Enzymatic Repair of Deoxyribonucleic Acid. IV. Mechanism of Photoproduct Excision*

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ABSTRACT: The enzymatic mechanisms controlling the incision and excision of UV-irradiated *Escherichia coli* DNA have been examined. The UV-endonuclease, which catalyzes the incision step in repair, hydrolyzes a single phosphodiester bond, 5′ to the photoproduct region. This step is associated with the formation of a 3′-phosphoryl group on the DNA and a 5′-hydroxyl group either at, or one nucleotide 5′, to the photoproduct. The release of either nucleotides or photoproducts does not occur to any significant extent under these circumstances. The excision of nucleotides, and those containing photoproducts, from incised DNA is dependent on the unique exonucleolytic properties of a second enzyme, the UV-exonuclease. This enzyme catalyzes the release of approximately seven nucleotides for each phosphodiester bond hydrolyzed during the incision of irradiated *E. coli* DNA. There are ten nucleotides released during the excision for each incision event of irradiated *Micrococcus luteus* DNA. The cavity of the excised *E. coli* DNA is terminated by two phosphoryl groups. A 3′-phosphoryl group arises from incision and after the excision event a 5′-phosphoryl group remains. Chromatographic analyses of the products released during the initial phases of excision revealed that oligonucleotides were liberated and subsequently degraded exonucleolytically by the same enzyme. It is concluded that the primary hydrolytic event during excision is endonucleolytic in nature. Moreover, the distribution of nucleotides 3′ to the photoproduct in the excised fragment from irradiated *E. coli* DNA is enriched in adenine and thymine containing deoxynucleoside 5′-monophosphates. As the irradiation is intensified this distribution is modified to include cytosine-containing deoxynucleotides. The significance of these findings is discussed.

The recovery of organisms from the effects of ultraviolet irradiation during dark repair is mediated by a multistep enzymatic process, which is presumably initiated by the excision of pyrimidine dimers. Setlow and Carrier (1964) and Boyce and Howard-Flanders (1964) investigated this mechanism and showed that pyrimidine dimers were selectively removed from irradiated DNA during *in vivo* dark repair in *Escherichia coli*. Dimer excision has now been observed in UV-resistant strains of many bacteria including: *Hemophilus influenzae* (Setlow *et al.*, 1968), *Bacillus subtilis* (Strauss *et al.*, 1966), and *Micrococcus luteus* (Kaplan *et al.*, 1969). In the human disease xeroderma pigmentosum sensitivity to sunlight, which is expressed as a high incidence of skin lesions and cancer, has been correlated with a defect in one of the early steps of dark repair (Cleaver, 1969). In fact, Setlow has shown that these cells excise pyrimidine dimers with difficulty (Setlow *et al.*, 1969).

Both of the current models which explain excision–repair (Howard-Flanders and Boyce, 1966; Haynes, 1966) involve incision of the damaged DNA strand by an endonuclease and either immediate ("cut and patch") or delayed excision of the photoproduct-containing regions ("patch and cut"). We have shown previously that the excision of pyrimidine photoproducts *in vitro* requires two enzymes from *M. luteus* (Kaplan *et al.*, 1969; Grossman *et al.*, 1968). The properties of the UV-endonuclease and UV-exonuclease have been described in a separate paper (Kaplan *et al.*, 1971). In this communication we describe the enzyme-catalyzed *M. luteus* photoproduct excision system and its relation to overall excision–repair mechanisms.

Experimental Section

Materials

The UV-endonuclease and UV-exonuclease from *M. luteus* are prepared as described previously (Kaplan *et al.*, 1971). Polynucleotide kinase (kinase) was the generous gift of Dr. C. C. Richardson. Yeast-photoreactivating enzyme was kindly donated by Dr. J. K. Setlow. *E. coli* DNA polymerase was generously provided by Dr. A. Kornberg. Crystalline pancreatic DNase I, micrococcal nuclease, snake venom phosphodiesterase, calf spleen phosphodiesterase, and bacterial alkaline phosphatase were all products of the Worthington Biochemical Corp.

^{32}P-Labeled DNA is prepared from either *E. coli* B or *M. luteus* by the method of Grossman (1967). [^{3}H]Thymine-labeled DNA is obtained from *E. coli* B/r thymine$^-$ by the method of Mahler (1967). UV-irradiated double-stranded DNA and heat-denatured DNA are prepared as previously described (Kaplan *et al.*, 1971). [^{3}H]Thymine-labeled poly(dA:dT) is synthesized enzymatically (Riley *et al.*, 1966).

Methods

Enzyme Assays. UV-Endonuclease and UV-exonuclease are assayed as described previously (Kaplan *et al.*, 1971). Pancreatic DNase I is assayed with native ^{32}P-labeled *E. coli*

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DNA as a substrate in which the reaction course is followed by the standard UV-endonuclease assay (Kaplan *et al.*, 1971). The incubation mixture contains 7.7 nmoles of ^{32}P-labeled native DNA (specific activity 20–100 cpm/pmole), 3 μmoles of sodium acetate buffer (pH 5), and 1.5 μmoles of $MgSO_4$ in 0.3-ml volume. *One unit of activity is defined as that amount of enzyme catalyzing the release of 10 pmoles of [^{32}P]P_i phosphomonoester groups by bacterial alkaline phosphates In 30 min at 37°.* Micrococcal nuclease is assayed in the same manner. Each reaction mixture includes 10 μmoles of sodium borate buffer (pH 8.8), 0.5 μmole of $CaCl_2$, and 7.7 mμmoles of ^{32}P-labeled native *E. coli* DNA (specific activity 20–100 cpm/pmole of nucleotide equivalent). The enzyme is diluted immediately before use in a solution of bovine serum albumin (1 mg/ml in H_2O). One unit is defined in the same manner as described for pancreatic DNase I.

Polynucleotide kinase is assayed according to the method of Richardson (1965). The yeast-photoreactivating enzyme is assayed using the procedure developed for the UV-endonuclease. The incubation mixture (0.5 ml) contains 6 mμmoles of UV-irradiated ^{32}P-labeled native *E. coli* DNA (specific activity 20–40 cpm/pmole of nucleotide equivalent, total incident UV dose = 1.3×10^5 ergs/mm^2 (precalibrated 15-W GE germicidal lamp)), 25 μmoles of Tris-HCl buffer (pH 7.5), 5 μmoles of $MgCl_2$, and 1 μmole of EDTA. Following a 10-min incubation in the dark at 37°, the solution is incubated for 2 hr at 37° in front of a 150-W General Electric photoflood lamp. The photoreactivated DNA is then examined under the standard UV-endonuclease reaction conditions to measure the disappearance of UV-endonuclease-susceptible pyrimidine–pyrimidine dimers. *One unit of activity is defined as that amount of enzyme catalyzing the disappearance of 10 pmoles of UV-endonuclease-susceptible photoproducts under standard assay conditions.*

^{32}P-Labeled DNA Incised with Pancreatic DNase I. Pancreatic DNase I catalyzes the formation of single-strand breaks in DNA producing 5′-phosphoryl and 3′-hydroxyl end groups (Laskowski, 1961). The incubation mixture (0.3 ml) contains 7.7 nmoles of native ^{32}P-labeled *E. coli* DNA (specific activity 50–80 cpm/pmole), 3 μmoles of sodium acetate buffer (pH 5.0), 1.5 μmoles of $MgSO_4$, and 8 units of pancreatic DNase I. The enzyme is diluted immediately before use in distilled water. After incubation for 30 min at 37° the reaction is stopped by adding 0.2 ml of calf thymus DNA (2.5 mg/ml) and 0.3 ml of 7% perchloric acid. The precipitated DNA is collected by centrifugation at 12,500*g* for 10 min and washed with cold distilled water. For determination of the number of single-strand breaks, the DNA is dissolved in 0.5 ml of 0.1 N NaOH and assayed using the second part of the standard UV-endonuclease assay (Kaplan *et al.*, 1971).

^{32}P-Labeled DNA Incised with Micrococcal Nuclease. Micrococcal nuclease cleaves phosphodiester bond to yield 3′-phosphoryl and 5′-hydroxyl groups (Cunningham *et al.*, 1956). The reaction mixture is similar to that described for the micrococcal nuclease assay described above and includes 8 units of enzyme. A 30-min incubation at 37° is followed by treatment as described in the previous section.

UV-Irradiated ^{32}P-Labeled DNA Incised with UV-Endonuclease. The incubation mixture (0.3 ml) contains 7.7 nmoles of UV-irradiated ^{32}P-labeled DNA (specific activity 50–80 cpm/pmole of nucleotide equiv), 5 μmoles of potassium phosphate buffer (pH 7.5), 3 μmoles of $MgCl_2$, 5 μmoles of 2-mercaptoethanol, and 4–10 units of UV-endonucleaes. After incubation at 37° for 30–45 min, the enzymatic reaction is terminated by heating at 65° for 15 min. Large batches of incised UV-irradiated DNA are prepared by pooling individual tubes. In some cases the DNA was precipitated by procedures already described for pancreatic DNase I.

UV-Irradiated ^{32}P-Labeled Excised DNA. Excised DNA refers to UV-irradiated ^{32}P-labeled DNA which is first treated with UV-endonuclease and then with UV-exonuclease. Sufficient time is permitted for complete removal of those photoproduct regions recognized by the UV-endonuclease. The initial incubation is identical with that described for incised UV-irradiated DNA. After the UV-endonuclease activity is terminated by heating at 65° for 15 min and the reaction tubes chilled, 10.0 units of UV-exonuclease are added, and the mixture incubated for 2 hr at 37°.

The isolation of excision products is accomplished in one of three ways. The reaction sequences may be terminated by perchloric acid treatment followed by solubilization arising from neutralization with alkali. The acid-soluble fractions are chromatographed directly. In some cases the reaction mixtures are chromatographed directly without acid precipitation, or the reactions are stopped with 2 M NaCl and ethanol in the presence of carrier DNA. In all cases the nature and distribution of excision products are chromatographically similar.

Analysis of Excision Products from UV-Irradiated DNA. Two types of chromatographic systems were employed for measuring levels of oligonucleotides and mononucleotides. Mononucleotide resolution is best obtained by spotting reaction mixtures on Whatman No. 3MM chromatography paper which is developed for 24 hr in a descending direction in isobutyric acid–NH_4OH–H_2O (99:1:33, v/v) (Carrier and Setlow, 1966). Resolution of the oligonucleotides according to chain length is achieved in a two-step DEAE-chromatography system developed by Kelley *et al.* (1969, 1970).

Excision products are categorized into two major classes according to their chromatographic mobility with standard oligonucleotide markers. Mononucleotide excision products (MEP)[1] consist of the four deoxynucleoside monophosphates and oligonucleotide excision products (OEP) consist of resolvable nucleotides of chain length $(pX)_2$–$(pX)_7$. Large quantities of excision products are obtained by combining individaul reaction mixtures.

Digestion of Incised ^{32}P-Labeled DNA with Snake Venom Phosphodiesterase. Incised ^{32}P-labeled *E. coli* DNA, which is prepared with pancreatic DNase I, micrococcal nuclease, or UV-endonuclease, is dissolved with gentle vortex mixing in 0.01 N NaOH following perchloric acid precipitation. These DNA samples are then brought to 100° for 10 min and rapidly chilled. The pH is adjusted to 8.8 by adding 6 μmoles of Tris-HCl (pH 6.45). The reaction mixture (0.6 ml) also contains 3 μmoles of $MgCl_2$, 1 μmole of 2-mercaptoethanol, and 1 unit of snake venom phosphodiesterase. The reaction is stopped at various times with perchloric acid precipitation as described for incision with pancreatic DNase I. The supernatant fluid is counted in Bray's–dioxane solution.

Digestion of Incised ^{32}P-Labeled DNA with Calf Spleen Phosphodiesterase. Incised ^{32}P-labeled *E. coli* DNA is prepared as described previously and dissolved with gentle vortex mixing in 0.01 N NaOH. The pH is adjusted to 6.5 by adding 2.5 μmoles of succinic acid and 12.5 μmoles of sodium succinate (pH 6.5). The incised DNA is denatured by heating. The final mixture (0.605 ml) also contains 3 μmoles of $MgCl_2$, 10 μmoles of 2-mercaptoethanol, and 1.0 unit of calf spleen

[1] Abbreviations used are: MEP, mononucleotide excision products; OEP, oligonucleotide excision products.

phosphodiesterase. The reaction is terminated as described in the previous section.

Digestion of Oligonucleotide Excision Products with Snake Venom Phosphodiesterase. Each reaction mixture (0.1 ml) contains 3 μmoles of Tris-HCl buffer (pH 8.8), 0.5 μmole of $MgCl_2$, 0.2 μmole of 2-mercaptoethanol, 20–100 nmoles of OEP, and 0.5 μg of snake venom phosphodiesterase. The solution is incubated for 4 hr at 37° and then spotted directly onto either DEAE- or Whatman chromatography paper.

Digestion of OEP with Calf Spleen Phosphodiesterase. The reaction mixture (0.1 ml) contains 5 μmoles of potassium phosphate buffer (pH 7.0), 0.5 μmole of $MgCl_2$, 0.2 μmole of 2-mercaptoethanol, 20–100 nmoles of OEP, and 30 μg of calf spleen phosphodiesterase. The solution is incubated for 4 hr at 37° and then spotted directly onto DEAE- or Whatman No. 1 chromatography paper.

Norit Assay. Following a 2-hr excision of UV-irradiated DNA as described previously, 60 μmoles of Tris-HCl buffer (pH 8.0) and 2.0 units of bacterial alkaline phosphatase are added to each reaction mixture. After a 30-min incubation at 45°, 0.1 ml of 1 N HCl, 0.2 ml of 20% Norit, and 0.12 ml of H_2O are added. The resulting mixture is shaken vigorously for 20 min. At this point 0.2 ml of bovine serum albumin (5.0 mg/ml) is added and the solution (1.0 ml) shaken for another 10 min. The supernatant fraction obtained from a 10-min centrifugation is counted in Bray's–dioxane scintillation fluid.

Dephosphorylation of Excision Products. The reaction mixture contains 10 μmoles of Tris-HCl buffer (pH 8.0), 20–100 nmoles of excision products, and 0.2 unit of bacterial alkaline phosphatase. After an incubation of 3 hr at 45°, the reaction is terminated by spotting onto either DEAE- or Whatman chromatography paper.

Results

Site of Initial Phosphodiester-Bond Breakage. SPLEEN PHOSPHODIESTERASE SENSITIVITY. The introduction of single phosphodiester-bond breaks either 3′ or 5′ to the photoproduct by the UV-endonuclease is presumably the first step in dark repair. These two possibilities were examined by measuring the rate of calf spleen phosphodiesterase catalyzed hydrolysis. This enzyme digests single-stranded DNA exonucleolytically initiating hydrolysis from the 5′-hydroxyl terminus liberating deoxynucleoside 3′-monophosphates (Razzell and Khorana, 1958). The course of its exonucleolytic action is inhibited by the presence of photoproducts (Pearson and Johns, 1966). The action of the UV-endonuclease results in 5′-hydroxyl and 3′-phosphoryl groups during incision (described in a subsequent section). It is to be expected, therefore, that the spleen phosphodiesterase should catalyze at a normal, or unimpaired, initial rate of hydrolysis on denatured incised UV-irradiated DNA if the initial break is 3′ to the photoproduct. On the other hand, the reverse situation involving a break 5′ to the photoproduct would be expected to interfere with the enzymatic reaction. In Figure 1 it can be seen that spleen phosphodiesterase was in fact unable to digest incised irradiated DNA after it had been denatured, although it readily hydrolyzed denatured DNA bearing 5′-hydroxyl and 3′-phosphoryl groups at its termini following micrococcal nuclease treatment. The inhibition of spleen phosphodiesterase activity indicated, therefore, that the initial incision was not only 5′ to the photoproduct but was fairly close to its site.

The lack of a 5′-phosphoryl group on the damaged portion of the DNA seems not to be responsible for the retardation of exonucleolytic rates by the spleen phosphodiesterase. The

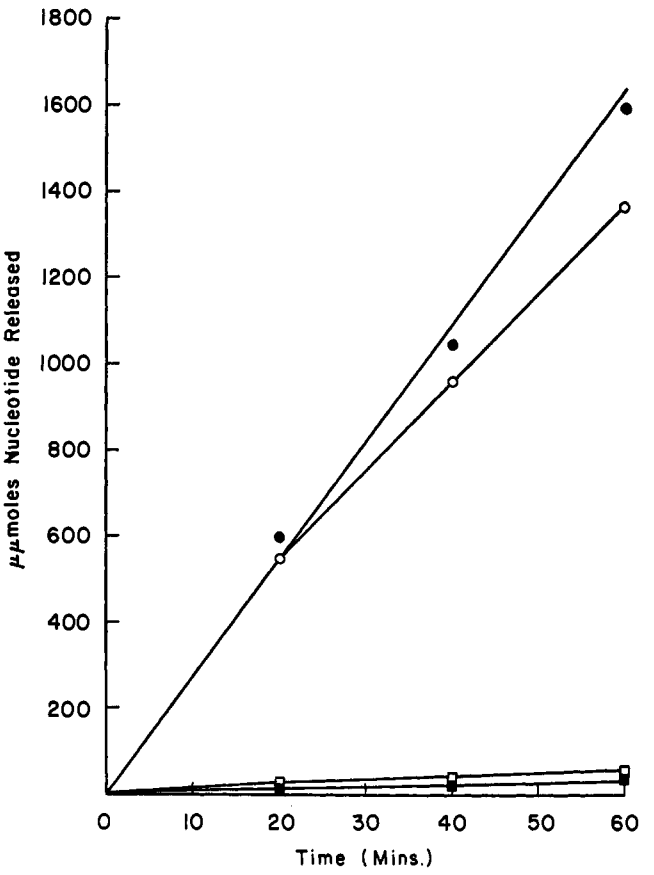

FIGURE 1: Digestion of incised ^{32}P-labeled DNA with spleen phosphodiesterase. 5′-Hydroxyl and incised irradiated DNA were prepared according to procedures outlined in the Methods section; similarly, the specific requirements for spleen phosphodiesterase activity are listed in the same section. The samples to be photoreactivated were treated with 150 μmoles of Tris-HCl buffer (pH 7.4); 7.5 μmoles of EDTA and 3.5 units of yeast-photoreactivating enzyme (PR) are added in a final volume of 3.0 ml. The conditions in photoreactivation are described in the legend to Table II. 5′-Hydroxyl-terminated DNA (●), incised irradiated DNA (■), incised irradiated DNA treated with photoreactivating enzyme in the presence of visible light (○), and incised irradiated DNA treated with photoreactivating enzyme in the absence of light (□).

block to hydrolysis if due exclusively to the presence of thymine-containing dimers should be relieved by enzymatic photoreactivation of the lesion prior to the action of spleen phosphodiesterase. Incised DNA was subjected to enzymatic photoreversal of the thymine-containing dimers prior to hydrolysis by the spleen enzyme. Enzymatic photoreactivation, requiring visible light, produced an active substrate for the spleen enzyme, whereas an equivalent dark incubation (in the presence of the photoreactivating enzyme but in the dark) did not provide a sensitive substrate.

PHOSPHOMONOESTER STOICHIOMETRY. Further proof concerning the position of the photoproduct relative to the initial break was obtained from the stoichiometry of $[^{32}P]P_i$ release by bacterial alkaline phosphatase following incision and then excision. UV-endonuclease action produces one phosphomonoester group which is sensitive to bacterial alkaline phosphatase. Since the UV-exonuclease produces 5′-mononucleotides (Kaplan *et al.*, 1971), a second bacterial alkaline phosphatase susceptible phosphomonoester group should, in fact, appear after excision. If the initial phosphodiester break by

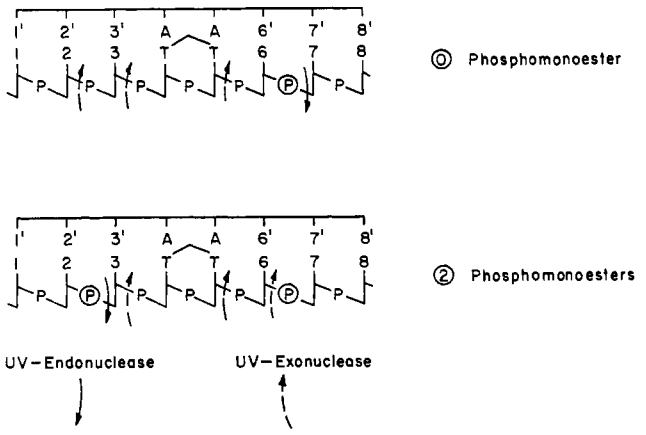

FIGURE 2: The structural basis for phosphomonoester stoichiometry.

the UV-endonuclease was 3′ to the photoproduct, then excision would result in a quantitative loss of phosphomonoester groups from the incised DNA (Figure 2). Experiments which determined the number of phosphodiester bonds after incision alone and incision plus excision clearly demonstrated that there was a stoichiometric appearance of a second phosphomonoester group (Table I). This apparent doubling of phosphomonoester groups could only be accounted for if the initial break was, in fact, 5′ to the site of damage in the DNA.

The incision process, therefore, includes an initial phosphodiester-bond break 5′ to the photoproduct. Since a negligable number of nucleotides were released by spleen phosphoesterase, the initial break may be adjacent to or possibly one nucleotide removed from the dimer region.

LOCATION OF PHOSPHOMONOESTER GROUP AFTER INCISION. Polynucleotide kinase catalyzes the phosphorylation of the γ-phosphorous from [γ-^{32}P]ATP specifically into 5′-hydroxyl groups of DNA (Richardson, 1965). Incorporation of labeled phosphorous into incised DNA would suggest phosphodiester cleavage resulted in the formation of 5′-hydroxyl groups. However, if pretreatment with bacterial alkaline phosphatase is required in order to achieve quantitative labeling, then it may be concluded that the original incision produced a 5′-phosphomonoester. When UV-irradiated incised DNA was treated with polynucleotide kinase under conditions in which pancreatic DNase I treated DNA was quantitatively phosphorylated after treatment with bacterial alkaline phosphatase, incorporation of the terminal phosphate from [γ-^{32}P]ATP into internal breaks was not observed. These negative findings were inconsistent with the single-stranded nature of the breaks produced during incision (Grossman *et al.*, 1968; Carrier and Setlow, 1970).

The possibility that thymine-containing photoproducts

TABLE I: Effect of UV-Exonuclease on the Apparent Number of Phosphodiester Bonds Broken.[a]

	Phosphomonoester Groups Detected after:	
Incubn Time (min)	Incision (pmoles)	Excision (pmoles)
90	22.3	43.6
150	36.2	68.9
270	58.0	112.0

[a] Incised and excised DNA were prepared as described in Methods. The number of phosphomonoester groups was determined as described in the previous publication (Kaplan *et al.*, 1971).

TABLE II: Location of Phosphomonoester Group after Incision.[a]

Exptl Condn	Phosphodiester Bonds Broken (pmoles)	^{32}P-Labeled 5′-OH Groups (pmoles)
A. Native *E. coli* DNA		
1. Pancreatic DNase I	21.2	
2. (1) + kinase[b]		0.4
3. (1) + BAP[c] + kinase		17.0
B. UV-Irradiated *E. coli* DNA		
1. UV-Endonuclease	234.0	
2. Photoreactivation + UV-endonuclease	20.0	
3. (1) ± BAP + kinase		25.0
4. (1) + photoreactivation ± BAP + kinase		206.4

[a] DNA substrates were treated sequentially with the listed enzymes. The incubation mixture in part A (0.3 ml) contained 20 μmoles of Tris-HCl buffer (pH 8.0), 1.5 μmoles of $MgCl_2$, 78.6 nmoles of native *E. coli* DNA, and 1.0 unit of pancreatic DNase I. After a 30-min incubation at 25°, the DNA was collected by adding 0.3 ml of 1.0 M NaCl and 1.2 ml of 95% EtOH, and centrifuging at 12,500*g* for 20 min. The precipitate was dissolved in 0.1 ml of 0.1 N NaOH and brought to pH 8.0 with 20 μmoles of Tris-HCl buffer (pH 6.45). For dephosphorylation 0.2 unit of bacterial alkaline phosphatase was added and the mixture incubated for 30 min at 45°. For reaction with polynucleotide kinase the above solution was chilled and 1.0 μmole of potassium phosphate buffer (pH 7.5), 3 μmoles of $MgCl_2$, 5 μmoles of 2-mercaptoethanol, 20 μmoles of γ-^{32}P-labeled ATP (specific activity 2.2 × 10^3 cpm/nmole), and 2.0 units of enzyme were added. The incubation at 37° for 60 min was terminated by adding 3.0 ml of cold 5% trichloroacetic acid and collecting the DNA on GF/C glass filters. The reaction mixture in part B (3.0 ml) contained 5.0 μmoles of Tris-HCl buffer (pH 7.5), 3.0 μmoles of $MgCl_2$, 5.9 μmoles of 2-mercaptoethanol, 83.4 nmoles of UV-irradiated *E. coli* DNA (1.3 × 10^5 ergs/mm^2), and 23 units of UV-endonuclease. After an incubation at 37° for 30 min, the reaction was stopped by heating at 65° for 15 min. To the chilled solutions were then added 150 μmoles of Tris-HCl buffer (pH 7.4), 7.5 μmoles of EDTA, and 3.5 units of yeast-photoreactivating enzyme. An initial 15-min incubation at 37° in the dark was followed by 2.5 hr at 37° in front of a 150-W photoflood lamp. The DNA was collected by alcohol precipitation as described in part A. The dephosphorylation and polynucleotide kinase labeling procedures were identical with those described in part A. The number of phosphodiester bonds broken was determined with ^{32}P-labeled *E. coli* DNA as described previously (Kaplan *et al.*, 1971). [b] Polynucleotide kinase. [c] Bacterial alkaline phosphatase.

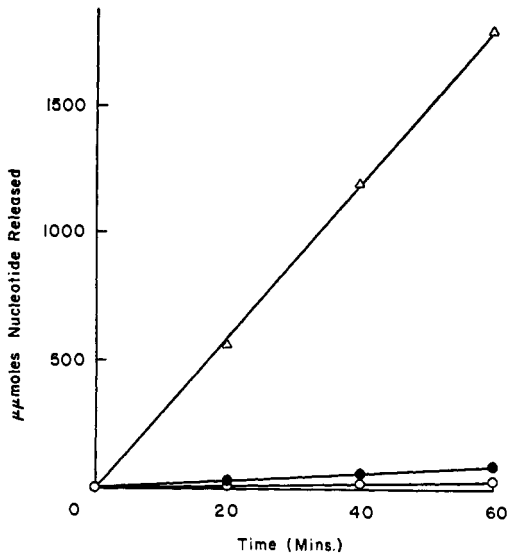

FIGURE 3: Digestion of incised DNA with venom phosphodiesterase. 3′-Hydroxyl-terminated, 3′-phosphonyl-terminated, and incised UV-irradiated *E. coli* DNA were prepared according to the procedures described in the Methods section. The number of phosphodiester bonds hydrolyzed in the various substrates were as follows: 3′-hydroxyl-terminated DNA (250 pmoles), 3′-phosphoryl-terminated DNA (140.2 pmoles), and incised UV-irradiated DNA (171.1 pmoles). Digestion of the heat-denatured incised DNAs with venom phosphodiesterase was carried out according to the procedures described in the Methods section. The figure illustrates the kinetics of digestion of 3′-hydroxyl-terminated DNA (△), 3′-phosphoryl-terminated DNA (○), and incised UV-irradiated DNA (●). Nucleotide release was corrected for self-absorption.

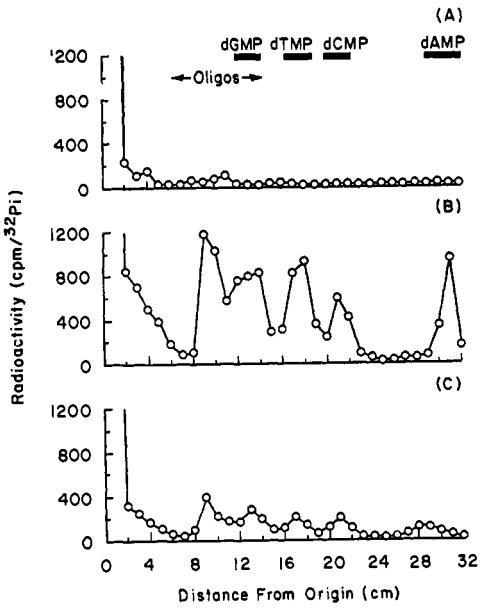

FIGURE 4: Excision as a two-step process. Incised and excised ^{32}P-labeled UV-irradiated *E. coli* DNA were prepared as described in Methods. Prior to precipitation with perchloric acid, the reaction mixtures (0.3 ml) were spotted directly on Whatman No. 3MM paper and developed as described in Methods. Radioactivity was determined as described in Methods. The bars on the graph represent reference compounds. The figure illustrates irradiated DNA plus UV-endonuclease (A), irradiated DNA plus UV-endonuclease and UV-exonuclease (B), and irradiated DNA plus UV-exonuclease (C).

might be interfering with the action of the polynucleotide kinase was investigated. Incised DNA was incubated with the yeast-photoreactivating enzyme and visible light—conditions which monomerize thymine dimers *in situ* (Cook and McGrath, 1967). Under these circumstances stoichiometric phosphorylation was obtained with the polynucleotide kinase independent of bacterial alkaline phosphatase pretreatment (Table II). These results clearly indicated that the endonucleolytic incision produced a 5′-hydroxyl group. Surprisingly, however, the action of the relatively nonspecific polynucleotide kinase was sensitive to the presence of pyrimidine photoproducts. It can be concluded, furthermore, that the photoproduct must be fairly close to the site of the initial phosphodiester bond hydrolyzed by the UV-endonuclease to impose this kind of restriction on the course of action of the polynucleotide kinase.

The presence of a 3′-phosphomonoester group was identified by examining the effects of dephosphorylation on the rates of exonucleolytic hydrolysis by snake venom phosphodiesterase. This enzyme initiates hydrolysis of denatured DNA from the 3′ terminus liberating 5′-deoxynucleoside monophosphates. In Figure 3 it can be seen that the enzyme degrades 3′-hydroxyl-terminated DNA at a rate faster than 3′-phosphoryl-terminated substrates. When incised DNA was exposed to the action of venom enzyme, this rate of hydrolysis coincided with that of the 3′-phosphoryl-terminated denatured DNA. Incised UV-irradiated DNA after dephosphorylation with bacterial alkaline phosphatase was digested normally by the enzyme. Since it has been shown that the initial break is 5′ to the photoproduct, this result is in direct support for a phosphomonoester group at the 3′ terminus. Other indirect support for the presence of a phosphomonoester located at this position is derived from the phosphomonoester stoichiometry in Table II.

Mechanism of Excision. DEMONSTRATION OF A TWO-STEP EXCISION PROCESS. The initial steps of the dark repair process involve the enzymatic recognition of DNA which contains photoproducts followed by the removal of pyrimidine dimers from such molecules. The primary incision by the UV-endonuclease does not result in the release of photoproducts (Kaplan *et al.*, 1971). However a second enzyme, the UV-exonuclease, can excise the photoproduct-containing region from the DNA molecule once the initial break has been made by the UV-endonuclease (Kaplan *et al.*, 1969, 1971). A chromatographic analysis of the two-step excision process is shown in Figure 4. When UV-irradiated DNA was treated sequentially, first with UV-endonuclease and then UV-exonuclease, extensive release of mono- and oligonucleotides occurred (Figure 4B). As expected from previously reported results (Kaplan *et al.*, 1971) very small amounts of radioactivity appeared in the oligonucleotide region when UV-irradiated *E. coli* DNA was reacted with UV-endonuclease alone (Figure 4A). At the dosage of irradiation employed, some pyrimidine dimers may have been sufficiently close so that successive single-strand breaks could release some oligonucleotides. For example, irradiation of [^{3}H]thymine-labeled poly(dA:dT) leads to extensive degradation by the UV-endonuclease under similar conditions (unpublished results).

Products obtained from an identical incubation of DNA with UV-exonuclease alone contained small amounts of mono- and DNA, probably arising from digestion of DNA termini (Figure 4C). The excision system, therefore, consists of two distinct steps, incision by UV-endonuclease and excision by UV-exonuclease. Further demonstration of this process is discussed and seen in Figure 5.

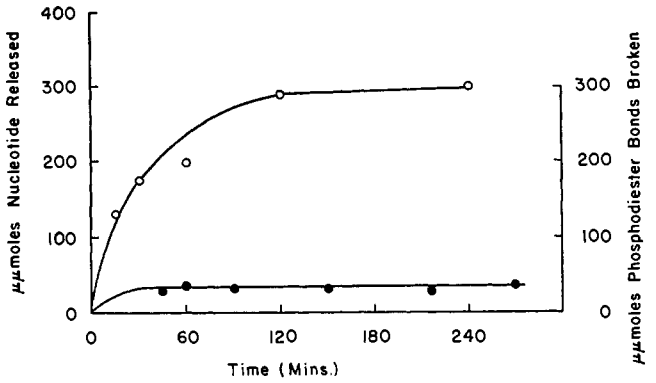

FIGURE 5: Excision of nucleotides from UV-irradiated ^{32}P-labeled native *M. luteus* DNA. The number of phosphodiester bonds broken and the amount of nucleotides released were measured by the standard UV-endonuclease assay (see Methods). The figure illustrates the number of phosphodiester bonds broken by the UV-endonuclease (●) and the amount of nucleotide released resulting from UV-exonuclease action (○).

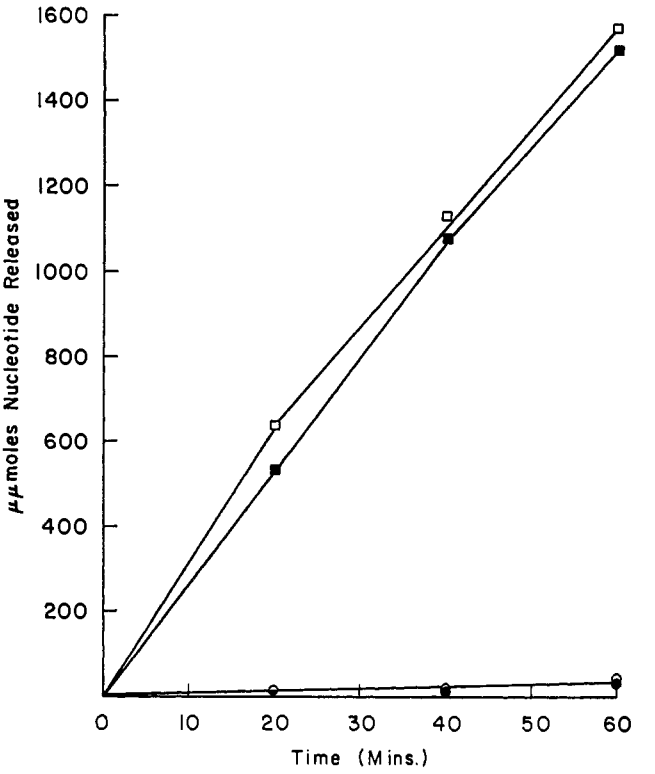

FIGURE 6: Digestion of excised ^{32}P-labeled DNA with spleen phosphodiesterase. 5′-Hydroxyl, 5′-phosphorylated, and excised DNAs were prepared according to the procedures described in the Methods section. Conditions describing the techniques for dephosphorylation of excised DNA are the same as those described in the legend associated with Table II. The experimental procedures followed for spleen phosphodiesterase digestion are described in the Methods section of this paper. The figure illustrates the kinetics of digestion of 5′-hydroxyl-terminated denatured DNA (□), 5′-phosphorylated denatured DNA (○), excised denatured DNA (●), and dephosphorylated excised denatured DNA (■).

EXTENT OF DNA EXCISION. The ability of the UV-exonuclease to degrade substrates containing photoproducts makes it uniquely suited as an excision enzyme. After UV-irradiated ^{32}P-labeled *E. coli* DNA is incised with the UV-endonuclease, treatment with the exonuclease produces a rapid but limited release of ^{32}P-labeled nucleotides (Kaplan *et al.*, 1969). Similar results are obtained when UV-irradiated *M. luteus* DNA is exposed to the two enzymes (Figure 5). A size estimate of the excised region was obtained by comparing the ratio of ^{32}P released to the number of phosphodiester bonds broken by the UV-endonuclease. A ratio of 6 is obtained for *E. coli* DNA at all doses of irradiation between 1.0×10^4 and 1.5×10^5 ergs/mm^2 (Table III). In the case of *M. luteus* DNA a significant increase to a ratio of 10 was obtained (Figure 5).

The limited degradation of incised DNA by the UV-exonuclease is attributable to its ability to act only on denatured DNA. This implies that the excisable region is of limited size and single-stranded in character. In addition, preliminary evidence suggests that the UV-exonuclease does not cleave the phosphodiester bond between the pyrimidine dimer moieties. These findings imply that the exonuclease can in fact act endonucleolytically under certain structurally constrained conditions.

POSITION OF THE SECOND PHOSPHOMONOESTER GROUP ON EXCISED DNA. The UV-exonuclease hydrolyzes both unirradiated and irradiated denatured DNA substrates to produce 5′-mononucleotides (Kaplan *et al.*, 1969). Similarly nucleoside 5′-monophosphates are released during excision of incised UV-irradiated native DNA. It is expected as a result that a 5′-phosphomonoester should remain on the DNA after excision by this same enzyme.

The incision step results in the formation of a 3′-phosphomonoester group, consequently the appearance of a second phosphomonoester group as a consequence of the excision step implies that its specific location is likely to be at the 5′ position. Verification of the 5′-phosphoryl group in the cavity of excised DNA is provided from exonucleolytic hydrolysis rates catalyzed by the spleen phosphodiesterase which is sensitive to the presence of a 5′-phosphomonoester at the site of its initiation. The resistance of excised ^{32}P-excised DNA to hydrolysis by the spleen phosphodiesterase before treatment with bacterial alkaline phosphatase implicates the presence of a phosphomonoester at this specific site on excised DNA (Figure 6).

NATURE OF THE PRIMARY EXCISION PRODUCTS. Chromatographic analyses of the excision products released as a function of time revealed that the initial events of the excision process appear to be endonucleolytic in nature (Figure 7). The larger oligonucleotide excision products are liberated from the irradiated DNA substrate and can be identified directly from the reaction milieu—from either an alcohol-soluble fraction of the reaction or from an acid-soluble supernatant fraction arising from termination of the reaction with 7% perchloric acid. As the DNA becomes further damaged by UV-irradiation, it can be assumed that the possibility exists for damaged

TABLE III: Effect of Ultraviolet Dose on the Size of Excised Regions.

UV Dose (ergs/mm^2 × 10^{-3})	Phosphodiester Bonds Broken (pmoles) Incision	[^{32}P]Nucleotides Released (pmoles) Excision	Released Nucleotides: Phosphodiester Bonds
9	19.5	120.1	6.15
36	63.0	359.2	5.7
108	85.1	471.7	5.5

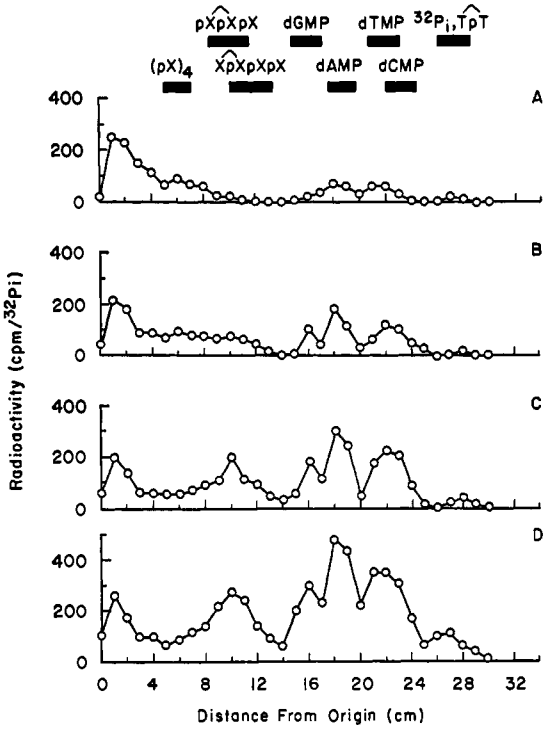

FIGURE 7: Composition of excision products as a function of time. Excision products from UV-irradiated *E. coli* DNA (3.6×10^4 ergs/mm^2) were isolated as described in Methods. The figure illustrates excision products at 5 min (A), 10 min (B), 40 min, (C), and 130 min (D).

regions becoming correspondingly closer exists. If this phenomenon is involved then acid denaturation of excised DNAs should liberate acid-soluble fragments between and including excised regions. If this in fact were the case, a rise in the ratio of acid-soluble nucleotides released per phosphodiester bond broken by the endonuclease might be observed as a function of dose. This is not the case, however, as seen in Table III in which this ratio remains relatively constant over a 10-fold increase in the dose range of ultraviolet light.

During the course of excision, the large oligonucleotides are degraded to smaller fragments. At 5 min, 68% of the excised oligonucleotides were at least as large as a pentanucleotide. Only 9% appeared as either a trinucleotide or a tetranucleoside triphosphate (Table IV). As the time course of excision increases, a marked shift in the size of the OEP shifted to shorter chain lengths. For example, at 130 min of excision 60% of the original radioactivity in the oligonucleotide region was converted into trinucleotides or tetranucleoside triphosphates. Shorter oligonucleotides such as XpXpX and pXpX were indistinguishable from the mononucleotides.

The UV-exonuclease appears, therefore, to initially act endonucleolytically on the incised DNA molecule. The released fragment is subsequently degraded exonucleolytically by the same enzyme. The ability of the UV-exonuclease to hydrolyze the single-stranded DNA and oligothymidylates exonucleolytically has been previously discussed (Grossman *et al.*, 1968; Kaplan *et al.*, 1971).

The 5′→3′-exonuclease activity associated with the homogeneous *E. coli* DNA polymerase also acts endonucleolytically on nicked irradiated poly(dA:dT) (Kelly *et al.*, 1969).

OLIGONUCLEOTIDE:MONONUCLEOTIDE RATIOS. The ratio of phosphodiester bonds to phosphomonoester groups in excision products is presented in Figure 8. At early times of excision, 81% of the excised material was insensitive to bacterial alkaline phosphatase, while at 90 min this percentage had dropped to 53% (Table V). The UV-exonuclease was therefore degrading the fragments, but the extent of hydrolysis appeared to decrease with the size of the fragments.

TABLE IV: Size Distribution of Oligonucleotide Excision Products.[a]

Excision Time (min)	Pentanucleotide or Longer (%)	Tetranucleotide or Pentanucleoside Tetraphosphate (%)	Trinucleotide or Tetranucleoside Triphosphate (%)
5	68	23	9
10	54	24	22
40	39	11	49
130	33	7	60

[a] The size estimates were obtained from the data shown in Figure 6. Size assignments based on chromatographic mobility were as follows: pentanucleotides, 0–4 cm; tetranucleotides, 5–7 cm; trinucleotides, 8–13 cm. Corrections were made for overlapping of peaks. The radioactivity appearing in strips 0–13 was taken as the total amount of oligonucleotide excision products.

Results from chromatographic analyses of the oligonucleotide to mononucleotide ratio are summarized in Table VI. Because small oligonucleotides such as XpXpX or pXpX migrated with the mononucleotides, the level of mononucleo-

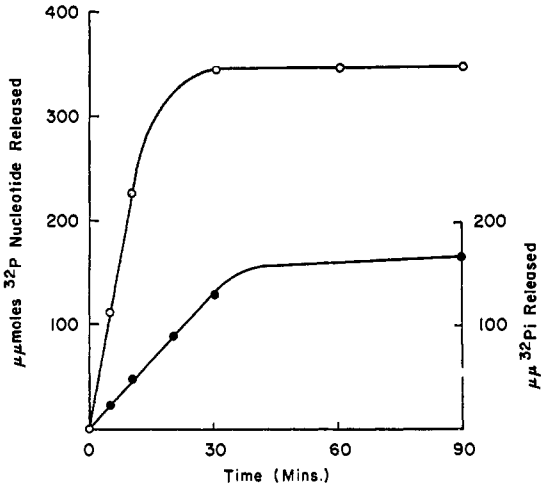

FIGURE 8: ^{32}P-Labeled nucleotide release *vs.* [^{32}P]P$_i$ release by bacterial alkaline phosphatase from excision products: a kinetic comparison. Incised ^{32}P-labeled UV-irradiated *E. coli* DNA was prepared as described in Methods. To each tube was added 4.0 units of UV-exonuclease and excision allowed to take place at 37°. At specified times tubes were removed and heated at 100° for 5 min. After chilling, one-half of the tubes were precipitated as described in Methods and the supernatant fluid counted in Bray's–dioxane scintillation fluid. The other tubes were treated with the Norit assay (see Methods). All values were corrected for self-absorption. The figure illustrates ^{32}P-labeled nucleotide release (○) and [^{32}P]P$_i$ release by bacterial alkaline phosphatase from the ^{32}P-labeled excision products (●).

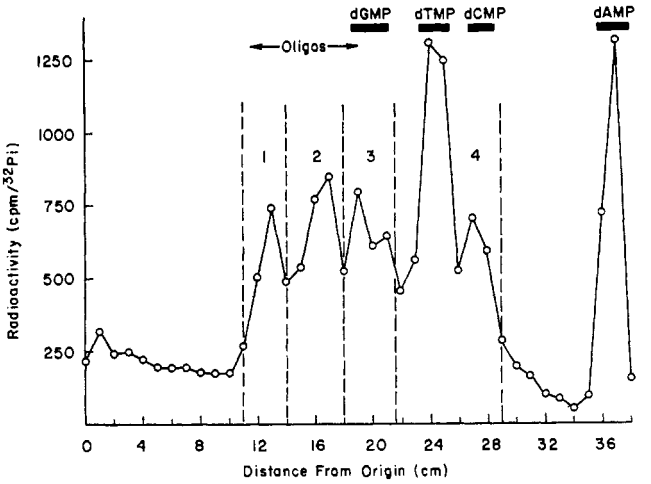

FIGURE 9: Chromatographic analysis of excision products. Excision products from ^{32}P-labeled UV-irradiated *E. coli* DNA (3.6×10^4 ergs/mm^2) were prepared as described in Methods and chromatographed as described in Figure 1. The dotted vertical lines indicate those regions which were eluted for further analysis.

tides arising from extended excision times may have been artifically increased. However, even after extended periods of excision the limit digest product, accounting for 40–45% of the total oligonucleotide excised, is not larger than a di- or trinucleotide. Even at 130 min not all of the fragments had been digested. The level of 40–45% for oligonucleotide excision product concentration, which correlates well with the data presented in Figure 7, was therefore taken to mean that at 130 min the average chain length of the photoproduct containing fragments was between two and three nucleotides in length.

IDENTIFICATION OF RELEASED MONONUCLEOTIDES. A comparison was made of the mononucleotides released by the UV-endonuclease from DNA irradiated as a function of dose. At a dose of 9×10^3 ergs/mm^2, 46.8% of all the nucleotides released were dTMP-5, indicating that initial dimer formation occurred in thymine-rich regions (Table VII). The second most prevalent nucleotide was dAMP-5′. As the dose was increased to 108×10^3 ergs/mm^2, the level of dTMP dropped significantly to 30.7% while dAMP-5′ and dCMP-5′ increased to 35.8 and 24.2%, respectively. At the higher doses, detectable dimer formation occurred in less homogeneous areas of the DNA molecule.

A similar compositional analysis of nucleotides released from *M. luteus* DNA (GC:AT = 2.7) showed a different spectrum of excised nucleotides. At a comparable dose the excised region of *M. luteus* DNA is more pyrimidine rich than is the same area from *E. coli* DNA. Moreover, the region appears to be less GC rich than the overall base composition of this DNA.

PERCENTAGE OF THYMINE IN THE EXCISED REGION. We have shown previously that approximately three thymine-containing nucleotides are released for every single-strand break (Kaplan *et al.*, 1969). Accordingly, if a total of 7 nucleosides (molar ratio of 6 phosphates:phosphodiester bond break) were released, 43% of them would have been thymine. When excision products of [^{3}H]thymine-labeled *E. coli* DNA were examined chromatographically, approximately 45% of the total released nucleotides appeared as dTMP-5′ with the remainder as oligonucleotides. Chromatographic analysis of ^{32}P-labeled excision products, such as shown in Figure 9, showed an overall composition of the excision products to be: oligonucleotide, 45.2%; dGMP-5′, 7.2%; dAMP-5′, 16.1%; dCMP-5′, 10.8%; and dTMP-5′, 20.7%. Since this amount of dTMP-5′ represented 45% of the thymine released, the total amount of thymine excised was equivalent to 46% of the entire excised regions, a value which was in excellent agreement with the percentage calculated previously.

NUCLEOTIDES 3′ TO PYRIMIDINE DIMER. The excised fragment (Figure 10A) was isolated and analyzed for the nucleotides 3′ to the photoproduct region by means of snake venom phosphodiesterase, which initiates hydrolysis from 3′-hydroxy termini liberating 5′-deoxynucleoside monophosphates. As shown in Figure 10B, digestion resulted in the appearance of large amounts of mononucleotides as expected from the 5′ position of the dimer in the excised fragment. Moreover, the

TABLE VI: Distribution of Excision Products.

Excision Time (min)	A $(pX)_6$–$(pX)_8$ (%)	A % Change in Concn	B $(pX)_3$–(pX_5) (%)	C $(pX)_1$ (%)	%[a] Change in Concn of B + C
5	51.8	–	18.0	27.4	–
10	29.6	−22.2	24.1	43.7	+22.4
40	16.2	−13.4	26.1	53.7	+12.0
130	12.3	−4.1	24.6	57.0	+3.3

[a] This is an incremental change calculated as a sum of differences B + C from one time period to the next later period.

TABLE V: Susceptibility of Excision Products to Phosphomonoesterase.

Excision Time (min)	Extent of Excision (%)	Phosphomonoesterase Insensitive: Sensitive (Ratio)
5	32.5	5.4
10	65.0	4.7
20	93.0	3.6
30	100	2.6
90	100	2.1

TABLE VII: Excised Deoxynucleotides Located 3′ to Photoproducts.

UV Dose (ergs/mm^2 $\times 10^{-3}$)	% Distribution of TMP-5′	dAMP-5′	dCMP-5′	dGMP-5′
A. *E. coli* DNA (GC:AT = 1.0)				
9	46.8	23.7	19.9	9.6
36	39.0	30.9	17.4	12.6
108	30.7	35.8	24.2	9.2
B. *M. luteus* DNA (GC:AT = 2.7)				
108×10^3	35.1	18.1	34.3	12.4

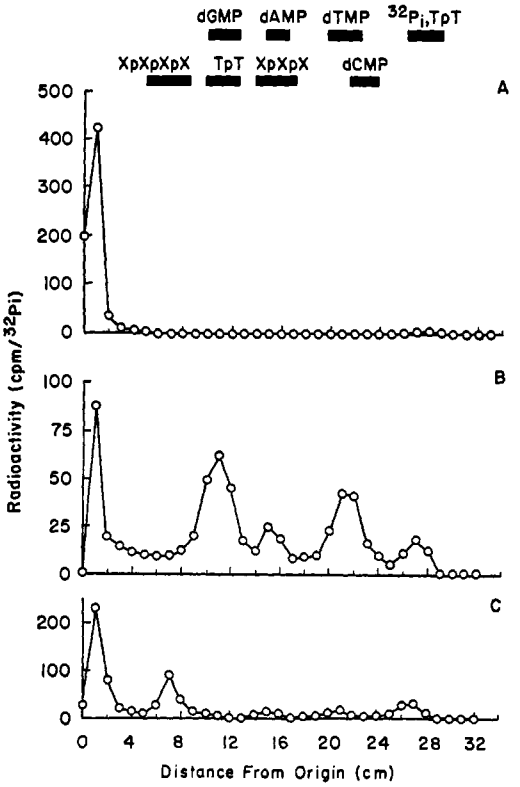

FIGURE 10: Digestion of early excision products with snake venom phosphodiesterase or calf spleen phosphodiesterase. Excision products from a 5-min excision of incised ^{32}P-labeled UV-irradiated *E. coli* DNA (3.6×10^4 ergs/mm^2) were prepared and isolated as described in Methods. All radioactivity which appeared prior to the region 1 of Figure 8 was eluted with H_2O and digested with snake venom phosphodiesterase or calf spleen phosphodiesterase (see Methods). The reaction mixtures (0.1 ml) were spotted on DEAE-cellulose chromatography paper and developed as described in Figure 9. The figure illustrates undigested excision products (A), snake venom phosphodiesterase digestion (B), and calf spleen phosphodiesterase digestion (C).

distribution of nucleotides was similar to that released by the UV-exonuclease during excision (Table VII). The apparent release of small amounts of radioactivity from the excised fragment by spleen phosphodiesterase (Figure 10C) indicates the occasional occurrence of nucleotides 5′ to the photoproduct. This possibility is presently under investigation.

Discussion

The excision of pyrimidine dimers occurs in several stages. As shown in Figure 11, the UV-endonuclease catalyzes a single phosphodiester-bond break proximate to the photoproduct. Because of the ready reversibility of lesions catalyzed by the photoreactivating enzyme, it is assumed that the primary photoproduct formed at these doses is probably a thymine dimer. The incision which is 5′ to this dimer leaves a 5′-hydroxyl and a 3′-phosphomonoester group. In the second step, catalyzed by the UV-exonuclease, initial cleavage results in the release of a large photoproduct-containing fragment. This process leaves a 5′-phosphomonoester on the DNA molecule and a 3′-hydroxyl group on the fragment. The large fragment is subsequently degraded by the UV-exonuclease. The size of the limit product is dependent on the time of the excision reaction. After 2 hr the major products are trinucleotide or tetranucleoside triphosphates and deoxynucleoside

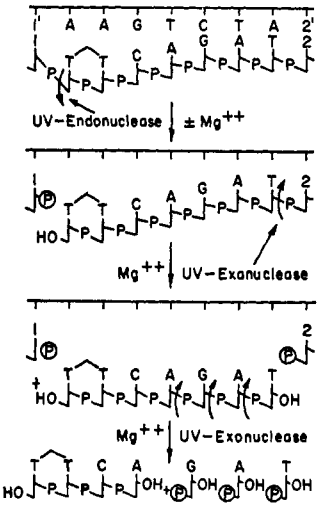

FIGURE 11: Model of initial steps in the enzymatic repair of UV-irradiated DNA.

5′-monophosphates. A summary of this repair cycle is depicted in Figure 11.

The proposed model for the enzymatic excision of photoproducts is consistent with the "cut-and-patch" mechanism currently proposed to explain dark repair (Howard-Flanders and Boyce, 1966). The UV-endonuclease and UV-exonuclease would catalyze the first two steps. The presence, however, of a 3′-phosphomonoester blocking a potential nucelophilic site for DNA polymerase initiation may require the participation of either a polynucleotide phosphomonoesterase or an exonuclease-III-type digestion in preparation for polymerization by the *M. luteus* polymerase. Resynthesis would then be finalized by a joining reaction mediated by DNA ligase to complete the repair process.

However, the recent work by Kelley and coworkers (1970) indicates that the "patch-and-cut" model (Haynes, 1966) may be a more accurate description of the repair process. The ability of the 5′→3′-exonuclease activity of the *E. coli* DNA polymerase to excise photoproduct-containing regions suggests that the DNA polymerase complex may start polymerization immediately after the initial incision has been made, excising the photoproduct after resynthesis. While an enzyme resembling the UV-endonuclease has not been isolated from uninfected *E. coli*, such an activity has been detected in bacteriophage T_4 infected *E. coli* (Friedberg and King, 1969; Yasuda and Sekuguchi, 1970).

This same type of patch-and-cut system may function in *M. luteus*, but under slightly different conditions.

A mechanism of polymerization as proposed by Kelly *et al.*, (1970) requires the participation of an enzyme possessing 5′→3′-exonuclease activity. The UV-exonuclease could fulfill such a role although it has been shown to prefer denatured DNA while the nuclease activity associated with the polymerase from *E. coli* is specific for native DNA. It might also be noted that the UV-exonuclease could be an integral part of the DNA polymerase *in vivo*, but appears as an independent activity *in vitro* presumably due to limited proteolysis during purification from this organism. In short, the model presented in Figure 11 could also fit a patch-and-cut system by which polymerization would start immediately following incision.

Both the UV-exonuclease and the 5′→3′-exonuclease activity of the DNA polymerase represent a third class of deoxyribonuclease activity since they act either as endo- or

exonucleases under certain conditions. The nature of the enzymatic attack is a function of the substrate and its structure. With incised UV-irradiated DNA the UV-exonuclease acts as an endonuclease, while with denatured DNA it functions exclusively as an exonuclease. It appears that a redefinition of nuclease classification is needed in order to better describe these multifunctional enzymes. Thus, an exonuclease is a phosphodiesterase requiring a terminus for its action whereas an endonuclease does not and can therefore initiate hydrolysis randomly.

The studies on the composition of the excised region indicate that the phenomenon of UV resistance in certain organisms is in part a function of DNA base composition. Fewer dimers are recognized in *M. luteus* than in *E. coli* DNA under equivalent conditions. Furthermore, dimer formation which is recognized by the excision–repair system occurs in thymine-rich regions. Since the UV-endonuclease action releases some oligonucleotides even at low doses of ultraviolet irradiation, some of the dimers must be fairly close to each other (Kaplan *et al.*, 1971). Regions must exist in the *E. coli* chromosome which are highly susceptible to ultraviolet damage. Also it is likely that only those photoproducts which result in large distortions of the DNA helix, that is, those formed in AT-rich regions are recognized by the UV-endonuclease. Further work is now in progress to determine whether the excision system of *M. luteus* is capable of acting on lesions other than those produced by ultraviolet light. These studies will help determine the generality of the cellular excision–repair system.

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