DNA DEGRADATION IN *ESCHERICHIA COLI* 15 T^-L^- INDUCED BY FAST PROTON BOMBARDMENT

D. C. HUSTON *and* E. C. POLLARD

From the Department of Biophysics, The Pennsylvania State University, University Park, Pennsylvania 16802

ABSTRACT DNA degradation and its temperature dependence as a function of linear energy transfer were studied in *Excherichia coli* using fast proton irradiation as the initiating agent. The data indicate that radiation-induced DNA degradation can proceed by two processes. The first, or fast component, begins immediately after irradiation with ^{60}Co γ-rays or with fast protons at doses less than 10^{10} protons/cm^2. The rate is high and involves a maximum of about 50% degradation. It is elicited more efficiently by protons of high linear energy transfer. The second, or slow component, results from higher doses of fast proton bombardment. There is a delay between irradiation and the initiation of this slower component, but 100% of the DNA complement is degraded. The data indicate that both processes are enzyme-mediated, the first probably by normal DNA-related activity and the second by DNAase activity.

INTRODUCTION

Treatment of microorganisms with X-rays or with ^{60}Co γ-rays results in the breakdown of cell DNA. Degradation has been observed under a variety of conditions in a wide selection of bacterial species by Stuy (1960, 1961), Kos and Drakulic (1961), Miletic, Kucan, and Novak (1964), Miletic, Kucan, and Sasel (1964), Pollard and Achey (1964), and Emmerson and Howard-Flanders (1965). It has also been observed by Kuzin (1963) in mammalian tissues. DNA degradation induced by the action of ionizing radiation appears to be a universal phenomenon and of considerable interest to our understanding of radiation action on living cells.

When it has been possible to study radiation effects utilizing heavy particles to produce the ionization, a second aspect of information has become available. If the sensitivity of the system to radiation depends only on the ionizations absorbed in the cell, regardless of their distribution, this suggests that some simple sensitive element is involved. On the other hand, if the resulting effect becomes considerably greater when the ionizations are grouped closely, as for fast heavy particles, some other aspect of sensitivity is deduced. Thus, for mammalian cells the survival curve, measured as a function of Linear Energy Transfer (LET), strongly suggests that the nucleus of the cell is the element which is radiosensitive (Barendsen, 1961).

The objective of this work was to examine DNA degradation as initiated by ionizing radiation in the form of fast protons. The experiments show that the process elicited differs in some aspects from the process resulting from gamma irradiation. The features of the proton-induced degradation have been found to merit study independently of the objective of exploiting different LET'S to deduce information relevant to the sensitive elements in the cell. However, the results suggest that there is an abnormal sensitivity relationship as LET increases—a relationship which is reminiscent of chromosome damage.

Accordingly, utilizing fast proton irradiation as the initiating agent we have studied DNA degradation and its temperature dependence as a function of LET in *Escherichia coli*. The principal results of our investigations are as follows: (*a*) Degradation can proceed by two processes. (*b*) The first process, or fast component, may be the result of action of an enzyme, on or adjacent to the DNA. This enzyme is inactivated at incubation temperatures above 42°C. (*c*) The second process, or slow component, is probably the result of DNAase activity. (*d*) The initial process which starts degradation probably requires the cooperative action of more than one ionization.

MATERIALS AND METHODS

The degradation of DNA can be observed readily with the thymine- and leucine-requiring mutant *Escherichia coli* $15T^{-}L^{-}$. When these cells are given labeled thymine, the subsequent fate of the label can be studied by determining whether thymine is incorporated into macromolecules which are precipitated by cold TCA or whether it is released into the cold TCA-soluble fraction. The technique that we have employed, with the exception of the method of irradiating bacterial cultures, is similar to the procedure outlined by Pollard and Achey (1964).

Cells were grown with aeration at 37°C in minimal medium (NH_4Cl, 2 g; Na_2HPO_4, 6 g; KH_2PO_4, 3 g; NaCl, 5 g; $MgCl_2 \cdot 6H_2O$, 62 mg; Na_2SO_4, 80 mg; glucose, 5 g per liter distilled water) supplemented with 40 mg leucine and 4 mg thymine-^{14}C (Sp. Act., 30 mc/mmole) per liter of medium. When the cell concentration was 10^8 to 3×10^8 cells/ml, the pool of thymine-^{14}C was "chased" by diluting 100-fold with normal thymine and continuing growth for 15 min. The cells in 10 ml of culture were collected on filters and washed with 10 ml of minimal medium. The cells were kept in a moist environment during irradiation by placing the filter on an absorbant pad soaked in minimal medium.

Cells on filters were irradiated in air with fast protons from a Van de Graaff accelerator. Control cultures, also maintained on filters attached to absorbent pads, were placed inside the bombardment chamber during irradiation but were not exposed to the proton beam.

Cells were resuspended in minimal medium supplemented with leucine and normal thymine and restored to the preirradiation incubation conditions (aeration at 37°C). Periodically 1 ml of irradiated culture was transferred to 1 ml of cold 10% TCA. The subsequent precipitate was collected on filters and the amount of ^{14}C it contained was determined by radioactivity measurements. Nonirradiated controls were also assayed in this manner. Degradation was seen as a loss of ^{14}C activity in the cold TCA-insoluble fraction.

The growth, the preirradiation treatment, the irradiation procedure, and the assay for the temperature dependency studies were as described. Resuspension of cells was carried out in

medium prewarmed to the postirradiation incubation temperature. The air used in bubbling the culture was also prewarmed to the incubation temperature by passing it through glass tubing submerged in the incubation water bath prior to bubbling through the culture.

Degradation experiments in which DNA synthesis was inhibited prior to irradiation and incubation required preirradiation preparations. We used a technique originally described by O. Maaløe and P. Hanawalt (1961) which permits DNA synthesis to continue (in the absence of protein synthesis) until the replicative cycle is completed. This was done as follows. A population of cells grown to a titer of 10^8 cells/ml in the presence of leucine and thymine-^{14}C were collected on a filter, washed, resuspended in minimal medium supplemented with only

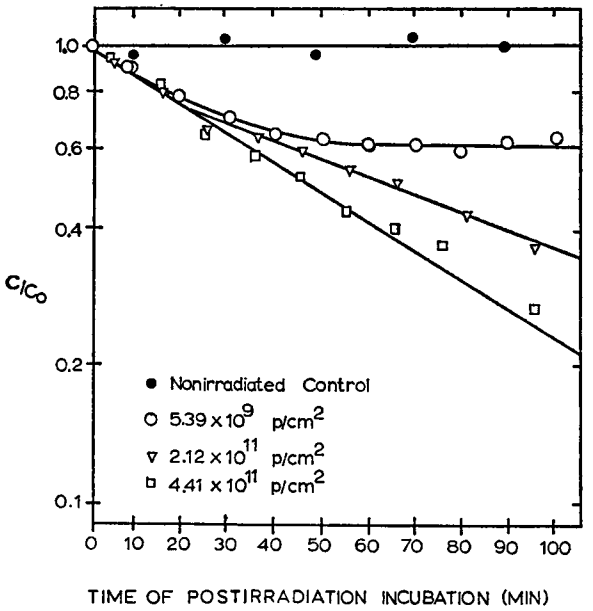

FIGURE 1 Time course of DNA degradation in *Escherichia coli* 15T⁻L⁻ irradiated in air with 2.75 Mev protons. The amount of ^{14}C in the fraction insoluble in cold TCA of irradiated cells expressed as a fraction of the corresponding ^{14}C level of nonirradiated cells (C/C_0) as a function of the time of postirradiation incubation; p = protons.

thymine-^{14}C, then incubated for 2 hr with aeration at 37°C. After this extended incubation period the cells were collected on a filter, irradiated, resuspended in unsupplemented minimal medium and assayed for loss of ^{14}C label from the acid-insoluble fraction as a function of time of postirradiation incubation.

RESULTS

Fig. 1 shows the effect of different doses of 2.75 Mev protons on the degradation of cell DNA. It should be noted that the cells which had not been irradiated continued to have a constant amount of their labeled thymine in the cold TCA-insoluble fraction. Irradiated cells began to lose ^{14}C label from the cold TCA-insoluble fraction

during postirradiation incubation immediately following irradiation. The loss reached a fixed amount for doses up to approximately 1×10^{10} protons/cm^2.

At higher doses two components of the degradation process are observed. ^{14}C label is initially lost at a rapid rate followed by a slow diminution of the labeled thymine activity. By subtracting the amount degraded by the slow process from the composite degradation line, the amount of DNA degraded by the initial process can be estimated. Several experiments similar to Fig. 1 are needed for each estimate of this amount. At still higher doses the level of ^{14}C label decreases at a constant rate for the duration of the postirradiation incubation period.

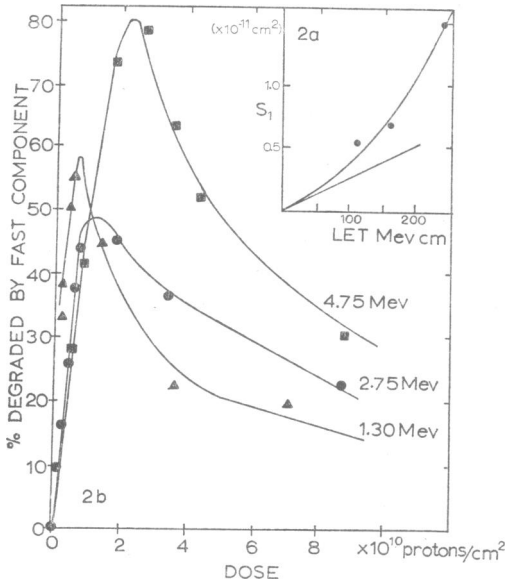

FIGURE 2 Per cent of the ^{14}C-labeled DNA component degraded by the initial component of the degradation process as related to the number of protons (p) per square centimeter. Two processes, one to start and one to limit degradation, are present. Using the relation $f = [1 - e^{(-S_1 D)}]\, e^{(-S_2 D)}$ values of S_1 and S_2 can be found. S_1 as a function of LET is shown in 2a. There is a nonlinear relationship suggesting that multiple ionization is more effective than single ionization.

The amount of DNA degraded by the initial process after irradiation with various doses of protons of three different energies is shown in Fig. 2. The maximum amount degraded by the initial process occurs at doses as indicated. In terms of D_{37} values at 2.75 Mev this dose corresponds to five times the D_{37} for colony-forming ability. The maximum is shifted toward higher doses if protons of lower LET are used.

The observed decrease in the amount of DNA degraded by the initial degradation process as a result of higher proton doses is not nearly as great as that with ^{60}Co γ-ray irradiation, even at doses of 100 kr. Emmerson and Howard-Flanders (1965) have reported that DNA breakdown in *Escherichia coli* K-12 after X-irradiation goes through a maximum at 8.5 kr. Data taken in our laboratory for *E. coli* $15T^-L^-$ irradiated by ^{60}Co γ-rays on filters are shown in Fig. 3 for comparison.

The time course of DNA degradation at different postirradiation incubation temperatures is shown in Figs. 4 a and b. Over the temperature range for which obser-

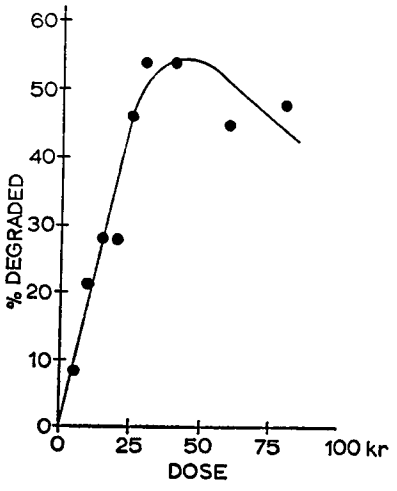

FIGURE 3 Per cent of ^{14}C-labeled DNA degraded in *Escherichia coli* 15 T^-L^- cells by different doses of ^{60}Co γ-irradiation.

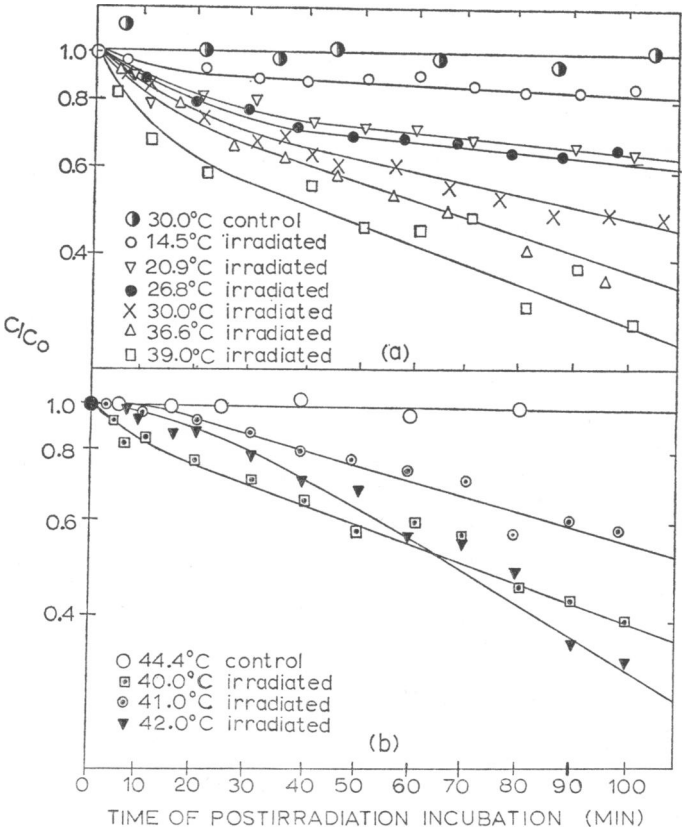

FIGURE 4 Time course of DNA degradation in *Escherichia coli* 15 T^-L^- exposed to 10^{10} protons/cm^2 at 2.75 Mev at different postirradiation incubation temperatures over a range between (a) 14.5° and 39.0°C and (b) 40.0° and 42.0°C.

vations were made, the level of ^{14}C label remained constant in nonirradiated cultures during the postirradiation incubation period. For irradiated cells the rate constant of the initial degradation process rapidly decreases above 40°C with relatively slow increase from 15°C up to 37°C. The rate constant for the slow degradation process increases uniformly with increasing temperature up to 44°C.

An Arrhenius plot (log of the rate constant vs. the reciprocal of Kelvin temperature) for both degradation processes is shown in Fig. 5.

The results of a series of experiments in which DNA synthesis was inhibited prior to irradiation and during postirradiation incubation are shown in Fig. 6. Included for comparison are data representing nonirradiated and irradiated cul-

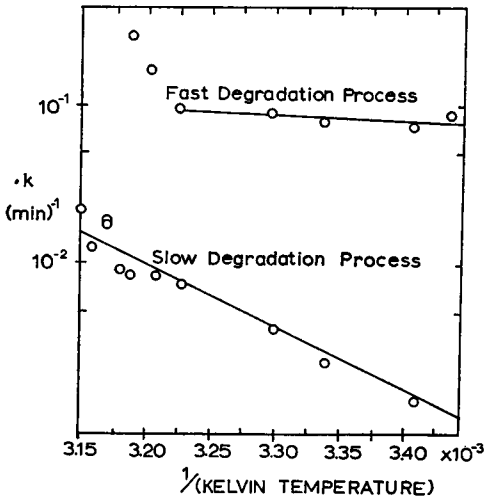

FIGURE 5 Arrhenius plots of degradation rate constants as a function of the reciprocal of postirradiation incubation temperature expressed in degrees Kelvin.

tures that were maintained under conditions permitting the simultaneous synthesis of proteins and DNA (samples I and V, respectively).

The technique of "completing the chromosomes" has no observed effect on nonirradiated cells when assayed for loss of ^{14}C label from the acid-insoluble fraction (sample II). For irradiated populations this procedure arrests the initial rapid degradation component although the slow component persists, as is indicated by the amount of sample IV.

The cells from which the data of sample III were obtained, in addition to being subjected to inhibition of DNA synthesis, were heated to 70°C for 10 min before irradiation. During 2 hr of postirradiation incubation the amount of ^{14}C label in the acid-insoluble fraction decreased only 10%, indicating that both rapid and slow degradation processes were diminished by inhibition of DNA synthesis and heat treatment. For comparison the results of colony-forming ability survival experiments are shown in Fig. 7. The 37% survival dose is 2.2×10^9 protons/cm^2.

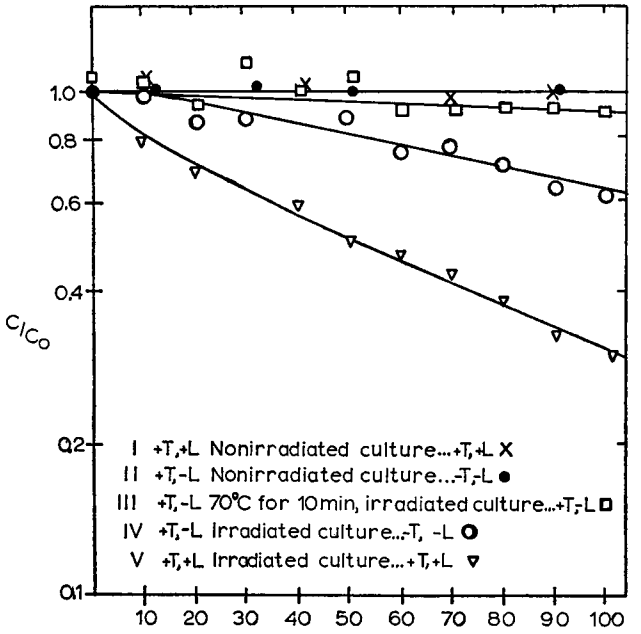

FIGURE 6 Time course of DNA degradation in *Escherichia coli* 15 T^-L^- illustrating the response of the radiation-induced degradation processes to heat and to DNA synthesis inhibition. Doses 2.11×10^{11} protons/cm² at 2.75 Mev.

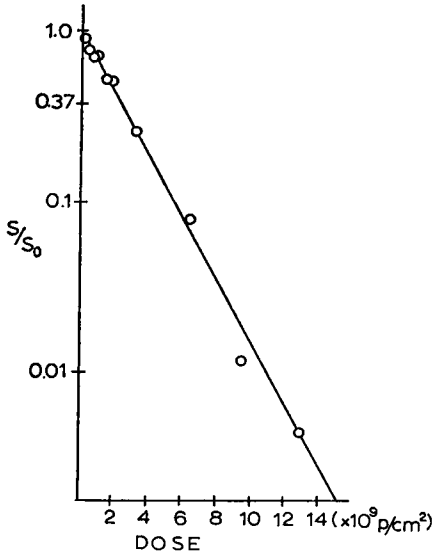

FIGURE 7 Colony-forming ability of *Escherichia coli* 15 T^-L^- as a function of dose of 2.75 Mev protons. S/S_0, the ratio of colony counts for irradiated cells to colony counts for control cells.

DISCUSSION

The time course of degradation of *Escherichia coli* 15 T^-L^- DNA irradiated with ^{60}Co γ-rays is comparable to our results obtained with low doses of fast protons. Pollard and Achey (1964) found in experiments on the effect of radiation dosage a maximum of approximately 50% of the DNA can be degraded and a dose of 15 kr (about five times the D_{37} for colony-forming ability) was the maximum dose required to degrade half the DNA complement. We find that the initial degradation process degrades approximately 50% of the DNA complement when a dose of protons equivalent to five times the D_{37} for colony-forming ability is administered.

The increase, followed by decrease for the "fast" degradation follows a pattern which suggests that two processes, one to initiate and one to terminate degradation, are present. Since strain 15 is known to carry a defective prophage (Endo et al., 1965), the possibility that the induction of the prophage plays a part needs to be

TABLE I

"CROSS-SECTIONS" FOR VARIOUS LET'S

Proton energy	Linear Energy Transfer	S_1	S_2
Mev	*Mev/cm*	cm^2	cm^2
1.30	240	1.50×10^{-10}	0.3×10^{-10}
2.75	160	0.70×10^{-10}	0.2×10^{-10}
4.75	110	0.55×10^{-10}	0.2×10^{-10}

*Initial slope deduced from ^{60}Co data of Fig. 3 is 0.25×10^{-10} cm^2 per 100 Mev/cm (Pollard *et al.*, 1955).

considered. A study by Rappaport (1958) shows a rather similar rise and fall for the induction of lysogenic *E. coli* (C-18) by deuterons and alpha particles. The dose dependence is different, being more sensitive. Our experiments do not exclude the idea that the induction of prophage is in some way concerned with the cessation of the fast process of degradation.

Rappaport's analysis employs a relation of the form

$$f = [1 - e^{(-S_1 D)}]e^{(-S_2 D)},$$

where f is the fractional effect, D the dose in particles per square centimeter, and S_1 and S_2 are two probability parameters having the dimensions of area and called "cross-sections" by analogy with similar terms in atomic and nuclear physics.

In Table I we show values of these parameters obtained for the three cases shown in Fig. 2. The values for S_2 are considerably less accurate than for S_1.

The variation of the rate constant for the slow process shows relatively normal enzyme kinetics over the range of temperatures for which observations were made.

The fast component behaves differently with a phenomenally sharp cutoff above 42°C.

In order to see whether the mechanism which synthesized DNA behaves similarly, over the temperature range 15° to 45°C, to the mechanism responsible for the rapid degradation process, the uptake of labeled thymine into the acid-insoluble fraction was studied as a function of incubation temperature. The results are indicated in Fig. 8 where we have plotted the rate constant for thymine uptake versus the incubation temperature. The rate constant for the initial degradation process as a function of postirradiation incubation temperature is also plotted in Fig. 8. The two are similar but not identical.

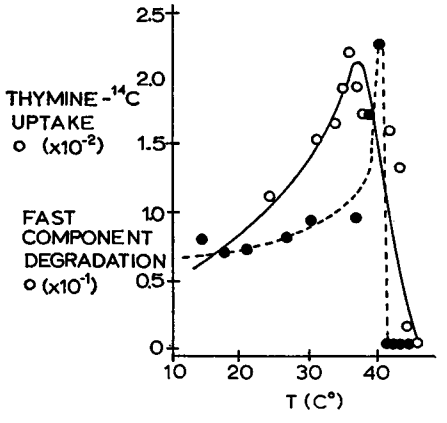

FIGURE 8 Effect of incubation temperature (T) on rate constant of ^{14}C-thymine uptake and of fast component degradation in *Escherichia coli* 15 T⁻L⁻.

Heating cells to moderately high temperatures (65° to 70°C) for a period of time sufficient to inhibit DNAase activity prior to irradiation has been observed by others to protect against degradation (Pollard and Achey, 1964; Emmerson and Howard-Flanders, 1965). Our results demonstrate the effectiveness of heating as an agent for arresting both fast and slow degradation processes.

The rapidity with which radiation-induced degradation commences after irradiation would seem to demand that the enzyme (or group of enzymes) responsible for degradation be on or adjacent to the DNA. Lack of a rapid degradation component in irradiated cells under conditions of DNA synthesis inhibition is indicative of the possibility that the DNA synthetic or transcription apparatus might be involved in radiation-induced DNA degradation.

One striking feature of proton irradiation is the very marked character of the maximum in degradation by the fast component. The way in which S_1, the cross-section for generation of initial DNA degradation, depends on linear energy transfer can be seen from Table I. The data of Fig. 4 can be used to deduce the initial slope of the cross section versus LET by the following reasoning. If radiation of low LET is used so that ionizations may miss the target even though a particle has gone

through it, a relation of the form

$$S = S_0 (1 - e^{-it})$$

(Pollard et al., 1955) holds. Here S is the observed cross section, S_0 is the true cross section, i is the number of ionizations per unit length, and t is the thickness of the sensitive region. For low values of i we have

$$S = S_0 t i$$

and $S/i = S_0 t$ = Volume, V, of sensitive region. From ^{60}Co irradiation we deduce V which tells us S/i, the slope near the origin. This has been included in Table I and the composite four pieces of data plotted in Fig. 2 a. It can be seen that the increase in the value of S_1 with LET is rather more rapid than proportional. This is not common in bacterial radiation studies but is reminiscent of chromosome aberration studies. Our data would fit with a requirement that three or four ionizations spaced closely together are necessary for the initiation of the fast degradation process (Pollard et al., 1955).

The data for the reduction of the amount degraded are much less good; S_2 is harder to measure than S_1. However, S_2 seems to vary less with LET, which could suggest that a rather thick sensitive region (about 100 Å thick) is involved in causing the loss in degrading ability.

Drakulic and Kos (1963) have advanced the hypothesis that the breakdown of DNA in irradiated *Escherichia coli* is the result of an unsuccessful attempt at replication and enzymes involved in DNA synthesis may be responsible for degradation. Their suggestions are based on the evidence that compounds which prevent the degradation of DNA after irradiation also inhibit DNA synthesis. Our data do not contradict their ideas, although the presence of two forms of degradation complicates the hypothesis.

SUMMARY AND CONCLUSION

The data we have presented clearly indicate that radiation-induced DNA degradation can proceed by two processes. The first of these processes has the following characteristics: (*a*) degradation is initiated by irradiating with ^{60}Co γ-rays or with doses of fast protons less than 10^{10} protons/cm^2; (*b*) degradation commences immediately after irradiation; (*c*) the rate of degradation is high; (*d*) a maximum of approximately 50% of the DNA complement is degraded; (*e*) degradation is inhibited at incubation temperatures above 40°C; (*f*) degradation does not occur in irradiated cells in which DNA synthesis is inhibited prior to, during, and after irradiation; and (*g*) protons of high LET are more effective in initiating the process. The second process has the following characteristics: (*a*) degradation is activated at fast proton doses greater than 10^{10} protons/cm^2; (*b*) there is a delay between the

time of irradiation and the onset of degradation; (*c*) the rate of degradation is slow compared to the first process; (*d*) the process degrades 100% of the DNA complement; (*e*) normal enzyme kinetics are observed over the temperature range of 14° to 44°C; (*f*) degradation is inactivated at high temperatures (70°C for 10 min); and (*g*) degradation is not affected by DNA synthesis inhibition.

We suggest that the first process is the result of a malfunction of normal DNA-affiliated activity such as replication or transcription.

If the magnitude of the radiation-induced lesions is severe, the DNA becomes susceptible to attack by enzymes which degrade the total DNA complement. Therefore, we believe the second process to be the result of DNAase activity.

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