BREAKDOWN OF DNA IN X-IRRADIATED *ESCHERICHIA COLI*

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ABSTRACT A comparison of differences in incorporation and loss of radioactivity between two strains of *Escherichia coli* shows that: (*a*) three times as much irradiation is necessary to produce the same reduction in incorporation of H^3-thymidine in B/r, the resistant strain, as in B_{s-1}, the sensitive one; (*b*) radioactivity is lost from the DNA of previously labeled bacteria during the first few cell generations after X-ray exposure, and even though the initial rate of loss is similar for all strains, the sensitive one loses much more label; (*c*) loss of DNA is a complicated function of dose. Losses increase with dose up to 25 or 50 kr in both strains; with higher doses, losses decrease in B_{s-1} but are unchanged in B/r. Since in both strains labeled RNA is retained in irradiated cells, lysis has not occurred but the DNA is broken down into small pieces which leak from each cell. Losses from either strain do not occur at ice-bath temperature, indicating that breakdown is a function of metabolic processes. A proposed mechanism for X-ray damage and repair is advanced.

INTRODUCTION

Exposure to ionizing radiation damages cells and alters their normal biological function. In a number of systems recovery occurs either by repair or by circumvention of the damage [(see Powers, Adler and Engle, Oster, and others, (1)]. Without excluding other effects of irradiation, it is tempting to assume that repair of damaged DNA may occur either during or as a prerequisite to the recovery of normal biological function. It is known, for example, that DNA is lost from irradiated bacteria (2, 3), that nucleosides and nucleotides leak from the cells (4), and that the amount of loss is strain-dependent (5).

Results consistent with the repair concept can be extracted from studies on mouse intestinal crypt cells (6), onion root tips (7; see also 8), and slime mold (9, 10), in which peculiar labeling of DNA was seen after irradiation. More direct evidence for repair was suggested from studies on grasshopper neuroblasts (11, 12) and mammalian cells in tissue culture (13), which, after exposure to X-rays, incorporate H^3-thymidine into DNA during cell stages when normal DNA sythesis does not occur.

This study deals with the DNA of *Escherichia coli* during the first few cell generations after X-ray exposure. H^3-thymidine was used to study the incorporation and loss of radioactivity in radiosensitive (B_{s-1}) and radioresistant (B/r) cells after X-ray exposure. Significant differences were observed between them.

MATERIALS AND METHODS

Two strains of *Escherichia coli,* B/r (Oak Ridge National Laboratories) and B_{s-1} (obtained from Dr. Ruth Hill), were used. The bacteria were grown overnight at room temperature without aeration. A dilution of culture was then grown with aeration at 37°C to approximately 2×10^8 cells/ml. A 1:10 dilution of this log-phase culture (which will be called *bacterial suspension*) was chilled in ice and subsequently used experimentally. The liquid culture medium used in this study was M-9 which contained 0.25% casamino acids (Difco Laboratories, Inc., Detroit) (14).

A General Electric Maxitron was used as a source of X-rays. Operating conditions were: 250 kvp, 30 ma, target to object distance 11 cm, added filtration 1.0 mm Al, exposure rate (measured in air with a Victoreen dosimeter, Victoreen Instrument Co., Cleveland) approximately 6 kr/min. Exposures up to 250 kr were used. Bacteria were at ice-bath temperature during irradiation.

To study incorporation of H^3-thymidine into DNA, 0.2 ml samples of irradiated bacterial suspension were added to chilled tubes which contained 5 μl of H^3-thymidine (New England Nuclear Co., Boston, 1 mc/ml, specific activity 6.7 c/mmole, and 10 μl of adenosine (2.5 mg/ml) (14). This mixture was incubated at 37°C and 10 μl samples were withdrawn at different times. Samples were mixed with a drop of water on stainless steel planchettes, a drop of 2% formaldehyde was added to kill the cells, and the sample was dried on the planchette at 50°C. To remove radioactivity not incorporated into the acid-insoluble fraction (presumably DNA), planchettes were immersed in 3:1 ethanol-acetic acid, washed for 10 min in 10% trichloroacetic acid, and passed through a series of ethanol-water solutions (15). Planchettes were again dried at 50°C, and the remaining radioactivity on the planchettes was determined by counting in a gas flow counter.

To study loss of radioactive material, the bacterial suspension was first labeled for 2 to 2½ hr at 37°C (bacterial suspension 0.4 ml, H^3-thymidine 10 μl, adenosine 20 μl). Cells in the suspension were collected on a type HA Millipore filter (Millipore Filter Corp., Bedford, Massachusetts) (pore size 0.45 μ), washed 3 to 4 times with culture medium, resuspended in fresh medium, and chilled in an ice bath (final concentration $\sim 2 \times 10^8$ cells/ml). The suspension of prelabeled bacteria was irradiated at ice-bath temperature; it was then incubated at 37°C and 10 μl samples were removed at different times. Planchettes were prepared, washed, and counted as described above.

Descending paper chromatography was carried out on: (*a*) Whatman No. 1 paper (Balston Ltd., Maidstone, Kent, England) with isobutyric acid, 100 ml; water, 55.8 ml; ammonia (specific gravity 0.9), 4.2 ml: EDTA (0.1 M, pH 4.6), 1.6 ml. Duration of development was 16 to 17 hours (16). (*b*) DEAE paper with ammonium bicarbonate, 0.25 M; potassium tetraborate, 0.005 M; EDTA, 0.001 M. Duration of development was 3 to 4 hours (17). Ascending chromatography was carried out on Whatman No. 1 paper with water at PH 6.5. Duration of development was 3 hr (16). The positions of known compounds were determined by their absorbance of ultraviolet light, and the position of radioactive spots was determined by counting in a gas-flow strip scanner.

RESULTS AND CONCLUSIONS

The effect of different exposures of X-rays on the incorporation of H^3-thymidine into bacterial DNA[1] is shown in Fig. 1*a* and *b*. It is clear that at 60 min B_{s-1} is affected to about the same degree by 2.5 kr as B/r is by an exposure to 10 kr. A direct comparison of sensitivities is shown in Fig. 1*c*, where the incorporation at 40 min after irradiation is plotted against X-ray exposure. B_{s-1} is about 3 times as sensitive as

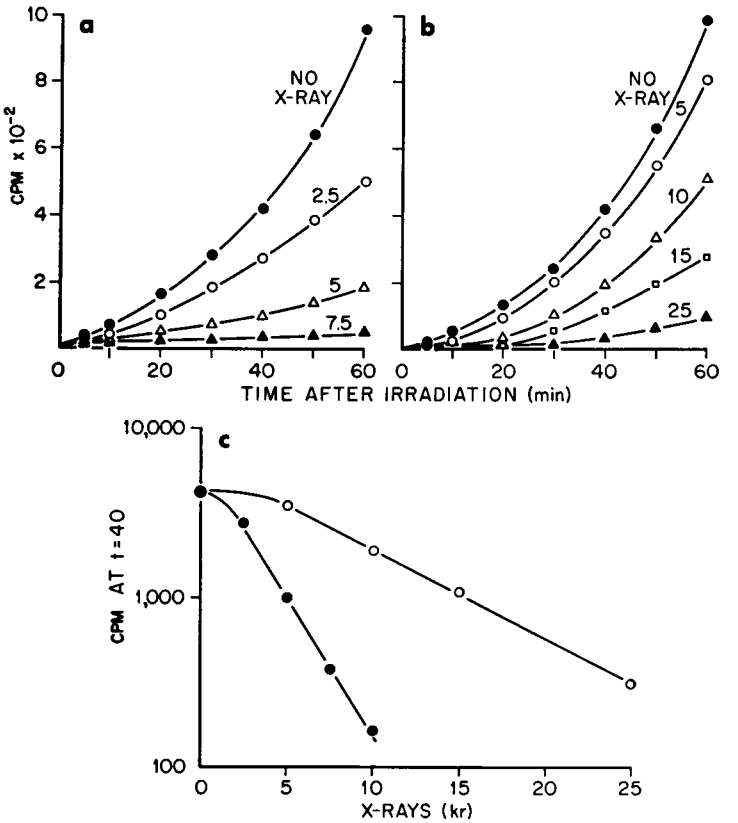

FIGURE 1 Incorporation of H^3-thymidine into *E. coli;* (*a*) Relationship between counts per minute per 10 μl of B_{s-1} cell suspension and minutes of incubation at 37°C. Details are given in the text. (*b*) Like (*a*) but for strain B/r. (*c*) Relationship between counts per minute per 10 μl of suspension and X-ray exposure. All samples taken 40 min after irradiation (○ B/r; ● B_{s-1}).

B/r. Percentage survival after exposure to X-ray (on the basis of colony formation on nutrient agar) was 75% for B/r at 10 kr, 1.8% at 50 kr; 0.3% for B_{s-1} at

[1] Autoradiographic studies have shown that radioactivity, as incorporated H^3-thymidine, is removed from bacteria by treatment with DNase (15).

10 kr, 0.00002% at 50 kr. Slopes of survival curves differ by a factor of about 3, in agreement with the findings of Hill and Simson (18).

Loss of labeled material from bacteria irradiated with 50 kr is shown in Fig. 2*a*. Total losses were 30 to 40% from B/r and 80 to 90% from B_{s-1}. The ratio of material lost (B/r : B_{s-1}) did not change significantly between cell concentrations of 1×10^8 to 6×10^8. The concentration used in this study was 2×10^8. After irradiation with lower doses, less material was lost from each strain. After exposure

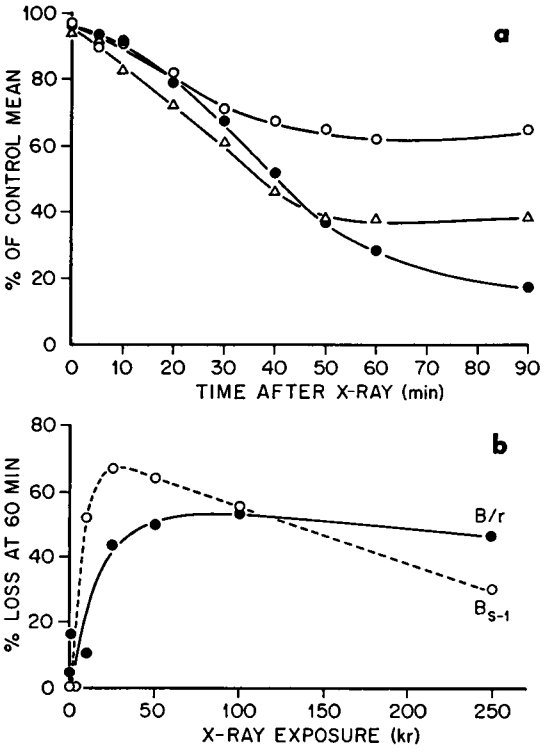

FIGURE 2 (*a*) Percentage of acid-insoluble radioactivity in labeled bacteria plotted against time at 37°C after exposure to 50 kr. 100% represents approximately 4000 CPM/10 μl sample and is the average of all unirradiated samples from 0 to 90 min. ● B_{s-1}; ○ B/r; △ *E. coli* B-94. The last is included to show the extent of loss observed with another strain. (*b*) The loss of radioactivity from irradiated B/r and B_{s-1} compared with unirradiated cultures. Samples taken 60 min after irradiation.

to 100 or 250 kr, however, the rate of loss of radioactivity form B_{s-1} decreased but no large differences were apparent in B/r (Fig. 2*b*). No losses were seen from irradiated preparations kept at ice-bath temperature for up to 120 min. When such preparations were subsequently incubated at 37°C, loss of labeled material occurred to the same extent and at the same rate as in preparations which had not been kept chilled.

In order to determine whether loss of labeled DNA was due to lysis (or permeability to large molecules), bacterial RNA was prelabeled with H^3-uridine (Schwarz BioResearch Inc., Orangeburg, New York), cells were washed, resuspended in nonradioactive medium, and exposed to 25 kr of X-rays. Samples were taken at

15 min intervals and counted on planchettes. Table I shows counts per minute in TCA-insolube H^3-uridine–labeled RNA of B/r and B_{s-1} at different times after X-ray exposure. Values in the table, which are averages of duplicate samples from a typical experiment, show that some loss of labeled material occurs. In the irradiated B_{s-1} a 17% loss was observed, whereas a 10% loss occurred in the unirradiated samples. The difference between these values, i.e. 7%, is a maximum estimate of losses due to lysis. Sixty min after radiation with 25 or 50 kr, B_{s-1} cultures labeled

TABLE I

CPM PER 10 μl SAMPLE OF H^3-URIDINE–LABELED *E. COLI*

	B/r		*B_{s-1}*	
	Nonirradiated	25 kr	Nonirradiated	25 kr
min				
0	11,760	11,050	10,599	10,452
15	11,344	11,485	10,440	10,293
30	11,148	11,103	10,746	9,943
45	11,107	10,784	10,087	9,601
60	10,918	10,311	9,583	8,663

with H^3-thymidine showed losses of 60 to 70%, whereas cultures labeled with H^3-uridine showed only 7% loss; it is therefore evident that losses are not due to lysis of the bacteria, but to partial degradation of the DNA. This conclusion is consistent with findings of an autoradiographic study which showed that similar percentages of radioactivity are lost from DNA of all cells in the population after X-ray exposure (19).

To study material released from DNA into the culture medium (4), bacteria were prelabeled with H^3-thymidine, washed, resuspended in nonradioactive medium, and exposed to 25 kr of X-rays. Cells were incubated for 30 min at 37°C and then chilled at ice-bath temperature. Samples of the suspension were dissolved in formic acid and counted in a scintillation counter. The remaining suspension was centrifuged with unlabeled carrier bacteria, and samples of supernatant were mixed with formic acid and counted. A comparison of irradiated and nonirradiated suspensions and their supernatants is shown in Table II. These data show that approximately 39% (B/r) and 58% (B_{s-1}) of the original radioactivity was lost from irradiated bacteria into the aqueous culture medium. Eight % (B/r) and 6% (B_{s-1}) of the original radioactivity was lost from unirradiated samples.

Radioactive material released into the medium after irradiation was studied chromatographically. Cultures of labeled B_{s-1} and B/r were filtered, washed with fresh culture medium, and resuspended in medium containing no thymidine. A 0.2 ml sample was used as a no X-ray control, another 0.2 ml sample was exposed to 25 kr of X-rays. Both samples were incubated at 37°C for 50 to 60 min, filtered through

TABLE II

CPM PER SAMPLE OF H^3-THYMIDINE-LABELED *E. COLI* 50 TO 60 MIN AFTER X-RAY EXPOSURE

	B/r		B_{s-1}	
	Nonirradiated	25 kr	Nonirradiated	25 kr
Suspension	392	496	501	720
Supernatant	33	196	30	415

a Millipore filter (type HA), and the filtrate was collected. Fifty μl of filtrate was applied to Whatman No. 1 paper and chromatographed overnight in the ammonia–isobutyric acid solvent along with nonradioactive markers. Results from a typical experiment with B_{s-1}, shown in Fig. 3, indicate that in irradiated preparations a large amount of radioactive material has a chromatographic mobility similar to thymidine. A smaller amount of material in both irradiated and nonirradiated samples moves chromatographically between thymidine monophosphate (TMP) and thymidine triphosphate (TTP). Material from the thymidine region was eluted

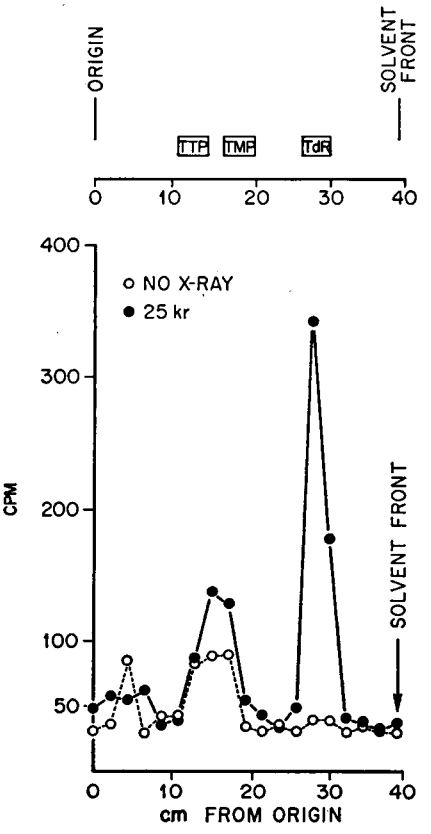

FIGURE 3 Counts per minute at various positions on a chromatographic strip. Table at top shows position of nonradioactive markers, determined by absorption of UV light. See text for additional details.

and rechromatographed with nonradioactive markers in water at pH 6.5 to distinguish between thymine and thymidine. All radioactivity, determined by gas-flow strip counting, was again found to correspond to the position of thymidine. Similar results were found with B/r.

To determine whether any abnormal products were formed *immediately* after irradiation, washed, prelabeled bacteria (both B/r and B_{s-1}) were exposed to 50 kr of X-rays at ice-bath temperature. The suspensions were centrifuged in the cold, and the supernatant was filtered through a Millipore filter and chromatographed on DEAE paper with ammonium bicarbonate solvent. No significant radioactivity was seen in any supernatant fraction.

Morphological evidence for loss of material from individual bacteria is given in Fig. 4, a series of electron micrographs depicting fields which contain either irradiated or nonirradiated cells. Suspensions of bacteria were irradiated and incubated for 45 min at 37°C, then fixed by a modified Kellenberger method (20) embedded, sectioned, and stained with uranyl acetate. Loss of heavily stained material from the nuclear bodies occurs in both B/r and B_{s-1}, with obviously greater losses from B_{s-1}. These results agree with the autoradiographic findings previously mentioned (19).

DISCUSSION

After X-ray exposure, the DNA of *E. coli* is solubilized and degradation products leak into the aqueous culture medium (2-5, 21). We find that when strains B/r and B_{s-1} are studied under conditions of exponential growth the amount of DNA degraded after a given X-ray exposure is greater in B_{s-1} by a factor of 2 to 3. Emmerson and Howard-Flanders (22) reported that the capacity to degrade DNA after X-irradiation of K-12 strains of *E. coli* was not affected by mutation at any one of the genetic loci which are known to control thymine dimer excision. Although it is known that B/r can excise dimers and B_{s-1} does not, the two studies under consideration differ for the following possible reasons: (*a*) our study was conducted with exponentially growing cells whereas the previous one (22) was done with near-stationary phase cells; (*b*) different sensitive strains were used (sensitive strains of both *E. coli* K-12 and B are unable to excise dimers from their DNA, but the K-12 strain may be able to complete the subsequent repair steps [replication and rejoining] whereas B_{s-1} cannot do so).

Evidence from autoradiographic studies of irradiated B/r and B_{s-1} shows that virtually all cells lose labeled material from DNA and that degradation is not a result of complete loss from some cells and negligible losses from others in the population (19). Combined with the electron microscopic evidence in this study (see Fig. 4), data from the autoradiographic work suggest that in some strains, such as B/r, the survivors must either replace the degraded DNA or continue to function after an appreciable loss of genetic material. We propose that a fraction of

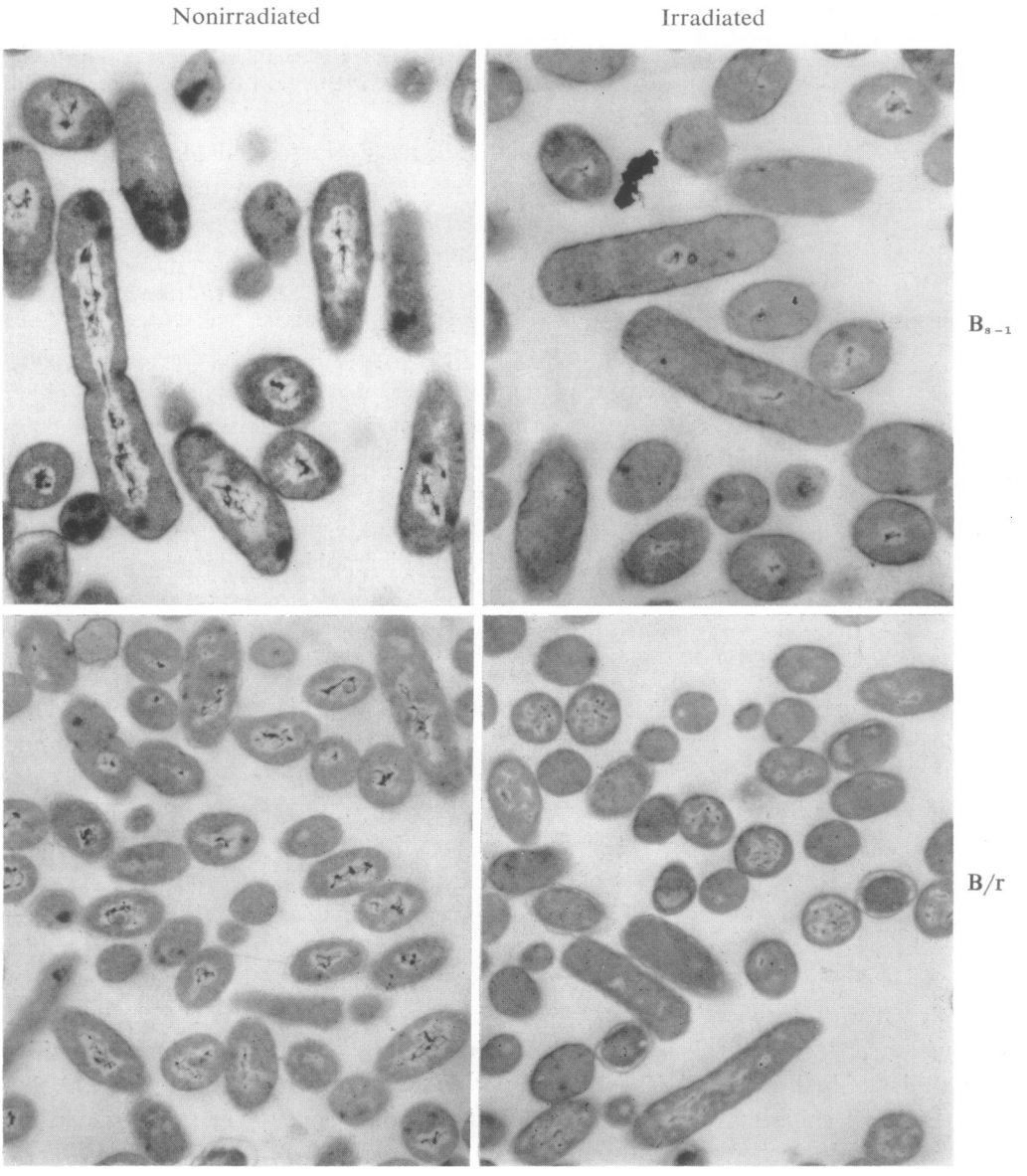

FIGURE 4 Electron micrographs of irradiated and nonirradiated bacterial suspensions fixed by a modified Kellenberger method (20) and embedded in Epon 812. An X-ray exposure of 50 kr was used. Approximate magnifications, B_{s-1} — 12,000 ×; B/r — 8000 ×. Loss of heavily stained material from irradiated cells is evident.

the DNA degraded after irradiation is indeed replaced, utilizing nondegraded complementary regions as templates for "replacement synthesis" (perhaps similar to the "repair replication" suggested by Pettijohn and Hanawalt (23) in cells exposed to UV light or to the perturbed replication seen in irradiated *E. coli* by Billen et al. [24]) If both a single chain region and its complement were degraded, double chain breaks would remain in the DNA. It seems reasonable to suppose, on the basis of our degradation data, that in B_{s-1} replacement synthesis and/or rejoining of broken strands does not occur; as a result there is extensive degradation and the cells do not survive. Some recent observations (25) support this hypothesis. The molecular weight of denatured DNA from B/r and B_{s-1} was measured both before and after X-ray exposure. Although the molecular weight of DNA from both strains is reduced equally by equal X-ray exposures of 20 kr, the molecular weight of DNA from irradiated B/r returns to near normal values during 40 min incubation at 38°C whereas that of B_{s-1} does not. We interpreted these results as evidence for rejoining of broken pieces of DNA in B/r. It seems reasonable to suppose that less degradation occurs from radioresistant cells such as B/r, because the broken pieces of DNA are rejoined, thereby removing sites at which degradation can start. In B_{s-1}, rejoining is ineffective and degradation can progress much further.

It is difficult to explain the reduced degradation seen in B_{s-1} after exposure to large (100 to 250 kr) amounts of irradiation (Fig. 2*b*). Although we have no evidence bearing on this point, it is possible that the effectiveness of one or more kinds of degradative enzyme may be reduced at this exposure level, either by partial destruction or by complexing with an excess of substrate.

With respect to the repair mechanisms considered, it seems important to distinguish between replacement synthesis and the repair of broken strands by rejoining. X-ray–induced incorporation of H^3-thymidine into DNA of cells not in their normal period of DNA synthesis has been reported both in grasshopper neuroblasts (11, 12) and in mammalian cells in tissue culture (13). It is possible that these and other cases of peculiar DNA synthesis in other organisms (6-10) may reflect replacement synthesis. Rejoining of broken pieces, on the other hand, may be more analogous to the well known breaking and rejoining or exchange of chromosome segments that occurs in cells of higher organisms.

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