ACTION OF IONIZING RADIATION ON SENSITIVE STRAINS OF *ESCHERICHIA COLI* B

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ABSTRACT Strain B_{s-11} has been found to be very sensitive to postirradiation DNA degradation. Up to 98% of the DNA is degraded at optimum doses. The amount of residual DNA correlates with the retention of colony-forming ability (CFA). Studies of rates of degradation as a function of dose agree with the concept that a degrading lesion causes a definite rate of degradation and that increased numbers of lesions produce proportionally faster rates. By observing the burst size of T7 phage which uses host DNA it has been established that DNA degradation occurs in an all-or-nothing fashion in a unit which is present two or three times per cell. Degradation is enzymatic and the enzyme system is already present in the cell as evidenced by the rapid onset of degradation. DNA synthesis continues in cells that have lost some chromosomes by degradation. Single-cell division patterns show that recovery from "sublethal" damage can occur even in this sensitive cell. Recovery in preirradiation oxygenated cells differs from that in nitrogenated cells.

INTRODUCTION

The effect of ionizing radiation on the various aspects of the molecular biology of bacteria has been under study in our laboratory for several years (Pollard, 1970; Pollard and Davis, 1970). One important aspect is the rapid degradation of the DNA of the irradiated cells. All cells so far studied have a fraction of the DNA which is not degraded, and this fraction can be influenced by the presence of a defective prophage (Grady and Pollard, 1968; Pollard and Weller, 1968). Recently we have made observations on strain B_{s-11}, which shows the property of degradation to a remarkable degree, having only about 2% residual DNA after optimum doses of radiation and sufficient incubation. Several other aspects of the response of the B_{s-11} cell proved to be different, notably the near absence of an oxygen effect for degradation of the DNA, combined with an appreciable oxygen effect for CFA. Because of these findings we decided to make a special study of the sensitivity of this strain of cells, observing the rates of DNA degradation, the division pattern of single cells, and the capacity of cells to sustain phage growth after degradation. We conclude that ioniz-

ing radiation produces a characteristic lesion in DNA that can be recognized by a system of degradation enzymes, which we call the nucleolysin, and which is already present in the cell. Once the nucleolysin starts to work, it continues until a very large part of the genome is destroyed. The action partakes of an all-or-nothing character, in that a population of cells which have only 25% of their DNA undegraded will contain a high proportion of cells having no residual DNA together with a number with all DNA intact. We believe that the survival of the cell depends on whether, having received the primary damage, the DNA can carry the lesion through the replication fork before the nucleolysin begins to function. This "race" is responsible for most of the oxygen effect: the initial rate of degradation is faster in oxygen and thus fewer genomes bring the lesion to the replication fork in time. In addition, we find that irradiation in the anoxic condition yields a class of cell capable of assembling one viable daughter, or even one viable granddaughter, to produce colonies; this is less so for oxygenated cultures. In the discussion we examine our data in the light of an all-or-nothing hypothesis for the loss of various cellular functions and point out reasons why there should be marked differences in radiation sensitivity between strains, as is observed.

MATERIALS AND METHODS

The strain of *Escherichia coli* B_{s-11} was obtained from Dr. James R. White of the Department of Biochemistry, University of North Carolina. It is described by Donch and Greenberg (1968) as probably *rec*$^-$ in addition to the properties of B_s strains, notably the lack of the "cut and patch" mechanism for the dark removal of dimers and of cross-links in the DNA. In the work of White and White (1968) on the production of DNA degradation by streptonigrin, this strain exhibited considerable DNA degradation. This suggested its investigation to us.

The cells were grown in nutrient broth, transferred to Roberts' C-minimal medium (NH_4Cl, 2 g; Na_2HPO_4, 6 g; KH_2PO_4, 3 g; NaCl, 3 g; $MgCl_2 \cdot 6H_2O$, 62 mg; Na_2SO_4, 80 mg; glucose, 5 g/liter) and grown for about 3 hr for labeling. We found that unless a very small amount of nutrient broth was present, the cells divided much too slowly. In general we found that repeated transfers of B_{s-11} in minimal medium could not be made, possibly because of a tendency for spontaneous DNA degradation, which in nutrient broth presents no problem. To label the cells in the DNA we employed the deoxyadenosine technique of Boyce and Setlow (1962). The minimal medium was supplemented with 250 μg/ml of deoxyadenosine and 0.5 μg/ml thymine-2-^{14}C (New England Nuclear Corp., Boston, Mass.; 30 mCi/mmole). The labeled cells were chilled on ice, irradiated for the appropriate dose in a ^{60}Co source (Gammacell 200, Atomic Energy of Canada, Ltd., Pinawa, Manitoba) at dose rates from 12,000 R/min to 1000 R/min, as determined by attenuators. The cells were then returned to 37°C, incubated, and sampled at appropriate times by adding 1 ml cultures to 2 ml ice-cold 10% trichloroacetic acid (TCA). After 30 min the samples were filtered through Millipore filters (Millipore Corporation, Bedford, Mass.; HAWP 02500, average pore size 0.45 μ), and washed with about 5 ml of cold 5% TCA containing 2 μg/ml thymine. The filters were then glued to metal planchets, dried, and counted with an end window G-M counter (Nuclear-Chicago, Des Plaines, Ill.). The relative number of counts was taken as a measure of the nondegraded DNA.

For observation of the growth of single cells we employed disposable trays with 96 10-

mm cups per tray, sterilized under a UV lamp for 3–5 min before use. The cells were diluted to give approximately one cell per 0.05 ml drop and 96 such drops were placed in the cups. The tray was then covered, placed in a 37°C incubator for the appropriate time, and then 1–2 ml warm (47°C) nutrient agar pipetted into each cup. The agar hardened and the trays were left in the incubator for the colonies of cells to develop.

The studies with T7 phage were made with an original stock obtained from Dr. F. W. Studier of Brookhaven National Laboratory. Fresh lysates were prepared as described by him (1969) and were found to have a reasonably good attachment rate. Phage titer was measured by dilution of the lysate in nutrient broth and plating on indicator bacteria of $B_{s\text{-}11}$ grown in nutrient broth. Single-step growth curves were obtained by adding 2×10^8 phage to 2×10^9 bacteria, sampling at appropriate intervals, centrifuging to remove the infected but not yet burst cells, and plating. Single-cell bursts were observed by sampling diluted attached cultures which contained an average of less than one infected cell per tube, incubating for sufficient time to allow a burst to develop, and then plating the contents of the whole tube. The single agar layer technique was used. The phage were pipetted directly on to the bottom of a plastic Petri dish and the agar plus indicator bacteria was poured over and well mixed. Plaques were readily observed after 4 hr incubation.

RESULTS

DNA Degradation in E. coli $B_{s\text{-}11}$ for Different Radiation Doses and Previous Oxygenation and Nitrogenation

Fig. 1 shows a characteristic time course of DNA degradation as measured by the amount of TCA-precipitable material (^{14}C counts) left in each sample after the appropriate times. The cells were previously oxygenated by bubbling with oxygen for 1 min, and nitrogenated by bubbling for 2 min with nitrogen. The results are remarkable for two reasons: first, the high degree of degradation for so small a dose, and second, the apparent lack of an oxygen effect. We have repeated this kind of experiment over a wide range of doses and the simplest analysis we have found is illustrated in Fig. 2. The dose administered in this case is much less, 1150 R. The graph is plotted with the ordinate on a logarithmic scale. It can be seen that for previous oxygenation the limit of degradation is at 59%. If this base line is deducted from the values of the experimental points, expressed as a ratio to the unirradiated case, the points appear as in the line below in the figure. There is thus a reasonable fit to the relation

$$\frac{df}{dt} = -k_1 f,$$

where f is the fraction undegraded, t is time, and k_1 is a rate constant; both conditions yield the value 1.8×10^{-2} fractions/min for k_1. All our curves can be analyzed in this way so that the two parameters of significance are the rate and the fraction undegraded. It should be noted that the analysis which yields the rate constant can only be accurate if the data are extremely consistent. Thus, the more rapid rate constants reported are almost certainly the summation of several different rates and not single

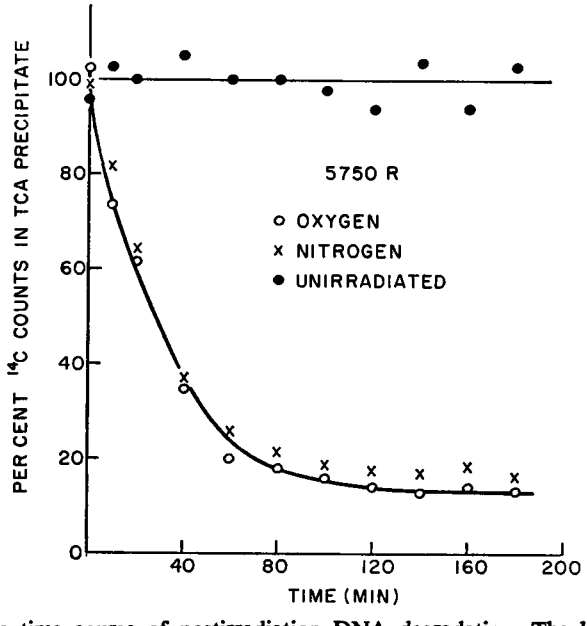

FIGURE 1 The time course of postirradiation DNA degradation. The loss of precipitability by TCA is used as a measure of the degradation of the DNA. Previous labeling with thymine-^{14}C is used and the degradation is followed by counting the activity in the precipitate. The dose is 5750 R and both previous oxygenation and nitrogenation have been used.

values. This is taken up in the discussion. While every effort is made to keep the state of the culture the same from day to day, the fact that the experiments are done on different days means that there will be some expected variability in the figures.

The rate constants for the previously oxygenated case are shown in Fig. 3. The open circles refer to theoretical calculations discussed later. The rate constant has nearly a fixed value up to about 1200 R and advances slowly after that. The same is observed for previous nitrogenation with a delay in the slow rise to about 4000 R. Fig. 4 shows the per cent DNA degraded as a function of dose for both previous oxygenation and nitrogenation. It can be seen that the fraction degraded advances to a very shallow maximum at 98%, reached at about 15,000 R. While the oxygen effect for DNA degradation is much less marked in this cell than in all others studied in this laboratory, it is nevertheless true that there is a difference between the results found in the two conditions. At low doses the amount degraded is greater for the oxygen case, and the increase in the rate constant shows for lower doses. The reason for the increase in the rate constant is discussed later.

The Degradation Enzyme System (Nucleolysin) Is Already in the Cell

If the enzyme system responsible for DNA degradation has to be induced, then the kinetics of derepression require that the time necessary for enzyme production be

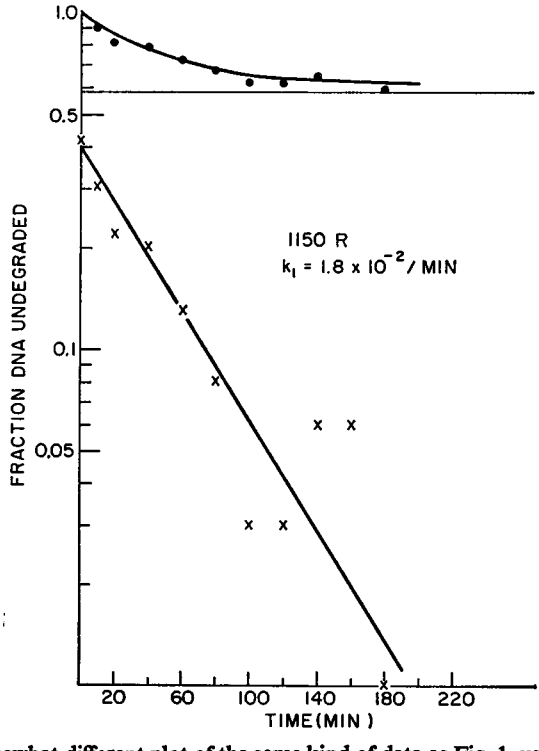

FIGURE 2 A somewhat different plot of the same kind of data as Fig. 1, using a logarithmic scale for the ordinate. This permits the deduction of the undegraded part and a determination of the rate constant for degradation.

about 3 min (Kepes, 1969). Previous work (Pollard and Weller, 1968) suggested that labeled thymine is observed in the medium within a minute of irradiation, indicating that the enzyme is already available in the cell and does not have to be induced. To make a sharper test of this idea we developed a method by which the presence of degradation could be observed at very short times after the cells were at the normal temperature for degradation. Since the amount of label should be high for good sensitivity we used the thymine-requiring strain $B_{s-1}T^-$ (which shows 90% degradation, nearly as much as B_{s-11}) for most of these experiments. Cells were labeled as described, irradiated at ice temperature, and then brought rapidly to 37°C by adding an amount of medium at 60°C designed to give the right final temperature. Samples were then put into cold TCA, filtered, and the filtrate counted in a scintillation counter. The results of one experiment are shown in Fig. 5. It can be seen that when only 30 sec have elapsed there is an appreciable amount of TCA-soluble material and hence of DNA degradation. No such presence of TCA-soluble material is seen for unirradiated cells. The same result was found for B_{s-11} and strain 15JG151. No difference between previous oxygenation and nitrogenation could be seen. We therefore believe that we have established the important point that the nucleolysin is already in the

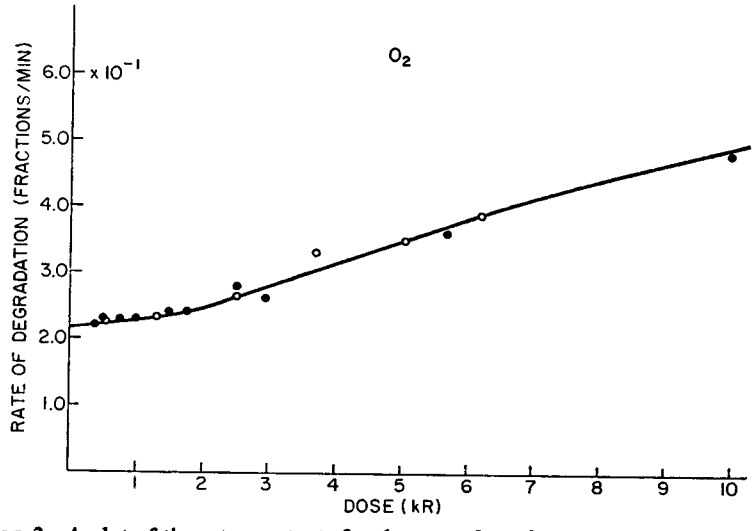

FIGURE 3 A plot of the rate constants for the case of previous oxygenation as a function of dose. The rate varies only slightly up to 1200 R and then rises as the dose increases. A comparison with theory, based on the concept that for each degradation-eliciting lesion there is one rate and for two there is double the rate, is shown in the open circles. The agreement suggests that the idea has some merit.

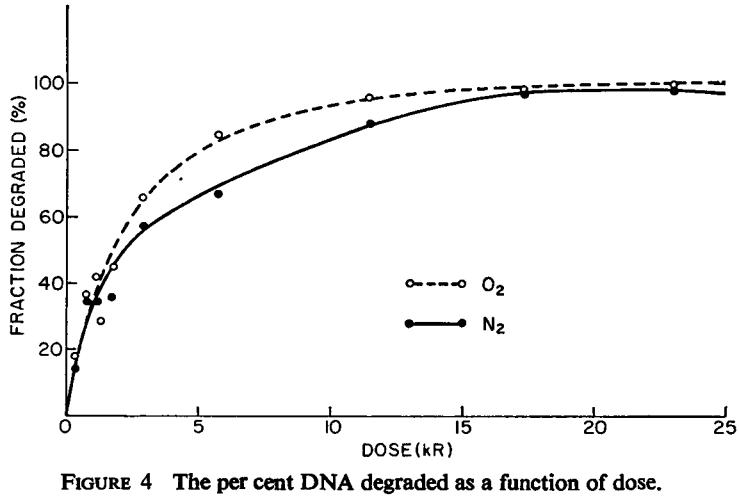

FIGURE 4 The per cent DNA degraded as a function of dose.

cell. This is in agreement with Grady and Pollard (1968) who showed that chloramphenicol does not inhibit degradation and with Pollard and Weller (1968) who found that rifampicin enhanced degradation.

The Sensitivity of the DNA-Synthesizing System in B_{s-11}

In a cell such as B_{s-11} which undergoes rapid and extensive DNA degradation there are two major problems to be solved before good data on the sensitivity of DNA syn-

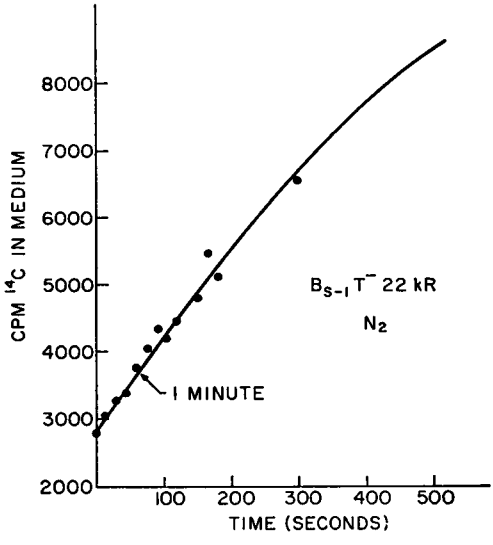

FIGURE 5 The initial kinetics of DNA degradation obtained by rapid warming of the culture to 37°C. Degradation has begun in less than 30 sec, which is too soon for the process of induction of an enzyme to have taken place.

thesis to ionizing radiation can be secured. The first and obvious problem is that degradation and synthesis may be going on together and so the kinetics of synthesis cannot be readily inferred. The second problem is the presence of the pool of degraded bases which will act to cause some dilution of the label in the case of observation of the incorporation of a DNA precursor, such as thymine. Both these act to give the impression that there is much less DNA synthesis present than is actually the case. In order to overcome this difficulty we tried heating the cells gently to block the nucleolysin, hoping that DNA synthesis might not be destroyed. This is to some extent possible in strain 15JG151, but we were unsuccessful with B_{s-11}.

In order to obtain some information on the sensitivity of DNA synthesis, we accordingly decided to wait after irradiation until the degradation process was complete before testing for the residual amount of incorporation of labeled thymine. Such an experiment is extreme, in that many concomitant processes, such as respiration, may be affected. Thus any DNA synthesis observed represents clear evidence that some of the synthetic machinery is operating. The results of one such experiment are shown in Fig. 6. The control cells were given label at the time of irradiation. The irradiated cells were incubated for 85 min to permit degradation to become complete, then spun down and resuspended in the labeled medium. In the case of Fig. 6 the cells were previously oxygenated. Similar data were obtained for previous nitrogenation and a summary of the experiments is shown in Table I. The cells are more sensitive in the oxygen case.

These results suggest that the effect of radiation on DNA synthesis in these cells is certainly no greater than the effect of producing DNA degradation. The fraction of

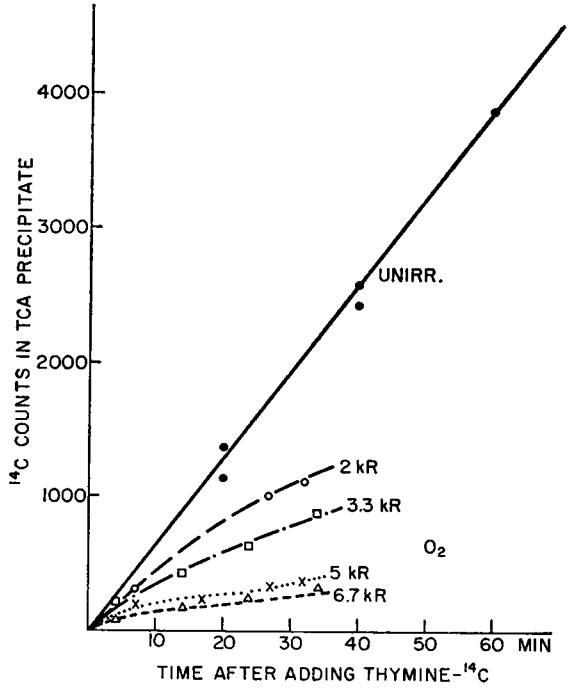

FIGURE 6 The amount of DNA synthetic ability left after cells have completed their DNA degradation and have had the label removed from the medium. The synthesis observed is more than would be expected for wholly surviving cells and suggests that one of two genomes can remain intact and still synthesize DNA.

TABLE I

DNA SYNTHESIS OBSERVED AFTER 85 MIN INCUBATION; RATIO TO CONTROL, WITH NUMBER OF UNDEGRADED CELLS FOR COMPARISON

Dose	Condition	Ratio	Fraction undegraded cells
R			
2000	O_2	0.59	0.33
	N_2	0.78	0.43
3300	O_2	0.36	0.18
5000	O_2	0.19	0.08
	N_2	0.32	0.12
6700	O_2	0.14	0.03
	N_2	0.22	0.06
10,000	N_2	0.11	0.015

cells escaping degradation can be estimated from the kinetic data as exemplified in Fig. 2. If it is supposed that the cells which give synthesis are those which have escaped the process of degradation, then the effect, as indicated in Table I, should be larger than observed. The difference could be due to the growth of the surviving cells

in the 85 min, but, especially for the higher doses, the irradiated cells show a lag after irradiation and therefore do not grow. It is also possible that a certain number of cells undergo degradation in one chromosome while the other growing point is intact.

Single-Cell Division Studies

Observations of the division of single cells after irradiation were carried out by Yeisley and Pollard (1969). These showed that cells which were able to generate colonies, and thus were normally scored as survivors in a colony-forming study, were often not able to divide on time and produced progeny which were very different from each other, even to the point of having only one viable daughter. As done by the above authors the experiments are very tedious and expensive; accordingly we sought a simpler and cheaper method. The availability of disposable multiple-cup plastic dishes suggested a method. The cells were diluted to an average of about one per $\frac{1}{10}$ ml. Drops of this size were then introduced into the cups, allowed to incubate for the desired time, varied between 30 and 120 min, and then "fixed" by pouring in warm molten nutrient agar at 47°C, which in a 1 ml volume sets immediately. The agar in the cups was then incubated until colonies appeared. The colonies were then counted.

A typical distribution of numbers of colonies for a control culture is shown in Table II, where we show the analysis of a single day's data. The control cells showed 37 cups with no cells on them. Such cups, of course, will always show zeros, so they can be used to estimate, by the Poisson formula, the average number of cells per cup. In this case $P(0)$, for an average x, is e^{-x} and so x is found to be 0.86. The dilution factor for the cells was 3.3×10^6, so there were 2.8×10^6 cells per drop of $\frac{1}{10}$ ml. The same distribution formula states that the probability of obtaining one cell in a cup is $P(1) = xe^{-x}$ and the calculated number is shown as 32. For two cells per cup the probability $P(2)$ is $(x^2/2)^{-}e^{x}$ and this yields the number 14. The probability for three cells is $(x^3/6)e^{-x}$ giving 4, and so on. The appropriate values are shown. In the early stages of the experiment we ran checks to see whether an unincubated control gave numbers which agreed with the Poisson formula. Typical of several checks is the following: we observed 28 0's; from Poisson we deduced $x = 1.19$ so that we expected 33 1's, 20 2's, 8 3's, $2\frac{1}{2}$ 4's. We found 32, 18, 9, and 4. Since there is no obvious reason to question the randomness of the numbers we took this as adequate verification.

Now the experimentally found numbers reflect the fact that in 120 min the cells divide and thus the number 32 with one cell originally gives way to numbers with two, three, four cells, etc. Thus one very good method of estimating the potential for division is to take the difference between the expected number of single cells and the actually found number and divide by the expected value. This gives a kind of first-division potential. We call this $\Delta(1)/P(1)$. A second estimation focuses on the

TABLE II

STATISTICS OF SINGLE-CELL GROWTH

Experiment	Cups	Cells/ cup	Expected No. cups incubated 120 min (Poisson from zeros)	Dilution factor	Estimated division potential	
					$\Delta(1)/P(1)$	$>5/[P(1) + P(2)]$
Control			$x = 0.86$	3.3×10^6 $n_0 = 0.86 \times (3.3 \times 10^6) = 2.8 \times 10^6$	0.75	0.5
	37	0	37			
	8	1	32			
	5	2	14			
	6	3	4			
	5	4	1			
	3	5	—			
	4	6	—			
	6	7	—			
	2	8	—			
	4	9	—			
	1	10	—			
	2	12	—			
	1	13	—			
	1	14	—			
	1	16	—			
	1	20	—			
2512 R (N_2)			$x = 0.74$	1.77×10^6 $n = 1.31 \times 10^6$ $n/n_0 = 0.47$	0.57	0.05
	40	0	40			
	13	1	30			
	12	2	11			
	8	3	3			
	6	4	0.5			
	4	5	—			
	2	7	—			
5025 R (N_2)			$x = 0.62$	3.3×10^5 $n = 2.05 \times 10^5$ $n/n_0 = 7.3 \times 10^{-2}$	0.44	0.05
	49	0	49			
	17	1	30			
	6	2	9			
	10	3	2			
	5	4	—			
	1	5	—			
	1	6	—			
	1	7	—			

TABLE III

SUMMARY OF SINGLE-CELL GROWTH IN ALL EXPERIMENTS

Condition	Dose	Survival ratio	Ratio to control	
			First-division index	Many-division index
	R			
O_2	3150	3.5×10^{-1}	0.89	1.1
	5025	1.5×10^{-1}	0.68	0.7
	7800	1.6×10^{-2}	0.53	0.24
	12,000	1.3×10^{-3}	0.47	0.13
N_2	2512	1.4×10^{-1}	0.6	0.05
	5025	1.5×10^{-1}	0.4	0.10
	7000	1.7×10^{-2}	0.4	0.05
	10,500	3.6×10^{-3}	0.4	0.1
	14,000	1.6×10^{-3}	0.4	0.0
	15,700	3.7×10^{-5}	0.4	0.0

number of cells that divide many times, takes the number of cases which have more than five cells, and divides by the Poisson probability for one plus that for two. This we indicate as $>5/[P(1) + P(2)]$. We call it the "many-division index." It is, in one way, a very sensitive measure of the persistence of division potential, but it is very demanding experimentally. If the value of x in two comparisons is different by more than about 30% the ratios obtained clearly represent more than the effect of radiation. This is because the higher average value has numbers per drop which may need to have only one division before registering in the category six or higher, while one with lower x can produce samples in this class only by three divisions. Nevertheless, clear trends can be observed with careful attention to cell concentration.

The growth of irradiated cells is profoundly altered. In order to obtain cells that will form colonies at all, the dilution factor must be diminished. In addition, the number of cells dividing out of the single-cell case is less, and very definitely the number of more than five cells is reduced. Two irradiations are shown in Table II. Both are for previous nitrogenation. It can be seen that both measures of division potential are down and the many-division index is drastically down.

We repeated this type of experiment many times using different doses and with previous oxygenation and nitrogenation. In order to permit some kind of summary we show, in Table III, the averages for all the experiments, giving the calculated value of the colony survival ratio, n/n_0, and the ratio of the two measures of division potential to the values found for the unirradiated control taken on the same culture. In the case where previous oxygenation was applied, the first measure of division potential falls steadily with the dose and the second shows a similar trend. The many-division index, which requires at least two divisions in about normal time, is not diminished for small doses but falls quite definitely as the dose is increased. This last index can be thought of as a measure of what can be termed "total escape"; it

suggests that up to 5025 R the cells which survive are in that class. As the dose increases the number of cells that give colonies which fit this criterion drops and at 12,000 R, where the colony survival is 1.3×10^{-3}, only 13% can be said to have totally escaped. Two very interesting suggestions arise. The first is that the process of "recovery" which may include enzymatic repair, or which may be no more than the operation of the DNA polymerase, is observed for oxygen irradiation only if a relatively large number of potentially lethal hits have been sustained. The second is that if it is possible for even 13% of the surviving cells to have completely escaped a dose of 12,000 R, which introduces nearly 40 direct ionizations in the DNA and 12,000 primary ionizations in the cell, there must be some critical sensitive region in which action is of the all-or-nothing character. If about one in five of the ionizations in the DNA were of the type that killed the cell and one in five of these were capable of recovery, the data can be understood.

The observations for previous nitrogenation are somewhat different. Again it is clear that the cell which is able to divide and form a colony is not capable of division on time and so has some kind of damage that must be able to delay division and probably introduce an asymmetry in the daughters after division, as found by Yeisley and Pollard (1969). The index of first division shows a progressive diminution with dose, as was observed for the oxygen case. On the other hand, the multiple-division index is depressed for the lowest dose, suggesting that there is something inherently different about the initial lesion produced by radiation in the presence of nitrogen. The damage sustained by cells that have been previously nitrogenated is of the kind from which cells recover, if time is allowed. The removal of oxygen from the cells thus seems to have a dramatic effect, even on the expression of damage due to events that carry an average energy of 55 ev.

A very informative test of this kind of recovery is the type initiated by Elkind and Sutton (1960). The concept is to observe the effect of a split dose, with an interval for recovery, in contrast to that of a single dose of the same total amount. We did this, using the trays for observation of the ability of cells to divide and deducing survival ratios as described previously. The technique is very convenient, for the observation of the response of the cells after the first part of the split dose permits an estimate of the number of cells which can divide. This then can be applied as a correction to the survival ratio for the split-dose finding. For example, in one experiment the following numbers were recorded after the initial 5000 R dose: 31 0's, 27 1's, 20 2's, 10 3's, 5 4's, and 1 6. From the number of zeros and the Poisson formula the average number per cup is found to be 1.14. By counting the actual number of cells, the average, after 90 min incubation, is 1.40. Thus growth has been used to reduce the survival ratio of the split-dose case, to allow for the increased number of cells after the first 90 min incubation.

The data of six experiments are summarized in Table IV. The over-all survival ratio has been used as the parameter for comparison. Correction for the growth of cells in the split-dose case has been made as described above. It is quite clear that in

TABLE IV

SUMMARY OF SIX EXPERIMENTS

Nitrogen	5000 R	12,500 R	5000 R, 90 min incubation +7500 R; corrected for growth
n/n_0	1.6×10^{-1}	7.8×10^{-4}	3.1×10^{-3}
	5.0×10^{-2}	2.3×10^{-4}	2.9×10^{-3}
	1.0×10^{-1}	5.1×10^{-4}	3.5×10^{-3}
Mean	1.03×10^{-1}	5.1×10^{-4}	3.2×10^{-3}
			Recovery factor 32/5.1 = 6.3
Oxygen	**3600 R**	**8200 R**	**3600 R, 90 min incubation +4400R**
n/n_0	1.0×10^{-1}	7.9×10^{-3}	7.2×10^{-3}
	1.1×10^{-1}	9.1×10^{-3}	6.3×10^{-3}
	1.04×10^{-1}	10.0×10^{-3}	9.1×10^{-3}
	7.7×10^{-2}	8.5×10^{-3}	11.8×10^{-3}
Mean	1.0×10^{-1}	8.9×10^{-3}	8.6×10^{-3}
			Recovery factor 8.6/8.9 = 0.97

every case of nitrogenated irradiation there is a recovery factor, averaging 6.3, while for the oxygen case there is scarcely any evidence of recovery at all. These cells have a low proportion of recoverable damage.

Because of the difference in the recovery factors we had difficulty in obtaining a comparison of the multiple-division index. For this to have any meaning the average initial number of cells must be nearly the same; this was not secured often enough to give satisfactory data for comparison. The first-division index was found to be less in the sudden-dose case than for the split-dose for oxygen (mean 0.3 vs. 0.8), but reversed in the nitrogen case (0.6 vs. 0.3). This suggests that the cells which might recover had not received full expression in the 90 min incubation in the nitrogen case.

That this suggestion has merit can be seen from Fig. 7. Here the comparison between the value of n/n_0 taken from the single-cell growth in the separate cups and the value found by direct plating without any incubation in the drop culture is shown. In the case of previous oxygenation there is complete agreement between the two methods, and the single-cell growth data for nitrogen also fit. On the other hand, the immediate plating after nitrogen-mediated irradiation gives a markedly higher survival. This strongly suggests that the nitrogen case produces a primary lesion which is potentially as drastic as the oxygen case, but that opportunity for repair or recovery is much greater when the cells have been previously in a non-oxygen-containing condition.

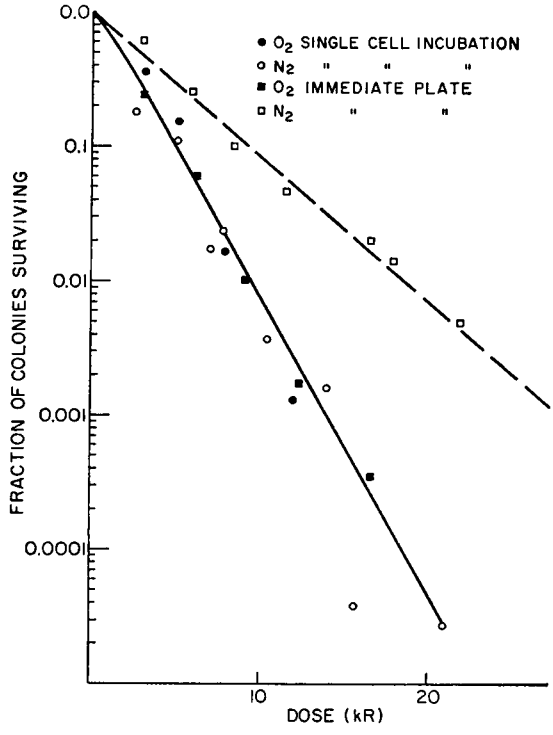

FIGURE 7 Comparison of survival of CFA obtained by direct plating (which gives essentially infinite time for cellular recovery processes to take place) with single-cell growth measurements of CFA in which 120 min postirradiation incubation is allowed, followed by fixing in agar. In the case of previous oxygenation there is no difference; in the case of nitrogenation the direct plating technique gives greater cell recovery.

Phage Infection Studies

The response of a cell that has lost almost all its DNA to infection by phage is of the greatest interest. The capacity of B_{s-1} to develop T4 phage was observed by Marsden et al. (1970) with the finding that postirradiation incubation markedly modifies the capacity of the cells. The work of Labaw (1953) and Studier (1969) has shown that nearly all the DNA incorporated into T7 phage is derived from DNA already in the cell. If the cell has lost its DNA there is presumably no material from which to assemble phage and the burst from that cell should be zero. Since it is readily possible to measure the burst size of individual cells, this technique offers a powerful method of testing the important question as to whether the DNA degradation is all-or-nothing, that is whether cells in which the average amount of DNA remaining is 30%, for example, are made from a population of cells some of which are fully degraded and some others which are not degraded at all. The study of the individual burst sizes should give information on this point.

One problem with T7 is its relative temperamentality about attachment. Unless

the preparation from the lysate is spun off quite soon, with added salt, the attachment is not nearly 100%. By following the procedure suggested by Studier (1969) we were able to obtain 75% attachment in a few minutes. We tested the attachment of phage to cells by adding phage to cells, centrifuging after various times, and observing the number of unattached phage in the supernatant. Irradiated cells served equally well for attachment as normal cells. For times of incubation after irradiation exceeding 120 min, the attachment was diminished but not eliminated.

The first comparison we made involved the plotting of a single-step growth curve. 0.1 ml of phage of titer 2×10^9/ml were added to 9.9 ml of B_{s-11} log phase (2×10^8/ml) bacteria. After incubation for 5 min at 37° C the mixture was centrifuged at 10,000 *g* for 3 min, the supernatant poured off, the infected cells resuspended in 10 ml fresh broth, and 1 ml samples plated at intervals. After 22 min, the expected time for occurrence of a burst, the samples were diluted 10-fold. The irradiated cells received various doses and were incubated for times up to 120 min after irradiation. The results of one such experiment are shown in Fig. 8. The average burst size, under these conditions, is seen to be 100 for the unirradiated cells. It is actually slightly higher for the irradiated and incubated (60 min) cells. The difference between the two cases is seen to be a lower initial number of phage. Since the same number of phage were added, and since the attachment process is unchanged, this reduced number suggests that there are cases where infection starts but cannot produce phage. The drop due to this effect is about a factor of 2. We found that if more radiation were given the drop was considerably more and there appeared

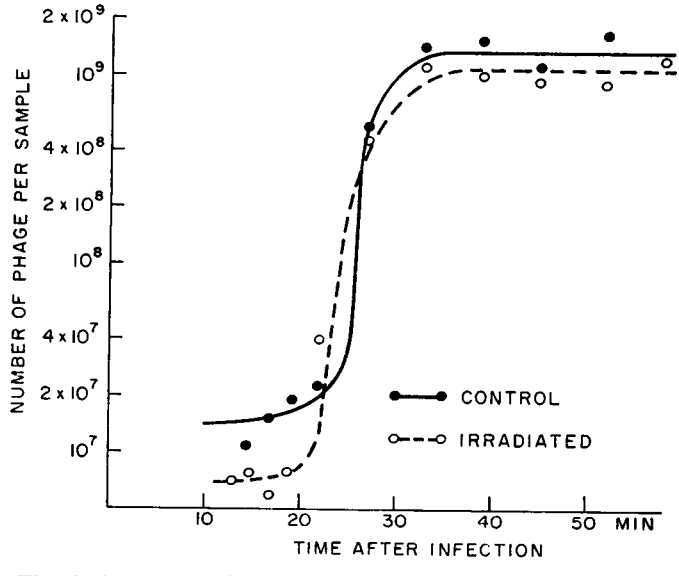

FIGURE 8 The single-step growth curve for infection of unirradiated and irradiated cells by T7 phage. The average burst size is unchanged, but the number of cells successfully infected is less. This suggests that some cells cannot sustain phage growth at all.

to be a rough correlation between the amount of DNA remaining in the cells at the time of infection and the loss of initial phage count; however, the average burst size remained the same, even when only 1 phage in 10 was successfully infecting. These experiments strongly suggest that some cells have about the normal complement of DNA, even though most have nearly none.

To follow this up we conducted a number of single-cell burst experiments. The design is very similar to the previous experiments, differing only in that the infected cells are diluted so that there is, on the average, only about one cell per sample plated. 100 samples are taken in each experiment, and the results of phage infection and bursting give a measure of the yield per cell. If it is true that many cells have lost their DNA while some have retained all their DNA and this fact is reflected in the phage yield, there should be a large number of zero bursts in the case of the irradiated cells, accompanied by normal size bursts. Since we believe the conclusion reached from the data is very important to the understanding of the radiobiology of bacteria, we give the full data from one experiment in Table V. In the unirradiated case the number of samples which show no burst is 44, being those cases in which either no phage appeared, or the number was much too small to correspond to an infection. From this number it is possible to predict that 36 samples should have contained one infected cell, 15 with two, 4 with three, and 1 with four. Looking at the actual observations suggests that the separation between one and two cells giving bursts is at 200, between two and three at 300, and four and over above that figure. Since T7 uses only the cellular DNA it is reasonable to treat the samples indicated as being couples, triplets, etc., and the comparison with the expected Poisson distribution is shown. It is biased on the high side, but only to the extent of seven plates. Accordingly the numbers on the plates estimated to correspond to two cells have been divided by 2 and added to the distribution at the appropriate number. These appear in the column marked "add."

The data for the irradiated case show two striking things. First, the number of zero burst cases is up sharply. Second, there are six samples with bursts in excess of 150 out of a total of 14 samples with any burst at all. Of even more interest is the presence of one four-cell burst. If the cells degraded DNA in an average way, the average burst size should be 22% of 116, or 25, since there is expected to be 22% DNA undegraded and the average burst size is 116. The predicted four-cell case should then contain only 100. The only way in which a four-cell burst can readily be accounted for is to suppose that a cell which was unusually large escaped any DNA degradation, but was inhibited from division so that it carried an unusually large complement of DNA into the process. The conclusion we draw is that degradation does *not* spread around an average figure, but that the average is obtained from many wholly degraded cells and a few with a large fraction, even including all, of the genome intact.

In Fig. 9 we show a plot of the data. The number of zeros plotted in the irradiated case has been taken to be the excess over the unirradiated case. If it is supposed

TABLE V

DATA FOR ONE SINGLE-CELL BURST EXPERIMENT*

No. plates	Burst size	Add.	No. plates	Burst size	Add.	No. plates	Burst size	Add.
Unirradiated								
23	0		1	100		1	188	
16	1		1	102		1	191	
3	2		1	105		2	193	
2	3		1	106		1	242	
1	26		1	108		1	246	
1	39		1	114	4	1	251	
1	45		1	120		1	253	
1	48		1	124	6	1	262	
1	51		1	127		1	263	
1	52		1	129	8	2	264	
1	60		1	135	3	1	283	
2	62		1	139	3	1	406	
1	68		1	140	2	1	414	
1	80		1	144		1	467	
1	81		2	149				
1	84	2	1	169		44 no burst $x = 0.82$		
1	86	2	1	172		$N(1) = 36$; found 33		
1	88	6	1	175		$N(2) = 15$; found 10		
1	94	4	2	177		$N(3) = 4$; found 9		
1	98	4	1	180		$N(4) = 1$; found 3		
Irradiated								
72	0		1	29			124	2
12	1		1	49			130	2
1	2		1	54			144	2
1	3		1	67		1	159	
1	15		1	69		1	218	
1	20			97	3	1	259	
				110	4	1	289	
			1	120		1	293	
						1	442	

* Dose 6000 R, 0_2. 60 min incubation. 22% DNA expected undegraded.

that the samples which gave counts under the main peak of the distribution have wholly intact genomes, that those to the left have half genomes and the zeros have none, then the estimated per cent DNA left is 16.5/60, or 27%, which compares with 22% predicted from the dose and conditions. The agreement is as good as the statistics of small numbers will permit. It is possible that some of the DNA degraded in the cell was still present as nucleotides and so available for making phage.

The experiment was performed 15 times with variation in dose, time of incubation, and nitrogenation vs. oxygenation. If the time of incubation was very short, the distribution was very much like the control. If the time was lengthened greatly,

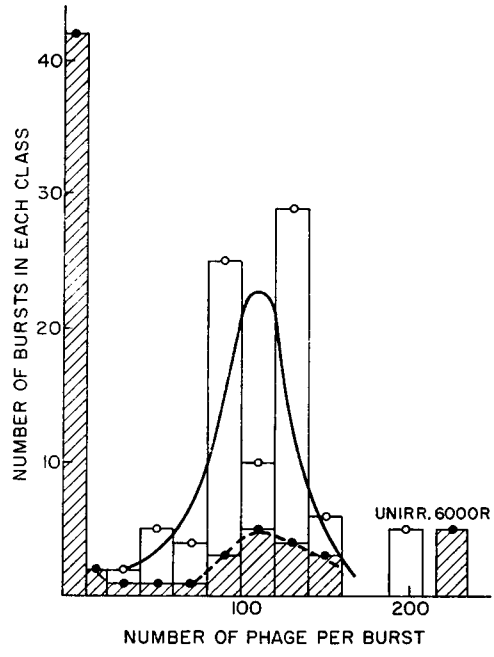

FIGURE 9 The distribution of single-cell bursts for unirradiated cells and cells which have received 6000 R and been incubated for 90 min. The irradiated cells show many more than expected zeros and have a good number of normal bursts. This is evidence for an all-or-nothing process for radiation damage.

beyond 120 min, the infectivity began to fall and no confidence in attachment could be retained. The same kind of distribution pattern was always observed if the incubation time was between 60 and 120 min. Two experiments can be selected for comment. In one, given 7500 R, 20 extra zeros appeared, and of the seven actual bursts, the smallest was 125 and there were two over 300. An irradiation of 20,000 R which should have left no more than 4% DNA showed only two bursts, one of size 10 and the other, 177. No clear difference was found for previous nitrogenation, other than a somewhat greater tendency for intermediate size bursts to show.

It should be possible to analyze the data to determine whether the whole genome, or a specific fraction of the genome, is the unit which is degraded all-or-nothing. Our best conclusions are that the whole genome represents too great a simplification. We prefer to suggest that there are three separate units for degradation and that each is either degraded by radiation action with the operation of the nucleolysin or not. It is very difficult to make an over-all presentation of the findings because it is hard to control the exact multiplicity of infection and the kinetics of degradation. We have made an attempt to present a more inclusive picture in Fig. 10. This summarizes four additional experiments of the type of Table V. The unirradiated distribution is shown in the upper part, together with the distribution from two experiments in which 2700 R was given with 60 min incubation time. In the lower

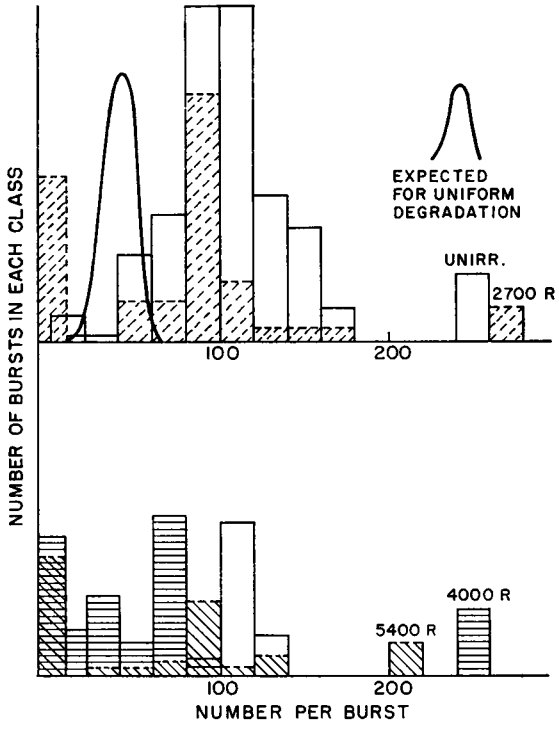

FIGURE 10 An attempt to pool four experiments with different doses. The same characteristic of normal bursts vs. zeros is seen. There is some evidence that intermediate size bursts show at larger doses and this can be taken to suggest that the "degradation unit" of the DNA is multiple and not single.

part, two distributions for 4000 and 5400 R are shown. In the upper figure we show the expected distribution if all the cells had degraded in the manner of the average, with a distribution of the same proportional width as the control. It occupies almost the only vacant space on the figure. The lower part indicates why we suggest that more than one unit is involved in the degradation process. In the 4000 R case, while there are clearly some cells with the whole genome intact, there is a grouping at lower yields. We believe that enough data of this kind would make it possible to determine the number of separately degradable segments, but for two reasons we are hesitant to make the attempt. The first is that the physiological state of the cells, which is known to modify the number of replicating forks (Maaløe and Kjeldgaard, 1966), is almost certainly a factor in fixing the number, and the second is the expense in plates and medium. Our best estimate is three.

DISCUSSION

In Fig. 11 is shown the comparison between the residual fraction of DNA left at each dose for previous oxygenation expressed as a ratio to the unirradiated value,

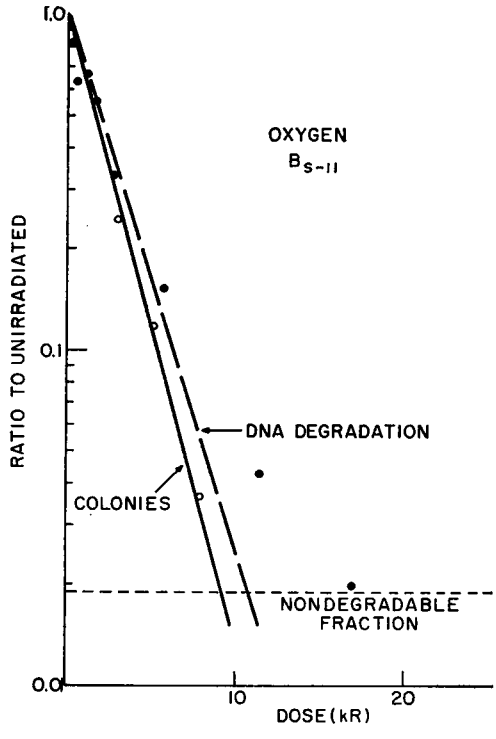

FIGURE 11 A comparison between the survival ratio for CFA and the proportion of cells which give no DNA degradation for previous oxygenation. If allowance is made for the presence of a small resistant fraction of DNA, the two go together, suggesting that the loss of CFA and escape from degradation of DNA are related.

and the survival ratio for colonies obtained by plating, as shown in Fig. 7. A very similar comparison is found for previous nitrogenation. It can be seen that the two very nearly go together, and if some allowance is made for the nondegradable fraction, the fit is almost perfect. This suggests that DNA degradation, in this cell, is an excellent measure of the ability of the cell to divide and form a colony. It is interesting because in no other cell that we have studied can the comparison be made with such confidence. We wish to stress that the degradation of the DNA may be an indicator and not the actual cause of the loss of CFA: for example, if the suggestion of White and White (1968) that DNA degradation is due to the cessation of DNA synthesis is true, then the cause of both the loss of CFA and the DNA degradation might be the loss of DNA synthesis, which we consider to be a sensitive element of the cell. This question is not yet resolved.

Because there is such an excellent parallel between the process of DNA degradation and the loss of CFA, it seems safe to say that the factors which influence DNA degradation in this cell are also the factors which influence sensitivity. Accordingly, the findings from the phage burst distribution experiments can be considered first,

as they make it possible to interpret the whole of the remainder of the experimental studies, with the exception of the enzyme induction work.

The data shown in Table V and Figs. 9 and 10 convince us that there are a small number of units of DNA in each cell which, upon receiving a specific kind of damage, become substrate for a degradation system, the nucleolysin, which is already in the cell. Other than to say that the kind of lesion represents about one in five of the primary ionizations within the mass estimated for the DNA, we do not know the nature of this lesion. A different type of study, in which the immediate action of radiation is undertaken, is needed. Once the DNA becomes substrate, the enzyme system must find the initial point for starting the degradation process. This cannot be instantaneous, and it must depend on the number of nucleolysin molecules in the cell. Since the process is random, the onset of degradation in a large culture appears immediately since fortunate collisions with no time delay can occur. The whole course of degradation permits some estimates to be made of the type of reaction rates involved. The rate constant for very low doses, which presumably correspond to one lesion and one enzyme at work, is 1.86×10^{-2} fractions/min. Assuming that the genome is 3×10^9 daltons, approximately 10^7 bases, this means that the initial rate of degradation corresponds to the removal of the whole genome in 43 min, which is at the rate of 3000 bases/sec, quite comparable to the rate of synthesis at the replication fork.

It is possible to make a test of the hypothesis that the association of a nucleolysin molecule with the radiation lesion causes the degradation of a whole unit of the DNA, as suggested by the phage burst work. If this is so then it should be possible to estimate from the amount of undegraded DNA at any dose what is the probability of escape of the DNA from receiving any degradation lesion. This then gives us the probability of a single lesion, two lesions, etc., using the Poisson formula. If we take the rate of degradation for a single lesion to be fixed by the low dose value, then we can calculate, for any dose, the proportion of single lesions, doubles, and so on. The rate for two lesions should be twice that for one, and for three, three times. If this analysis is applied to one single degradation unit then the increase in rates predicted is faster than that observed. The T7 bursts suggest at least two separate units. Accordingly we have supposed that there are two units. With an average of x lesion-producing hits per unit then we have the following: probability of escape in one, e^{-x}; probability of both receiving hits $(1 - e^{-x})^2$; probability of one hit in one genome, xe^{-x}; probability of two hits, $x^2e^{-x}/2$; and of three hits, $x^3e^{-x}/6$. If we now associate the basic rate with one hit, double the rate with two hits of any kind, and triple the rate for three hits, we obtain estimates of relative rates. Taking the basic rate (Fig. 3) as 1.86×10^{-2} we obtain the circles on Fig. 3. Considering the difficulty in measuring accurate values on cultures for different days, the agreement is reasonably good. This analysis suggests the rate should continually increase whereas it does not do so. To account for the diminution at higher doses we can adopt the suggestion, made by Huston and Pollard

(1967), that the presence of too many lesions in one degradation unit diminishes the rate and amount of degradation. If there were only a very few nucleolysin molecules per cell, this might be expected purely in terms of the utilization of the enzymes.

The phage studies suggest that there are about three degradation units per cell. The above analysis is for two units. In neither case are the data sufficiently accurate in detail to permit the firm statement that two or three units are involved. We feel sure that more than one unit and less than four units are involved in our cells.

We can now turn to the data on DNA synthesis. If the degradation process is of the all-or-none type, then after 85 min all the cells that are going to lose their DNA have done so. A first assumption is that these cells can not synthesize DNA. Table I shows that it is not that simple. Either the cells which have totally escaped have multiplied in the 85 min interval and so account for more DNA synthesis, or those cells which have lost one of the units that degrade can synthesize DNA normally. We recoil from the idea that a cell which has lost all its DNA can synthesize any DNA at all. The first hypothesis is rendered unlikely by the single-cell growth studies. Here it was found that in 90 min incubation time the cells multiplied no more than 40% after irradiation. This is not sufficient to account for the factor of more than two in many cases. The second hypothesis comes much closer to fitting the data. For example, at 5000 R, if 80% had suffered degradation in both units, then 20% could be thought of as having a degradation lesion in one unit, yielding a 10% over-all residual DNA, not far from what is found. If, in the 85 min of incubation, these undegraded units were able to add initiators and increase the speed of synthesis, a fair agreement is obtained.

The observations on degradation and synthesis do enable one to examine one hypothesis. It has been suggested (Pollard, 1970) that all the actions of ionizing radiation are all-or-nothing in character and that the sensitivity parameters are such that they can be added to give the over-all sensitivity. If this is so, then the sensitivity of DNA synthesis to gamma radiation in this cell must be very low, with a D_{37} of more than 18,000 R. Alternatively, as suggested by White and White (1968), the two processes are coupled together. They cannot be exactly coupled, as Table I shows more than the requisite fraction of synthesis. Clearly much more work needs to be done on DNA synthesis under conditions where DNA degradation is not a factor.

Whatever the reason for the process, the action of the nucleolysin needs further investigation. One very attractive suggestion can be made. The nature of the restriction enzyme in *Hemophilus influenzae* has been elucidated by Smith and Wilcox (1970) and by Kelly and Smith (1970). This endonuclease R recognizes a special pattern of bases and there causes a sharp double-strand break. Subsequently a very rapid degradation of the DNA occurs by an enzyme mechanism not yet fully understood. If it were true that the enzyme system which goes to work on the doubly severed DNA in the restriction system and the DNA in the radiation system

were the same, then the degradation lesion produced by radiation would be a double-strand break which left the two ends as left by the restriction endonuclease. Kaplan (1966) has suggested that a double-strand break may be the significant radiation lesion that kills cells. All of this would fit together very nicely if the above hypothesis were right.

The observations on the difference between the growth of cells after nitrogenation and oxygenation are in line with a suggestion made by Alper (1968) that type O damage is different from type N damage. Whether the specific idea that one type acts on a membrane and the other on DNA is right we cannot say.

We can conclude with some remarks on the strain dependence of sensitivity. The sensitive property of the extremely sensitive cell B_{s-11} resides in the rapid degradation of DNA which follows an initial lesion, possibly a double-strand break. Even in this cell, with so drastic a means of magnifying initial damage, there is evidence that cells can recover, even when damage has been incurred in oxygen. A cell strain which lacked the nucleolysin or in which there was present an inhibitor of degradation (defective prophage, for example) would be one in which recovery could be much greater. Thus the factors we can see as influencing strain dependence of sensitivity are at least three, as follows: (*a*) presence of and speed of action of nucleolysin (B_{s-1}, B_{s-11}, JG151), (*b*) presence of and speed of action of a defective prophage transcribing an inhibitor of the nucleolysin (K12λ, B3, B/r), (*c*) physiological state encouraging multiple genomes which increase the probability of a restored intact genome. We stress that this list is incomplete; certainly repair enzymes need to be added to the list.

Thus dependence of sensitivity on strain is not unexpected and there is hope of understanding how it takes effect.

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Dr. Paul Horan contributed in many discussions.

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