THE STUDY OF IONIZING RADIATION EFFECTS ON *ESCHERICHIA COLI* BY DENSITY GRADIENT SEDIMENTATION

C. E. HILDEBRAND *and* E. C. POLLARD

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

ABSTRACT Density gradient sedimentation of bacterial cells in cesium chloride has been used to separate cells which have been irradiated with ^{60}Co gamma rays and have lost an appreciable amount of their DNA by subsequent degradation. Irradiated cells are found to band mainly at two characteristic densities, one corresponding to normal unirradiated cells and the other at a considerably lower density. The region corresponding to normal density cells is the only one that contains cells which will form colonies. Cells capable of synthesizing DNA following irradiation are found mainly at the region of normal density cells with some spreading into the lower density region. Cells in the lower density region contain less DNA than normal density cells. From an analysis of the relative numbers of cells in the two regions, it is suggested that the process of DNA degradation either takes place to a considerable extent in the genome or not at all. Analysis of the data in terms of numbers of cells having intact DNA and those having degraded DNA indicates a strong correlation between DNA degradation and cell death in this strain, JG151, and suggests that DNA degradation is a major but not the only cause of cell death.

INTRODUCTION

Early work by Stuy (1) showed that DNA degradation in bacteria following ionizing radiation could be readily observed. The extent and rate of DNA breakdown have been found to depend on a number of factors: for example, different strains are differently affected; postirradiation incubation conditions have an effect; and there is an increase in extent of breakdown for irradiation in the presence of oxygen. Several attempts have been made to determine whether DNA degradation and loss of viability in irradiated bacterial cells are related. The most recent of these attempts is a study done by Trgovčević and Kućan which shows a striking correlation between radiation-induced DNA degradation and radiosensitivity of *Escherichia coli*. This correlation held whether the variations in radiosensitivity between different strains or variations during the various growth phases of a single strain were considered (2).

One important question to answer concerns whether degradation occurs uniformly in all cells of an irradiated population or to a considerable extent in some and not at all in others. A radioautographic study has been performed by Shaffer and McGrath (3) on X-irradiated *E. coli B/r* and *E. coli* B_{s-1}. In both strains the loss of DNA in cells throughout the irradiated population did not show a great variability. A similar investigation by Achey and Pollard (4) using *E. coli* 15 T^-L^- supports the findings of Shaffer and McGrath. It should be mentioned that in both of these studies it was impossible to determine the distribution of cell viability throughout the irradiated population.

Recent studies by Grady and Pollard (5) indicate that gamma ray induction of a defective prophage is an additional factor which, although influencing DNA degradation, has not been considered in a majority of previous radiobiological studies. This phenomenon has been studied in detail by Frampton and Brinkley (6) in strains of *E. coli* 15 T^-. Ishibashi and Hirota have designated one of the strains of 15 T^- modified in regard to a colicinogenic factor as JG151 (7). The modification of the colicinogenic factor in this cell provides a system from which one variable influencing DNA degradation has been eliminated (5).

The technique of separating irradiated bacterial cells on the basis of differences in buoyant density using sedimentation equilibrium in a cesium chloride (CsCl) density gradient has been developed by Pollard and Grady (8). They found that unirradiated *E. coli* 15 T^-L^- banded at a characteristic density in a CsCl density gradient, while the irradiated cells banded mainly at two densities, a very narrow band at the density characteristic of unirradiated cells and a second rather wide band at lower density.

The method of buoyant density centrifugation of intact bacterial cells in CsCl density gradient has provided a means of separating the population of irradiated bacterial cells into two or more subpopulations. This study is concerned specifically with the classification of the separated populations of irradiated cells on the following bases: (*a*) viability as measured by colony-forming ability, (*b*) ability to synthesize DNA during postirradiation incubation, and (*c*) the relative content of intact DNA per cell in each population. In an attempt to explain the distribution of irradiated bacteria in the density gradient, three additional factors were considered. These included changes in distribution of cells in the density gradient at various times during postirradiation incubation, the extent of postirradiation breakdown of RNA, and changes in cell morphology during postirradiation incubation. Finally, several models for the relationship between DNA degradation and cell death are presented and discussed in light of the results of this investigation.

MATERIALS

Bacterial Strain

The bacterial strain used exclusively in this investigation was JG151, a derivative of the thymine-requiring auxotroph *E. coli* 15 T^-, which was cured of the defective prophage. JG151

was isolated by Dr. M. Ishibashi of Osaka University, Osaka, Japan, and was obtained through Dr. Leo Grady of the Department of Terrestrial Magnetism of the Carnegie Institution of Washington.

Growth of Bacteria

The C-minimal salts medium described by Roberts et al. (9) was used for the growth of all bacterial cultures. To fulfill the thymine requirement of JG151 2 μg/ml thymine (Calbiochem, Los Angeles; A grade) was supplied to the growth medium.

Viability assays were performed by plating the bacteria on C-minimal agar which contained 1.5% Difco Bacto-Agar in addition to the growth medium described above.

Radiochemicals

Thymine-2-^{14}C, specific activity 56.5 mc/mM, L-lysine-^{14}C, specific activity 223 mc/mM, and thymine-methyl-^{3}H, specific activity 6.7 c/mM, were obtained from the New England Nuclear Corp., Boston, Mass. Uracil-2-^{14}C, specific activity 22 mc/mM, was purchased from Calbiochem.

Cesium Chloride Centrifugation

Each 13.5 ml polyallomer centrifuge tube contained 3.80 g CsCl (Trona UV-grade, American Potash and Chemical Corp., Los Angeles) dissolved in 6.6 ml phosphate buffer, pH 8.0. After chilling these solutions to 4°C, 0.5 ml of cell suspension was added to each centrifuge tube and mixed thoroughly with the CsCl solution giving a starting density of 1.39 g/cm^3. The tubes were filled with mineral oil to prevent their collapse during centrifugation and then capped. The sample tubes were placed in a type 40 fixed-angle rotor cooled to 4°C and centrifuged at 4°C in a Beckman Model L preparative ultracentrifuge (Beckman Instruments, Inc., Palo Alto, Calif.) at 35,000 rpm for approximately 48 hr.

At the end of the centrifugation period each sample tube was removed from the rotor, fitted with an apparatus designed to regulate flow rate during fraction collection, and punctured. For each sample tube, approximately 35 four-drop fractions were collected in chilled tubes. The density gradient was determined on the basis of refractive index measurements of fractions collected from a tube containing no cells.

Viability Assay

In order to perform viability assays on samples collected after CsCl centrifugation it was necessary to dilute the samples slowly in order to prevent osmotic rupture of the cells. Each fraction was diluted dropwise at 0°C with 0.4 ml of 3x concentrated Roberts' C-minimal medium without glucose. All fractions were then diluted slowly with two 0.5 ml portions of Roberts' C-minimal medium without glucose. The number of viable cells was determined by plating a series of dilutions of each fraction. The plates were incubated at 37°C for 24–36 hr, and colonies were scored.

So that the radiation survival of colony-forming ability could be measured, samples were removed from an irradiated culture after various doses and diluted in ice-cold growth medium. Viability was measured by plating as described above.

Radioactivity Analyses

To determine the amount of cold acid-precipitable material remaining in labeled cells at various times before and after gamma irradiation, a 1.0 ml sample of a given culture was

taken into 1.0 ml of 10% trichloroacetic acid (TCA) and held at ice-bath temperature for at least 1 hr. Each sample was then filtered through a 0.45 μ pore size, 25 mm diameter Millipore filter and washed with 20 ml of 5% TCA. Filters that held only ^{14}C-containing precipitate were glued to planchets and allowed to dry. The radioactivity in each of these samples was measured with a gas flow counter (Nuclear Chicago, Des Plaines, Illinois) operated in the G-M range. Filters retaining ^{3}H- or ^{3}H- and ^{14}C-labeled precipitates were placed in polyethylene scintillation vials. After the filters had dried, 15 ml of dioxane scintillation fluid were added to each vial. The vials were then capped, shaken, and chilled in the dark for at least 1 hr before counting. The radioactivity was measured in a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., La Grange, Illinois). Appropriate corrections were made for ^{3}H- ^{14}C double-label counting.

In order to determine the amount of acid-precipitable RNA in cells labeled with uracil-2-^{14}C, it was necessary to make a correction for the amount of ^{14}C-label incorporated into the DNA by way of intracellular conversion of uracil to cytosine. So that this correction could be performed, duplicate 1.0 ml samples were taken at each sampling time: one sample was taken into 1.0 ml of ice-cold TCA and the other into 1.0 ml of 0.6 M potassium hydroxide (KOH) at 3°C. The KOH-treated samples were incubated at 37°C for 15 hr at which time they were acidified with ice-cold 10% TCA. This amount of time was sufficient to hydrolyze the RNA leaving precipitable label in the DNA only. Filtering, washing, and counting were performed as described aove. The amount of ^{14}C-label incorporated into the DNA was determined by subtracting the counts in the alkaline-hydrolyzed sample from the counts in the duplicate untreated sample.

Experimental Procedures

The bacteria were grown at 37°C with aeration in 50–100 ml cultures, inoculated from an overnight culture. Cell titer was monitored by measuring the optical density at 420 mμ in a Bausch and Lomb Spectronic 20 spectrophotometer. Optical density measurements were calibrated in terms of cell titer using a Coulter counter Model B (Coulter Electronics, Hialeah, Florida).

In the initial experiments the cells were grown to midlog phase in the presence of either thymine-2-^{14}C for labeling DNA or L-lysine-^{14}C for labeling protein. When the cell titer reached 10^{8}/ml, the culture was harvested by rapid filtration on a 0.22 pore size, 142 mm diameter Millipore filter, washed with an equal volume of isotope-free medium, and resuspended in an equal volume of nonradioactive medium at 37°C. The suspension was oxygenated vigorously for 1 min and divided into two equal portions, one to be irradiated and the other to serve as the control. Irradiations were done in a ^{60}Co gamma ray source (Gammacell 200, Atomic Energy of Canada, Ltd.) using a lead attenuator of known dose reduction factor for low doses. Following irradiation both control and irradiated cultures were incubated at 37°C with aeration for 120 min. In cases in which postirradiation DNA synthesis was to be examined, 500 μc of thymine-methyl-^{3}H was added to both control and irradiated cultures immediately following irradiation. Sampling for radioactivity analysis of both cultures was done at regular intervals starting just before irradiation. At the end of the postirradiation incubation period, cells in both control and irradiated cultures were harvested by centrifugation, washed in 15 ml of phosphate buffer, pH 8.0, and resuspended in a volume of phosphate buffer one-tenth the volume of the postirradiation cultures. These final cell suspensions were prepared for CsCl centrifugation as described above.

The radiation survival of colony-forming ability was determined using unlabeled cells grown to midlog phase, oxygenated, and irradiated as described above.

EXPERIMENTAL RESULTS AND DISCUSSION

DNA Degradation and Banding in CsCl

The dose dependency and time course of DNA degradation are shown in Fig. 1. To demonstrate this the cells were prelabeled by growth in ^{14}C-labeled thymine (56 mc/mM) for three generations, irradiated and sampled as shown in the figure. JG151 shows immediate and extensive DNA breakdown for doses up to 14,000 R. In Fig. 2 we show the distribution of irradiated and unirradiated cells after equilibrium centrifugation in CsCl. In Fig. 2 the dose chosen is such that the average degradation is only 30% so that the lack of degradation is more likely than the reverse. It can be seen that the cells appear to band about two densities, one of which is at the normal density (1.41 g/cm^3) and the other at a lower density (1.38 g/cm^2). Also the distribution about the normal peak is more spread out than in the unirradiated case. We designate the two peaks as I and II, respectively, as shown.

Colony-Forming Ability

In Fig. 2 *b* the distribution of those cells which will yield colonies when plated after irradiation and centrifugation is shown. It can be seen that effectively all of the colony-forming ability is found under peak I. Also there is a very narrow and symmetrical distribution of colony-forming ability that strongly resembles the distribution of control cells. We found a plating efficiency of up to 38% for unirradiated cells diluted and plated after being centrifuged in CsCl.

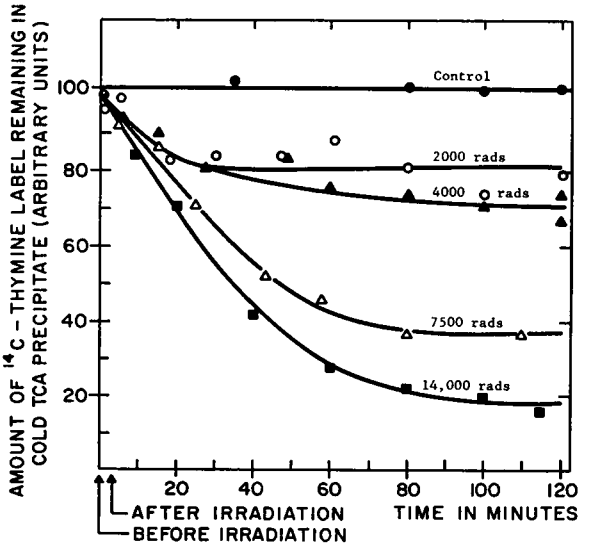

FIGURE 1 Postirradiation DNA degradation. For each dose the culture was oxygenated, irradiated, and incubated at 37°C with aeration. The unirradiated control shows no DNA degradation.

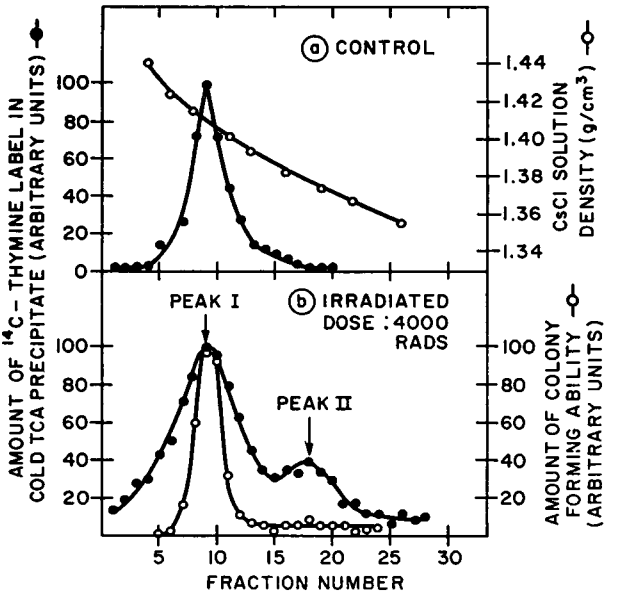

FIGURE 2 Colony-forming ability among distribution of irradiated cells in density gradient. The same CsCl density was used in each case and the samples were centrifuged simultaneously. In (*a*) the control (unirradiated) cells show a single symmetrical band. Irradiated cells in (*b*) display a spread-out distribution with the cells banding predominantly about two densities, with colony-forming units appearing almost entirely under the peak corresponding to unirradiated cells.

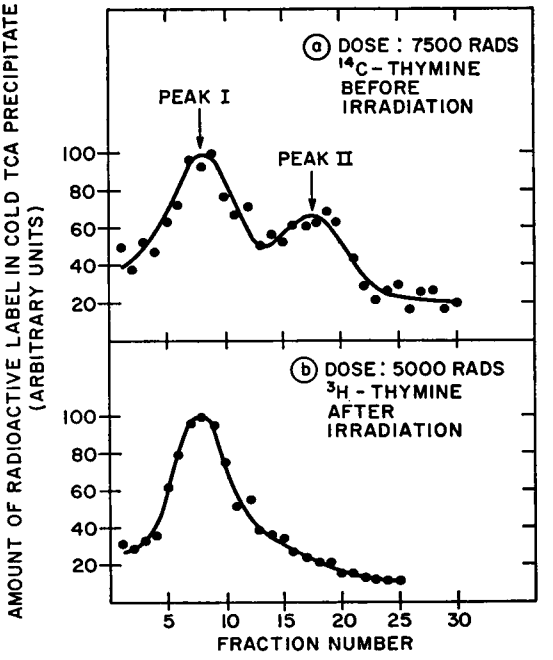

FIGURE 3 Postirradiation DNA synthesis among distributions of irradiated cells in density gradient.

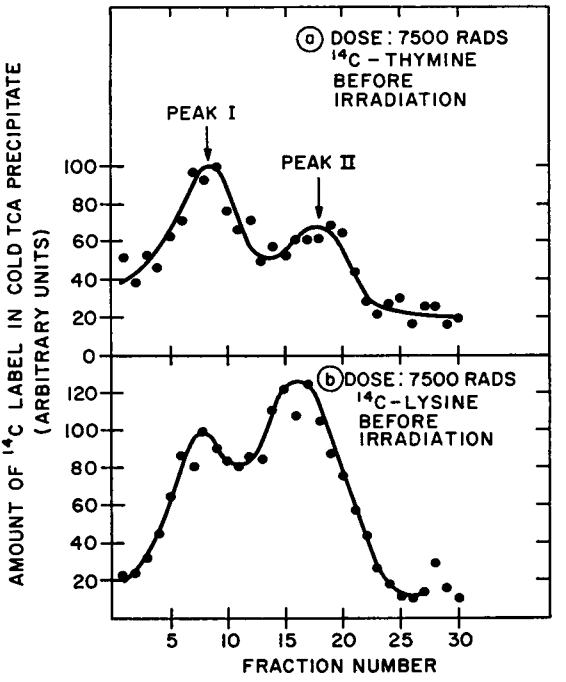

FIGURE 4 Comparison of density gradient profiles of irradiated cells labeled with ^{14}C-thymine (*a*) and ^{14}C-lysine (*b*).

DNA Synthesis

In Fig. 3 we show the effect of a higher radiation dose on the density gradient sedimentation of cells. In the upper part of the figure the distribution of ^{14}C-thymine label obtained as previously described is used to mark the cells. In the lower figure the cells were given ^{3}H-thymine *after* irradiation, allowed time for its incorporation, and then centrifuged. The cells which appear under peak I certainly retain their ability to synthesize DNA. However, the conclusion is also reached that very few and possibly none of the cells under peak II can synthesize DNA. The distribution of the ^{3}H label under peak I is not narrow as is the case for the formation of colonies and suggests that some cells which cannot divide (and so plate out colonies) nevertheless can still produce new DNA.

Relation between DNA Degradation and Colony-Forming Ability

These experiments suggest that those cells which band under peak II are those which have undergone DNA degradation and that they are also those cells which are unable to form colonies. In order to see if a quantitative relationship between the numbers under the two peaks and the survival ratio could be obtained, it is necessary to use a label that does not alter in amount in the two classes of cells—

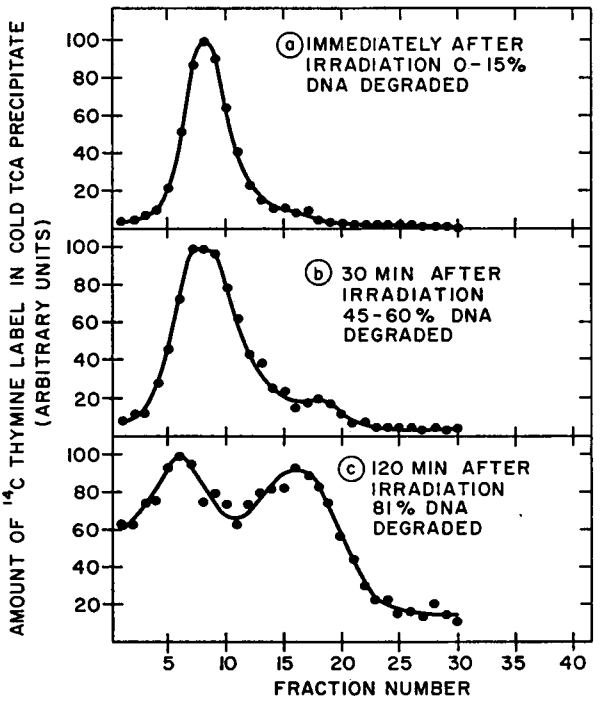

FIGURE 5 Comparison of density gradient profiles of irradiated cells harvested at various times after irradiation. A portion of an incubated culture of irradiated cells was removed from the culture and chilled to 0°C at each of the three times indicated. Simultaneous density gradient centrifugation was performed on the three preparations. Radiation dose was 10,000 rads.

normal and degraded. For this reason we labeled the cells with ^{14}C-lysine. We found in trial experiments that no loss of this protein label was observed after doses of the kind used in the DNA degradation work. This is in agreement with observations by Drakulic and Kos (10). Therefore, we concluded that quantitative observation of the relative numbers of cells in the two peaks with a lysine label should enable the proper comparison to be made. In Fig. 4 we show the results of centrifugation with the usual prelabel with ^{14}C-thymine in addition to a lysine label. The two peaks show as before, only in the case of the lysine label peak II is greater. This indicates, as expected, that peak II contains cells in which a considerable amount of the DNA has been degraded. A confirmation of this idea can be obtained by observing the density gradient profiles for different times of incubation after irradiation when different amounts of DNA have been degraded. Fig. 5 shows the results of this work. It can be seen that as the time after irradiation increases, and the observed per cent of DNA degraded increases, the size of peak II also increases. However, it is not possible to relate banding density and DNA content of a cell, because in Fig. 5 *b*

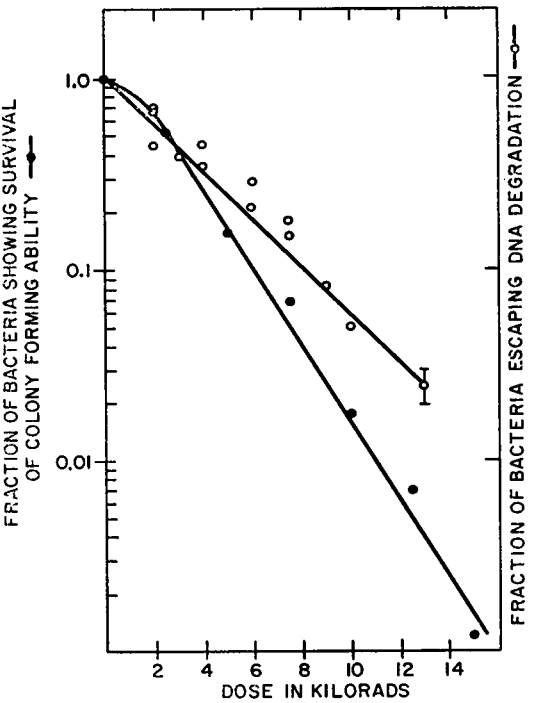

FIGURE 6 Comparison of survival of colony-forming ability with survival from DNA degradation. Survival of colony-forming ability as a function of dose was determined by plating experiments. The fraction of bacteria escaping DNA degradation was calculated as described in the text for centrifugation data similar to Fig. 2 *b* obtained for various doses. Each open circle represents an independent experiment.

there should be a much larger peak II than is observed, since the amount of DNA degraded is about 50%.

We made attempts to see whether the above discrepancy was due to RNA degradation. It is unlikely that it is simply related to the breakdown of RNA. We base this on the lack of evidence that RNA degradation products have been lost from these cells within the first hour. Experiments in this laboratory indicate that transcription of the DNA is affected by ionizing radiation (11). If this is so then formation of the RNA which sediments at very high density is reduced. If long-lived messenger RNA continued to permit the translation of protein, then the cells would become lighter because of the unbalance introduced by the lighter protein addition. We think this process is a factor in the development of the lighter peak at long times.

Fig. 5 *c* also shows that a fraction of cells are of greater than the control density. Observation of an irradiated culture shows short filamentous cells of between 5 and 20 normal cell lengths. We found these in higher than normal numbers in the high density part of peak I, and we suggest that this fraction accounts for the spread toward the high density side in peak I.

Taken together these results indicate that cells which are known to have undergone extensive DNA degradation can be divided into two, probably three, classes. The first has normal density, ability to plate colonies, and is, in our judgment, composed of cells which have not undergone detectable degradation. The second contains cells which are not viable, is of lighter density, and, we believe, is composed of cells which have undergone sufficient radiation to develop extensive DNA degradation. The third we tentatively identify as cells which have not lost appreciable DNA, but which have lost the ability to form colonies (filaments).

In order to check the idea that *all* loss of colony-forming ability is due to DNA degradation we analyzed our centrifugation data to establish the ratio of bacteria escaping degradation at each dose. So that we could find this ratio it was necessary to make several assumptions, all of which are substantiated by our results. We assumed (*a*) that the distribution of cells in the density gradient could be separated into two groups according to the two peaks, and (*b*) that the cells under the lighter peak (II) had undergone various degrees of DNA degradation, whereas those under the denser peak (I) had essentially escaped degradation. By these assumptions the ratio of the area under peak I to that under peak I plus peak II, corrected for degradation, gives the ratio of the bacteria which escaped DNA degradation. This calculation was performed for the centrifugation data for cells which had their DNA uniformly labeled before irradiation. The results are shown in Fig. 6. On the same figure the survival of the colony-forming ability is also plotted. It can be seen that the two do not agree, though the line for the avoidance of degradation is not wildly different from the colony-forming ability line. Even if we allow for the idea that for the formation of a colony the degradation of part of one genome and none of another in a cell is permissible, the discrepancy is not removed. We are thus led to suggest that DNA degradation is not the sole cause of loss of colony-formation. It is, however, a major factor which correlates with that loss.

In conclusion, we call attention to the fact that the existence of a peak at normal density even when 81 % average degradation has taken place requires that a fraction of cells have undergone no degradation. Thus, we can state that a population effect exists, by which we mean that the average amount of degradation represents a state of affairs in which a large number of cells have undergone more than average degradation and some have escaped entirely. We stress that the element which can undergo the all-or-nothing degradation is most probably a genome, and not the whole DNA in a cell, which is probably two genomes on the average.

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