Chemical Nature of Radiation-Induced Single-Strand Breaks in the DNA of Dormant Barley Seeds in Vivo

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Gamma irradiation of dormant barley seeds has led to the production of DNA single-strand breaks, the liberation of inorganic phosphate, and the formation of phosphomonoester groups. For each single-strand break produced in DNA, 0.22 molecules of inorganic phosphate were liberated and 1.1 molecules of phosphomonoester groups were formed. Of the phosphomonoester groups formed, 40% were 5'-phosphomonoesters. Only 8.6% of the 5'-termini produced by radiation were 5'-hydroxyl termini. The chemical mechanisms involved in the induction of single-strand breaks in the DNA of dormant barley seeds are discussed.

INTRODUCTION

Sedimentation velocity studies of mammalian cells exposed to ionizing radiation and lysed immediately on the top of sucrose gradients, have shown that single-strand breaks in cultured mammalian cell DNA are caused by irradiation and that the inverse of molecular weight of mammalian DNA increase linearly with the radiation dose (1-5). This finding has also been extended to higher plants by the authors (6), i.e., a linear relationship was found between the number of induced single-strand breaks/g DNA of irradiated dormant barley seeds and radiation dose.

Although ultracentrifugation studies have provided considerable information about quantitative aspects of the induction of single-strand breaks, this technique does not provide information about the chemical mechanisms involved. Therefore, in the present paper an enzyme, polynucleotide kinase, was used to characterize the nature of 5'-termini of DNA breaks induced in dormant barley seeds as has previously been studied in aqueous solutions of calf thymus DNA (7, 8). Also, an attempt was made to determine the quantitative relationships between DNA breaks, inorganic phosphate yield, phosphomonoester group formation, and deoxyribose damage.

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MATERIAL AND METHODS

Dehusked dormant barley seeds (*Hordeum distichum*, cultivar Fuji 2-row) with a moisture content of about 11%, were exposed to doses of $^{137}$Cs gamma radiation ranging from 10 to 40 kR at a rate of 500 R/min.

Immediately after irradiation, the nuclei were isolated from irradiated as well as nonirradiated seed samples, each consisting of 2 g dormant embryos (6). The nuclear pellet was washed twice with cold saline–citrate (0.15 M NaCl and 0.015 M sodium citrate) and then suspended in 1 ml of saline–citrate and 0.01 M EDTA, pH 8. Three drops of 1% sodium dodecyl sulfate were added to lyse the nuclear suspension. RNA and protein were removed by the procedure described by Cleaver (9). The solution obtained here was referred to as the DNA sample and the DNA content was measured by the diphenylamine method of Burton (10).

**Determinaton of DNA Single-Strand Breaks**

DNA samples from either irradiated or nonirradiated seeds were denatured by the addition of an equal volume of 0.2 N NaOH. One milliliter of each sample (50 µg DNA/ml) was layered on top of 28 ml alkaline sucrose density gradient (5–20% sucrose in 0.1 N NaOH and 0.01 M EDTA). The gradients were centrifuged at 23,000 rpm for 7.5 hr at 4°C in the RPS 25 swinging-bucket rotor of Hitachi Model-65P ultracentrifuge. After centrifugation, 20-drop fractions were collected using a Hitachi "DGF" density gradient fractionator. The assessment of sedimented DNA in the gradients was done by reading its optical density at 260 nm, since it was difficult to label the DNA of barley seeds with radioactive materials in the resting state (6).

Each fraction was diluted up to 2 ml with distilled water and the absorbance at 260 nm was read in a Hitachi 124 spectrophotometer. The data were plotted as absorbance at 260 nm against fraction number. The first moment of each curve was used as an index of the sedimentation behavior. The number of single-strand breaks per molecule ($N$) was obtained from the following relationship:

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

where $M_0$ and $M_r$ are the number average molecular weight of the control and irradiated DNA, respectively. The ratio ($M_0/M_r$) was calculated according to the procedure of Kapp and Smith (7).

**Liberation of Inorganic Phosphate and Formation of Phosphomonoester Groups by Gamma Irradiation**

Inorganic phosphate yield measurement was carried out by the ascorbic acid method of Chen *et al.* (11), in which 6 N H$_2$SO$_4$ was used to precipitate DNA and to provide the proper pH for the final development of color. The total phosphate content of the DNA sample was determined by the micromethod of Meun and Smith (12).
After 2 ml DNA (35.2 µg P/ml) from the irradiated seeds was incubated for 30 min at 65°C in waterbath, the amount of liberated inorganic phosphate was determined. The formation of phosphomonoester groups after irradiation was measured by the treatment of the DNA from irradiated seeds with alkaline phosphatase (EC 3.1.3.1., Worthington Biochemical Co.) according to the procedure of Richardson and Weiss (13), followed by the determination of inorganic phosphate. To 2 ml of the DNA sample (35.2 µg P/ml) from irradiated seeds, 0.15 ml of 1 M Tris-HCl buffer (pH 8), was added and the mixture was incubated for 30 min at 65°C with 5 µl of alkaline phosphatase (30 units/mg, 1 mg/ml), and another 5 µl of the enzyme was added after 15 min. Inorganic phosphate was assayed as described above.

**Preparation of Polynucleotide Kinase**

The preparation of polynucleotide kinase was obtained from *Escherichia coli* infected with T₄ phage according to the procedure of Richardson (14). E. coli B and T₄⁺ phage strains were a generous gift from Dr. T. Beppu, Laboratory of Fermentation and Microbiology, The University of Tokyo.

*E. coli* was grown at 37°C under forced aeration (4.5 liter/min) in a fermenter with 54 liters of M-9 medium containing casamino acid (2 g/liter) and L-tryptophan to final concentration of 1 µg/ml. At a cell density of 2.4 × 10⁹ cells/ml, T₄⁺ phage were added at a multiplicity of 3.5. The culture was quickly cooled to 5°C after an additional 20-min incubation and the cells were harvested by centrifugation. The cell pack (140 g) was stored at -20°C.

The enzyme was extracted and partially purified from the infected cells through step IV. This fraction was found to have a specific activity of about 700 units/mg protein.

**Determination of End Group Products of Chain Breakage Induced by Gamma Irradiation**

The polynucleotide kinase method (7) was used for the identification of 5'-termini of irradiated dormant barley seed DNA. Alkaline phosphatase treatment was performed as described earlier. The alkaline phosphatase was then removed by the use of an equal volume of 4% sodium dodecyl sulfate (SDS). The mixture was then heated at 65°C for 30 min and allowed to cool at room temperature. An equal volume of 1 M KCl was added and centrifuged to precipitate the protein and the SDS. The supernatant fraction was dialyzed overnight against 0.15 M NaCl.

The dialyzed solution of DNA was then treated with polynucleotide kinase in the following manner. The enzyme was routinely diluted into a solution containing 0.05 M Tris buffer, pH 7.5, and 0.01 M 2-mercaptoethanol. The incubation mixture (0.1 ml) contained 30 µl of the DNA sample, 20 µmoles of Tris buffer (pH 7.5), 3 µmoles of MgCl₂, 0.5 µmoles of 2-mercaptoethanol, 2 nmoles ATP, and 20 nmoles γ-³²P-ATP (sp act 2 × 10⁸ cpm per µmole, The Radiochemical Center, Amersham, England), plus 0.2-0.4 units of the enzyme. Incubation was carried out in a 37°C waterbath for 30 min. After incubation, 0.2 ml of 0.1 M
FIG. 1. Sedimentation patterns of DNA of irradiated and nonirradiated dormant barley seeds; (A) nonirradiated sample (B) irradiated with 20 kR, and (C) irradiated with 40 kR. The short vertical bars indicate the position of the first moment of the distribution of $A_{260}$. The direction of sedimentation is from right to left.

$\text{Na}_2\text{P}_2\text{O}_7$, 0.05 ml of 10 mM ATP, and 0.1 ml of 0.1 $M \text{Na}_2\text{HPO}_4$ were added and the tubes were shaken gently. Carrier DNA (0.2 ml, 2 mg/ml) was added and 2 ml of 0.7 $N$ perchloric acid (PCA) was added to precipitate DNA. The samples were collected on Millipore filters (HAWP) and washed repeatedly with PCA. Then the amount of $^{32}\text{P}$ remaining on the filter was determined by Cerenkov counting (15), using a liquid scintillation spectrometer (Packard Tri-Carb 3375). The moles of acid-insoluble $^{32}\text{P}$ added by the polynucleotide kinase treatment of alkaline phosphatase-treated irradiated barley seed DNA, was calculated according to the procedure of Kapp and Smith (7).

RESULTS

Sedimentation patterns for the DNA of the irradiated and nonirradiated dormant barley seeds, in alkaline sucrose density gradients, are shown in Fig. 1. By comparing the position of the first moment of the irradiated seed DNA sample (Fig. 1B, C) with that of the nonirradiated seed DNA (Fig. 1A), it can be seen that irradiation produced a decrease in the sedimentation velocity of the irradiated seed DNA. Nonirradiated seed DNA samples showed a number average molecular weight of $1.53 \times 10^6$, when calculated according to the procedure of Van der Schans et al. (16). An estimate of the average chain length was determined by the ratio of terminal phosphate to the total phosphate (17) for the nonirradiated barley seed DNA. An average chain length of 3820 nucleotides/strand DNA was obtained, which corresponds to a molecular weight of about $1.2 \times 10^6$. This
The number of DNA single-strand breaks per molecule \((N)\) induced by radiation is presented in Table I. The efficiency of single-strand break induction was found to be 0.087 breaks/strand DNA/kR (or 0.057 breaks/\(10^6\) daltons/kR).

The amount of inorganic phosphate liberated after various doses of \(\gamma\)-irradiation and the yield of inorganic phosphate produced by subsequent alkaline phosphatase treatment are illustrated in Fig. 2. It can be seen from Fig. 2 that the amount of inorganic phosphate liberated with or without alkaline phosphatase treatment increased linearly with the radiation dose up to 40 kR. From the slopes of the various lines in Fig. 2, the amount of inorganic phosphate liberated after irradiation alone was 0.0101 nmoles \(\text{Pi/\(\mu\)mole DNA-P/kR}\) and after irradiation plus alkaline phosphatase treatment was 0.0594 nmoles \(\text{Pi/\(\mu\)mole DNA-P/kR}\).

### Table I

<table>
<thead>
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<th>Radiation dose (kR)</th>
<th>Experiment no.</th>
<th>Average</th>
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<tbody>
<tr>
<td>20</td>
<td>1.23 1.39</td>
<td>1.31</td>
</tr>
<tr>
<td>40</td>
<td>3.91 4.03</td>
<td>3.97</td>
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</table>

Fig. 2. Radiation-induced liberation of inorganic phosphate and the formation of phosphomonoester groups. Inorganic phosphate yield after irradiation (••••); inorganic phosphate yield after irradiation and alkaline phosphatase treatment (■■■■). Phosphate present in phosphomonoester groups, obtained from the difference in the slopes of above two curves (---). Open and closed symbols represent different experimental sets.
FIG. 3. Radiation-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'-PO$_4$ termini formed 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 nonirradiated controls. Open and closed symbols represent different experimental sets.

The amount of phosphomonoester groups formed (0.0493 nmoles Pi/µmole DNA-P/kR) was obtained by subtracting the yield of inorganic phosphate produced by irradiation alone, from that produced by irradiation plus alkaline phosphatase treatment.

Since polynucleotide kinase specifically catalyzes the transfer of one phosphate group from ATP to the 5'-hydroxyl termini of the polynucleotide chain, it provides a sensitive method for the identification of 5'-end groups. The moles of acid-insoluble $^{32}$P added by the polynucleotide kinase in irradiated seeds DNA (without alkaline phosphatase treatment) represent the number of 5'-hydroxyl termini produced. Their amount was very small and there was a slight increase with radiation dose. Most of $^{32}$P was incorporated after dephosphorylation of the irradiated seed DNA samples, indicating the formation of new 5'-hydroxyl termini, and would represent the total number of 5'-termini formed. The yield of 5'-phosphoryl termini was determined by subtracting the yield of 5'-hydroxyl termini from the total 5'-termini (Fig. 3).

By dividing the slope of the curve for the production of 5'-hydroxyl termini (0.0019 nmoles $^{32}$P added/µmole DNA-P/kR) by that for the production of total 5'-termini (0.022 nmoles $^{32}$P added/µmole DNA-P/kR) the relative percentage of 5'-termini that are 5'-hydroxyl was found to be 8.6%, the relative percentage of 5'-phosphoryl termini formed can be readily determined.

Also from the slope of the curve presented in Fig. 3, the production of 5'-phosphoryl termini is 0.0201 nmoles P produced/µmole DNA-P/kR. By comparing this value with the slope of the production of phosphomonoester groups (0.0493 nmoles P liberated/µmole DNA-P/kR), it can be estimated that approximately 40% of the total phosphomonoester groups formed after irradiation are 5'-phosphomonoesters.
A comparison between the number of single-strand breaks with the yield of inorganic phosphate, and phosphomonoester groups is given in Table II. Also the $G$ values of the various products are tabulated.

Also, we have attempted to determine radiation damage to the deoxyribose. It has been reported (7) that x-irradiation to 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. When the TBA reaction (18) was applied to the present samples of DNA of irradiated barley seeds, the formation of such compound was not detected.

**DISCUSSION**

In this paper we have attempted to clarify the biochemical nature of radiation-induced single-strand breaks in the DNA of irradiated dormant barley seeds, since once the nature of chain breakage in vivo is known, the nature of the requirement for the repair may be better understood.

In this experiment a rather high amount of DNA sample (50 µg) was layered on top of the sucrose gradient, which may result in the sedimentation rates being subject to an error of a few percent (2). However, in an earlier paper (6), we showed that reproducible results were obtained with such amounts of DNA. Also Van der Schans et al. (16) showed that gradient distortion as a consequence of a large amount of DNA was much less severe for denatured DNA, probably because of the reduced viscosity of the DNA in the gradients.

The number average molecular weight of the nonirradiated barley seed DNA samples was found to be $1.53 \times 10^6$. However, in comparing this value with that obtained previously (6) for the same material lysed immediately on the top of the sucrose gradients ($7.9 \times 10^6$), it can be deduced that mechanical breaks were introduced during the course of the extraction of DNA. On the other hand, the efficiency of single-strand break induction calculated from Table I, is in fair agreement with that obtained previously (6).

To compare quantitatively the yield of phosphomonoester groups and inorganic phosphate with the production of single-strand breaks, the value of single-strand breaks obtained from Table I ($0.087$ breaks/strand DNA/kR) must be converted
to chain breaks/µmole DNA-P/kR. In the present experiment, an average chain length of about 3820 nucleotides/strand, and 3.12 × 10^{14} strands/µmole DNA-P, were obtained. According to these data there were 0.087 breaks/strand DNA/kR, and 0.271 × 10^{14} breaks/µmole DNA-P/kR for the DNA samples from irradiated seeds. The results summarized in Table II indicate that for every single-strand break induced, about 0.22 molecules of inorganic phosphate were liberated and 1.1 molecules of phosphomonoester groups were formed.

As to the reduced G values of DNA single-strand breaks and the other chemical species formed after irradiating dormant barley seeds, this may be, in part, due to the dry state of the seeds. Collyns et al. (19) also showed reduced G values for the formation of phosphomonoester groups in irradiated solid DNA.

Employing the polynucleotide kinase technique (Fig. 3) to determine the nature of the 5'-end groups formed, about 91% of the total 5'-termini was found to carry 5'-phosphoryl termini. A similar percentage was obtained in irradiated aqueous solutions of calf thymus DNA (8) and in ultrasonicated DNA solutions (20). Corresponding to the liberation of phosphate in irradiated mononucleotide 5-phosphate (21) an oxidation of C'-5 in the deoxyribose of DNA may lead to the formation of unstable acyl phosphate and hence to a splitting of the nucleotide chain with the formation of a 3'-phosphoryl end group. In a similar way the 5'-phosphoryl termini may originate from an oxidation of the C'-3 of the deoxyribose and a subsequent splitting of the phosphoester bond.

The results of this experiment, regarding the yield of inorganic phosphate, phosphomonoesters and the ratio of 5'-hydroxyl to 5'-phosphoryl termini, are in good agreement with those obtained by Kapp and Smith (7) and Bopp and Hagen (8) in irradiated diluted solutions of calf thymus DNA, considering the difference in the conditions of the irradiation. In our case, barley seeds were irradiated and DNA was extracted thereafter, while in the other two papers, dilute solutions of calf thymus DNA were irradiated. Thus, neither the packaging of the DNA nor the water content appears to alter the type of single-strand breaks produced. Tanooka and Terano (22) also showed no effect for the water content in the induction of single-strand breaks in bacterial spores. Palec and Skarsgard (23) suggested that the production of single-strand breaks in the DNA of cultured mammalian cells after irradiation were due to the direct physicochemical action. This may be also true for the induction of single-strand breaks in the DNA of dormant barley seeds. However, a concrete reason cannot be given here because of the lack of experimental data on the efficiency of radiation-induced single-strand breaks in higher plants. On the other hand, the absence of deoxyribose damage after the irradiation of barley seeds may be due to the difference in the irradiation conditions.

Moreover, in comparing the amount of phosphomonoester groups formed (Fig. 2) with that of the 5'-phosphoryl termini (Fig. 3), it can be estimated that only about 40% of the total phosphomonoester groups formed are 5'-phosphomonoesters. One cannot rule out whether all the other phosphomonoesters are 3'-phosphomonoesters, since Daniels et al. (24) showed that radical attack on the C atom of the sugar moiety results in the formation of a labile phosphate group with the eventual release of inorganic phosphate. Therefore, additional studies, perhaps
employing a specific 3'-phosphomonoesterase will be necessary to identify the nature of the 3'-termini.

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REFERENCES


