pH 7.5, 2.4 nmoles), ^{32}P-labeled irradiated denatured DNA, and enzyme (10–100 ug/ml). Each point on the curve represents an average of three determinations.

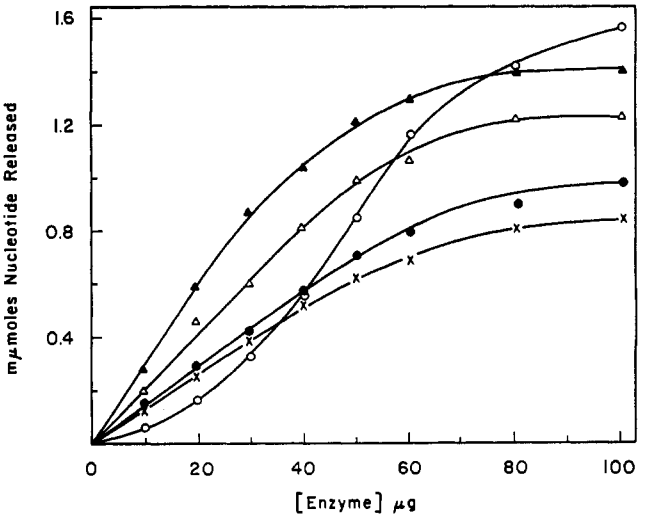

FIGURE 9: Effect of monovalent cations on the UV-exonuclease. The enzyme was assayed as described in Methods after it had been dialyzed against 0.01 M Tris-HCl (pH 7.5). The following salts were included in the incubation mixture: no salt (○), $NaSO_4$ (14 mM) (●), Li_2SO_4 (14 mM) (X), $(NH_4)_2SO_4$ (7.0 mM) (△), and K_2SO_4 (7.0 mM) (▲).

TABLE III: Distribution of Products after Limited Hydrolysis of Denatured DNA by UV-Exonuclease.[a]

Expt	Enzyme Added UV-Exonuclease (Units)	Hydrolysis (%)	Products (% Released)		
			Polynucleotide (at Origin)	Oligonucleotide (Chain Length 2–8)	pT
1	0.1	6	93	0	6
2	0.3	31	66	0	30
3	0.6	54	43	0	51
4	None	0.30	100	0	0

[a] The experimental procedure was carried out as described in Methods.

TABLE IV: Identification of Mononucleotides Released by the UV-Exonuclease Using 5′-Nucleotidase.[a]

Expt	Enzyme Added		Acid-Soluble [^{32}P]Nucleotides		^{32}P Released from Acid-Soluble Nucleotides	
	UV-Exonuclease	5′-Nucleotidase	nmoles	%	nmoles	%
1	None	None	0.05	0.16	0.05	100
2	0.1	2.0	1.9	6.3	1.7	91.6
3	0.2	2.0	3.8	12.7	3.54	93.2
4	0.4	2.0	8.2	23.9	7.73	94.3
5	0.4	None	8.0	23.3	0.10	0.43
6	None	2.0			0.28	0.93

[a] The experimental procedure was carried out as described in Methods.

sis, the acid-soluble products migrated as mononucleotides. The location of the phosphomonoester group on the mononucleotide digestion product was examined using 5′-nucleotidase, an enzyme that specifically hydrolyzes nucleoside 5′-monophosphates to nucleosides and inorganic orthophosphate. When ^{32}P-denatured DNA was digested to varying degrees with the UV-exonuclease, the digestion products (mononucleotides) were found to be quantitatively dephosphorylated by 5′-nucleotidase (Table IV).

Certain exonucleases hydrolyze unirradiated DNA primarily to mononucleotides yet produce both oligo- and mononucleotides when degrading irradiated DNA. The oligonucleotides contain pyrimidine dimers which are not hydrolyzed by these enzymes. Unirradiated denatured [^{3}H]thymine-labeled DNA, when hydrolyzed completely to acid-soluble products, was quantitatively converted into mononucleotides (Figure 10A). However, when irradiated denatured DNA similarly treated an oligonucleotide fraction with a mobility slower than the dinucleotides $d(\widehat{pT})_2$ or $d(pT)_2$ was also produced (Figure 10B). In order to determine the size of the oligonucleotide produced when the UV-exonuclease degraded an irradiated single-stranded DNA substrate, a model compound, ^{3}H-labeled polydeoxythymydylate ($d(pT)_{200}$), was used. However, when $d(pT)_{200}$ was irradiated and digested, a mixture of two kinds of oligonucleotides was produced along with a larger percentage of mononucleotides (Figure 11b). The oligonucleotides were chromatographically coincident with a trinucleotide $d(\widehat{pT})_3$ and a dinucleotide $d(\widehat{pT})_2$. Of the total radioactivity on the chromatogram, 5.0% migrated as the trinucleoside triphosphate and 6% as the dinucleoside diphosphate.

Site of Initiation of Hydrolysis. Most exonucleases studied which release 5′-deoxynucleoside monophosphates attack at the 3′ terminus of a DNA strand. When denatured DNA, terminally labeled at the 3′-hydroxyl end with [^{14}C]deoxyadenylic acid residues, was treated with the UV-exonuclease, under conditions of enzyme excess, the rapid release of radioactive deoxynucleoside monophosphates occurred before the bulk of the ultraviolet-absorbing polynucleotide material became acid soluble (Figure 12). Within 30 min when 56% of the ^{14}C-labeled had been released, only 1.5% of the unlabeled nucleotides had been removed. About 97% of the total label was liberated after 4 hr when 15% of the unlabeled nucleotides had become acid soluble. Similar results have been reported for the *E. coli* exonuclease 1 (Lehman, 1960). Since most of the 3′-terminal nucleotides were released before internal ones, it appeared that the UV-exonuclease was not degrading one DNA chain to completion before initiating attack on a second molecule. Under these conditions, the enzyme attacked all termini synchronously before digesting the remainder of the chain.

Although the UV-exonuclease appeared to belong to the class of exonucleases which degraded DNA from the 3′ terminus, because of its role in excision of incised DNA it was important to determine whether or not the enzyme could simultaneously attack the 5′ terminus. To test this possibility denatured DNA was specifically labeled at the 5′ terminus with ^{32}P and exposed to the UV-exonuclease. The radioactive terminal nucleotides were again released more rapidly than the internal unlabeled nucleotides (Figure 13). While the UV-exonuclease is able to attack DNA from either

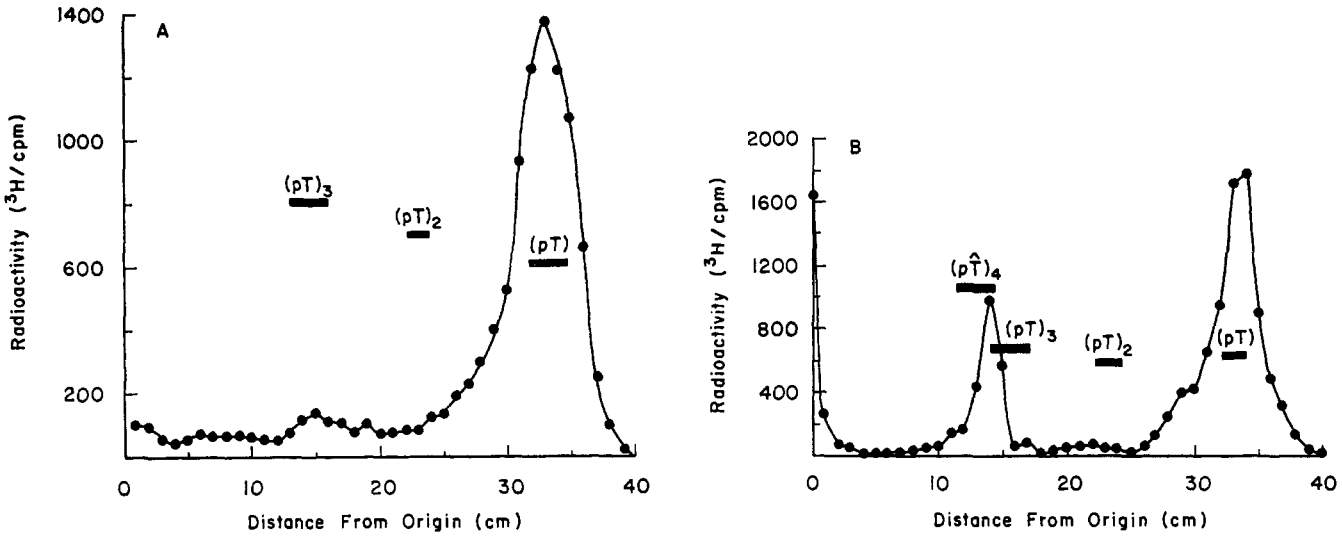

FIGURE 10: Chromatography of nucleotides produced by complete hydrolysis of unirradiated and irradiated denatured DNA. The reaction mixture (0.15 ml) containing: 8.0 mμmoles of either irradiated or unirradiated ^{3}H-labeled denatured DNA, 1.0 μmole of $MgCl_2$, 1.0 μmole of potassium phosphate buffer (pH 7.5), and UV-exonuclease (2.0 units) were incubated for 24 hr at 37°. The products were separated by DEAE-cellulose paper chromatography as described in Methods. (A) Illustrates the products arising from the hydrolysis of unirradiated denatured DNA and part B shows the products resulting from the hydrolysis of irradiated denatured DNA. The bars on the graphs represent the positions of reference marker compounds.

direction, the rate of release of nucleotides is greater from the 3′ than from the 5′ terminus. The rates of DNA hydrolysis are the same whether or not the 5′ terminus is phosphorylated.

Discussion

Although the UV-endonuclease is specific for UV-irradiated native DNA, it is not clear what specific aspect of irradiated DNA the enzyme recognizes. If it is able to act only on pyrimidine dimers other enzymes would be required to account for the observed recovery of organisms from treatment with such agents as methyl methanesulfonate, mitomycin C, and nitrous acid (Haynes, 1966). On the other hand, the formation of pyrimidine dimers may produce local helical distortions because of the dimer's radically altered stereochemistry. Recognition of this distortion, rather than the dimer *per se*, would give the UV-endonuclease a more general function in cellular repair.

From the results which have been presented in this paper it is clear that the UV-endonuclease recognizes pyrimidine dimer containing regions in *E. coli* and *M. luteus* DNA. The UV-endonuclease may recognize only certain types of distorted regions such as those containing thymine-thymine dimers. These would cause the largest helical distortion because of their likely occurrence in (AT)-rich regions. Distortions or alterations of a lesser magnitude may not present an immediate danger to cells providing the postreplication repair system were capable of repairing such damage (Rupp and Howard-Flanders, 1968). It is of interest in this regard that the activities of the photoreactivating enzyme and the UV-endonuclease do in fact overlap. The photoreactivating enzyme can restore 80% of the biological activity to irradiated transforming DNA indicating that the enzyme is highly selective for biologically important damage (Setlow and Setlow, 1963). Hence both enzymes seem to recognize either directly or indirectly those photoproducts which are of immediate danger to the cell. Work is now in progress to further delineate the recognition process of the UV-endonuclease to determine its relationship to other damage such as mitomycin C, alkylating agents, and nitrous acid. However, *E. coli* endonuclease II recently has been shown to be specific for alkylated DNA and has no activity on irradiated DNA (Friedberg *et al.*, 1969). It is possible that different enzymes do exist to repair different lesions.

It is appropriate at this point to review some of the distinctive properties of the UV-exonuclease. An important difference between the UV-exonuclease and other exonucleases like snake venom phosphodiesterase, calf spleen phosphodiesterase, and *E. coli* exonuclease 1 is its ability to degrade unirradiated and irradiated denatured DNA at the same rate (Grossman *et al.*, 1968). The presence of a pyrimidine dimer in irradiated DNA does not appear to inhibit its catalytic activity. A detailed analysis of the reaction products revealed that the degradation of irradiated denatured DNA by the UV-exonuclease resulted in the simultaneous production of both 5′-mononucleotides and small pyrimidine dimer-containing oligonucleotides. A limit trinucleotide or longer oligonucleotide containing the photoproduct arises from hydrolysis of irradiated denatured *E. coli* DNA whereas di- and trinucleotides are the limit digestion product from irradiated poly[$(dT)_{200}$].

The ability of the UV-exonuclease to both degrade irradiated denatured DNA and to excise pyrimidine dimers from irradiated native DNA certainly qualifies it as a possible dark repair nuclease and, moreover, its activity can be examined as a constituent in an enzymatic mechanism for the excision of irradiated DNA *in vitro*. Similarly, it has been shown that oligonucleotide-containing thymine dimers can also be excised from UV-irradiated DNA by the exonucleolytic activity associated with *E. coli* DNA polymerase. The involvement of this enzyme in excision, however, requires a preliminary endonucleolytic break for removal of damaged DNA fragments (Kelly *et al.*, 1970). These observations are consistent with the characterization of excision products *in vivo* with tri- and tetranucleotides as well as longer oligonucleotides.

Another important property of the UV-exonuclease is its ability to initiate hydrolysis at either terminus of denatured

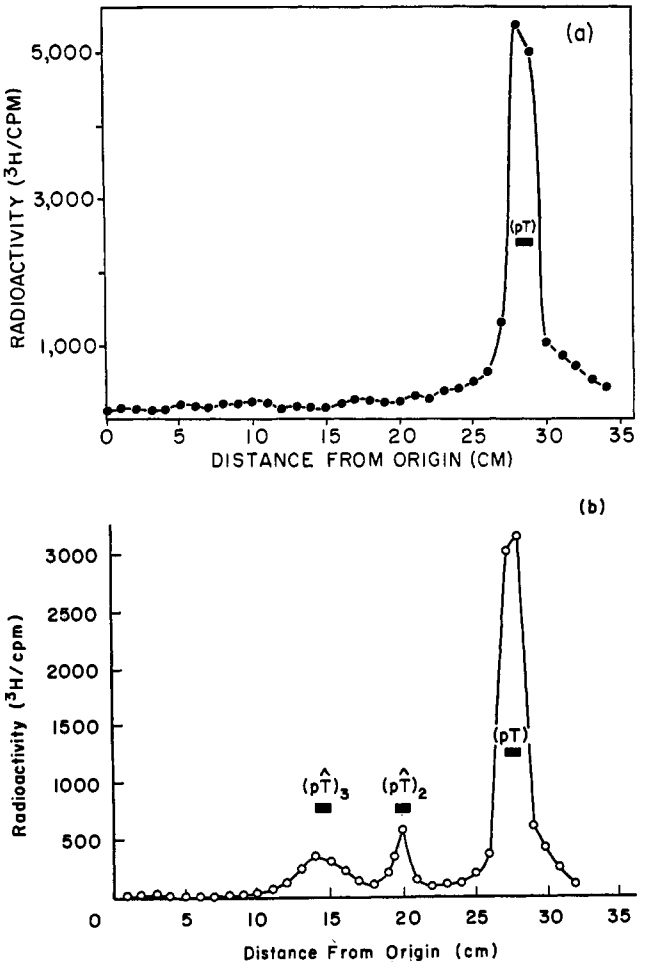

FIGURE 11: Chromatography of nucleotides produced by complete hydrolysis of unirradiated and irradiated polythymidylic acid. The reaction mixture (0.3 ml) containing: 24 nmoles of either unirradiated or irradiated ^{3}H-labeled polythymidylic acid (poly[d(pT)$_{200}$]), 2.0 μmoles of $MgCl_2$, 2.0 μmoles of potassium phosphate buffer (pH 7.5), and 6.0 units of UV-exonuclease was incubated for 24 hr at 37°. The products were separated by chromatography on DEAE-cellulose paper as described in Methods. (a) Illustrates the products resulting from the hydrolysis of unirradiated poly[d(pT)$_{200}$] and part b shows the products arising from the hydrolysis of irradiated poly[d(pT)$_{200}$]. The bars on the graphs represent the positions of reference marker compounds.

DNA, liberating 5′-mononucleotides. The only other nucleases that have been reported to degrade DNA from either end are *B. subtilis* exonuclease (Okasaki *et al.*, 1966; Kerr *et al.*, 1967), and the exonuclease activity associated with the *E. coli* DNA polymerase (Kelly *et al.*, 1970). In the case of *B. subtilis*, the site at which the nuclease initiates hydrolysis is dictated by the secondary structure of the polynucleotide substrate and 3′-mononucleotides are produced. The DNA polymerase associated exonuclease initiates hydrolysis at the 3′ end of single-stranded DNA and at the 5′ end of native DNA to yield 5′-mononucleotides. Most other known exonucleases, which release 5′-mononucleotides, attach at the 3′ terminus of the DNA strand. Conversely, those enzymes initiating hydrolysis at the 5′ terminus produce 3′-mononucleotides. Another exception to the above classification includes the λ-exonuclease (Radding, 1966; Little, 1967) which initiates degradation at the 5′ terminus of native DNA to yield 5′-mononucleotides.

It is difficult to interpret the distribution of digested products when an enzyme is hydrolyzing DNA from both ends since it reflects molecular average of all the released fragments. It would therefore be useful to compare the rates of UV-exonuclease initiation on DNA molecules that have been either blocked at the 3′ or 5′ terminus thereby forcing the enzyme to degrade from one end only or by selectively labelling polymers at both terminii. In fact, the 5′-hydrolytic activity associated with the *E. coli* DNA polymerase was uncovered in studies using a synthetic block polymer which was resistant to hydrolysis at the 3′ end of the chain (Klett *et al.*, 1968). According to our model for the enzymatic excision of thymine dimers in *M. luteus* the UV-exonuclease is forced to hydrolyze from the 5′ terminus as a result of the specific hydrolytic site catalyzed by the UV-endonuclease. Our data supports a two-step model for the excision of photoproducts from irradiated native DNA *in vitro*. Excision ceases after ap

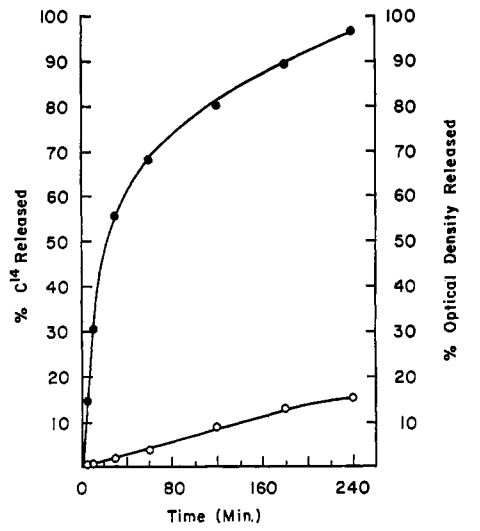

FIGURE 12: UV-exonuclease action on 3′-terminally ^{14}C-labeled DNA. The experimental procedure was carried out as described in Methods. In the figure, the percentage of end groups released (●) are plotted against the percentage of total nucleotide degradation (○).

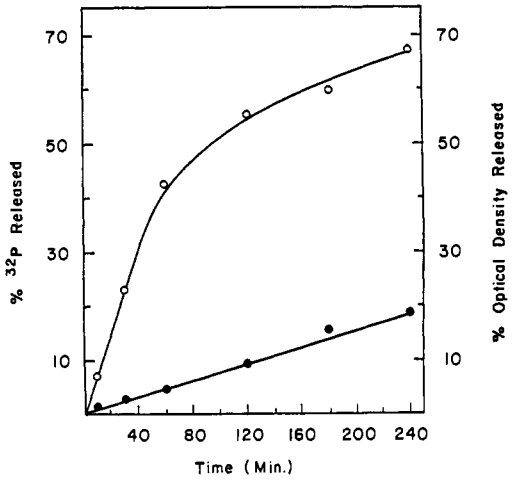

FIGURE 13: UV-Exonuclease action on 5′-terminally ^{32}P-labeled DNA. The experimental procedure was carried out as described in Methods. In the figure, the percentage of end groups released (○) was plotted against the percentage of total nucleotide degradation (●).

proximately 5–6 nucleotide equivalents have been removed for every single-strand break made by the UV-endonuclease (Kaplan *et al.*, 1969). Initial incision leaves the 5′-hydroxyl group next to the dimer. This end is now susceptible to the UV-exonuclease and the mechanism of the excision process will be the subject of the accompanying paper.

References

Adler, J., Lehman, I. R., Bessman, M. J., Simms, E. S., and Kornberg, A. (1958), *Proc. Nat. Acad. Sci. U. S. 214*, 641.

Boyce, R. P., and Howard-Flanders, P. (1964), *Proc. Nat. Acad. Sci. U. S. 51*, 293.

Bradley, D. F., and Wolf, M. K. (1959), *Proc. Nat. Acad. Sci. U. S. 45*, 944.

Carrier, W. L., and Setlow, R. B. (1970), *J. Bacteriol. 102*, 178.

Friedberg, E. C., Hadi, S. H., and Goldthwait, D. A. (1969), *J. Biol. Chem. 244*, 5879.

Garen, A., and Levinthal, C. (1960), *Biochim. Biophys. Acta 38*, 470.

Grossman, L. (1967), *Methods Enzymol. 12*, 700.

Grossman, L., Kaplan, J. C., Kushner, S. R., and Mahler, I. (1968), *Cold Spring Harbor Symp. Quant. Biol. 33*, 229.

Haynes, R. H. (1966), *Radiat. Res., Suppl. 6*, 8.

Heppel, L. A., and Hilmoe, R. J. (1955), *Methods Enzymol. 2*, 547.

Kaplan, J. C., Kushner, S. R., and Grossman, L. (1969), *Proc. Nat. Acad. Sci. U. S. 63*, 144.

Kelley, R. B., Cozzarelli, N. R., Deutcher, M. P., Lehman, I. R., and Kornberg, A. (1970), *J. Biol. Chem. 245*, 39.

Kelner, A. (1949), *Proc. Nat. Acad. Sci. U. S. 35*, 73.

Kerr, I. M., Chien, J. R., and Lehman, I. R. (1967), *J. Biol. Chem. 242*, 2700.

Khorana, H. G., and Vivsolyi, J. P. (1961), *J. Amer. Chem. Soc. 83*, 675.

Klett, R. P., Cerami, A., and Reich, E. (1968), *Proc. Nat. Acad. Sci. U. S. 60*, 943.

Kushner, S. R. (1970), Ph.D. Dissertation, Brandeis University.

Lehman, I. R. (1960), *J. Biol. Chem. 235*, 1479.

Lehman, I. R., Roussos, G. G., and Pratt, E. S. (1962), *J. Biol. Chem. 237*, 819.

Lieb, M. (1961), *Z. Vererbungslehre 92*, 416.

Little, J. W. (1967), *Advan. Enzymol. 12*, 263.

Okasaki, R., Okasaki, T., and Sakabe, K. (1966), *Biochem. Biophys. Res. Commun. 22*, 64.

Radding, C. M. (1966), *J. Mol. Biol. 18*, 235.

Richardson, C. C. (1966), *Progr. Nucl. Acad Res.*, 263.

Rupp, W. D., and Howard-Flanders, P. (1968), *J. Mol. Biol. 31*, 291.

Setlow, J. K., and Setlow, R. B. (1963), *Nature (London) 197*, 560.

Setlow, R. B., and Carrier, W. L. (1964), *Proc. Nat. Acad. Sci. U. S. 51*, 226.

Setlow, R. B., and Carrier, W. L. (1966), *J. Mol. Biol. 17*, 237.

Wacker, A. (1962), *J. Mol. Biol. 4*, 410.

Weiss, B., Live, T. R., and Richardson, C. C. (1968), *J. Biol. Chem. 243*, 4530.

Witkin, E. M. (1958), *Proc. Int. Congr. Genet., 10th, Mont.*, 280.