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END GROUP DETERMINATION IN γ-IRRADIATED DNA

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SUMMARY

Calf thymus DNA was irradiated in dilute solution with ^{60}Co-γ-rays. The number of single-strand breaks was determined by sedimentation analysis. In comparison the frequency of 5'-OH end groups was measured by specific phosphorylation with polynucleotide kinase. By applying this method to DNA before and after incubation with phosphomonoesterase, the frequency of 5'-phosphate groups besides the 5'-OH groups were obtained. The radiation-induced single breaks amount to about 90 % 5'-phosphate end groups. The number of breaks and simultaneously the number of 5'-phosphate groups rise after alkaline treatment of irradiated DNA (30 min 1 N NH_4OH) by a factor of about 1.5. The mechanism of chemical reactions leading to the single-strand break in DNA is discussed.

INTRODUCTION

Ionizing radiation causes breaks of the nucleotide chain in DNA. These breaks play an important role in the inactivation of biological functions of DNA. Single-strand breaks inhibit the RNA synthesis along the chain[1], whereas double-strand breaks are closely related to the inactivation of bacteria and double-strand viruses[2]. To obtain information on the mechanism involved in the breakage, a biochemical analysis of the end groups of the split chain was performed.

Corresponding to the liberation of phosphate in irradiated mononucleoside 5-phosphate[3], an oxidation of C-5' in deoxyribose of the DNA may lead to the formation of an unstable acyl phosphate and hence to a splitting of the nucleotide chain with the formation of a 3'-phosphate end group. In a similar way a breakage may occur by an oxidation of C-3', resulting in 5'-phosphate end groups. Further, we have to consider that irradiated DNA is not stable at pH values higher than 7, *i.e.* in alkaline solution new terminal phosphate groups are formed[4]. This may be due either to the release of an oxidized terminal sugar from the phosphate or to the fact that new breaks are formed during alkaline treatment[5].

The end groups in DNA may be characterized by polynucleotide kinase which phosphorylates specifically the 5'-OH groups:

$$5'\text{—OH—DNA} + [\gamma\text{-}^{32}\text{P}]\text{ATP} \xrightarrow{\text{enzyme}} [5'\text{-}^{32}\text{P}]\text{DNA} + \text{ADP}$$

Terminal 5'-phosphate groups can also be determined with this enzyme if DNA is pretreated with phosphomonoesterase (EC 3.1.3.2). This analysis was done before and after alkaline treatment of irradiated DNA. For comparison the frequency of single breaks was determined under these conditions.

MATERIALS AND METHODS

ATP was obtained from Boehringer, Mannheim, and [γ-^{32}P]ATP from Radiochemical Centre, Amersham. Each batch of ATP was tested chromatographically for the absence of hydrolytic products. Spleen acid phosphomonoesterase (EC 3.1.3.2) was a gift from Dr. Bernardi, Strasbourg. The preparation was free of exonucleases and endonucleases. Micrococcus nuclease (EC 3.1.4.7) was purchased from Worthington BC (Code N.F.C.P.). *Escherichia coli* B/1 and T4 bacteriophage were obtained from Dr. Hotz of this institute.

Polynucleotide kinase was isolated from *E. coli* B/1 20 min after infection with bacteriophage T 4, according to RICHARDSON[6]. The fractions used (Fraction V, ref. 6) had a specific activity of about 4000 and 8000 units/mg (defined according to ref. 6). The absence of nuclease activity was tested by incubation (30 min) of ΦX-174 DNA with our enzyme preparations. No breaks of the circular DNA could be detected. Furthermore, an addition of 3'-OH DNA, prepared by degradation with pancreatic deoxyribonuclease I (EC 3.1.4.5), had no influence on the incorporation of ^{32}P into the 5'-OH. Consequently, 3'-OH DNA was not effective as a competitive substrate, and any disturbing activities of nucleases or phosphatases leading to 5'-OH groups must be negligible.

DNA preparations

Calf thymus DNA was isolated according to KAY *et al.*[7]. It contained less than 1 % protein. 5'-OH DNA was prepared by partial degradation with Micrococcus nuclease according to NOVOGRODSKY AND HURWITZ[8] (about 25 % conversion to acid-soluble products). The DNA solutions were irradiated in glass stoppered tubes after N_2 saturation in a ^{60}Co-γ-source (Gammacell 220, Atomic Energy of Canada, Ltd.). The alkaline treatment of the DNA samples was performed by a 30-min incubation at 37° in 1 M NH_4OH. The DNA solution was neutralized by dialysis against 0.025 M phosphate buffer, pH 6.7, whereby the buffer was changed several times. In this way, aggregation of DNA molecules which occur after neutralisation with strong acids can be avoided. Subsequently, the DNA solutions were dialysed against the desired solvent (0.01 M NaCl, pH 7, or 0.2 M KCl, pH 7). To split off the 5'-phosphate groups, the DNA was incubated with phosphomonoesterase (0.025 unit/ml in 0.05 M acetate buffer, pH 5.35) for 15 h at 37°. After inactivation of the phosphomonoesterase by repeated shaking with chloroform–isoamylalcohol (5:1, v/v), the DNA solution was dialysed against 0.2 M KCl.

Chain length of DNA and frequency of breaks

The molecular weight of the DNA was determined by sedimentation in an analytical ultracentrifuge (Spinco E, Beckman). Centrifugation was performed at 24 000 or 30 000 rev./min; the concentration distribution of the denatured DNA was determined by the scanning system at 265 nm. The denaturation of DNA for sedimentation analysis was done with alkali and formaldehyde according to DAVISON *et al.*[9]. The DNA was dissolved in 0.2 M NaCl+0.025 M phosphate buffer (pH 7.3)+2 % formaldehyde. Some of our DNA samples were centrifuged in 0.01 M NaCl. From the shape of the boundary the distribution of sedimentation coefficients $s^\circ_{20,w}$ was calculated and transformed into a molecular weight distribution which allows the

computation of the weight average molecular weight M_w and the number average molecular weight M_n. Details of this procedure have been described previously[10,11,12]. From the molecular weight of the irradiated DNA samples the frequency of single-strand breaks per nucleotide (B_1) was calculated (*cf.* ref. 10).

Assay of polynucleotide kinase and labelling of 5'-OH DNA

The standard assay for polynucleotide kinase was accomplished according to RICHARDSON[6], except that 80 nmoles ATP were used per 0.3 ml incubation mixture. The reaction was stopped with 0.1 ml conc. $Na_4P_2O_7$. After the addition of 0.1 ml herring sperm DNA (1 mg/ml) and 0.5 ml trichloroacetic acid (1 M), the precipitate was collected on membrane filters (Sartorius SM 11303 and SM 11309) and thoroughly washed with 1% trichloroacetic acid. The samples were counted in a liquid-scintillation spectrometer (Tricarb, Packard).

When 5'-OH-DNA was incubated without enzyme, the acid insoluble material contained 0.05–0.1 % of the added radioactivity. For complete labelling of the 5'-OH groups, an excess of polynucleotide kinase was added (about 5 units of enzyme per 0.3 ml of incubation mixture). It was checked that the incorporation of ^{32}P into the precipitate did not increase during prolonged incubation. From the ratio of the incorporated ^{32}P to the total phosphorus content of the DNA the number of terminal [5'-^{32}P]phosphate groups per nucleotide was calculated.

To show the accuracy of our end group determination the frequency of 5'-OH groups in enzymatically generated 5'-OH-DNA was determined by polynucleotide kinase and compared with the molecular weight of these samples from sedimentation analysis. For instance, a sample of denatured 5'-OH-DNA had a number average molecular weight $M_n = 3.4 \cdot 10^4$, *i.e.* a chain length of 110. In this DNA 0.94 ± 0.068 nmole ^{32}P were incorporated per 100 nmoles total phosphorus which equals one 5'-OH group per 106 nucleotides.

RESULTS

Frequency of single breaks before and after alkaline treatment

The frequency of single-strand breaks in native DNA can be measured only after separation of the strands by denaturation. Since any kind of denaturation, heat or alkali, may lead to secondary reactions in the irradiated DNA, single-stranded DNA has to be irradiated in order to determine the number of primary single breaks, *i.e.* the number of breaks without further treatment of the DNA. To examine whether the number of breaks in double-stranded DNA was comparable to that in single-stranded DNA, both forms were irradiated and treated with alkali and formaldehyde before sedimentation.

As shown in Fig. 1a the breakage rate in the single-stranded DNA was slightly higher than in native DNA. From the frequency of single-strand breaks B_1 at various doses the G value (G_{B_1} = number of single-strand breaks per 100 eV absorbed energy) was calculated. After irradiation of native DNA, G_{B_1} amounted to 0.37, irradiation of denatured DNA led to $G_{B_1} = 0.41$. These G values are close to the G_{B_1} value of 0.395 determined previously[10].

To determine the number of primary single-strand breaks *i.e.* without alkaline treatment, DNA was denatured by heat in 0.01 M NaCl, irradiated and analysed in

the ultracentrifuge in 0.01 M NaCl. An aliquot was treated with alkali, dialysed against 0.01 M NaCl and analysed for single breaks in the same way. As shown in Fig. 1b there was a distinct increase in the frequency of breaks after alkaline treatment by a factor of about 1.5. Average values of G_{B_1} of 0.22 and 0.33, respectively, were calculated from the slope of the curves.

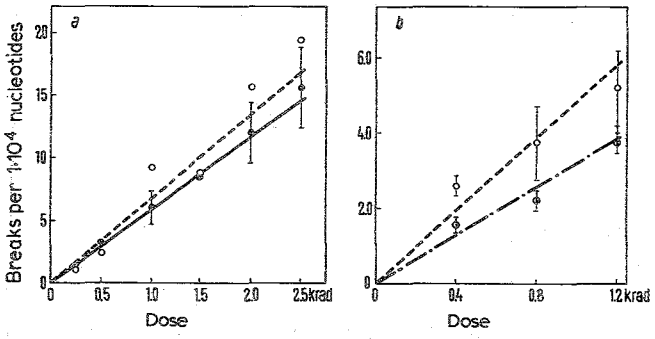

Fig. 1. Frequency of single breaks in irradiated DNA. a. Calf thymus DNA was irradiated at 200 μg/ml in 0.01 M NaCl either in the native or in the denatured state. Denaturation was performed by 10 min of heating to 90°, then cooling to 0°. After irradiation the DNA was treated with alkali and formaldehyde before the frequency of single breaks (B_1) was determined as described under methods. ●, irradiation of native DNA; ○, irradiation of denatured DNA. b. Calf thymus DNA was denatured by heating and irradiated at 200 μg/ml in 0.01 M NaCl. After irradiation the DNA was analysed in the ultracentrifuge either without further treatment or treated with alkali as described in the text. ◑, irradiation of denatured DNA, no further treatment; ○, irradiation of denatured DNA, treatment with alkali.

In irradiated double-stranded DNA alkaline treatment may have the same effect. Since the G_{B_1} value after alkaline denaturation amounted to 0.37 (*cf.* Fig. 1a), it can be assumed that the G_{B_1} value for the primary single breaks in native DNA was about 0.25.

Determination of terminal groups by polynucleotide kinase

After maximum phosphorylation only 0.04 % of the added radioactivity was incorporated into non-irradiated DNA. This value lies in the range of control values after incubation without any enzyme. The number of 5′-end groups in irradiated DNA is shown in Fig. 2. Without further treatment of the DNA samples the 5′-OH groups in the primary breaks are measured. Their amount was very small and there was only a slight increase with dose. After alkaline treatment of the DNA, the number of 5′-OH groups rose by a factor of about two.

Much more ^{32}P was incorporated after dephosphorylation of the irradiated DNA indicating the formation of new 5′-OH groups. This value represents the number of terminal 5′-phosphate groups before treatment with phosphomonoesterase. The frequency of 5′-phosphate groups rose linearly with the dose up to 50 krad. Examining in the same way DNA samples which were pretreated with alkali, an additional increase of the 5′-phosphate groups was observed.

Simultaneously with these measurements, the molecular weight of some of the alkali-treated denatured DNA samples was determined and the frequency of single breaks was calculated (Fig. 2). The numbers of single breaks are comparable with

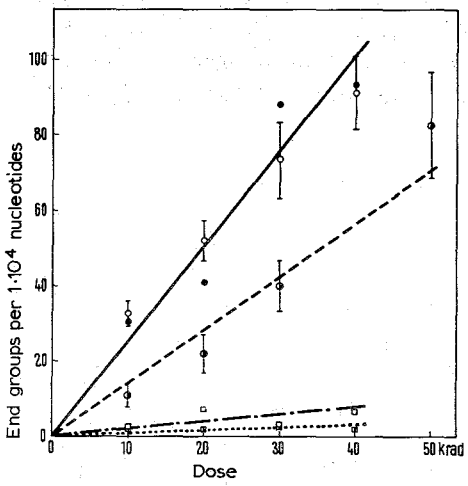

Fig. 2. Single-strand breaks and end groups in irradiated DNA. Native calf thymus DNA was irradiated at 500 μg/ml in 0.01 M NaCl. ◧ . . . ◧, frequency of 5′-OH groups in irradiated DNA which are phosphorylated by polynucleotide kinase; □–·–□, frequency of 5′-OH groups after alkaline treatment of irradiated DNA (alkaline treatment as described under METHODS); ◑- - -◑, frequency of 5′-OH groups in irradiated DNA after dephosphorylation (for details see METHODS); ○–○, frequency of 5′-OH groups after alkaline treatment and dephosphorylation; ●, frequency of single-strand breaks, determined in the alkali-treated denatured DNA samples. The frequency of 5′-OH groups represents average values±S.E. (8 measurements).

the numbers of 5′-OH groups after dephosphorylation, indicating that most of the radiation-induced breaks contain either 5′-OH- or 5′-phosphate groups. The *G* values for the formation of these end groups as calculated from the dose-effect curves (Fig. 2) are summarized in Table I. Obviously, about 90 % of the radiation-induced breaks contain 5′-phosphate groups, both before and after alkaline treatment.

TABLE I

G-VALUES FOR THE OCCURRENCE OF SINGLE-STRAND BREAKS AND END GROUPS IN IRRADIATED DNA

G value = number of events per 100 eV of energy absorbed.

Event	*Untreated DNA*	*DNA after alkaline treatment*	*Difference*
Single-strand break	0.25	0.37	0.12
5′-OH groups	0.02	0.04	0.02
5′-OH groups after dephosphorylation	0.22	0.39	
5′-Phosphate groups*	0.20	0.35	0.15

* Difference of 5′-OH groups before and after dephosphorylation.

DISCUSSION

The determination of the breakage rate as well as of the number of 5′-end groups in γ-irradiated DNA gives some information concerning the chemical mech-

anism of DNA breakage. Without further treatment of the irradiated DNA, about 90% of the breaks carry 5′-phosphate groups. A similar percentage of 5′-phosphate end groups was found by WEISS AND RICHARDSON[13] after degradation of DNA with ultrasonics. 5′-phosphate end groups may originate from an oxidation of the C-3′ of the deoxyribose and a subsequent splitting of the phosphoester bond. Only a small percentage of the breaks carry intact 5′-OH groups; it cannot be excluded that a few other end groups exist in irradiated DNA which are not detected by polynucleotide kinase treatment.

There is a distinct increase of the breakage rate by alkaline incubation of the irradiated DNA. As suggested by RHAESE and FREESE[5], this increase may be due to the fact that the furanose configuration is destroyed by irradiation leading to a splitting of the nucleotide chain under alkaline conditions. It was proposed that at the site of a strand break a nucleotide base is eliminated. Our observations are in agreement with this hypothesis, since the increase of the breakage rate and of the 5′-phosphate groups after alkaline treatment (difference of the G values = 0.12 and 0.15, respectively) corresponds to the formation of free nucleotide bases in irradiated DNA (G_X = 0.17 according to SIMON[4]).

The subsequent breakage of the nucleotide chain in alkali after the loss of the furanose configuration may occur either by β-elimination (*cf.* BAYLEY *et al.*[14]) or by the formation of cyclic phosphoesters (*cf.* TAMM *et al.*[15]). β-Elimination would cleave the 3′-phosphate linkage but produce 5′-phosphate end groups on the adjacent nucleotide. This mechanism seems to be predominant as the increase of 5′-phosphate groups after alkaline treatment nearly corresponds to the increase in the breakage rate. To a small extent additional 5′-OH groups are formed by alkaline treatment. Possibly they originate from a formation of 3′, 4′-cyclophosphates. The existence of cyclophosphates in alkali-treated irradiated DNA was demonstrated recently by SIMON[4].

As shown by various authors[16,17], single breaks formed in the DNA of irradiated organisms, will be repaired *in vivo*. The 5′-phosphate end groups of these breaks are suitable substrates for polynucleotide ligase proposedly involved in the repair.

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