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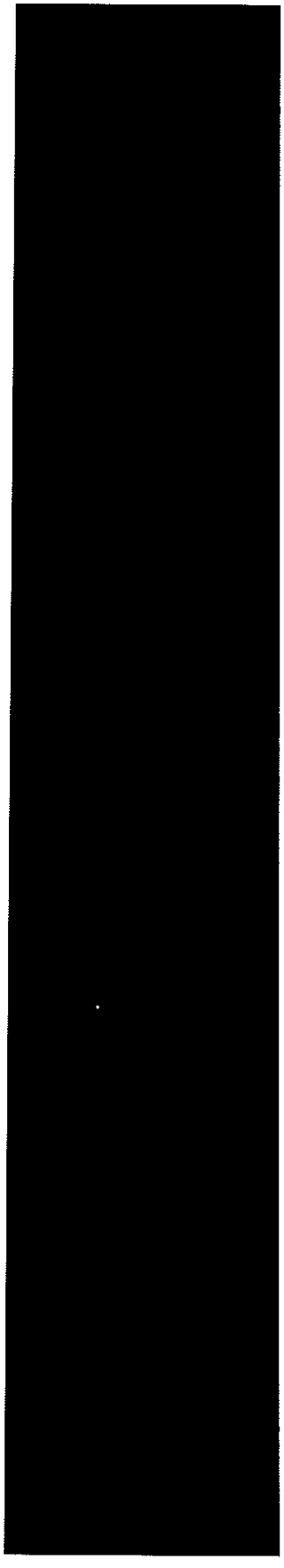

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In vitro repair of single-strand breaks in γ-irradiated DNA by polynucleotide ligase

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The ability of polynucleotide ligase to eliminate single-strand breaks in γ-irradiated DNA was studied. The irradiated DNA was first labelled at the 5′-end of the break with ^{32}P-phosphate and treated *in vitro* with polynucleotide ligase. In phosphoester-bonds joined by the ligase, the incorporated ^{32}P-phosphate is resistant to subsequent phosphatase treatment. The number of sealed breaks, detected in this way, was compared with the total number of single-strand breaks measured in the analytical ultracentrifuge. In DNA with 0·4–2·0 radiation-induced single-strand breaks per 1000 nucleotides, up to 40 per cent of the breaks can be joined directly by the ligase.

1. Introduction

Single-strand breaks, induced in DNA by ionizing radiation, are repaired *in vivo* very rapidly, as shown by various authors for bacteria or mammalian cells (McGrath and Williams 1966, Humphrey, Steward and Sedita 1968, Ormerod and Stevens 1971). The elimination of the breaks may be performed by the polynucleotide ligase, either directly or as a final step after several enzymatic reactions. Kapp and Smith (1969) did not observe a decrease in the number of single-strand breaks after treating x-irradiated DNA with polynucleotide ligase *in vitro*, whereas Gasiev, Fomenko, Sakrschevskaja and Kuzin (1970) observed an *in vitro* repair of the radiation-induced inactivation of transforming DNA by ligase treatment.

Our investigations about the nature of the single-strand break in γ-irradiated DNA (Bopp and Hagen 1970, Ullrich and Hagen 1971, Bopp, Carpy, Burkart and Hagen 1972) revealed that most of the 5′–ends carry 5′-phosphate groups. The 3′-end consists of various types of end-groups: the sugar of the 3′-end may be destroyed, or the nucleoside is liberated leading to a 3′-phosphate end. It was not known, however, if γ-irradiation also leads to a simple hydrolysis of the phosphoester-linkage forming 3′OH- and 5′P-end, the only break subject to direct rejoining by the polynucleotide ligase. To test this, the effect of polynucleotide ligase was studied on irradiated DNA with 5′-^{32}P-labelled end-groups, a method sensitive enough to detect even a small amount of joined breaks.

2. Materials and methods

2.1. *Enzymes*

Deoxyribonuclease I from pancreas (E.C.3.1.4.5.) and alkaline phosphomonoesterase (E.C.3.1.3.1.) was obtained from Boehringer, Mannheim. Spleen acid phosphomonoesterase (E.C.3.1.3.2.) was a gift of Dr. Bernardi, Paris.

The preparation was free of exonucleases and endonucleases. Polynucleotide kinase was isolated from T4-infected *E. coli* cells according to the method of Richardson (1965). Further details have been described by Bopp and Hagen (1970). NAD-dependent polynucleotide ligase was isolated according to Weiss, Jacquemin-Sablon, Live, Fareed and Richardson (1968) from an endonuclease I-deficient strain (E.c. 1100, F^-, prototroph, endonuclease I^-). The bacteria were obtained from E. Merck, Darmstadt. The polynucleotide ligase was free of DNA-polymerase I (E.C.2.7.7.7.). In most of the ligase fractions used, there was no detectable activity of exonucleases and of phosphatases. [γ-^{32}P] ATP was obtained from Radiochemical Centre, Amersham.

2.2. *DNA preparations*

Calf-thymus DNA was isolated according to the method of Kay, Simmons and Dounce (1952). 3'OH-5'-phosphate DNA was prepared by degradation with DNase I. DNA solutions of 500 μg/ml were incubated with this enzyme (0·04 μg/ml) in 0·05 M Tris (pH 8·0) and 0·01 M $MgCl_2$ for various times at 37°. The degradation of the DNA was followed by a simultaneous measurement in a Couette-viscosimeter and the reaction was stopped by adding 1/10 of the volume chloroform/isoamylalcohol (5 : 1 v/v) and shaking for 5 min. After centrifuging at 3000 rev/min for 5 min, the supernatant fluid was treated again with chloroform/isoamylalcohol. The final supernatant fluid was dialysed three times against 1·0 M KCl, once against 0·02 M KCl and once against 0·01 M NaCl, for 12 hours each.

For γ-irradiation, DNA solutions of 420 μg/ml in 0·01 M NaCl were saturated with nitrogen, sealed and irradiated in a ^{60}Co-γ-source (Gammacell 200, Atomic Energy of Canada, Ltd). The frequency of both double-strand (B_2 = dsb per nucleotide pair) and single-strand breaks (B_1 = ssb per nucleotide) was calculated from sedimentation analysis of the DNA samples in the analytical ultracentrifuge before and after denaturation with alkali (Hagen 1967, Lücke-Huhle, Braun and Hagen 1970). The frequency of internal breaks amounts to $B_i = B_1 - B_2$.

2.3. *Ligase assay*

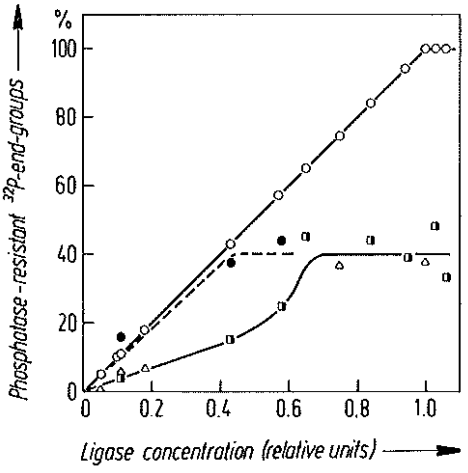
The following enzymatic reactions were necessary to test irradiated DNA as well as 3'OH-5'P-DNA for enzymatic joining: (1) dephosphorylation of both ends with spleen phosphomonoesterase; (2) phosphorylation of the 5'OH-group by polynucleotide kinase with [γ-^{32}P] ATP to a 5'-^{32}P end; (3) treatment with polynucleotide ligase; and (4) dephosphorylation with alkaline phosphomonoesterase.

The primary dephosphorylation of the various samples was performed with spleen acid phosphomonoesterase according to Bopp and Hagen (1970), the phosphorylation with polynucleotide kinase to 5'-^{32}P DNA according to Weiss *et al.* (1968). Samples of both substrates (3'OH-5'P DNA or irradiated DNA) were incubated with polynucleotide ligase according to Weiss *et al.* (1968), except that 6.6 mμmoles NAD per 0·3 ml incubation mixture were used. The pH was changed to 8·0 according to Zimmermann and Oshinsky (1969). The reaction was stopped with 0·1 ml conc. $Na_4P_2O_7$. 0·1 ml herring-sperm DNA

(0·5 mg/ml) was added, and the DNA was precipitated with 0·5 ml 1 M trichloroacetic acid. After washing with 0·01 M HCl, the precipitate was dissolved, brought to pH 10 with alkali and incubated with alkaline phosphomonoesterase at 65°c according to the method of Weiss *et al.* (1968). For the final precipitation, 0·5 M trichloroacetic acid was used. The precipitate was collected on membrane filters (0·45 μ) and counted in a liquid scintillation spectrometer. From the ^{32}P-activity, measured in the acid-insoluble precipitate, the number of phosphatase-resistant end-groups per nucleotide can be calculated. This number was compared with the total number of internal single-strand breaks measured by sedimentation analysis, leading to the percentage of single-strand breaks, joined by the polynucleotide ligase.

3. Results and discussion

DNA samples were irradiated in solution with ^{60}Co γ-rays and the percentage of joined single-strand breaks was determined after ligase treatment as described above. Enzymatically-degraded DNA with about the same amount of internal breaks (B_i between 0·4 and 2·0 10^{-3}) was treated in the same experiment. The results are shown in figure. With increasing concentrations of polynucleotide ligase up to 100 per cent of the single-strand breaks of the 3'OH-5'P DNA can be joined, whereas in irradiated DNA up to 40 per cent of all possible single-strand breaks (including the alkaline labile lesions) can be sealed under the experimental conditions applied. This maximum value of sealed breaks in irradiated DNA is independent of the extent of degradation.



Joining of single-strand breaks in DNA by polynucleotide ligase: effect of various ligase concentrations on the percentage of ^{32}p- end-groups made resistant to alkaline phosphatase by treatment with polynucleotide ligase. ○—DNA, degraded by DNase I (3'OH-5'P DNA); ●γ-irradiated DNA containing 0·4 ssb per 10^3 nucleotides (1 krad); ▯—γ-irradiated DNA containing 0·7 ssb per 10^3 nucleotides (2 krads); △γ-irradiated DNA containing 2·0 ssb per 10^3 nucleotides (5 krads).

The polynucleotide ligase seems to be less effective when acting on irradiated DNA than on enzymatically-degraded DNA. This becomes evident on studying the effect of low enzyme concentrations on DNA irradiated with 2 or 5 krads. Under these conditions, the number of joined breaks in irradiated

DNA is distinctly lower than in 3'OH-5'P DNA. This may be due to the fact that in irradiated DNA other types of end-groups exist which bind polynucleotide ligase but cannot be joined. Such competitive inhibition of the ligase by irradiated DNA was not observed, however, after 1 krad. In our assay for the number of sealed single-strand breaks, the end-groups have first been dephosphorylated before labelling the 5'end with ^{32}P-phosphate. Thus, not only the 3'OH-5'P breaks in the irradiated DNA are joined, but also breaks that were originally in the inverse configuration (3'P-5'OH) or those, originally with 3'OH-5'OH end-groups. Single-strand breaks with 5'OH end-groups were detected by the polynucleotide kinase. In irradiated DNA, 10 per cent of the total amount of breaks can be phosphorylated with this enzyme, i.e. carry 5'OH end-groups (Bopp and Hagen 1970). As 40 per cent of the single-strand breaks are sealed in irradiated DNA under our experimental conditions, we may conclude that at least 30 per cent of the breaks are directly joinable by the polynucleotide ligase, i.e. without preceding dephosphorylation and phosphorylation.

Our observations are in agreement with the result of Gasiev *et al.* (1970) who found a repair in the transforming activity of γ-irradiated DNA by ligase treatment. This repair was observed, however, only after low doses of γ-irradiation. As the loss of transforming activity is mainly due to single-strand breaks (Thorsett and Hutchinson 1971), the results of Gasiev *et al.* (1970) are also an indication for joining of radiation-induced breaks by polynucleotide ligase. Our results do not agree with those of Kapp and Smith (1969). These authors, however, determined the number of single-strand breaks by sucrose sedimentation before and after treatment with polynucleotide ligase. This technique may not allow detecting a small percentage of joined breaks. Furthermore, in the experiments of Kapp and Smith, the DNA samples were irradiated in air, whereby other end-groups may be formed than under our irradiation condition, i.e. in absence of oxygen.

The percentage of single-strand breaks joined by the ligase is also consistent with the reaction scheme for the radiation-induced chain breakage proposed by Ullrich and Hagen (1971). In this scheme, about 30 per cent of the breaks are formed by an attack of the radiation energy on the carbon 3' of the deoxyribose, 30 per cent by liberation of a nucleoside, and 30 per cent by subsequent alkaline treatment of the irradiated DNA. About 10 per cent of the breaks carry 5'OH-3'P end-groups. According to the results of this study, the first fraction of breaks, induced by an attack on the 3' end of the sugar may be sealed directly by polynucleotide ligase.

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On a étudié la capacité de la polynucléotide ligase à effacer les cassures monofilaires dans de l'ADN irradié aux rayons γ. L'ADN une fois irradié, a été marqué à l'extrémité 5' de la rupture avec du ^{32}P, puis traité *in vitro* par la polynucléotide ligase. Dans les liaisons phosphoester reformées par celle-ci, ce ^{32}P devient résistant au traitement à la phosphatase. On peut ainsi comparer à l'aide de cette technique, le nombre de cassures réparées, au nombre de cassures monofilaires mesuré à l'aide de l'ultracentrifugation analytique. Dans un ADN irradié présentant 0,4–2,0 ruptures, monofilaires par 1000 nucléotides, la ligase peut en réparer directement jusqu' à 40 par cent.

Die Wirkung von Polynukleotidligase auf die Einzelstrangbrüche in γ-bestrahlter DNS wurde untersucht. Dazu wurde bestrahlte DNS zunächst am 5′-Ende mit ^{32}P-Phosphat markiert und anschließend in vitro mit Polynukleotidligase behandelt. Wird der Bruch durch Ligase geschlossen, so widersteht das ^{32}P-Phosphat einer anschließenden Behandlung mit Phosphatase. Die Anzahl der auf diese Weise geheilten Brüche wurde mit der Gesamtzahl der durch Ultrazentrifugation gemessenen Einzelstrangbrüche verglichen. Es ergab sich, daß bei DNS mit 0,4–2,0 strahleninduzierten Brüchen pro 1000 Nukleotiden bis zu 40 Prozent der Brüche direkt geschlossen wurden.

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