A33

Reprinted from RADIATION RESEARCH, Volume 42, No. 1, April 1970

RADIATION RESEARCH **42**, 34–49 (1970)

Chemical Nature of Chain Breaks Produced in DNA by X-Irradiation *in Vitro*[1]

DANIEL S. KAPP AND KENDRIC C. SMITH

Department of Radiology, Stanford University School of Medicine, Stanford, California 94305

KAPP, DANIEL S., AND SMITH, KENDRIC C., Chemical Nature of Chain Breaks Produced in DNA by X-irradiation *in Vitro*. *Radiation Res.* **42**, 34–49 (1970).

X-irradiation of aqueous solutions of calf thymus DNA leads to the production of chain breaks, the liberation of inorganic phosphate, the formation of phosphomonoester groups, and the production of a malonic aldehyde-like product due to damage to deoxyribose residues.

For each single-chain break produced in DNA by x-irradiation in 0.15 *M* NaCl, 0.60 molecules of malonic aldehyde-like product were formed; 0.33 molecules of inorganic phosphate were liberated and 1.35 molecules of phosphomonoester groups were produced. Of the phosphomonoester groups liberated, 29% were 5′-phosphoryl groups. The ratio of newly formed 5′-phosphoryl termini to 5′-hydroxyl termini was 32:1. The presence of 0.001 *M* *l*-histidine reduced the number of single-chain breaks by a factor of 0.39, the yield of inorganic phosphate by 0.24, the formation of phosphomonoester groups by 0.42, and deoxyribose damage by 0.41.

INTRODUCTION

Internucleotide chain breaks in DNA induced by x-irradiation have been implicated as the primary lethal events in x-irradiated bacteria (*1*, *2*). Studies on the irradiation of DNA in aqueous media have shown that inorganic phosphate is liberated (*3*) and that phosphomonoester groups are formed (*4*). Such studies suggest that chain breakage occurs at the phosphodiester bond when DNA is irradiated in aqueous media. However, significant damage to the deoxyribose moiety has also been reported (*5*) suggesting that another site of chain breakage is at the C3′–C4′

[1] This investigation was supported by USPHS research grant CA-02896, research program project grant CA-10372, and research career development award CA-3709 (to K.C.S.) from the National Cancer Institute and a Medical Scientist Fellowship (to D.S.K.) from the Life Insurance Medical Research Fund.

bonds. This is in accord with earlier suggestions that the radiation-induced lability of nucleotides is due to the formation of carbonyl groups at the carbon atom C3′ (*6*) and that a similar labilization of phosphate groups by the oxidation of the sugar moiety in DNA may occur causing fragmentation of polynucleotide chains (*7*). In addition, the failure of the polynucleotide joining enzyme to repair (in one step) the single-strand chain breaks produced in DNA by x-irradiation in aqueous media implies that chain breakage involves a more complicated mechanism than a simple rupture of the phosphodiester bond to produce polynucleotide chains with 3′-hydroxyl and 5′phosphoryl groups in juxtaposition (*8*).

It was the purpose of this investigation to elucidate the locations and end products of polynucleotide chain breakage produced by the x-irradiation of aqueous solutions of calf thymus DNA. We have attempted to determine quantitative relationships between chain breakage, inorganic phosphate yields, phosphomonoester group formation, and deoxyribose damage. The effect of *l*-histidine (10^{-3} *M*) on the above parameters of x-ray damage was also determined.

MATERIALS AND METHODS

Materials. Calf thymus DNA and alkaline phosphatase were purchased from Worthington Biochemical Corporation. *l*-Histidine dihydrochloride was obtained from Mann Research Labs, Inc. DNA solutions of 2.5 mg/ml were prepared either in 0.15 *N* NaCl or in 0.15 *N* NaCl containing 0.001 *M* *l*-histidine. The solutions were then dialyzed for 24–48 hours against the same solvent. The final solutions contained 176 μg P/ml.

The polynucleotide kinase and γ-^{32}P-ATP were kindly supplied by Drs. Olivera and Lehman (Stanford University). The malonic aldehyde diacetal was purchased from K & K Laboratories. The 2-thiobarbituric acid (TBA) was obtained from Eastman Organic Chemicals and was recrystallized three times from hot water. The ascorbic acid and ammonium molybdate were from J. T. Baker Chemical Co. and the ATP was purchased from Pabst Laboratories.

Irradiation of DNA. DNA solutions (176 μg P/ml) in 0.15 *M* NaCl (±0.001 *M* *l*-histidine), exposed to air, were irradiated with x-rays (50 kV; 50 mA; 48 mA; 0.3-mm Al filters) employing the twin-tube 50 kVp beryllium window x-ray unit developed by Loevinger and Huisman (*9*). For each set of experimental conditions, the radiation dose rate was determined by the use of ferrous sulfate dosimetry (*10*) using a *G* value of 14.8 (*9*). The dose rate was 11.1 krads/minute in the chain-break experiments, 8.66 krads/minute in the phosphate studies, 7.92 krads/minute in the deoxyribose destruction studies, and 9.40 krads/minute for the polynucleotide kinase experiments. The different dose rates employed resulted from differences in sample volumes (*9*).

Determination of single-chain breakage. Irradiated DNA solutions (0.15 ml) were denatured by the addition of an equal volume of 0.2 *N* NaOH. After a 10-minute

delay to permit denaturation of the DNA, 0.2 ml of each sample was layered on top of a 4.6-ml alkaline sucrose gradient (5–20% sucrose in 0.1 *N* NaOH and 0.01 *M* EDTA). The gradients were centrifuged at 37,000 rpm for 10.5 hours at room temperature in the SW-39 swinging bucket rotor of a Spinco Model-L ultracentrifuge. After centrifugation, 5-drop fractions were collected and diluted to about 0.3 ml by the addition of 0.18 ml of double-distilled water. The diluted fractions were then transferred to microcuvettes and the absorbance at 260 nm was read in a Gilford spectrophotometer. Data are plotted as absorbance at 260 nm (A_{260}) against fraction number. The first moment of each curve was used as a convenient index of sedimentation behavior (*1*) and the ratios of the first moments were employed in the calculation of the number of single-chain breaks produced.

Phosphate determinations. Inorganic phosphate was measured using a modification of the ascorbic acid method of Chen *et al.* (*11*). The modification consists of the initial use of 6 *N* H_2SO_4 both to precipitate the DNA and to provide the proper pH for the final development of color. This eliminates the necessity of TCA precipitation of the DNA and the further dilution of the sample.

The total phosphorus content of the DNA solutions was determined by the microphosphate method of Meun and Smith (*12*).

Liberation of inorganic phosphate and formation of phosphomonoester groups by x-irradiation. The amount of inorganic phosphate liberated by x-irradiation was determined after the irradiated DNA solutions (2 ml) were incubated for 30 minutes at 65°C in a water bath after the addition of 0.15 ml of 1 *M* Tris–HCl (pH 8.0).

The formation of phosphomonoester groups after irradiation was measured by treatment of the irradiated DNA with alkaline phosphatase [under conditions similar to those employed by Richardson and Weiss (*13*)], followed by determination of inorganic phosphate. To 2 ml of the irradiated DNA solutions was added 0.15 ml of 1 *M* Tris–HCl (pH 8.0) and the mixture was incubated for 30 minutes at 65°C with 5 μl of alkaline phosphatase [23 units/mg, 1 mg/ml; assayed as described by Garen and Levinthal (*14*)]. Another 5 μl of alkaline phosphatase was added after 15 minutes. Under these conditions internal as well as external phosphomonoesters can be removed (*13*). Inorganic phosphate was then assayed for as described in the preceding section.

Determination of the nature of end groups after chain cleavage by x-irradiation. Polynucleotide kinase (*15*, *16*) was employed to elucidate the nature of the end groups formed by DNA chain breakage. Since polynucleotide kinase specifically catalyzes the transfer of one phosphate group from ATP to the 5′-hydroxyl termini of polynucleotides, it provides a sensitive method for the identification of 5′-end groups. By employing polynucleotide kinase before and after alkaline phosphatase treatment of irradiated DNA the number of 5′-termini produced by x-irradiation was quantitatively determined. By a comparison of the results of the alkaline phosphatase studies with those of the polynucleotide kinase studies, the relative number of the 5′-phosphomonoester groups formed were estimated.

Alkaline phosphatase treatment was performed as described earlier. The alkaline phosphatase was then removed by the use of detergent and KCl precipitation as follows: an equal volume of 4% sodium lauryl sulfate (SLS) was added to the enzyme–DNA mixture and shaken gently. The mixture was heated at 65°C for 30 minutes and allowed to cool at room temperature for 30 minutes. An equal volume of 1 *M* KCl was then added to precipitate the protein and the SLS (*17*). The mixtures were shaken for 30 minutes and centrifuged at 2000 rpm for 30 minutes. The supernatant liquid was filtered through Whatman No. 42 filter paper. The filtrate was dialyzed overnight against 0.15 *N* NaCl.

The dialyzed DNA solutions were then treated with polynucleotide kinase in the following manner: an enzyme cocktail was prepared containing 0.5 ml of 1 *M* Tris (pH 7.5); 0.75 ml of 0.1 *M* $MgCl_2$; 0.125 ml 1 *M* β-mercaptoethanol; 0.05 ml 11 m*M* ATP, 0.025 ml γ^{32}P-ATP (1.08 m*M*, 1.9×10^8 cpm/μmole), and 1.05 ml H_2O. DNA samples (30 μl) were added to 25 μl of the enzyme cocktail followed by the addition of 20 μl of H_2O. To this reaction mixture 0.2 to 0.4 units (*16*) of polynucleotide kinase were added. Incubation was carried out in a 37°C water bath for 30 minutes. After incubation 0.2 ml of 0.1 *M* $Na_4P_2O_7$, 0.05 ml 10 m*M* ATP, and 0.1 ml of 0.1 *M* Na_2HPO_4 were added and the tubes were mixed. Carrier DNA (0.2 ml of 2.5 mg/ml) was added, the tubes were again mixed thoroughly, and 2.0 ml $HClO_4$ was added to precipitate the DNA. The tubes were shaken again and put in an ice bath for 10 minutes.

The samples were filtered, washed, and counted in the following manner: the solutions were washed out of the test tubes with 1 *M* HCl onto glass filters (Whatman GF/A glass paper, 2.4-cm diameter) placed on a stainless-steel vacuum filter assembly. The tubes containing the samples were then washed an additional seven times with 1 *M* HCl and the washes were filtered. The filters were washed with 10 ml of 0.1 *M* Na_2HPO_4 in 1.0 *M* HCl; 5 ml of 0.1 *M* $Na_4P_2O_7$ in 1.0 *M* HCl, 5 ml of 1.0 *M* HCl, and 7 ml 95% EtOH; mounted on pins; and dried under heat lamps. The dry filters were then placed in glass counting vials with 5 ml of toluene scintillation mixture (*18*) and the amount of ^{32}P present was determined in a liquid scintillation counter.

Detection of damage to deoxyribose by reaction with 2-thiobarbituric acid. It has been reported (*19*) that the x-irradiation of aqueous solutions of DNA produces a compound which forms a pink pigment in its reaction with 2-thiobarbituric acid (TBA). It has been postulated that this compound is malonic aldehyde and that it is formed as the result of a break in the C3′–C4′ bond in deoxyribose (*5*).

The concentration of this substance was determined by the use of the TBA reaction [*20*]. The DNA solutions (176 μg P/ml) were irradiated, and aliquots (0.4 ml) were transferred to 12-ml graduated centrifuge tubes and 0.6 ml of double-distilled water was added. Then 2.0 ml of TBA reagent (0.6%, pH 2.0) was added to the diluted samples and the contents of the tubes were mixed. The tubes with marbles placed over their mouths were heated in a boiling water bath for 20 minutes. The

tubes were cooled for 2 minutes in a water bath at room temperature. The absorbance of the samples was read at 457 nm, 496 nm, and 532 nm in a Gilford spectrophotometer. In addition, the absorption spectra of the samples between 420 and 600 nm were determined on a Beckman DB recording spectrophotometer. An aqueous solution of malonic aldehyde diacetal was employed as a standard. It was assumed that the malonic aldehyde diacetal was converted quantitatively to malonic aldehyde under the acid conditions of the reaction.

Effect of histidine. Irradiation of DNA in 0.001 *M* solutions of *l*-histidine helps to counteract the indirect effects of radiation (*21*). The effect of 0.001 *M* *l*-histidine on chain breakage, inorganic phosphate yields, phosphomonoester formation, and deoxyribose damage was studied employing the techniques described in the above sections.

Curve fitting. The data in Figs. 2–5 and 7 were fitted with straight lines passing through the origin by the method of least squares.

RESULTS

Single-chain breakage. By comparison of the position of the first moment of the x-irradiated DNA samples (Fig. 1B, C) with that of the control (Fig. 1A) it can

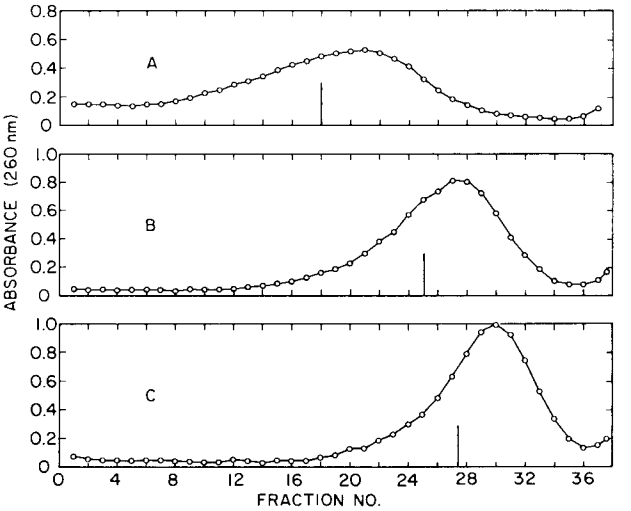

FIG. 1. Sedimentation patterns for unirradiated and x-irradiated calf thymus DNA. Samples of calf thymus DNA were layered on sucrose gradients (5–20%) containing 0.1 *N* NaOH and 0.01 *M* EDTA. Sedimentation was performed in a Spinco SW39 rotor for 10.5 hours at 37,000 rpm at room temperature. (A) unirradiated DNA; (B) DNA irradiated with 11.1 krads, and (C) DNA irradiated with 33.3 krads. The short vertical bars under the peaks indicate the position of the first moments of the distribution of A_{260}. The direction of sedimentation is from right to left.

be seen that irradiation has produced a decrease in molecular weight. To obtain a quantitative estimate of the number of breaks per molecule (N) we have employed the following relationship:

$$N = \frac{M_0}{M_r} - 1, \tag{1}$$

where M_0 and M_r are the number average molecular weights of the control and irradiated DNA respectively. The ratio (M_0/M_r) is approximated (*22*) by the relationship between sedimentation distance D and the molecular weight of the DNA (M):

$$\frac{D_r}{D_0} = \left[\frac{M_r}{M_0}\right]^a \tag{2}$$

The value of 0.40, determined by Studier (23) for alkaline-denatured DNA was employed for the exponent a. The D values were approximated by the use of normalized distance from the meniscus to the location of the first moment of each curve. Hence, by substituting the values obtained for M_0/M_r from the sedimentation distances (Eq. 2) into Eq. (1) one can directly estimate the number of single-strand breaks.

The number of single-chain breaks produced by irradiation in the presence and absence of 0.001 M *l*-histidine is given in Fig. 2. The ratio of single-chain breaks produced in the presence of histidine to the number produced by irradiation in the absence of histidine was 0.39.

Liberation of inorganic phosphate and formation of phosphomonoester groups. The liberation of inorganic phosphate after various doses of irradiation in the presence and absence of histidine is illustrated in Figs. 3 and 4. These figures also show the yields of inorganic phosphate produced by x-irradiation and alkaline phosphatase

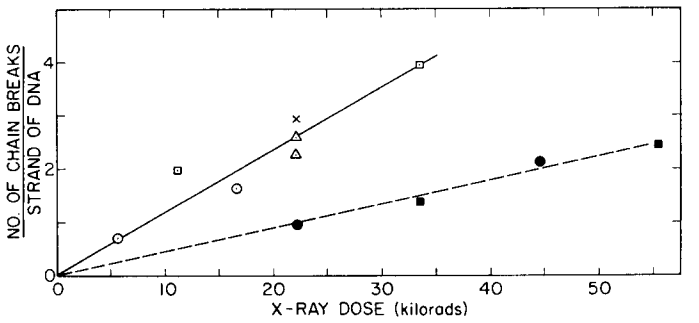

FIG. 2. Production of single-chain breaks in x-irradiated calf thymus DNA (176 μg P/ml). Irradiated in 0.15 *M* NaCl in the absence of *l*-histidine (——); irradiated in 0.15 *M* NaCl and 0.001 *M* *l*-histidine (– – –). The different symbols indicate separate experiments.

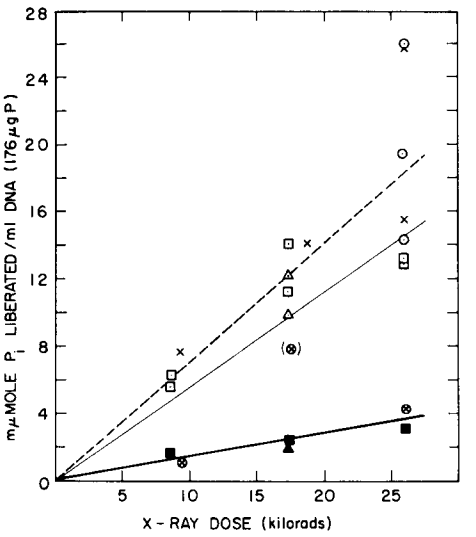

FIG. 3. X-ray-induced liberation of inorganic phosphate and the formation of phosphomonoester groups in the absence of *l*-histidine. Inorganic phosphate yield after irradiation (——); inorganic phosphate yield after irradiation and alkaline phosphatase treatment (– – –); phosphate present in phosphomonoester groups (obtained from difference in the slopes of above two curves) (——). Inorganic phosphate yields of 3.58 mμmoles P_i/ml DNA (176 μg P) and 1.22 mμmoles P_i/ml DNA (176 μg P) where obtained for the unirradiated control samples treated and untreated with alkaline phosphatase, respectively. The inorganic phosphate liberated from the unirradiated samples has been subtracted from all subsequent samples so that the values plotted represent the amounts liberated due to the irradiation alone. The methods are described in the text. The different symbols indicate separate experiments. The point (⊗) was not employed in the calculation of the slope of the bottom curve.

treatment. The amount of phosphomonoester groups produced by x-irradiation was obtained by subtracting the yield of inorganic phosphate produced by irradiation alone from that produced by irradiation plus alkaline phosphatase treatment. The slopes of the various lines in Figs. 3 and 4 are presented in Table 1. The ratios of the yields of inorganic phosphate and phosphomonoester groups in the presence and absence of histidine are also given.

The number of 5′-hydroxyl and 5′-phosphoryl termini formed by x-irradiation. The nature of the 5′-termini after x-irradiation was determined in a manner similar to that employed by Richardson (*24*) in his determination of the nature of the end groups produced by the sonic irradiation of DNA. The moles of acid-insoluble ^{32}P added by the polynucleotide kinase treatment of alkaline phosphatase-treated irradiated DNA represents the total number of 5′-termini present. The acid-insoluble ^{32}P added by the polynucleotide kinase treatment of irradiated DNA (no alkaline phosphatase treatment) represents the number of 5′-hydroxyl termini pro-

duced. By subtracting the yield of 5′-hydroxyl termini from the yield of total 5′-termini the yield of 5′-phosphoryl termini is determined (Fig. 5). By dividing the slope of the curve for production of 5′-OH termini [6.01 × 10^{-3} mμmoles ^{32}P added/ml DNA (176 μg P)/krad] by that for the production of total 5′-termini [1.71 ×

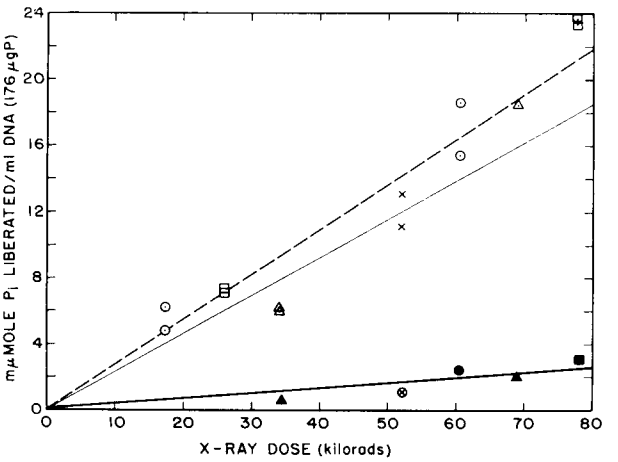

FIG. 4. X-ray-induced liberation of inorganic phosphate and the formation of phosphomonoester groups in the presence of 0.001 *M* *l*-histidine. Inorganic phosphate yield after irradiation (——); inorganic phosphate yield after irradiation and alkaline phosphatase treatment (– – –); phosphate present in phosphomonoester groups (obtained from difference in slopes of above two curves) (——). Inorganic phosphate yields of 0.774 mμmoles P_i/ml DNA (176 μg P) and 1.24 mμmoles P_i/ml DNA (176 μg P) were obtained for the unirradiated control samples treated and untreated with alkaline phosphatase, respectively. The inorganic phosphate liberated from the unirradiated samples has been subtracted from all subsequent samples so that the values plotted represent the amounts liberated due to irradiation alone. The methods are described in the text. The different symbols indicate different experiments.

TABLE I

X-RAY-INDUCED LIBERATION OF INORGANIC PHOSPHATE AND THE FORMATION OF PHOSPHOMONOESTER GROUPS IN THE PRESENCE AND ABSENCE OF 0.001 *M* *l*-HISTIDINE

Treatments performed	*Slope (mμmole P/ml DNA (176 μg P)/krad)*		*Slope ratio* = $\frac{\text{with histidine}}{\text{without histidine}}$
	Absence of histidine	*Presence of histidine*	
Inorganic phosphate yield after irradiation	0.14	0.03	0.24
Inorganic phosphate yield after irradiation and treatment with alkaline phosphatase	0.70	0.27	0.38
Phosphomonoester group formation	0.56	0.24	0.42

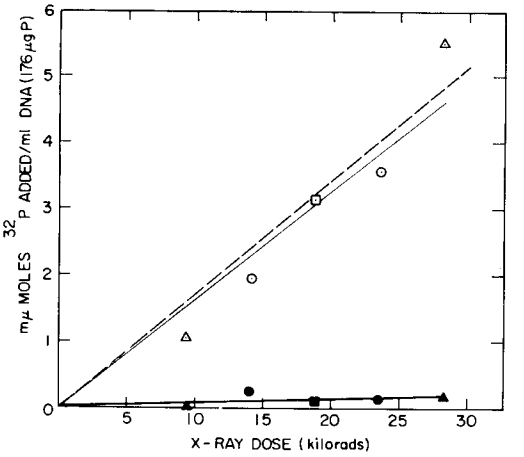

FIG. 5. The x-ray-induced formation of 5′ phosphoryl and 5′ hydroxyl termini as measured by ^{32}P uptake subsequent to polynucleotide kinase treatment. 5′-OH termini produced by irradiation (——); total number of 5′ termini formed by irradiation (5′-OH + 5′-PO_4) (– – –); 5′-PO_4 termini produced by irradiation (obtained from the difference in the slopes of the above two curves) (——). The above values have been corrected for the ^{32}P-phosphorylation in unirradiated DNA controls. The methods are described in the text. The different symbols indicate different experiments.

10^{-1} mμmoles ^{32}P added/ml DNA (176 μg P)/krad] the relative percentage of 5′-termini that are 5′-OH is found to be 3.5%.

Distribution of phosphomonoester groups formed in DNA by x-irradiation. The relative percentage of 5′-phosphomonoester groups formed can readily be determined from the results presented in the two preceding sections. From Fig. 5, the slope of the curve for the production of 5′-phosphoryl termini is 0.165 mμmole P produced/ml DNA (176 μg P)/krad. By comparison of this value with the slope of the curve for the production of phosphomonoester groups (0.563 mμmole P liberated/ml DNA (176 μg P)/krad; Fig. 3) it can be seen that approximately 29% of the total phosphomonoester groups formed during irradiation are 5′-phosphoryl esters.

Since cyclization of the phosphomonoester groups (25) and fragmentation of the sugar residues might occur as a consequence of irradiation, one can not be certain that the other phosphomonoesters are all 3′-phosphomonoesters. Additional studies, perhaps employing a specific 3′-phosphomonoesterase such as exonuclease III, will be necessary to identify the nature of the 3′-termini.

Detection of radiation damage to deoxyribose by reaction with TBA. Figure 6 illustrates the absorption spectrum of the product produced by the reaction of TBA with (A) malonic aldehyde diacetal, (B) unirradiated calf thymus DNA, and (C)

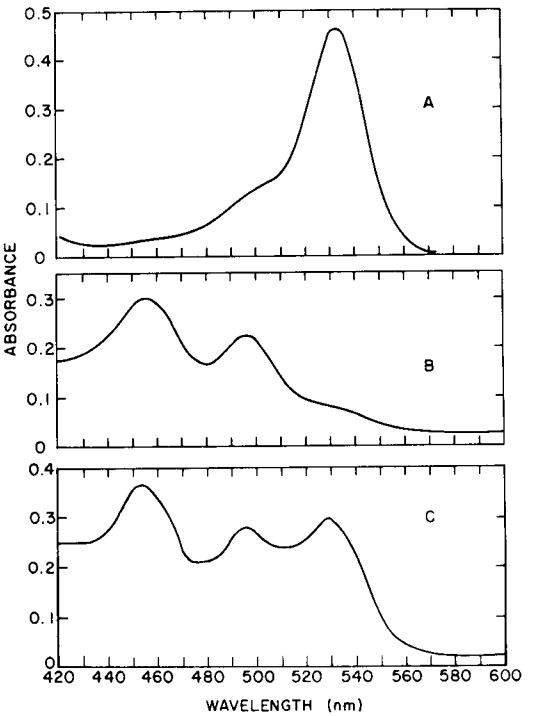

FIG. 6. Absorption spectra obtained for the reaction of DNA and malonic aldehyde diacetal with 2-thiobarbituric acid. (A) 2 μg of malonic aldehyde diacetal, (B) 1 ml of unirradiated calf thymus DNA (176 μg P/ml); and (C) 0.4 ml of calf thymus DNA (176 μg P/ml) irradiated with 23.8 krad (absorbance multiplied by 2.5). The methods are described in the text.

irradiated calf thymus DNA. Our absorption spectrum for the malonic aldehyde diacetal-TBA reaction product agrees with the spectra previously reported for the malonic aldehyde-TBA complex (*26*, *27*). The absorption spectrum of the irradiated DNA showed the formation of the characteristic absorption peak at 532 nm as reported by Krushinskaya and Shal'nov (*5*). The height of the peak at 532 nm increased with increasing radiation dose and the peak was not present in the unirradiated sample. However, two additional absorption peaks were seen at 457 nm and 496 nm for unirradiated DNA. The height of both of these peaks increased with increasing radiation dose.

By comparison of the absorbance of the irradiated DNA samples at 532 nm with that of the malonic aldehyde diacetal standards (both treated with TBA), one can determine the amount of malonic aldehyde formed in irradiated DNA. Yields of malonic aldehyde were determined assuming 1 μg of malonic aldehyde diacetal (molecular weight taken to be 164.2) corresponds to 0.438 μg malonic aldehyde.

However, the slope of the standard curve, reproducible within ±2.5%, was approximately 73% of that reported in the literature (0.517 vs 0.709 $A_{532}/\mu g$) (*10*). We are at present unable to explain this discrepancy. Figure 7 shows the malonic aldehyde yields for DNA irradiated in the presence and absence of 0.001 *M* *l*-histidine. The slope in the absence of histidine is 0.25 mμmole malonic aldehyde produced/ml DNA (176 μg P)/krad. The slope ratio of malonic aldehyde yield in the presence of histidine to that in the absence of histidine is 0.41.

It should be noted that malonic aldehyde is not split free from the DNA as an immediate consequence of the irradiation. If one irradiates the DNA sample and alcohol precipitates it, malonic aldehyde can be quantitatively recovered by reacting the washed and resuspended precipitate with the TBA reagent. Hence, some combination of the acid reaction conditions, heating, and the presence of TBA is necessary for the liberation of malonic aldehyde and the production of the color.

Estimation of number of chain breaks per ml DNA (176 μg P). To quantitatively compare the yield of phosphomonoester groups, inorganic phosphate, and malonic aldehyde with the production of single chain breaks, the values for chain breaks determined from the alkaline sucrose gradient studies (chain break/strand DNA/krad) must be converted to chain breaks/ml DNA (176 μg P)/krad. To be able to make this conversion, the number of strands of DNA present per ml DNA must

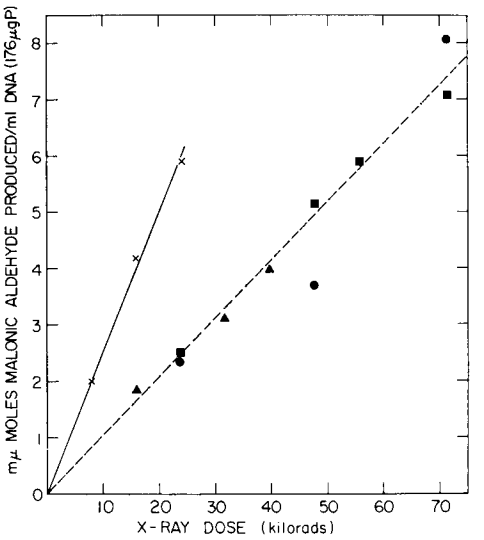

FIG. 7. Liberation of malonic aldehyde from irradiated DNA as determined by its reaction with 2-thiobarbituric acid. DNA irradiated in absence of *l*-histidine (——); DNA irradiated in the presence of *l*-histidine (– – –). The methods are described in the text. The different symbols indicate separate experiments.

TABLE II

PRODUCTION OF SINGLE-STRAND BREAKS IN DNA BY X-IRRADIATION

DNA employed	*Solvent*	*Number of single-strand breaks/rad/10^6 daltons*
Calf thymus DNA (2.5 mg/ml)	0.15 *M* NaCl	2.37×10^{-4}
B3 DNA (20 μg/ml)	0.01 *M* PO_4 buffer (pH 7.8)	3.3×10^{-4} [a]
Calf thymus DNA (2.5 mg/ml)	0.15 *M* NaCl, 10^{-3} *M* *l*-histidine	0.92×10^{-4}
B3 DNA (20 μg/ml)	0.01 *M* PO_4 buffer, 10^{-3} *M* *l*-histidine	1.25×10^{-4} [a]

[a] Data of Freifelder (30).

first be calculated from a knowledge of the number average chain length of the DNA molecules present.

An estimate of the average chain length was determined by the ratio of terminal phosphorus to total phosphorus (*28*). Terminal phosphorus was determined by treating unirradiated DNA with alkaline phosphatase. The value obtained was 0.11 μg P/ml DNA. Using a value of 176 μg P/ml DNA for the total phosphorus content, we obtained an average chain length of 1586 nucleotides/strand of DNA. The number of strands/ml DNA is equal to the total number of nucleotides present/ml DNA (176 μg P) divided by the nucleotides/strand, or 2.16×10^{15}.

For DNA irradiated in absence of histidine (Fig. 2), there were 0.12 chain breaks/strand DNA/krad and 2.50×10^{14} chain breaks/ml DNA (176 μg P)/krad. For DNA irradiated in the presence of 0.001 *M* and *l*-histidine, there were 0.045 chain breaks/strand DNA (176 μg P)/krad, and 0.97×10^{14} chain breaks/ml DNA (176 μg P)/krad. Employing a value of 308.6 for the average molecular weight of a monomeric unit of calf thymus DNA (*29*), the production of single-chain breaks can be expressed in terms of number of breaks/rad/10^6 molecular weight. These results are presented in Table II. The values obtained by Freifelder (*30*) for single-chain breakage in DNA isolated from bacteriophage B3 are presented for comparison.

DISCUSSION

The results of this series of investigations permit us to compare quantitatively the number of single-chain breaks with the yields of inorganic phosphate, phosphomonoester groups and sugar damage. Such a comparison is given in Table III, in which G values for the various products are also tabulated. Table IV gives G values taken from the literature for similar measurements. It should be kept in mind, however, that G values depend strongly on conditions of irradiation, e.g., pH, presence of buffer, concentration, etc., so that the G values taken from the literature cannot be compared exactly with the G values obtained in these investigations.

For every single-chain break produced when DNA is irradiated in the absence of histidine approximately 0.60 malonic aldehyde-like molecules were produced; 1.35 phosphomonoester groups were formed; and 0.33 molecules of inorganic phosphate were liberated. These data roughly fit the scheme for the production of single-chain breaks proposed by Krushinskaya and Shal'nov (*5*) (see Fig. 8). These authors hypothesize that malonic aldehyde is formed by cleavage of the C3′–C4′ bonds of

TABLE III

EFFECTS OF IONIZING RADIATION ON DNA IN AQUEOUS SOLUTION

Effect on DNA	*Number of breaks or molecules formed/ml DNA (176 μg P)/krad*[a]		*G value*		
	No histidine	*10^{-3} M Histidine*	*No histidine*	*10^{-3} M Histidine*	*Ratio: $\frac{\text{with histidine}}{\text{without histidine}}$*
No. single-strand breaks	2.50×10^{14}	0.97×10^{14}	0.40	0.16	0.39
Inorganic phosphate yield	0.84×10^{14}	0.20×10^{14}	0.13	0.03	0.24
Total inorganic phosphate yield after irradiation and alkaline phosphatase treatment	4.23×10^{14}	1.63×10^{14}	0.68	0.26	0.38
Phosphomonoester group production	3.39×10^{14}	1.43×10^{14}	0.54	0.23	0.42
Deoxyribose damage[b]	1.52×10^{14}	0.62×10^{14}	0.24	0.10	0.41

[a] Irradiation performed in air.

[b] Assayed by reaction with 2-thiobarbituric acid (a test for the production of malonic aldehyde).

TABLE IV

G VALUES FROM LITERATURE FOR EFFECTS OF IONIZING RADIATION ON DNA IN AQUEOUS SOLUTION

Change in DNA	*Concn DNA (%)*	*Method*	*G value*	*Reference*
Single-strand breakage	0.43	Light scattering	1.5	(*33*)
	0.1	Viscosity and	0.61	(*34*)
	0.5	sedimentation	0.83	
	1.0		0.40	
	5.0		0.31	
	10.0		0.32	
	50.0		0.22	
	80.0		0.43	
Yield of inorganic phosphate	0.2		0.05	(*35*)
	1.0		0.09	
	0.15		0.04	(*19*)
Phosphomonoester formation	0.1	Acid phosphatase	0.4	(*4*)
	0.5	treatment	0.8	
Yields of malonic aldehyde	0.015	TBA reaction	0.1	(*5*)

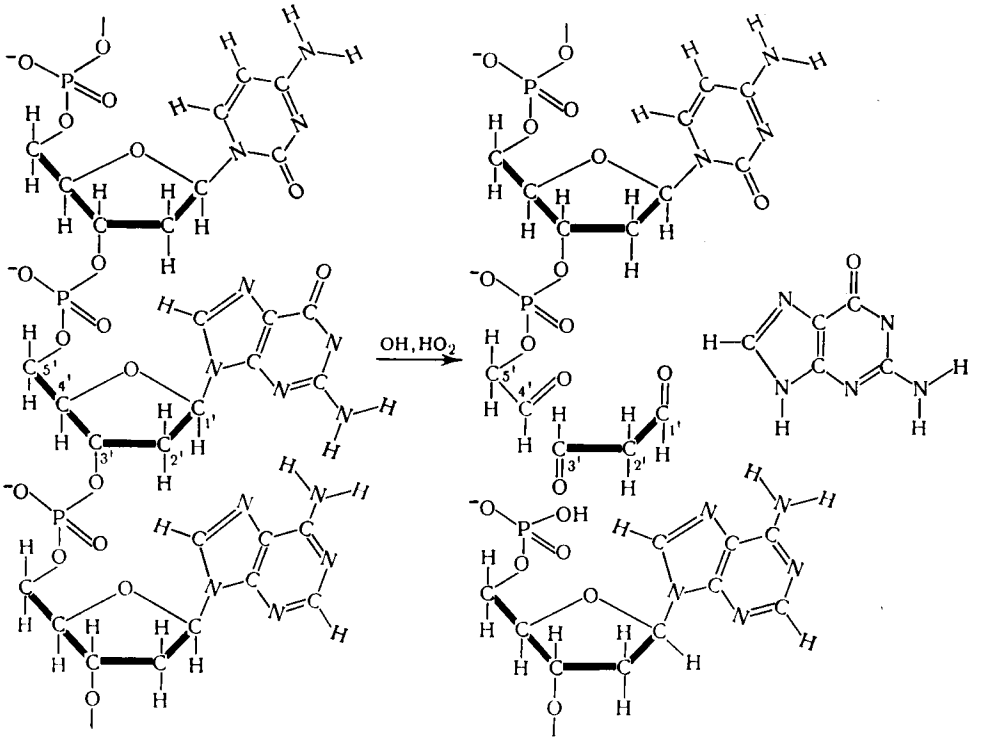

FIG. 8. Hypothetical chemical reaction which produces breaks in the DNA chain, with the formation of malonic aldehyde [from Krushinskaya and Shal'nov (*5*)].

deoxyribose producing single-chain breaks in DNA. This reaction also leads to the splitting of glycoside bonds, with the release of bases. (It should be noted that the sum of the G values for base release from irradiated DNA solutions is 0.228 (*31*) which is in excellent correspondence with the G value of 0.24 that we have determined for malonic aldehyde production). It has also been postulated (*32*) that inorganic phosphate is released by the labilizing effect that C3′-oxidation has on the 5′-phosphate group. If all of the free phosphate arises from the liberation of 5′-phosphate groups one would then expect the number of 5′-hydroxyl groups to equal the yield of inorganic phosphate. However, the yield of inorganic phosphate was found to be in excess of the 5′-OH yield and so some other mechanism must be proposed to explain the yield of inorganic phosphate. Also, if the proposed scheme represents the complete story of chain breakage, the yield of malonic aldehyde would be expected to equal the yield of single chain breaks, but this was not found to be so.

The effects of 0.001 M l-histidine on the x-irradiation of aqueous solutions of DNA are summarized in Table III. The value of 0.39 for reduction of single-chain

breaks in DNA is in excellent agreement with the value of 0.38 reported by Freifelder (*30*) for the irradiation of free phage DNA in buffer vs buffered histidine (0.001 *M*).

Additional investigations into alternative mechanisms of sugar damage and chain breakage are in progress. Once the nature of chain breakage is known, the biochemical requirements for repair of x-irradiated DNA may then be better understood.

RECEIVED: July 14, 1969

REFERENCES

1. H. S. KAPLAN, DNA-strand scission and loss of viability after x-irradiation of normal and sensitized bacterial cells. *Proc. Natl. Acad. Sci. U.S.* **55**, 1142–1446 (1966).
2. R. A. MCGRATH and R. W. WILLIAMS, Reconstruction *in vivo* of irradiated *Escherichia coli* deoxyribonucleic acid; the rejoining of broken pieces. *Nature* **212**, 534–535 (1966).
3. G. SCHOLES and J. WEISS, Chemical action of x-rays on nucleic acids and related substances in aqueous systems. *Exptl. Cell Res., Suppl.* **2**, 219–244 (1952).
4. B. COLLYNS, S. OKADA, G. SCHOLES, J. J. WEISS, and C. M. WHEELER, Chain scission and hydrogen bond breakage on irradiation of DNA. *Radiation Res.* **25**, 526–536 (1965).
5. N. P. KRUSHINSKAYA and M. I. SHAL'NOV, Nature of breaks in the DNA chain upon irradiation of aqueous solutions. *Radiobiology* **7**, 36–45 (1967). (English translation by U.S.A.E.C.)
6. M. DANIELS, G. SCHOLES, and J. WEISS, Chemical action of ionizing radiations in solutions. XVI. Formation of labile phosphate esters from purine and pyrimidine ribonucleotides by irradiation with x-rays in aqueous solution. *J. Chem. Soc. London* 3771–3779 (1956).
7. J. WEISS, Chemical effects of ionizing radiations on nucleic acids and related compounds. In *Progress in Nucleic Acid Research and Molecular Biology* (J. N. Davidson and W. E. Cohn, eds.), Vol. 3, p. 136. Academic Press, New York (1964).
8. D. S. KAPP and K. C. SMITH, Lack of *in vitro* repair of x-ray induced chain breaks in DNA by the polynucleotide-joining enzyme. *Intern. J. Radiation Biol.* **14**, 567–571 (1968).
9. R. LOEVINGER and P. HUISMAN, A twin-tube 50-Kvp beryllium-window x-ray unit for microbial radiobiology. *Radiation Res.* **24**, 357–367 (1965).
10. J. WEISS, A. O. ALLEN, and H. A. SCHWARZ, Use of the Fricke ferrous sulfate dosimeter for gamma-ray doses in the range 4 to 40 Kr. *Proc. Intern. Conf. Peaceful Uses Atomic Energy*, 1st Geneva, 1955 **14**, 179–181 (1956).
11. P. S. CHEN, JR., T. Y. TORIBARA, and H. WARNER, Microdetermination of phosphorus. *Anal. Chem.* **28**, 1756–1758 (1956).
12. D. H. C. MEUN and K. C. SMITH, A micro phosphate method. *Anal. Biochem.* **26**, 364–368 (1968).
13. C. C. RICHARDSON and B. WEISS, Enzymatic breakage and joining of deoxyribonucleic acid. I. Repair of single-strand breaks in DNA by an enzyme system from *Escherichia coli* infected with T4 bacteriophage. *Proc. Natl. Acad. Sci. U.S.* **57**, 1021–1028 (1967).
14. A. GAREN and C. LEVINTHAL, A fine-structure genetic and chemical study of the enzyme alkaline phosphatase of *E. coli*. I. Purification and characterization of alkaline phosphatase. *Biochim. Biophys. Acta* **38**, 470–483 (1960).
15. A. NOVOGRODSKY and J. HURWITZ, The enzymatic phosphorylation of ribonucleic acid and deoxyribonucleic acid. I. Phosphorylation at 5′-hydroxyl termini. *J. Biol. Chem.* **241**, 2923–2933 (1966).

16. C. C. RICHARDSON, Phosphorylation of nucleic acid by an enzyme from T4 bacteriophage-infected *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.* **54**, 158–165 (1965).

17. K. C. SMITH, Dose dependent decrease in extractability of DNA from bacteria following irradiation with ultraviolet light or with visible light plus dye. *Biochem. Biophys. Res. Commun.* **8**, 157–163 (1962).

18. R. J. MANS and G. D. NOVELLI, A convenient, rapid and sensitive method for measuring the incorporation of radioactive amino acids into protein. *Biochem. Biophys. Res. Commun.* **3**, 540–543 (1960).

19. M. I. SHAL'NOV and N. P. KRUSHINSKAYA, Najboleje verojatnyje radikalnyje sostojanija v cepjach nukleinovych kislot pri oblucenii vodnych rastvorov. *Tr. MOIP* (*Trans. Moscow Soc. Naturalists*) **16**, 99–105 (1966).

20. V. S. WARAVDEKAR and L. D. SASLAW, A sensitive colorimetric method for the estimation of 2-deoxy sugars with the use of the malonaldehyde—thiobarbituric acid reaction. *J. Biol. Chem.* **234**, 1945–1950 (1959).

21. D. FREIFELDER, Mechanism of inactivation of coliphage T7 by x-rays. *Proc. Natl. Acad. Sci. U.S.* **54**, 128–134 (1965).

22. E. BURGI and A. D. HERSHEY, Sedimentation rate as a measure of molecular weight of DNA. *Biophys. J.* **3**, 309–321 (1963).

23. F. W. STUDIER, Sedimentation studies of the size and shape of DNA. *J. Mol. Biol.* **11**, 373–390 (1965).

24. C. C. RICHARDSON, The 5′-terminal nucleotides of T7 bacteriophage deoxyribonucleic acid. *J. Mol. Biol.* **15**, 49–61 (1966).

25. C. R. BAYLEY, K. W. BRAMMER, and A. S. JONES, The nucleotide sequence in deoxyribonucleic acids. V. The alkaline degradation of apurinic acids. *J. Chem. Soc. London* 1903–1907 (1961).

26. S. PATTON and G. W. KURTZ, 2-Thiobarbituric acid as a reagent for detecting milk fat oxidation. *J. Dairy Sci.* **34**, 669–674 (1951).

27. W. G. JENNINGS, W. L. DUNKLEY, and H. G. REIBER, Studies of certain red pigments formed from 2-thiobarbituric acid. *Food Res.* **20**, 13–22 (1955).

28. E. SEAMAN, Chain length determination by end to total phosphorus. In *Methods in Enzymology* (L. Grossman and K. Moldaue, eds.), Vol. XIIB pp. 218–220. Academic Press, New York (1968).

29. U. HAGEN, Bestimmung von Einzel- und Doppelbrüchen in bestrahlter Desoxyribonukleinsäure durch die Molekular-Gewichtsverteilung. *Biochim. Biophys. Acta* **134**, 45–58 (1967).

30. D. FREIFELDER, DNA strand breakage by x-irradiation. *Radiation Res.* **29**, 329–338 (1966).

31. G. HEMS, Chemical effects of ionizing radiation on deoxyribonucleic acid in dilute aqueous solution. *Nature* **186**, 710–712 (1960).

32. M. I. SHAL'NOV, Radiacionno-chimiceskije povrezdenija DNK *in vitro* i *in vivo*. *Nukleinovyse Kysloty*, 28–44 (1967).

33. A. R. PEACOCKE and B. N. PRESTON, The action of x-rays on sodium deoxyribonucleate in solution. II. Degradation. *Proc. Roy. Soc. Ser. B* **153**, 90–102 (1960).

34. U. HAGEN, Untersuchungen über die Strahlenempfindlichkeit derDeoxyribonukleinsäure, II. Die Länge der Nukleotidketten nach Bestrahlung *in vitro*. *Strahlentherapie* **124**, 428–437 (1964).

35. J. SCHOLES and J. WEISS, Chemical action of x-rays on nucleic acids and related substances in aqueous systems. I. Degradation of nucleic acids, purines, pyrimidines, nucleosides and nucleotides by x-rays and by free radicals produced chemically. *Biochem. J.* **53**, 567–578 (1953).