Mechanism of Inactivation of Transforming Deoxyribonucleic Acid by X Rays

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Transforming deoxyribonucleic acid (DNA) from *Haemophilus influenzae* was exposed to X rays either in phosphate buffer or in 10% yeast extract. Relations between determinations of biological inactivation, DNA uptake by competent *H. influenzae*, integration of DNA into the competent cell genome, and induced single- and double-strand breaks indicate that transforming DNA is inactivated by the direct and the indirect effect of X radiation primarily because integration of DNA is prevented as a result of the production of double-strand breaks.

The biological effects of X irradiation of cells are commonly attributed to deoxyribonucleic acid (DNA) damage. X irradiation of cells results in single-strand breaks, double-strand breaks, nucleotide damage in the DNA, and damage to other parts of the cell (1). Energy from ionizing radiations, unlike properly selected wavelengths of ultraviolet, is absorbed equally by DNA and other cellular constituents. However, in vitro X irradiation of purified transforming DNA is a means of investigating the biological and physico-chemical effects of X rays on a single cellular constituent. We attempted to determine the mechanism of X-ray inactivation of transforming DNA by measuring the following properties of DNA as functions of X-ray dose: (i) inactivation of transforming ability, (ii) entrance of the DNA into the cell, (iii) physical integration of the DNA into the recipient genome, and (iv) single- and double-strand breaks. Our results indicate that transforming DNA is inactivated by X rays primarily because the cell does not integrate DNA containing X-ray-induced double-strand breaks, although such DNA is mostly taken up by the cell.

MATERIALS AND METHODS

Microorganisms and transforming DNA. The strains of *Haemophilus influenzae*, methods of growing cells, preparation of transforming DNA, and transformation assays were described previously (10). Radioactively labeled transforming DNA was made from cathomycin-resistant cells grown in the presence of 250 µg of adenosine per ml and either 50 µCi of [^{3}H]thymidine per ml (specific activity, 11.2 Ci/mmole) or 1 µCi of [^{14}C]-thymidine per ml (specific activity, 50 mCi/mmole). The specific activities of the [^{3}H]DNA and [^{14}C]DNA were 1.6 × 10^6 and 1.9 × 10^3 counts per min per µg, respectively. We added 10^{-3} M 2-mercaptoethanol (Eastman Chemical Co.) to the labeled DNA preparations to reduce radiation damage from decaying radioisotope.

DNA uptake. *H. influenzae* strain Rd cells (0.35 ml at a concentration of 10^9 cells/ml), made competent in MIV medium (12, 13), were exposed to 0.05 ml of irradiated or unirradiated [^{3}H]DNA (0.25 µg/ml) for 15 min at 37 C. A 0.1-ml amount of deoxyribonuclease (1 mg/ml) was then added, and the mixture was incubated another 5 min, followed by centrifugation and resuspension of the cells in 0.5 ml of MIV medium. Two 0.1-ml samples were placed on Whatman 3MM paper discs (2.3 cm) which were then washed three times in cold 5% trichloroacetic acid and twice in alcohol and counted with toluene-2,5-bis-2-[(5-*tert*-butylbenzoxazolyl)]-thiophene scintillation fluid in a scintillation counter. Control experiments with exponentially growing cells which are unable to take up a significant amount of DNA from the medium (12) showed no uptake with this method.

Integration. The method of measuring integration which was described previously (9) is based on the work of Steinhart and Herriott (13), who showed that radioactively labeled competent *H. influenzae* cells release label into the medium equivalent to the amount of integrated nonlabeled transforming DNA which replaces some of the DNA of the recipient genome.

X irradiation. Transforming DNA was X-irradiated with a General Electric Maxitron 250 X-ray machine equipped with a Be window X-ray tube and operated at 250 kvp and 30 ma. An added filter of 0.5-mm Al was used. Under these conditions, the half-value layer thickness is 0.12 mm of Cu, and the homogeneity factor is 0.17. Exposure rates, determined from measurements with calibrated Victoreen ionization chambers, were 1 to 12 kr/min, depending on distance (10 to 35 cm) from the source. All irradiations of 1- to 15-ml samples were done in covered 5-cm diameter plastic petri plates in air at room temperature. Samples of 10 µg of DNA per ml were either in 0.07 M phosphate buffer (pH 7) or in 0.15 M NaCl with 10% yeast extract added as a protective agent.

Measurement of X-ray-induced single- and double-strand breaks in DNA. Single-strand breaks were estimated from sedimentation rates of DNA in 5 to 20%

alkaline (pH 12) sucrose gradients with 0.1 M NaCl and double-strand breaks from sedimentation in 5 to 20% neutral sucrose containing the same saline solution. Centrifugation was at about 18 C in a SW39 or SW50.1 rotor at 30,000 rev/min for 90 to 240 min. Fractions from the bottom of the centrifuge tube were collected on Whatman 3MM discs (2.3 cm) and processed as described in the section on DNA uptake. The radioactivity measured in the top and bottom samples was variable, probably because of artifacts such as incomplete removal of low-molecular-weight pieces of DNA which remain at the top of the gradients or aggregates which appear at the bottom. Therefore we have calculated the weight-average, M_w, and the number-average, M_n, molecular weights using only the middle 80 to 85% of the gradients. This procedure had little effect on the calculated value of M_w but could appreciably affect the value of M_n. Hence, all of our calculated values for M_n tend to be too large. The empirically based relations between sedimentation rate and molecular weight given by Studier (14) were used for the calculations. The constants of proportionality for our experimental conditions with alkaline and neutral gradients were obtained from analysis of separate sedimentation experiments with radioactively labeled DNA of bacteriophage lambda b₂ (kindly supplied by Grete Kellenberger-Gujer), for which we took the double-strand molecular weight at the gradient peak to be 2.7×10^7 (reference 15). Equal amounts (0.25 µg) of irradiated ³H-labeled transforming DNA and unirradiated ¹⁴C-labeled transforming DNA were placed on top of each gradient. For each gradient, the average number of X-ray-induced breaks, B, per single or double strand was calculated in three ways as follows

$$B = (C_n/T_n) - 1 \qquad (1)$$

$$B = (2 C_n/T_w) - 1 \qquad (2)$$

$$B = (C_w/T_w) - 1 \qquad (3)$$

where C_n and T_n are the number-average molecular weights of the unirradiated [¹⁴C]DNA and irradiated [³H]DNA, respectively, and C_w and T_w are the weight-average molecular weights. Charlesby (4) has shown that, for all molecular-weight distributions, equation 1 gives the average number of breaks induced in the number-average molecule and that, for a random distribution of molecular weights, equation 2 yields the same result. We use equation 3 as an estimate of breaks per weight-average molecule. For various conditions (e.g., single strand, direct effect), we plotted the numbers of breaks (calculated from these equations) as functions of exposure. The average slope, S_i, and the widths of their 95% confidence intervals, V_i, were obtained from least square fits. The average exposure per break, E, for weight-average molecules, was computed from

$$E = [(0.8 \, S_1\overline{M}_n/V_1{}^2\overline{M}_w) + (S_3/V_3{}^2)]/ \\ [(S_1{}^2/V_1{}^2) + (S_3{}^2/V_3{}^2)] \quad (4)$$

where $\overline{M}$ values are number and weight averages for unirradiated DNA. This procedure amounts to averaging the values of $1/S$ by using weighting factors $(S_i/V_i)^2$. The factor 0.8 was arbitrarily introduced to compensate for overestimating T_n in equation 1 when B is large.
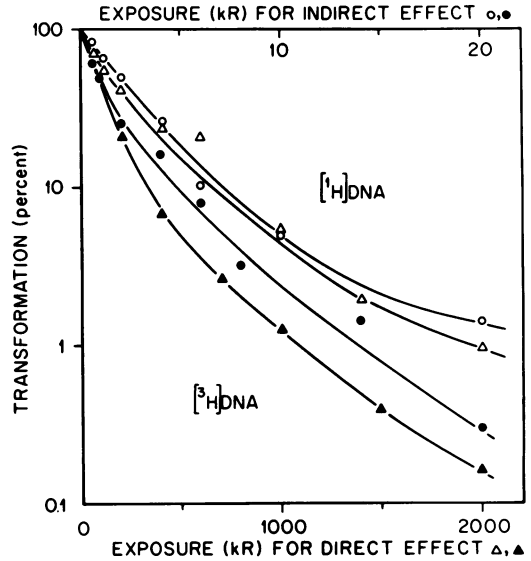

FIG. 1. *X-ray inactivation of the ability of H. influenzae DNA to transform H. infleunzae strain Rd to cathomycin resistance. DNA was irradiated in phosphate buffer (indirect effect) or in 10% yeast extract and 0.15 M NaCl (direct effect). Exposure scales for indirect effect (given at the top) and direct effect (given at the bottom) differ by a factor of 100. Open symbols are shown for the unlabeled DNA ([¹H]DNA) and solid symbols for the labeled DNA ([³H]DNA).*
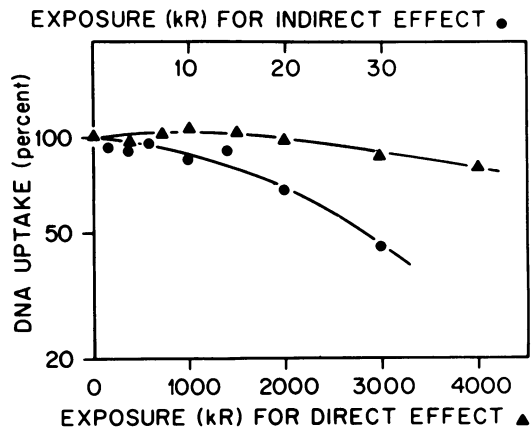

FIG. 2. *Effect of X-ray exposure of transforming DNA on uptake of this DNA by competent H. influenzae strain Rd. DNA was irradiated in phosphate buffer (indirect effect) or in 10% yeast extract and 0.15 M NaCl (direct effect). Exposure scales for indirect effect (given at the top) and direct effect (given at the bottom) differ by a factor of 100. All points are averages of at least two separate experiments. Approximately 75% of the input unirradiated DNA entered the cells. This was called "100% uptake."*

RESULTS

The loss of transforming ability of DNA resulting from X-ray irradiation is about 100 times as great when the DNA is irradiated in buffer as when it is irradiated in a protective compound, although the shapes of the dose effect curves are similar under the two conditions (Fig. 1). All of the curves are convex downward on a semilogarithmic plot. The radioactively labeled DNA is about twice as sensitive as the other DNA, which difference we believe reflects that the molecular weight of the labeled DNA preparation may be twice that of the other preparation. These measurements were completed within 3 weeks of the preparation of the labeled DNA. The double-strand molecular weight of this material decreased only about 30% in 8 months. Hence, strand breaks induced by isotope decay seem an unlikely cause for differences in radiosensitivity.

Figure 2 shows the direct and indirect effect of X irradiation of transforming DNA on the competent cells' uptake of DNA. There is little or no observable decrease in uptake except at the highest doses. Thus, the radiosensitivity as measured by uptake is much less than that for transformation as Lerman and Tolmach have shown for pneumococcal DNA (8). However, integration of transforming DNA into the recipient genome is decreased markedly by X irradiation, as shown by the data of Fig. 3 in which the release of radioactivity from radioactively labeled
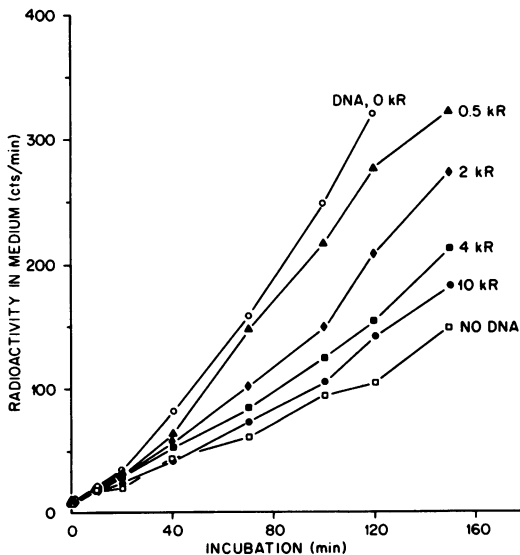

FIG. 4. *Effect of X-ray exposure of transforming DNA on integration into the DNA of competent H. influenzae strain Rd. DNA was irradiated in phosphate buffer (indirect effect, data of Fig. 3), or in 10% yeast extract and 0.15 M NaCl (direct effect). Exposure scales for indirect effect (given at the top) and direct effect (given at the bottom) differ by a factor of 100.*

TABLE 1. *Molecular-weight averages for unirradiated transforming DNA*

Type of average	Single strand	Double strand	Ratio of double/single
Weight	25×10^6	58×10^6	2.3
Number	8.5×10^6	12.4×10^6	1.5
Ratio of weight/ number average[a]	3	4.7	

[a] For a monodispersive molecular population, the weight-average (M_w) and number-average (M_n) should be equal. If an average of more than about five random breaks are introduced into a monodispersive population, the weight average should be twice the number average (4). A ratio of M_w to M_n significantly greater than 2 is evidence for a bimodal or polymodal distribution. From our method of calculation, the value of M_w/M_n should be less than the true one, but the observed ratio is greater than 2. Thus, the true ratio for this transforming DNA must be considerably greater than 2. The measured unirradiated distributions of molecular weights of either single- or double-strand molecules are therefore bi- or polymodal.

FIG. 3. *Release of radioactivity from ³H-labeled competent H. influenzae strain Rd with time of incubation, with or without unirradiated or X-irraidated transforming DNA. DNA irradiated in phosphate buffer (indirect effect).*
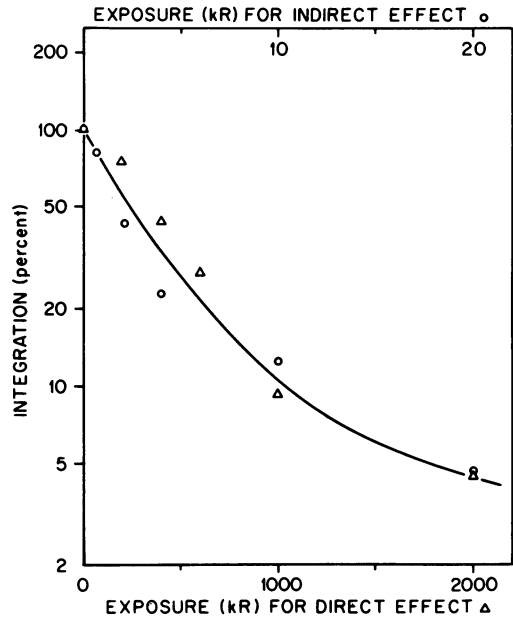
competent cells exposed to unirradiated DNA or DNA irradiated in buffer is plotted against time of incubation with the DNA. There is also a slow nonspecific release of label from competent cells
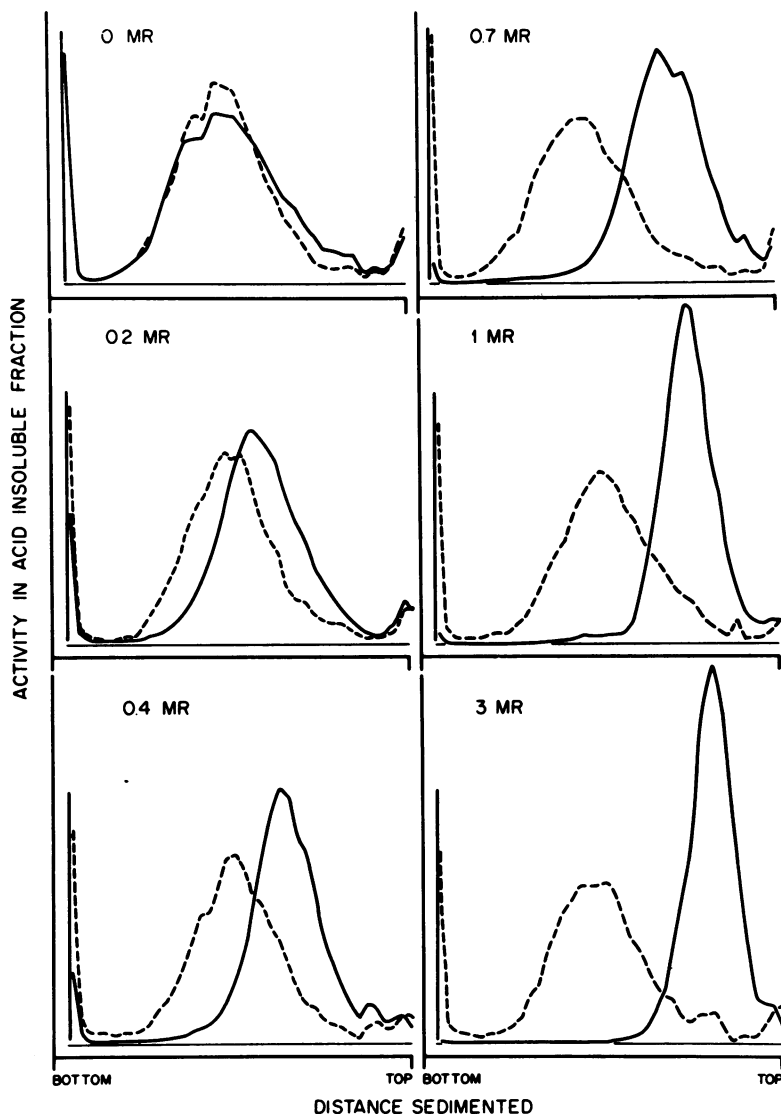
FIG. 5. *Sedimentation of transforming DNA in neutral sucrose after various X-ray exposures of the DNA. Centrifugation at 30,000 rev/min for 150 min was done with a SW50.1 rotor. In each panel, the solid line represents the ^{3}H-labeled DNA, and the dotted line represents the unirradiated ^{14}C-labeled DNA. Sedimentation goes from right to left. The height of the vertical bar at the left of each panel represents 10% of the total radioactivity found on the gradient and typically for these gradients composed of about 35 samples represents 8,000 ^{3}H counts or 1,000 ^{14}C counts.*

not exposed to exogenous DNA. The specific release of label (i.e., difference in release from cells exposed to and not exposed to DNA) is reduced by irradiating the DNA and approaches zero at the highest dose. Calculation from these data of the amount of integration, taken as proportional to specific release, as a function of dose is shown in Fig. 4, along with similar data obtained for DNA irradiated in yeast extract. Dose curves for reduction of integration by both the direct and

indirect effect are similar to those for inactivation (Fig. 1, [^{1}H]DNA; Fig. 4), although transformation appears to be slightly more sensitive.

The average molecular weights for the labeled unirradiated transforming DNA obtained from sedimentation velocity measurements are shown in Table 1. The double-strand molecular weights are about twice the single-strand ones. The weight-average molecular weights are more than twice as large as the number-average weights.
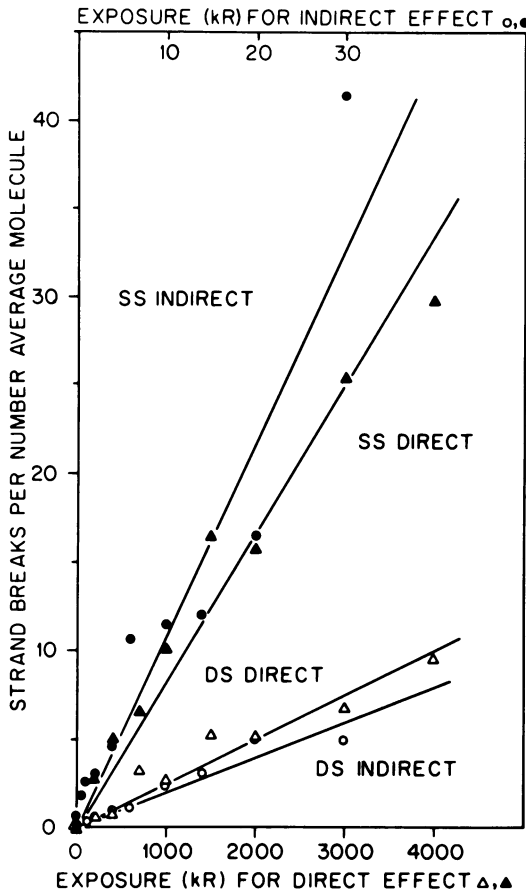
EXPOSURE (kR) FOR INDIRECT EFFECT o,●

FIG. 6. *Production of single-strand (SS) breaks (solid symbols), and double-strand (DS) breaks (open symbols) per number-average strand as a function of X-ray exposure of ³H-labeled transforming DNA. DNA was irradiated in phosphate buffer (indirect effect) or in 10% yeast extract and 0.15 M NaCl (direct effect). Exposure scales for indirect effect (given at the top) and direct effect (given at the bottom) differ by a factor of 100. Most points are averages of two different sedimentation velocity experiments. Equation (1) was used for calculation of breaks.*

Figure 5 shows typical gradients for DNA given a series of X-ray doses. Figure 6 shows a summary of such data as the production of single- and double-strand breaks per number-average strand (calculated from equation 1) induced by the direct and indirect effect of X-rays on the labeled DNA. The radiosensitivity for the indirect effect is about 100 times that for the direct effect. Per unit mass, single-strand breaks are produced 5 to 10 times as readily as double-strand breaks.

Table 2 is a summary of the doses required to reduce transforming activity and integration to

TABLE 2. *X-ray exposures (kr) of transforming DNA required to reduce transformation, DNA integration, and uptake to 37% and to make an average of one single-strand or double-strand break per weight-average molecule*

DNA	Direct effect	Indirect effect
[¹H] DNA		
Transformation	240	2.8
Integration	350	3.5
[³H] DNA		
Transformation	115	1.4
Single-strand break	42	0.6
Double-strand break	104	1.8
Integration[a]	170	1.7
Uptake	≫4,000	>30

[a] Calculated from the ratio of sensitivities of transformation and integration obtained for the [¹H]DNA, assuming the same ratio for [³H]DNA.

37% in the labeled and unlabeled DNA species and doses to make single- and double-strand breaks in weight-average molecules of the labeled DNA. Since the transforming activities of the two DNA species have different inactivation sensitivities, and since integration was measured only in the unlabeled DNA and strand breaks only in the labeled DNA, we calculated the values for integration in the labeled DNA, assuming a constant ratio of inactivation of transformation and integration. In the case of the labeled DNA, there is a close correspondence between the values for transformation and double-strand breaks, whereas the single-strand breaks show lower radioresistance.

DISCUSSION

The similarity of comparable curves for loss of transforming ability and for reduction of integration indicates that reduction of integration into the DNA of competent cells is the dominant factor in X-ray inactivation of transforming DNA. This applies for both direct and indirect effects. This conclusion is contrary to that obtained from a similar study of ultraviolet inactivation of H. influenzae transforming DNA (9) which showed that transforming activity is very much more ultraviolet-sensitive than is integration. In considering transformation here, we deliberately report results for only the least radiosensitive marker (cathomycin) and base our discussion on these because we believe that this inactivation is the result of a common underlying mechanism to which, for other markers, is added inactivation by secondary effects.

The fact that transformation appears to be

slightly more sensitive to X irradiation than does integration (Table 2) could indicate that a small amount of DNA damage is integrated into the recipient genome. However, the errors in measurement of integration leave the magnitude and even validity of this difference uncertain.

Our values for radiosensitivity of transforming DNA and generally our ratios of radiosensitivities for direct versus indirect effects and for single- versus double-strand breaks are comparable to values of others (3, 5, 7) for such effects in different bacterial systems.

The sensitivity of the labeled DNA to X-ray-induced double-strand breaks per weight-average molecule and the calculated values for the comparable sensitivity to loss of integration are the same within experimental error (Table 2). We thus believe that most of the inactivation of transforming ability results from double-strand breaks which prevent integration. However, because of the relatively small difference in radiosensitivities between single- and double-strand breakage per strand and the problems of averaging over wide molecular-weight distributions, distinguishing between single- and double-strand break mechanisms is difficult. Since X rays produce more single-strand than double-strand breaks, our finding that double-strand breaks and reduction of integration are related suggests that single-strand breaks are incorporated into the recipient genome and repaired there. The sensitivity of transforming DNA X-irradiated in yeast extract was observed previously (11) to be slightly greater when assayed in strain DB116 than in wild-type cells, although this has not been found for all preparations of transforming DNA. The observed sensitivity difference could result from a difference in repair of incorporated single-strand breaks, since it was earlier found that the rejoining of single-strand breaks is lower in DB116.

For *Bacillus subtilis*, Bodmer (2) found a direct relationship between transforming activity and single- rather than double-strand molecular weight of transforming DNA after deoxyribonuclease I digestion. He also found that single-strand breaks inhibit integration less than transformation. Our fundamental differences from his conclusions may be caused by differences between bacterial systems or (we think more likely) by differences between means of degrading DNA.

Our present results on X-ray inactivation of transforming DNA for *H. influenzae* are explic-

able in terms of strand breakage, independent of other (e.g., nucleotide) forms of damage (16).

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