# DENATURATION OF DNA AFTER X-IRRADIATION *IN VITRO*

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SUMMARY: X-irradiation of *E. coli* DNA caused partial strand-separation as judged by susceptibility of the DNA to nuclease specific for single-stranded DNA, namely an endonuclease from *Neurospora crassa* and exonuclease I of *E. coli*. The elution profile of X-irradiated DNA from hydroxyapatite column showed that single-stranded fragments were produced. These fragments had a sedimentation coefficient of 3-4s corresponding to a length of about 250 nucleotides.

The primary target of X-ray-induced lethal damage in bacteria is believed to be DNA (1). The main effects on DNA of X-irradiation both *in vivo* and *in vitro* are (a) strand scissions, (b) alteration and elimination of bases, (c) intra- and inter-strand cross links and (d) strand-separation (1-5). Among these phenomena, the strand-separation of DNA after X-irradiation *in vitro* was studied by ultraviolet spectroscopy, electrometric titration and viscosity (2-5). The measurement of viscosity could not distinguish strand-separation from strand-breakage because both cause similar changes in hydrodynamic parameters. An increase in the ultraviolet absorbance of DNA as a result of denaturation after X-irradiation could be offset by the destruction of bases (2). In the present communication, we wish to report the use of enzymatic assay and chromatography to demonstrate the formation of single-strand fragments from double-stranded DNA as a result of X-irradiation.

MATERIALS AND METHODS

$^{32}P$-labeled DNA was isolated from E. coli B by a slightly modified procedure of Marmur (6). The bacteria were grown in a low-phosphate medium (7) to which carrier-free $^{32}P$ (0.3 mC per 100 ml medium) was added. The specific activity of the DNA was 1-2 x $10^6$ cpm/μmole. The concentration of DNA was expressed as molar equivalent of phosphorus.

DNA samples were irradiated at about 0.2 μm/ml in 0.05M Na-phosphate, pH 6.8 in ordinary atmosphere at room temperature from a Mullard X-ray generator, MG 150 operated at 80 KVp without any filter. The dose as estimated by Fricke's $FeSO_4$ dosimetry method was 3.2 Krads per minute. After irradiation, the DNA samples were immediately chilled to $0^o$ and left for about an hour before further processing. Freifelder (8) showed that there was no after effect in the physical properties of DNA after X-irradiation.

Exonuclease I of E. coli was purified from E. coli B cells upto the stage of chromatography on hydroxyapatite according to Lehman (9). An endonuclease specific for single-stranded DNA was purified from Neurospora crassa mycelia according to the procedure of Linn (10). These nucleases degraded native DNA to acid-soluble products to an extent of 1-3% at an amount which was necessary to degrade 90 to 100% of denatured DNA.

The incubation conditions for the enzymes were as follows: For exonuclease I, $^{32}P$ DNA (irradiated, native or heat-denatured), 20 mμmoles; Na-glycinate buffer, pH 9.5, 30 μmoles; $MgCl_2$, 2 μmoles, β-mercaptoethanol 0.3 μmole and the enzyme in an

amount sufficient to degrade 95% of heat-denatured DNA in a total volume of 0.3 ml. In the case of Neurospora crassa endonuclease, the reaction mixture was, $^{32}$P-DNA, 20 mμmoles; Tris-Cl pH 7.5, 30 μmoles; $MgCl_2$, 3 μmoles and the enzyme in an amount necessary to degrade 90% of the denatured DNA in a total volume of 0.3 ml. In both cases, after incubation at 37° for 30 min, the reaction mixtures were chilled, 0.1 ml unlabeled and crude E. coli DNA (2.5 mg/ml) and 0.4 ml 7% $HClO_4$ were added. After 10 min. at 0°, the precipitated DNA was removed by centrifugation (10,000 x g, 10 min) in the cold and the radioactivity of an aliquot of the supernatant was counted in an end-window GM-counter (DAE, India).

Hydroxyapatite was prepared according to the method of Miyazawa and Thomas (11). Hydroxyapatite columns (3.5 cm x 1.2 cm) were used to separate double-stranded from single-stranded DNA either by stepwise elution or by elution with a linear gradient (total volume of 200 ml) of 0.05 to 0.4M Na-phosphate, pH 6.8. 1-2 ml of DNA corresponding to $6x10^4$ to $10^5$ cpm was loaded on the column. The recovery of radioactivity was about 95%. The denatured and the native DNA were eluted at about 0.19M and 0.26M Na-phosphate respectively.

Table 1. Susceptibility of X-irradiated DNA to nucleases specific for single-stranded DNA.

| X-ray dose (Kilorads) | Susceptibility to E. coli exonuclease I (Per cent) | Susceptibility to Neurospora crassa endonuclease (Per cent) |
|---|---|---|
| 9.6 | 4.7 | 5.6 |

RESULTS AND DISCUSSION

X-irradiated DNA was found to be partially susceptible to both Neurospora endonuclease and E. coli exonuclease I (Table 1). The extent of degradation by these two enzymes were comparable. Since the endonuclease hydrolyzes phosphodiester bonds anywhere in the single-stranded regions, while the exonuclease degrades single-stranded DNA only from the 3'-ends (9,10), it is clear that most of the single-stranded regions would have free 3'-ends. This is likely because X-ray causes extensive single-strand and double-strand breaks in DNA (1). Also, at the dose of X-ray used, it is unlikely that significant base destruction at least in the denatured regions of DNA has taken place because the exonulcease I should be expected to be unable to degrade such DNA. This comment is pertinent in view of the fact that exonuclease I could not degrade u.v.-irradiated single-stranded DNA or DNA partially denatured as a result of u.v. - irradiation, presumably because of the presence of thymine dimers and other photoproducts (Dasgupta and Mitra, Unpublished).

X-irradiated DNA was then chromatographed in hydroxyapatite (Fig.1). It is evident that, while there is no radioactivity corresponding to the position of denatured DNA in the unirradiated control DNA, 4.5% of the total DNA was eluted as denatured DNA when irradiated DNA was chromatographed. The fraction corresponding to the position of denatured DNA was concentrated and was found to be mostly susceptible (92%) to Neurospora endonuclease. Thus its single-stranded nature was confirmed. The correspondence of the amount of total irradiated DNA susceptible to the nucleases and the fraction eluted as single-stranded fragments from the hydroxyapatite column indicates that the frag-

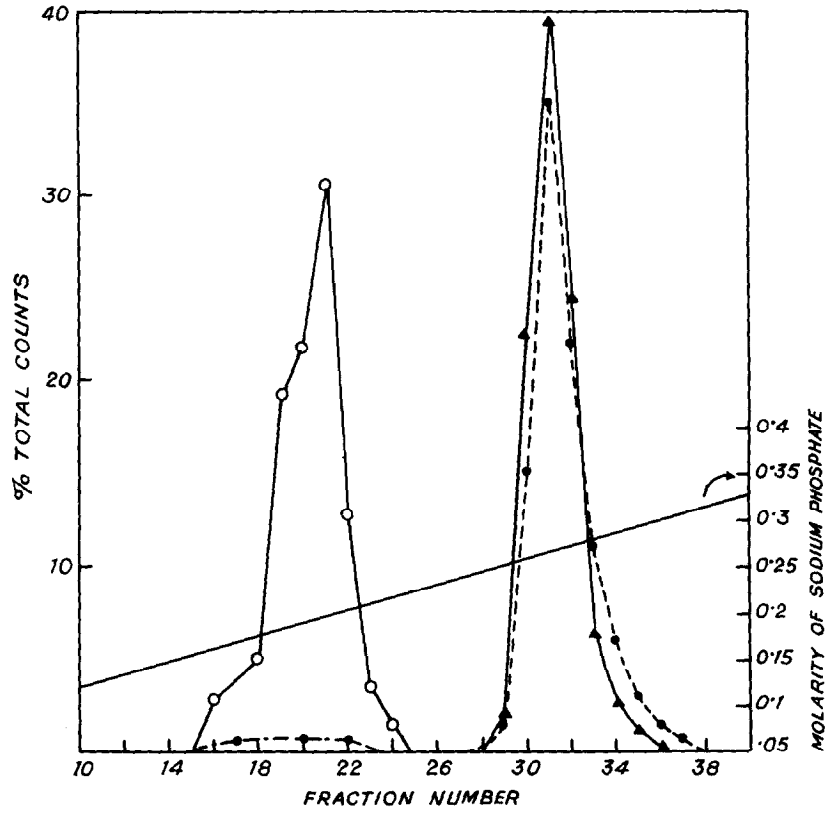


Fig.1. Gradient elution of E. coli DNA from hydroxyapatite column. Native DNA (▲—▲); Heat-denatured DNA (o—o); X-irradiated DNA (9.6 Krads), (●—●).

ments were produced by simultaneous denaturation and single-strand and/or double-strand breakage of irradiated DNA. In other experiments, particularly when homogeneous DNA of T7 phage was irradiated, beside single-stranded fragments, partially denatured DNA was obtained which was eluted from hydroxyapatite in between the position of double-stranded and single-stranded DNA. This DNA was also susceptible to degradation to a varying degree by the Neurospora endonuclease. Thus it is possible, that the single-stranded fragments were produced from DNA with pre-existing single-strand breaks, while in the case of high molecular weight DNA with very few single-strand breaks, single-

stranded fragments as well as double-stranded DNA with protruding single strands were obtained after X-irradiation.

The size of the single-stranded DNA fragments produced after X-irradiation was investigated by sucrose-density gradient centrifugation (Fig. 2). The native E. coli DNA had a broad distribution with about 26s as the average sedimentation coefficient as compared to the reference T7 phage DNA. After irradiation, with 9.6 Krads, the sedimentation coefficient of the native DNA was reduced to 6.5s while that of the single-stranded DNA fragments was about 3-4s. Applying Studier's formula (12), the molecular weight of the latter fragments was calculated to be in the range of 7 to $8 \times 10^4$ daltons, corresponding to about 250 nucleotides. X-irradiation at the dose level used here increased

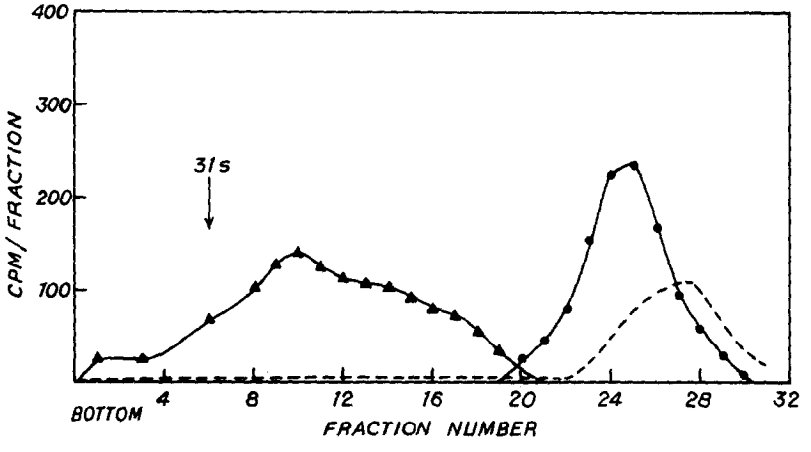


Fig.2. Sucrose-gradient centrifugation profile of X-irradiated DNA. Fractions corresponding to single-stranded and double-stranded DNA after hydroxyapatite chromatography were separately pooled and concentrated by dialysis against 60% sucrose in 0.05M Na-phosphate, pH 6.8 and was subsequently layered on 5-20% linear gradient of sucrose in 1M NaCl, 0.01M Tris-Cl, 0.00M EDTA, pH 7.5. Centrifugation was carried out in SW 39 swinging out rotor of Spinco ultracentrifuge, model L at 37,000 rpm for 240 min at 1°. Fractions of equal volume were collected from the bottom. The arrow indicates the position of T7 phage DNA ($S^0_{20,w}$ = 31s). X-irradiated DNA; (----), single-stranded DNA; (●—●), double-stranded DNA. The unirradiated E. coli DNA (▲—▲) is also shown.

the acid-soluble radioactivity only slightly and hence very small fragments could not have been produced.

The mechanism of X-ray induced denaturation over such a long stretch of polynucleotide chains is not understood. Scholes et al. (2) suggested that X-irradiation might cause the formation of single-stranded fragments but they estimated that the number of hydrogen bond pairs broken per chain break would be of the order of 14 to 16. Our figure is at least an order of magnitude higher than the above estimate. It is not clear whether the extent of denaturation is dependent on the wavelength of X-ray used.

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