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Endonuclease II of *Escherichia coli:* Degradation of γ-Irradiated DNA†

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ABSTRACT: Irradiation of DNA in a nitrogen atmosphere with ^{60}Co γ-radiation produces at least two types of damage. The first type leads to single strand breaks in the DNA observed *after* exposure to alkali. This type of alkali-labile bond will be designated a spontaneous break. The second type of damage to DNA is an alteration which makes the DNA susceptible to phosphodiester bond hydrolysis by a 1600-fold purified preparation of endonuclease II of *Escherichia coli* and is designated an enzyme-sensitive site. This site is not alkali-labile. After irradiation, preincubation of the DNA either for days at 0° or for 4 hr at 37° increases both the spontaneous breaks and the enzyme sensitive sites. There is a greater increase of spontaneous breaks when the preincubation is in O_2 compared to N_2. The increase of enzyme sensitive sites due to the preincubation is not altered significantly by O_2. The increase of spontaneous breaks during the preincubation is almost completely prevented by addition of either $NaBH_4$ or NH_2OH after the irradiation. The treatment can be before or after the preincubation. This effect indicates that these breaks are due to alkali-labile bonds possibly produced by depurination or depyrimidination reactions. That the spontaneous breaks are due primarily to alkali-labile bonds is supported by an experiment in which formamide gradients were used. Neither $NaBH_4$ nor NH_2OH has any effect on the enzyme sensitive sites. Addition of β-mercaptoethanol (0.5 M) at the start of the preincubation prevents in part the appearance of both spontaneous breaks and enzyme-sensitive sites. It has no effect when added at the end of the preincubation. Catalase added before the preincubation has no effect on either type of damage. It is postulated that the spontaneous breaks occur because purine or pyrimidine radicals are formed (possibly hydroxyl radicals) which can then interact with oxygen to produce unstable intermediates. These intermediates then undergo either depurination or depyrimidination. The subsequent alkali catalyzed β-elimination reaction of depurinated or depyrimindinated DNA is prevented by $NaBH_4$ or NH_2OH. An alternative hypothesis would involve damage to the sugar rather than to bases. The enzyme-sensitive sites represent another form of base damage which is not oxygen dependent. The chemical nature of either form of primary damage is not known.

Enzymatic repair of DNA treated with physical or chemical agents can occur through the replacement of altered bases. One of the first steps involves an endonuclease. Two endonucleases have been described to date which recognize such altered DNA. One endonuclease recognizes thymine dimers (Kaplan *et al.,* 1971) and probably also DNA reacted with one or more specific chemical carcinogens (Kondo and Kato, 1968, Van Lancker and Tomura, 1974). Endonuclease II of *Escherichia coli* recognizes DNA which has reacted with various alkylating agents (Kirtikar and Goldthwait, 1974). Both of these enzymes produce endonucleolytic phosphodiester bond hydrolysis near the altered bases.

Endonuclease II of *E. coli* has been purified 1600-fold (Hadi *et al.,* 1973) on the basis of its ability to hydrolyze phosphodiester bonds in DNA alkylated with methyl methanesulfonate. It also degrades DNA reacted with ni-

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trogen mustard, dimethyl sulfate, and methylnitrosourea (Friedberg *et al.*, 1969, Kirtikar and Goldthwait, 1974).

The enzyme preparation acts on depurinated DNA. The alkali-catalyzed β-elimination reaction which results in single strand breaks in depurinated DNA in 0.1 N NaOH can be prevented by reduction of the depurinated DNA with sodium borohydride ($NaBH_4$) or reaction with hydroxylamine (NH_2OH), and DNA so treated is still a substrate for the enzyme preparation (Hadi and Goldthwait, 1971).

A limited number of single-strand breaks are also made by the enzyme in native DNA (Friedberg *et al.*, 1969). In T7 DNA at 37°, an average of 2.4 breaks per single strand are produced, but this requires several 100-fold more enzyme than the amount needed to degrade alkylated DNA (Hadi *et al.*, 1973).

The enzyme preparation cleaves phosphodiester bonds in native DNA to give 5′-phosphomonoesters. Because polynucleotide kinase did not react with the depurinated reduced DNA after it had been incubated with endonuclease II and alkaline phosphatase it was concluded that the enzyme makes single-strand breaks on the 5′ side of the altered nucleotides (Hadi *et al.*, 1973).

Because the enzyme preparation was able to recognize both alkylated and depurinated DNA, its ability to depurinate alkylated DNA was tested. From DNA alkylated with methylnitrosourea, *O*-6-methylguanine, 3-methyladenine, and small amounts of 1-methyladenine and 7-methyladenine were liberated by the enzyme. No 7-methylguanine was released (Kirtikar and Goldthwait 1974). Recently the depurination activity has been separated from the methyl methanesulfonate activity and the latter has been shown to be active on γ-irradiated DNA (Kirtikar, unpublished results).

In this paper, evidence is presented which indicates that the enzyme recognizes some form of damage in DNA induced by γ-irradiation.

Materials and Methods

[^{3}H]Thymidine was purchased from the New England Nuclear Corporation, Boston, Mass. Sodium borohydride (98% pure) and hydroxylamine hydrochloride were from the Fisher Scientific Company, Fair Lawn, N.J. Irradiation was carried out with a US Nuclear Model GR-9 ^{60}Co-irradiator (3).

DNA. ^{3}H-labeled DNA from T7 bacteriophage was isolated from phage grown on *Escherichia coli* B-3 Thy-strain in the presence of [^{3}H]thymidine (Richardson *et al.*, 1964). The specific activity of the labeled DNA was 2.4×10^4 cpm/nmol of DNA nucleotide when counted with 50% efficiency. For [^{3}H]purine-labeled T7 DNA, *E. coli* B-96 Pur$^-$ strain provided by Dr. J. Gots was used.

Treatment of DNA. Detailed experimental procedures are given in the table legends. In general, the following procedure was adopted for the irradiation of DNA samples: ^{3}H-labeled T7 DNA was diluted with unlabeled T7 DNA to give 30–45 μg of DNA with a final specific activity of 2900–4000 cpm/nmol of DNA nucleotide in 1 ml of buffer containing 0.1 M KPO_4–0.005 M Tris-HCl (pH 7.0). This was the standard irradiation buffer unless stated otherwise. Nitrogen was bubbled gently through the DNA solution at 0° for 15 min. A screw cap was then placed on the vial which was held at 0° for 10–20 min until irradiation with the ^{60}Co γ-ray source at a dose rate of 7.5 krads/min. In early experiments, irradiated DNA was processed after 24–48 hr of storage at 0° but in later experiments the interval between irradiation and the analysis was shortened to 1–2 hr.

Enzyme. Endonuclease II, with a specific activity of 80 units/mg of protein (one unit equals 1 μmol of DNA nucleotide released/hr from an acrylamide gel as described by Friedberg and Goldthwait, 1969), was isolated from an endonuclease I minus strain of *E. coli* and stored in small aliquots as indicated previously (Hadi *et al.*, 1973). For experiments described in this paper, the enzyme was diluted to 3–8 units/ml in 0.05 M Tris-HCl (pH 8.0) containing 0.1 mM β-mercaptoethanol and 20% glycerol.

Enzyme Reactions. The incubation mixture contained, in 0.25 ml, 20–25 nmol of irradiated ^{3}H-labeled T7 DNA nucleotide, 1×10^{-4} M β-mercaptoethanol, 1×10 M 8-hydroxyquinoline, 5×10^{-2} M Tris-HCl, pH 8.1 (Tables I–III, and Figure 1) and pH 8.5 (Tables IV–VII, and Figure 3), and 0.072 unit of enzyme unless otherwise stated. After 60 min at 37° (the incubation as distinguished from the preincubation) the enzyme reaction was terminated by the addition of sodium dodecyl sulfate (SDS) and sodium ethylenediaminetetraacetate (NaEDTA) at final concentrations of 0.25% and 2×10^{-2} M, respectively. Sodium hydroxide was added to the DNA samples to a final concentration of 0.066 N and the samples were held at 37° for 10 or 20 min. An aliquot of 0.25 ml was centrifuged at 20° in a SW 56 rotor through 3.6 ml of a 5–20% alkaline sucrose density gradient containing 0.1 N NaOH, 0.9 N NaCl, and 10^{-2} M EDTA, for 3 hr at speeds of 40,000, 45,000, or 50,000 rpm. The gradients were fractionated by puncturing the bottom of each tube and collecting drops. Radioactivity in each fraction was determined and the number average molecular weight (M_n) was calculated (Cleaver *et al.*, 1972) using a computer program for

$$\frac{\Sigma C_1 + C_2 + C_3 \ldots}{\Sigma C_1/M_1 + C_2/M_2 + C_3/M_3 \ldots}$$

where C_1, C_2, C_3 are the amounts of radioactivity in each fraction and M_1, M_2, M_3, the molecular weights of the fractions. The molecular weight of the DNA in each fraction was calculated from the S value according to Studier (1965). *E. coli* rRNA was used as a reference marker. The number of single-strand breaks was determined from $(M/M_n) - 1$, where M is the molecular weight of untreated single-stranded DNA. For T7 single-stranded DNA, a molecular weight of 12.5×10^6 was used (Freifelder, 1970). All determinations of radioactivity were made using the Packard TriCarb liquid scintillation spectrometer.

Reaction of DNA with $NaBH_4$ or NH_2OH. $NaBH_4$ reduction of irradiated DNA was done as follows. To the irradiated ^{3}H-labeled T7 DNA sample, a fresh solution of 5 M $NaBH_4$ was added in three aliquots at 15-min intervals to give a final concentration of 0.25 M, and the sample was incubated at room temperature for a total of 1.5 hr. The pH of the DNA solution was then adjusted to the desired value with 0.2 M Tris-HCl (pH 8.0) containing 1.1 N HCl.

Hydroxylamine treatment of DNA was as follows. A fresh solution of NH_2OH [4.0 M] was added to the irradiated DNA sample to a final concentration of 0.2 M and the sample was incubated at 37° for 1 hr. The pH of the treated DNA was adjusted to the desired value with concentrated Tris solution.

Alkali-Catalyzed Phosphodiester Bond Breakage in Depurinated and γ-Irradiated DNA. The time of incubation of DNA in 0.066 N NaOH at 37° required to hydrolyze phosphodiester bonds at depurinated sites was deter-

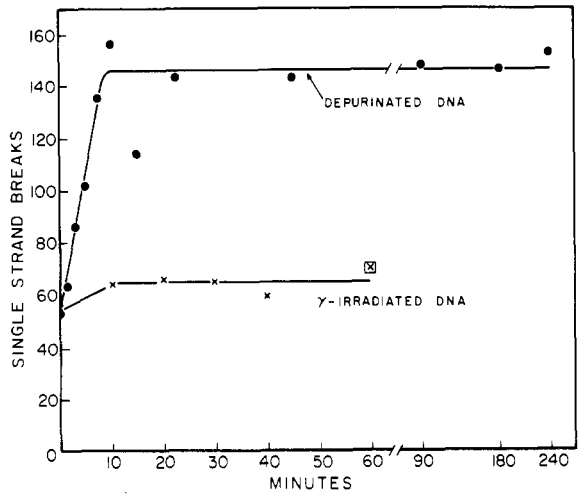

FIGURE 1: The effect of incubating depurinated DNA and irradiated DNA in 0.066 N NaOH at 37° on the number of single strand breaks. Depurinated DNA: 1094 nmol of ^{3}H-labeled T7 DNA (specific activity 500 cpm/nmol of DNA nucleotide) was incubated at 50° in 6.7 ml of 0.1 M sodium citrate (pH 3.5)–0.001 M NaEDTA for 30 min. The DNA was cooled and the pH was adjusted immediately to 6.5 with 10.0 M NaOH. Aliquots of depurinated DNA were incubated at 37° with NaCl–NaOH to final concentrations of 0.6 and 0.066 M, respectively, for the times indicated. Aliquots of 0.25 ml from each incubated DNA were analyzed for single strand breaks (see Materials and Methods). As a zero time control, to an aliquot of depurinated DNA, KPO_4 buffer (pH 6.5) was added to final concentration of 0.5 M, and the DNA was reduced with $NaBH_4$ and then analyzed. γ-Irradiated DNA: 324 nmol of [^{3}H]purine-labeled T7 DNA (1960 cpm/nmol of DNA nucleotide) in 3.0 ml of standard buffer, irradiated under standard conditions, was incubated at 37° for 4 hr. Aliquots of preincubated DNA were then incubated with NaCl–NaOH as above and analyzed for single strand breaks (see Materials and Methods).

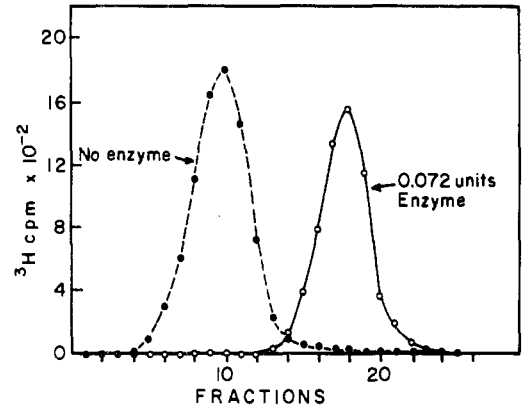

FIGURE 2: Degradation of γ-irradiated DNA by endonuclease II. ^{3}H-labeled T7 DNA [3500 cpm/nmol of DNA nucleotide], 44 μg in 0.1 M KPO_4–0.005 M Tris-HCl (pH 7.0) buffer, was irradiated under N_2 at 0° with 22.5 krad. After storage at 4° for 48 hr, aliquots of DNA were incubated at 37° without and with 0.072 unit of enzyme as described under Materials and Methods. Subsequent analysis, as described under Materials and Methods, involved alkaline sucrose gradients centrifuged at 20° for 3 hr at 45,000 rpm.

Table I: The Effect of Increasing Amounts of γ-Irradiation of T-7 DNA on the Number of Single Strand Breaks before and after Enzyme Treatment.[a]

Expt	Dose (krad)	Single Strand Breaks: No Enzyme	+Enzyme	Enzyme Induced
1	7.5	3	9	6
	15	5	15	10
	22.5	6	87	81
2	22.5	6	62	56
	38.5	17	77	60
	75	103	124	21
	150	155	178	23

[a] Aliquots of [^{3}H]DNA (4,000 cpm/nmol of nucleotide), 19 μg in 0.5 ml of 0.1 M KPO_4–0.005 M Tris-HCl (pH 7.0), were irradiated under N_2 as described under Materials and Methods at a dose rate of 7.5 krads/min. The incubation with or without 0.072 unit of enzyme at pH 8.1 and the subsequent treatment in 0.066 M NaOH at 37° for 10 min and analysis were as described under Materials and Methods.

mined as follows. A sample of DNA was partially depurinated by heating at 50° for 30 min in 0.1 M sodium citrate buffer (pH 3.5) containing 0.001 M EDTA. An aliquot of the DNA was reduced with $NaBH_4$ and served as the zero time control. To aliquots of the depurinated nonreduced DNA both NaOH and NaCl were added to final concentrations of 0.066 N and 0.66 M, respectively. These samples were incubated at 37° for varying times prior to centrifugation in alkaline sucrose gradients and analysis. Results, shown in Figure 1, indicate that by 10 min all of the depurinated sites are cleaved.

Irradiated DNA was incubated at 37° for 4 hr prior to exposure to 0.066 N NaOH at 37° for increasing periods of time. The number of single strand breaks was determined under the conditions noted above. As shown in Figure 1, the number of strand breaks reach a plateau by 10 min. Incubation of the irradiated DNA in 0.066 N NaOH at 37° for 10–20 min prior to analysis on alkaline sucrose gradients appeared adequate to convert depurinated sites as well as any other alkali-labile alteration of DNA to single strand breaks. The 10-min period was used for experiments in Tables I–V. For the experiments in the remainder of the tables as well as in Figures 3 and 4 a 20-min period was used.

Results

The relationship of the number of spontaneous breaks and enzyme sensitive sites observed in alkaline sucrose gradients to the dosage of γ-irradiation is shown in Table I and Figure 2. Samples of T7 DNA in solution, under nitrogen and 0°, were exposed to increasing doses of γ-irradiation. After storage, the samples were incubated for 60 min at 37° with or without endonuclease II. For all of the early experiments, incubation was at pH 8.1, a pH used for the enzyme with alkylated DNA. NaOH was then added to a final concentration of 0.066 M and the incubation at 37° was continued for 10 min. The second incubation in alkali was to convert any depurinated sites to single strand breaks, so that any depurinated sites present in the irradiated DNA would be registered as single strand breaks in the control samples without enzyme as well as in the enzyme-treated samples. The samples were then layered on alkaline sucrose gradients for centrifugation and analysis. Figure 2 is an example of an experiment done with DNA exposed to 22.5 krads and then incubated with and without 0.072 unit of enzyme. For doses up to 38.5 krads, enzyme-sensitive sites exceeded spontaneous single strand breaks. Above this dose, there was a rapid increase in spontaneous breaks and a concomi-

Table II: The Effect of Varying Amounts of Enzyme on the Number of Single Strand Breaks in Irradiated DNA.[a]

Enzyme Units	Single Strand Breaks	Enzyme Units	Single Strand Breaks
None	5	0.072	95
0.018	13	0.144	113
0.036	29	0.360	138

[a] Aliquots of ^{3}H-labeled T7 DNA (3500 cpm/nmol of DNA nucleotide), 44 μg ml of 0.01 M KPO_4-0.005 M Tris-HCl (pH 7.0), were irradiated under N_2 at 0° with 22.5 krad. After storage at 4° for 48 hr subsequent treatment with the exception of the enzyme concentration was as in Table I.

tant decrease in enzyme-sensitive sites. It is not known whether enzyme-sensitive sites are converted to spontaneous breaks at high doses. Since the chemical structures of both the enzyme-sensitive site and the alteration leading to spontaneous strand breaks are not clear, there is no obvious explanation for the unusual dose effect. The presence of an amine buffer during the incubation had an effect on the number of spontaneous single strand breaks. This was examined by irradiation of the DNA in potassium phosphate buffer without and with varying concentrations of Tris. The results indicated that Tris had a protective effect. A concentration of 5×10^{-3} M decreased the spontaneous breaks to one-half and a concentration of 10^{-1} M to one-quarter. For subsequent experiments, DNA was irradiated in 0.1 M KPO_4 buffer with 0.005 M Tris-HCl. This effect of Tris has been noted by others (Setlow and Carrier, 1973; Achey *et al.*, 1974).

The number of single strand breaks produced by the enzyme was examined next as a function of enzyme concentration (Table II). With 0.072 enzyme unit added to the reaction mixture, 95 single strand breaks were made per T7 single strand. Under the conditions used, this concentration of enzyme would not produce any single strand breaks in native T7 DNA. With more enzyme, slight nicking of native DNA was observed and a limit of 2.4 breaks per single strand of native T7 DNA has been reported (Hadi *et al.*, 1973). To avoid any action of the enzyme on native DNA, 0.072 unit of enzyme was added in subsequent experiments. Therefore the number of single strand breaks recorded in ensuing tables represents approximately two-thirds of the total number of enzyme-sensitive sites present. The amount of enzyme required for γ-irradiated DNA was 10-100-fold the amount needed to degrade depurinated reduced DNA (Hadi *et al.*, 1973). This suggest there may be separate enzymes, in this 1600-fold purified preparation.

Irradiation of the DNA at pH values of 7.0 and 8.0 and incubation with and without enzyme at pH 8.1 produced similar numbers of spontaneous breaks and enzyme sensitive sites, whereas irradiation at pH 6.0 produced the same number of spontaneous breaks, but one-half of the enzyme-sensitive sites. For subsequent experiments the DNA was irradiated in 0.1 M KPO_4-0.005 M Tris-HCl buffer at pH 7.0.

The number of spontaneous and enzyme-induced single strand breaks produced in the DNA by γ-irradiation was found to be variable and depended upon the length of time and conditions of storage of the DNA after irradiation. These conditions were not carefully controlled in the experiments in Tables I and II and Figure 2. When the DNA was irradiated under N_2 in the standard buffer and stored in sealed, screw-cap glass vials at 4° (Table III), an increasing number of spontaneous single-strand breaks was observed during periods of up to 30 days. Enzyme-sensitive sites increased during the first 20 days. When the DNA was irradiated and stored in 0.05 M Tris-HCl (pH 8.0) instead of in the standard buffer, there was after 30 days a slight decrease in the number of spontaneous breaks and a slight increase in the number of enzyme induced breaks compared to the results shown in Table III.

Table III: The Effect of Storage at 4° on the Number of Single Strand Breaks in DNA before and after Enzyme Treatment.[a]

Storage at 4°, days	Single Strand Breaks		
	No Enzyme	+Enzyme	Enzyme Induced
0	3	26	23
10	6	76	70
20	17	94	77
30	45	110	65

[a] Aliquots of ^{3}H-labeled T7 DNA (2900 cpm/nmol of DNA nucleotide) with 20 μg in 0.5 ml of 0.1 M KPO_4-0.005 M Tris-HCl buffer (pH 7.0) were irradiated at 0° under N_2 with 22.5 krad. The sealed vials were stored at 4° for the time noted and were then analyzed as in Table I.

If the DNA, after irradiation in nitrogen, was then kept at 37° for several hours (defined as the preincubation) there was also an increase in the number of nonenzymatic and enzymatic single strand breaks (Table IV). In experiments 1-5, each DNA aliquot was flushed with N_2 for 15 min prior to irradiation, sealed in a screw cap vial, irradiated, and either held at 0° or incubated at 37° in a water bath. This incubation is described in the subsequent tables as the preincubation. The vial was then opened, an aliquot of the DNA was added to a reaction mixture, incubated at 37° for 60 min with or without enzyme, and processed as in Table I. In experiments 1-4 there is some variation in the number of spontaneous strand breaks not at zero time but after the 4-hr preincubation. There is considerable variation in the number of enzyme-induced breaks both before and after preincubation. In experiment 5, there is a lag in the appearance of spontaneous single strand breaks and of enzyme-induced breaks. The reason for this is not known but that is discussed later. Incubation of a nonirradiated native DNA under the same conditions for 4 hr did not produce any spontaneous single strand breaks or enzyme-sensitive sites.

The optimum pH for the enzymatic hydrolysis of irradiated DNA was found to be 8.5 (Table V). A broad pH optimum from 8.0 to 9.0 was observed with alkylated DNA and reactions with that substrate were run at pH 8.1 (Friedberg *et al.*, 1969). The data in Tables I-IV were obtained with enzyme incubations at pH 8.1. Subsequent incubations were at pH 8.5. The slight differences between the pH curves for irradiated and alkylated DNA may indicate different enzymes, but may also be due to the different substrates. The optimum pH for the preincubation of the DNA *after* irradiation is shown in Table V. The preincubation was for 4 hr at 37° and the pH optimum for the pro-

Table IV: The Effect of Preincubation at 37° on the Number of Single Strand Breaks in DNA before and after Enzyme Treatment.[a]

Expt	Preincubation Time (hr)	Single Strand Breaks: No Enzyme	+Enzyme	Enzyme Induced
1	0	3	10	7
	4	20	85	65
2	0	4	15	11
	4	18	57	39
3	0	4	18	14
	4	25	56	31
4	0	4	17	13
	4	16	49	33
5	0	4	30	26
	1	6	41	35
	2	8	49	41
	3	12	103	91
	4	30	138	108

[a] Aliquots of ^{3}H-labeled T7 DNA, 22 μg in 0.5 ml of 0.1 M KPO_4–0.005 M Tris-HCl (pH 7.0), were irradiated at 0° under N_2 with 22.5 krads. One set of vials was opened and the DNA was incubated with and without 0.072 unit of enzyme and analyzed in the usual fashion. Another set of unsealed vials was preincubated in a water bath at 37° for the times indicated and then treated as above.

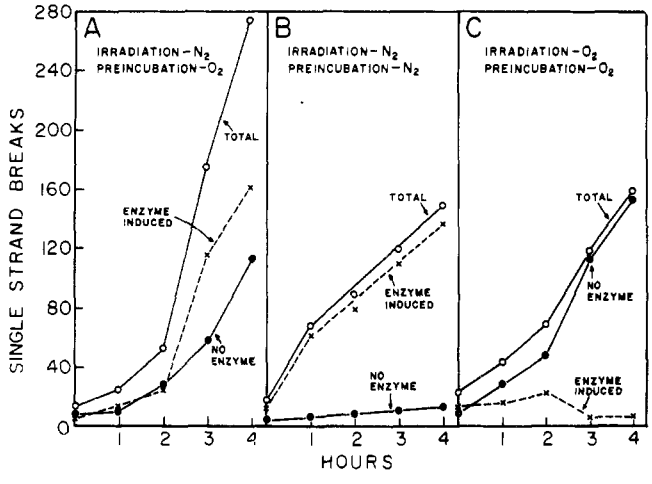

FIGURE 3: The effect of oxygen or nitrogen during a preincubation period at 37° following the irradiation. ^{3}H-labeled T7 DNA (specific activity 500 cpm/nmol of DNA nucleotide) was irradiated under standard conditions under N_2 (A and B) or under O_2 (C) and preincubated at 37° in an atmosphere of O_2 (A and C) or N_2 (B). The O_2 or N_2 gas was bubbled through the DNA solutions during the entire preincubation period. Aliquots were removed at 60-min intervals and assayed for single strand breaks without and with enzyme.

duction of enzyme sensitive sites was found to be approximately 8.6. In subsequent experiments all preincubations and incubations were done at pH 8.5.

The effect of incubation in oxygen or in nitrogen on DNA which had been previously irradiated in an atmosphere of nitrogen was examined. The results are shown in Figure 3. The DNA used for the experiments shown in graphs A and B was irradiated in N_2. Samples preincubated in oxygen (graph A) after irradiation showed, after an initial lag of 2 hr, a marked increase in the number of spontaneous single strand breaks. Samples preincubated in nitrogen (graph B) showed only a slight increase in nonenzymatic breaks over the 4-hr period. The increase in enzyme-sensitive sites shown in A and B was approximately the same at the 4-hr point. In graph B the increase in enzyme sensitive sites is linear if one adds the 1 hr of incubation at 37° used for the assay. In graph A, there was an initial lag. In a similar experiment, after a 4-hr preincubation in oxygen, the number of spontaneous single strand breaks was 100 and enzyme-induced breaks was 107, while after preincubation in nitrogen the numbers were 32 and 120, respectively. These experiments support the suggestion that the presence of oxygen after irradiation is responsible for the increase in the number of nonenzymatic single strand breaks observed during preincubation. However, the presence of oxygen does not affect the number of enzyme-sensitive sites.

The effect of O_2 present during irradiation and also during preincubation is shown in Figure 3, graph C. In this case there is almost a complete absence of enzyme-sensitive sites.

Since oxygen appeared to be involved in the formation of spontaneous single strand breaks, the effect of the reducing agent $NaBH_4$ was tested. Some results are shown in Table VI, experiment 1. Two conclusions can be drawn. First,

Table V: The Effect of Varying the pH of the Incubation or Preincubation of Irradiated DNA on the Number of Spontaneous and Enzyme-Induced Single Strand Breaks.

Expt	pH of Incubation	Single Strand Breaks: No Enzyme	+Enzyme	Enzyme Induced
1[a]	7.2	4	54	50
	7.6	4	85	81
	8.1	4	110	106
	8.5	4	131	127
	8.8	4	82	78
	9.5	4	72	68
Expt	pH of preincubation at 37° for 4 hr			
2[b]	7.0	22	61	39
	7.6	20	83	63
	8.2	19	95	76
	8.6	19	113	94
	9.0	19	98	79
	9.4	17	97	80

[a] ^{3}H-labeled T7 DNA (2900 cpm/nmol of DNA nucleotide), 40 μg/ml of 0.01 M KPO_4–0.005 M Tris-HCl, (pH 7.0), was irradiated under N_2 at 0° with 22.5 krads. Irradiated DNA was treated with $NaBH_4$ at room temperature (see Materials and Methods), and incubated at 37° for 4 hr. The pH of the final reaction mixtures was adjusted as indicated and the samples were analyzed as in Table I. [b] The DNA was irradiated in the same manner. The pH values of 0.5-ml aliquots were adjusted with 2.0 M Tris, nitrogen was bubbled through each sample for 10 min and the samples were incubated at 37° for 4 hr. The pH of all samples was then adjusted to 8.5 prior to the 60 min incubation with or without enzyme and analysis.

Table VI: The Effect of Addition of $NaBH_4$ or NH_2OH before Preincubation or before Incubation of Irradiated DNA on Spontaneous and Enzyme Induced Single Strand Breaks.

Expt	Preincubation	Additions		Single Strand Breaks		
		Before Preincubation	Before Incubation	No Enzyme	+Enzyme	Enzyme Induced
1[a]						
a	−			4	21	17
b	−		$NaBH_4$	2	22	20
c	+			18	110	92
d	+	$NaBH_4$		3	88	85
2[b]						
a	−			5	28	23
b	−		$NaBH_4$	2	23	21
c	−		NH_2OH	3	26	23
d	+			21	103	82
e	+	$NaBH_4$		4	103	99
f	+	NH_2OH		7	101	94
3						
a	−			8		
b	−		$NaBH_4$	4		
c	−		NH_2OH	4		
d	+			47		
e	+	$NaBH_4$		4		
f	+		$NaBH_4$	11		
g	+	NH_2OH		5		
h	+		NH_2OH	13		

[a] A set of four vials containing [^{3}H]thymine-labeled T7 DNA [2900 cpm/nmol of DNA nucleotide] was irradiated under standard conditions. The DNA samples b and d were treated with $NaBH_4$ (see Materials and Methods). Equivalent amounts of the standard buffer were added to samples a and c. The pH of all DNA samples was adjusted to 8.5. Samples a and b were analyzed immediately. Samples c and d were flushed with N_2 for 10 min at 0°, the screw capped vials were closed and incubated at 37° for 4 hr. The pH was again adjusted to 8.5 if necessary, all samples were incubated with and without enzyme, and then analyzed for single strand breaks as in Table I. [b] Experiments 2 and 3 were executed similarly to experiment 1, except for the use of [^{3}H]purine-labeled T7 DNA (1960 cpm/nmol of DNA nucleotide) in experiment 3. NH_2OH was added as indicated under Materials and Methods.

$NaBH_4$ reduces the number of spontaneous single strand breaks observed immediately after irradiation and the effect is more pronounced after the preincubation. Second, $NaBH_4$ does not decrease the number of enzyme-sensitive sites significantly, and in some cases appears to increase them slightly.

In previous studies (Hadi and Goldthwait, 1971) both $NaBH_4$ and NH_2OH when reacted with depurinated DNA were shown to prevent the alkali-catalyzed β-elimination reaction which results in cleavage of the phosphodiester bond. Since $NaBH_4$ prevented the nonenzymatic single strand breaks in irradiated DNA due to preincubation (Table VI, experiment 1), NH_2OH was also tested (Table VI, experiment 2). The results show that NH_2OH, when added at the start of the 4-hr preincubation (2f *vs.* 2d), decreases the number of nonenzymatic single strand breaks almost as effectively as $NaBH_4$ (2e *vs.* 2d). These results strengthen the hypothesis that the spontaneous strand breaks arise indirectly from base damage which during the preincubation leads to depurination or depyrimidination.

If this hypothesis is correct, one would predict that the single strand breaks occur not during the preincubation but because of the addition of alkali, and also that this strand breakage could be prevented by the addition of $NaBH_4$ or NH_2OH at the end of the preincubation as well as at the beginning. Experiment 3 of Table VI shows the results obtained when both agents were added either at the beginning or the end of the preincubation. With no additions, 47 breaks were observed (3d); with $NaBH_4$ or NH_2OH addition at the beginning 4 and 5 breaks were seen (3e and 3g) while with addition at the end, 11 and 13 breaks were observed (3f and 3h). The conclusion must be that approximately three-quarters of the spontaneous single strand breaks are alkali catalyzed. It is possible that the remaining breaks could arise from depurinated or depyrimidinated DNA during the 1-hr incubation.

To prove that many of the spontaneous single strand breaks were alkali catalyzed, the irradiated DNA was single stranded and examined either with alkali or formamide both before and after the 4-hr. preincubation. The results are shown in Figure 4A and B. When examined immediately after irradiation (Figure 4A), the sample exposed to alkali for 20 min and then run on an alkaline sucrose gradient migrated to approximately the same place as the same sample in the formamide gradient. The 23S marker in Figure 4A and B refers to the alkaline gradients only. After preincubation at 37° for 4 hr the irradiated DNA when examined in the alkaline gradient (at a higher speed) had been converted from a sample with five breaks (Figure 4A) to one with 56 breaks (Figure 4B). If either $NaBH_4$ or NH_2OH was added at the end of the preincubation prior to examination in the alkaline sucrose gradient, the DNA

Table VII: The Effect of β-Mercaptoethanol on the Spontaneous and Enzyme Induced Strand Breaks in Irradiated DNA.[a]

Expt	Pre-incubation	β-Mercaptoethanol after Irradiation: Before Preincubation	β-Mercaptoethanol after Irradiation: Before Incubation	β-Mercaptoethanol before Irradiation	Single Strand Breaks: No Enzyme	Single Strand Breaks: +Enzyme	Single Strand Breaks: Enzyme Induced
1							
a	−	−	−	−	6	38	32
b	−	−	+	−	6	41	35
c	+	−	−	−	26	110	84
d	+	+	−	−	10	56	45
e	−	−	−	+	2	14	12
f	+	−	−	+	2	24	22
2							
a	−	−	−	−	6	24	18
b	+	−	−	−	51	132	81
c	+	+	−	−	12	44	32
d	+	−	+	−	56	141	85

[a] For experiment 1, [^{3}H]thymine-labeled T7 DNA [500 cpm/nmol of DNA nucleotide] was used. For experiment 2, [^{3}H]-purine-labeled DNA was used [1960 cpm/nmol of DNA nucleotide]. Within 30 sec after irradiation, the β-mercaptoethanol was added to some DNA samples. The pH was then adjusted to 8.5 and the DNA solutions were flushed with N_2 for 10 min at 0°, and the vials were sealed. Preincubation, incubation, and analysis were done as described previously.

samples showed 9 and 13 breaks, respectively (the locations of the peak fractions are indicated by arrows in Figure 4B). The same preincubated sample of irradiated DNA, when examined in the formamide gradient, had a sedimentation coefficient considerably greater than that observed in the alkaline sucrose. These data provide further evidence for the statement that most of the spontaneous single strand breaks are alkali catalyzed.

Since sulfhydryl reagents can decrease radiation damage, β-mercaptoethanol was tested for its effect on the spontaneous breaks and enzyme-sensitive sites. This reagent was used because cysteamine was found to inhibit the enzyme. The results in Table VII, experiment 1, indicate that 0.5 M β-mercaptoethanol added after irradiation and before the preincubation decreased both the spontaneous breaks and the enzyme sensitive sites to approximately one-half the level observed in the control (experiment 1d *vs.* 1c). Addition before irradiation decreased both types of breaks even more extensively (experiment 1e *vs.* 1a, and 1f *vs.* 1c). In experiment 2, addition of β-mercaptoethanol 30 sec after the irradiation but before the preincubation again decreased both the spontaneous breaks and the enzyme sensitive sites (2c *vs.* 2b). However, when β-mercaptoethanol was added at the end of the preincubation, there was no protective effect (2d *vs.* 2b). This suggests that following irradiation and before preincubation there are several molecular species which can be converted to alkali-labile sites and enzyme-sensitive sites and that this conversion can be partially prevented by the sulfhydryl reagent. Since addition of β-mercaptoethanol at the end of preincubation had no effect, these species do not persist during the preincubation.

Finally, an experiment was done to rule out the presence of H_2O_2 as the agent responsible for the appearance of either the spontaneous or enzyme-induced single strand breaks. After irradiation but prior to preincubation, varying amounts of catalase (2–54 μg) were added to the aliquots of DNA. No significant change of either the spontaneous breaks or enzyme-induced sites was observed.

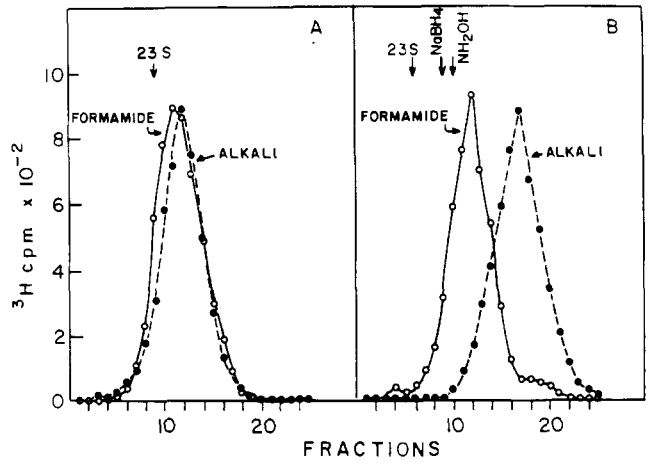

FIGURE 4: γ-Irradiated DNA in alkaline and formamide gradients before and after preincubation at 37° for 4 hr. [^{3}H]Purine-labeled T7 DNA (775 cpm/nmol of DNA nucleotide), 22 μg/ml in standard buffer, was irradiated under N_2 at 0° with 22.5 krad. (A) One aliquot of γ-irradiated DNA was incubated in alkali, 0.066 M NaOH, at 37° for 20 min and centrifuged through 3.6 ml of 5–20% sucrose density gradient solutions in 0.9 M NaCl–0.1 M NaOH–0.001 M NaEDTA, for 3 hr at 20° at 40,000 rpm in an SW 56 rotor. A second aliquot, without prior treatment, was centrifuged in a 70–100% formamide gradient under the same conditions. (B) γ-Irradiated DNA was preincubated in sealed vials for 4 hr at 37° and processed as in (A) but centrifuged at 45,000 rpm. Arrows labeled $NaBH_4$ and NH_2OH show the positions of the peak fractions of DNA treated with $NaBH_4$ and NH_2OH after the preincubation.

Discussion

The results reported in this paper can be summarized as follows. (1) Evidence is provided for at least two types of damage to DNA caused by γ-irradiation. The first type leads to spontaneous single strand breaks due primarily to alkali-labile bonds. The second type of damage produces enzyme-sensitive sites which are not alkali-labile. (2) After irradiation under nitrogen, preincubation of the DNA for 4 hr at 37°, prior to a 1-hr incubation at 37° with or without

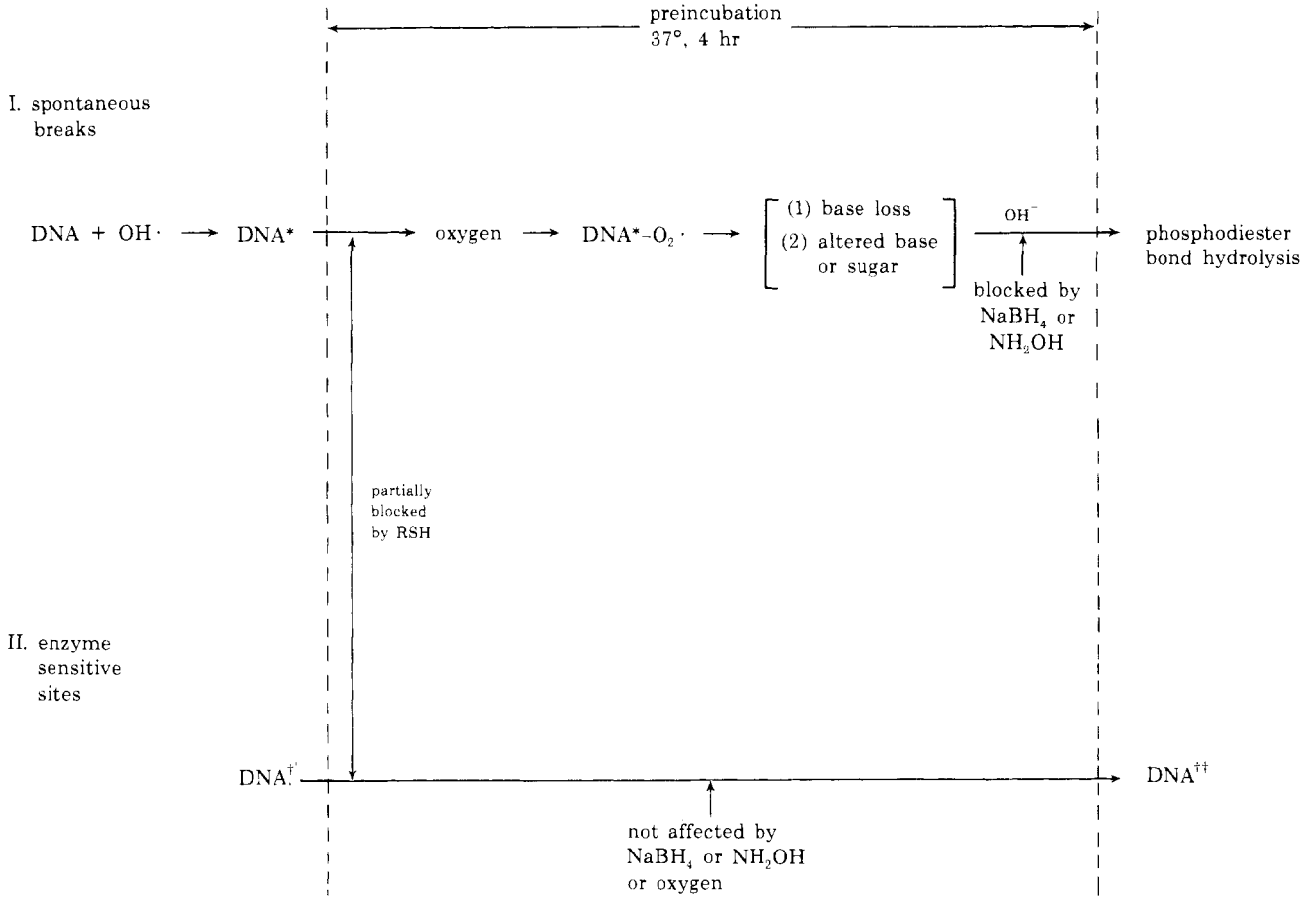

FIGURE 5.

enzyme, leads to an increase in both types of damage. (3) Preincubation in oxygen produces a marked increase in spontaneous single strand breaks compared to preincubation in nitrogen, but little if any change in enzyme-sensitive sites. (4) The increase in spontaneous breaks on preincubation can be prevented completely by addition of $NaBH_4$ or NH_2OH before the preincubation, and almost completely by the addition after the preincubation. Many less breaks are seen when the DNA is examined in formamide. (5) β-Mercaptoethanol partially prevents the increase in spontaneous breaks if added at the beginning of the preincubation, but has no effect when added at the end. (6) The increase in enzyme-sensitive sites during the preincubation is not prevented by $NaBH_4$ or the NH_2OH, but is partially prevented by β-mercaptoethanol added at the beginning of the preincubation. The scheme in Figure 5 illustrates one interpretation of these results when DNA is irradiated under nitrogen.

The spontaneous breaks (Figure 5) are thought to be due to alkali-labile bonds, the evidence for which has been presented. This evidence is indirect and is based on the fact that $NaBH_4$ or NH_2OH added at the end of the preincubation period prevented the appearance of three-fourths of the alkali-catalyzed spontaneous breaks and that the number of single strand breaks observed in alkali is greater than those observed in formamide. The production of alkali-labile bonds after irradiation has been observed by a number of groups (Bopp and Hagen, 1970; Kessler *et al.*, 1971; Achey *et al.*, 1971; Ward and Kuo, 1973). The increase in bond breakage due to alkali varied from 1.4- to 3-fold in these reports.

The nature of the alkali-labile bond is not clear. Two possibilities exist: loss of a damaged base or direct damage to the sugar. The loss of a damaged purine or pyrimidine by hydrolysis of the glycosidic bond leaves the phosphodiester bonds intact, but the free sugar is now susceptible to an alkali-catalized β-elimination reaction and phosphodiester bond hydrolysis (Tamm *et al.*, 153; Brown and Todd, 1955). In the experiments described in this paper, loss of one or several of the four bases is considered very probable, first, because the alkali-lability of the DNA at the end of the preincubation is prevented by $NaBH_4$ and NH_2OH (both are agents which react with depurinated DNA to prevent the alkali-catalyzed β-elimination reaction) and, second, because in preliminary experiments by one of us (D.K.), release of counts from both purine-labeled and thymine-labeled, irradiated DNA was demonstrated during the preincubation. Ullrich and Hagen (1971) have shown the release of adenine, guanine, cytosine, and thymine from irradiated DNA and Ward and Kuo (1973) have demonstrated that irradiation of pTp in oxygen leads to release of volatile ^{14}C from [2-^{14}C]pTp. The production of alkali-labile bonds after irradiation does not mean that *in vivo* strand breaks occur. However, if these alkali-labile bonds are due to loss of bases, then the depurinated or depyrimidinated sites would be substrates for an enzyme active on depurinated DNA and they could be converted enzymatically to strand breaks. Since the DNA in these experiments prior to analysis was incubated in NaOH, such potential enzyme sites are converted to spontaneous strand breaks.

The second possibility for radiation induced alkali-labile bonds involves damage to the sugar as suggested by the work of Krushinskaya and Shal'nov, 1967; Kapp and Smith, 1970; Ullrich and Hagen, 1971; Ward, 1972; and

Ward and Kuo, 1973. Damage to the sugar may produce an aldehyde or keto group which could react with $NaBH_4$ or NH_2OH. Therefore it should not be assumed that the evidence presented proves that the alkali-labile bonds are due to depurination or depyrimidination.

Incubation of the DNA after irradiation at 0° for days or at 37° for hours, particularly in the presence of oxygen, increased the number of spontaneous strand breaks. These results have a counterpart in the viscosity experiments of Daniels *et al.*, 1957, and the pTp degradation experiments of Ward and Kuo (1973). The post-irradiation changes were not seen when nitrogen replaced oxygen. The sensitizing effect of oxygen on radiation induced killing of bacteria and mammalian cells is well known (Alper, 1967; Setlow and Setlow, 1972) and the presence of oxygen compared to nitrogen increases the number of single strand breaks in DNA (Boyce and Tepper, 1968; Van der Schans and Blok, 1970; Lehnert and Moroson, 1971). In our experiments, the DNA was irradiated under nitrogen and then 10–15 min later either oxygen or nitrogen was bubbled through the sample for the preincubation period. Thus, the effect of oxygen, which increased spontaneous strand breaks, was separated from the immediate damage due to the irradiation. The immediate damage is thought by many workers to be due to a reaction of hydroxyl radicals with DNA (Figure 3, DNA*) (Roots and Okada, 1972). The subsequent reaction of oxygen with DNA* forms DNA*-O_2. In model systems with bases, this reaction is diffusion controlled (Willson, 1970). The peroxidation of nucleic acids by radiation in the presence of oxygen was noted by Scholes *et al.* (1956) and the hydroperoxides were found to be unstable on incubation (Schweibert and Daniels, 1971). Their decay over a 24-hr period by hydrolysis involved a fast and a slow reaction. The former was thought to be due to a decay of hydroperoxides of cytosine while the latter to decay of hydroperoxides of thymine. This may not be an explanation for the initial lag in the appearance of spontaneous breaks during the preincubation (Figure 2A and 2C, Table IV, experiment 5). One explanation might be the presence of at least two sequential steps leading from DNA*-O_2 to an alkali-labile form.

Minutes after the irradiation of DNA in an atmosphere of nitrogen, the exposure to oxygen increased the number of spontaneous strand breaks observed especially after preincubation at 37°. This indicated the presence of radiation-induced long-lived molecular species which were reactive. Brustad *et al.* (1972, 1973) observed ["DNA transients" of each of the four bases and noted that transients in DNA gradually decayed over a period of up to 1 hr. It is probable that β-mercaptoethanol, added after irradiation, reacted with some of the transients in competition with oxygen. The effect of addition of $NaBH_4$ or NH_2OH after irradiation was more likely due to their interaction with a breakdown product of the DNA–radical–oxygen adduct.

The molecular nature of the transients is not clear. The more stable forms of base damage due to irradiation are better understood for pyrimidines than for purines. The major thymine radiolysis products with oxygen involve the 5,6-double bond (Teoule *et al.*, 1974). Repair of some of these has been studied (Hariharan and Cerutti, 1972). Unlike the pyrimidine derivatives, there are no widely accepted purine derivatives produced by irradiation of DNA. 8-Hydroxyadenine, 4,6-diamino-5-formamidopyrimidine, and other compounds have been isolated after irradiation of adenine (Van Hemmen and Bleichrodt, 1971) and opening of the imidazole ring of guanylic acid has also been noted (Hems, 1958). There are no data on the stability of the glycosidic linkage of model compounds following radiation in nitrogen or oxygen.

The nature of the enzyme sensitive sites produced by γ-irradiation (Figure 3, $DNA^{\dagger}$) and also by the preincubation after the irradiation ($DNA^{\dagger\dagger}$) are completely unknown. Because of the specificity of endonuclease II for some alkylated purines (Kirtikar and Goldthwait, 1974) it is possible that the enzyme recognizes purines altered by the irradiation. Since alkylated bases are released by the enzyme, it may be possible to isolate and identify the altered bases responsible for the enzyme sensitive sites due to γ-irradiation.

A general conclusion regarding cell viability is that single strand breaks are in most instances not lethal, but that double strand breaks are (Setlow and Setlow, 1972). Not only do double strand breaks account for lethality as in T4 bacteriophage (Freifelder, 1968), but also base damage *per se* can be responsible for approximately one-half of the lethal effect as in T5, T7 and λ (Freifelder, 1968; Freifelder *et al.*, 1972). Base damage may also result *in vivo* in strand breaks indirectly by providing a substrate for an enzyme such as endonuclease II. In that case the enzyme might be involved in repair if there is minimal damage to the DNA, but in cell death if there is extensive damage.

Enzymatic repair *in vivo* of breaks in DNA strands induced by ionizing radiation has been demonstrated in many systems including *M. radiodurans* (Dean *et al.*, 1969) and *E. coli* (Paterson and Setlow, 1972). *In vitro* evidence for enzymatic recognition of damage in irradiated DNA was provided by Setlow and Carrier (1973) and by Wilkins (1973) who showed endonucleolytic activity in extracts of *Micrococcus luteus*. The work presented in this paper extends their observations by the use of a partially purified enzyme preparation which also has activity on other substrates.

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The Copper Coordination Group in "Blue" Copper Proteins: Evidence from Resonance Raman Spectra†

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ABSTRACT: Tunable dye laser excitation in the intense ~600-nm absorption band of azurin, plastocyanin, and ceruloplasmin provides resonance enhanced Raman spectra. They consist of a complex set of bands, at least three or four in number, between 350 and 470 cm^{-1}, which are assignable to Cu-N or Cu-O bond stretching, and a weak band near 270 cm^{-1}, which probably arises from Cu-S stretching. A weak band at 765 cm^{-1} found in plastocyanin may arise from C-S stretching. Analysis of the Raman intensity pattern, as well as of the nature of the resonant electronic transition, leads to a model of the "blue" copper site involving approximately trigonal-bipyramidal coordination, with a sulfur and two nitrogen ligands in the equatorial plane, and less strongly bound nitrogen or oxygen ligands at axial positions. This arrangement would be well poised for stabilization of Cu(I) upon reduction.

Among copper containing proteins, those with "blue" copper centers have attracted particular attention because of their intense light absorption ($\epsilon \sim 5 \times 10^3\ M^{-1}\ cm^{-1}$) near 600 nm, which gives rise to their deep blue color (Malkin and Malmstrom, 1970). While the energy of this absorption band, along with those of neighboring, less intense bands, is in line with expected "d-d" electronic transitions centered on Cu^{2+} ions, its intensity is some 50 times greater than is observed for simple complexes.

"Blue" copper centers are found in the copper oxidases which contain several copper atoms per molecule and it was once thought that the anomalous absorption arose from copper-copper interactions. Similar absorption was subsequently found for proteins containing isolated copper atoms, e.g., azurin, stellacyanin, and plastocyanin (the last of which contains two noninteracting copper atoms, one each in two identical subunits), demonstrating that the "blue" copper centers are a unique kind of mononuclear copper complex. The intense visible absorption is associated with copper(II) as shown by electron paramagnetic resonance

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