DEGRADATION OF DNA IN *HAEMOPHILUS INFLUENZAE* CELLS AFTER X-RAY IRRADIATION

I. EXPERIMENTAL RESULTS

M. L. RANDOLPH *and* JANE K. SETLOW

From the Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830. Dr. Setlow's present address is Brookhaven National Laboratory, Upton, Long Island, New York 11973.

ABSTRACT Sequential measurements of DNA in *Haemophilus influenzae* cells after X-ray irradiation show rapid initial degradation of DNA followed by a plateau after about 40 min at normal growth conditions. Both the initial rate and final amount of degradation increase with radiation exposure. Degradation is somewhat greater in stationary-phase than in log-phase cells, but colony-forming ability (CFA) is independent of cell stage. Distributions of single-strand lengths of DNA in unirradiated or irradiated cases, as measured by alkaline sucrose gradient techniques, are neither monodispersive nor random, and possible causes for nonrandomness are discussed. The energy dissipated in the DNA is estimated as 40–50 eV per single-strand break for log-phase cells. The fractions of initial DNA remaining in heavily irradiated cells after long incubation are much greater than either the residual CFA or the number of DNA strands free of breaks. Hence, we conclude that cellular degradation of DNA, after exposure to ionizing radiation, cannot be explained quantitatively or qualitatively by simple correlations to these measures of cellular damage, but rather requires a more complex theory.

INTRODUCTION

The immediate fate of a strand of cellular DNA in which a break has been induced may be innocuous repair, repair with modification which may lead to cell death or mutation, degradation of the strand, or merely unjoined parts of the strand remaining. Partial degradation may be followed by repair. Stuy (1960, 1961) first measured degradation of DNA in cells of several bacteria as functions of incubation time after X-ray irradiation. Many further studies on the subject have been reviewed by Pollard (1970). There is widespread agreement that cellular degradation of DNA during incubation after exposure of bacterial cells to ionizing radiation is initially rapid, but stops after a period of perhaps 40 min (Stuy, 1961; Pollard and Achey, 1964; McGrath et al., 1966; Pollard et al., 1966; Frampton and Billen, 1966; Grady and Pollard, 1967; Achey and Pollard, 1967; Stavrić et al., 1968; Chapman et al., 1968; Chapman and Pollard, 1969;

Trgovčević and Kućan, 1969; Town et al., 1971; Paterson et al., 1971; Pollard and Tilberg, 1972; Pollard and Krauss, 1973). Most of the degradation has been attributed to exonuclease V (Youngs and Bernstein, 1973; Marsden et al., 1974). The maximum degradation depends, among other variables, on radiation dose (Stuy, 1961; Pollard and Achey, 1964; McGrath et al., 1966; Frampton and Billen, 1966; Grady and Pollard, 1967; Stavrić et al., 1968; Chapman and Pollard, 1969; Pollard and Krauss, 1973); and the presence of inducible, degradation-inhibiting agent (Pollard and Randall, 1973; Marsden et al., 1974; Tolun et al., 1974); but is independent of temperature below 42°C (Chapman and Pollard, 1969), which also suggests the enzymatic character of degradation. The initial rate of degradation increases with both radiation dose (Stuy, 1960; Pollard and Achey, 1964; Frampton and Billen, 1966; Grady and Pollard, 1967; Stavrić et al., 1968; Trgovčević and Kućan, 1969; Chapman and Pollard, 1969; and Town et al., 1971) and incubation temperature (Achey and Pollard, 1967; Pollard, 1970, p. 69). With the exception of the work of Achey and Pollard (1967), detailed analyses of the shape of the degradation curve and mechanistic models for its explanation have not been reported.

In this paper, we report results of our measurements (*a*) of degradation of DNA by *Haemophilus influenzae* cells after exposure to ionizing radiation, and (*b*) of radiosensitivity for production of single strand breaks and colony-forming ability (CFA). Here in a preliminary way, and in an associated paper[1] in a more detailed way, we report the results of tests of several such models to fit our data on cellular degradation of DNA.

MATERIALS AND METHODS

Microorganisms and Their DNA

The strains of *Haemophilus influenzae*, methods and medium for growing these cells, and storage procedure have all been described previously (Setlow et al., 1968 *a*). From plots of growth curves at 37°C vs. time, we judge these cells to be in the logarithmic growth phase (log phase) at concentrations of less than about 10^9 cells/ml or at optical densities (OD) of less than about 0.5 at 675 nm (measured with a Bausch & Lomb Spectronic 20; Bausch & Lomb Inc., Rochester, N.Y.), and to be close to or in stationary growth phase at OD = 0.8 or more. During log phase the cell doubling time was 37 min. The cellular DNA was radioactively labeled with ^3H, or ^{14}C by growing cells for at least three generations in the presence of 250 μg of adenosine per ml and, typically 60 μCi of [^{3}H]thymidine per ml (sp act 11–17 Ci/mmol) or 1 μCi of [^{14}C]thymidine per ml (sp act 50–60 mCi/mmol).

Irradiations

Cell suspensions in air were irradiated in 5 cm in diameter plastic petri dishes or 5-ml plastic centrifuge tubes. In both cases a 1 mm-thick plastic cap was used for secondary equilibrium and the samples were surrounded by ice. All irradiations were done at 15 cm from the target of a GE Maxitron 250 X-ray machine equipped with a Be window X-ray tube and run at 250 kVp and 30 mA. Added filtration of 3 mm Al was used. Under these conditions the exposure rate, as measured by Victoreen ion chambers (Victoreen Instrument Div., VLN Corp., Cleve-

[1] M. L. Randolph. Submitted for publication.

land, Ohio) which were compared with ones calibrated at the Bureau of Standards, was 3 kR/min, the half-value thickness was 0.47 mm of Cu, and the homogeneity coefficient (ratio of first to second half-value layer thickness) was 0.36.

Degradation of DNA

Either log- or stationary-phase cells, their DNA radioactively labeled with ^3H, were centrifuged and resuspended three times in the enriched growth medium (Brain Heart Infusion [Difco Laboratories, Detroit, Mich.] plus supplements [Setlow et al., 1968*a*]). The cells were then irradiated. During incidental storage and irradiation the cells were kept at 4°C. Irradiated cells were incubated at 37°C. At various times up to 180 min after the start of incubation, 10-μl samples were removed, put on 3/4-in in diameter Whatman No. 1 filter-paper discs, and within seconds immersed in 5% trichloroacetic acid (TCA), 5–10 ml/disc, at 4°C. After 10 or more min the discs were washed twice in ethanol and once in acetone, dried, put in vials containing toluene with 16 g/gal of 2,5-bis-2(5-*t*-butylbenzoxazolyl)-thiophene (BBOT), and the TCA insoluble radioactivity on each disc was counted with a Packard Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Downers Grove, Ill.).

Simultaneous Measurements of DNA Degradation and Cell Survival

Occasionally we simultaneously measured both DNA degradation and cell survival of log- and stationary-phase cells by the following technique: Cells with resistance to a specific drug (i.e., streptomycin or novobiocin) were grown to log phase (OD_{675} = 0.4), while others with specific resistance to the other drug were grown to stationary phase. The DNA of one phase was labeled with ^{14}C and the other phase with ^3H. The cells were then pooled, centrifuged, and resuspended three times, irradiated, incubated, sampled, and the TCA insoluble radioactivity counted as usual for measurement of degradation except that the spectrometer pulse-height discriminators were set to resolve between counts from decay of the two radioisotopes. Then, using "spillover" corrections, we determined the remaining DNA in the two cell types independently. We also measured CFA for the two cell types independently by plating separate samples in the presence of each of the two drugs. The survival data were similar to those obtained by the usual method (Setlow et al., 1968 *a*).

Molecular Weights of Single-Stranded DNA

Single-strand molecular weights of the cellular DNA were measured using the alkaline sucrose gradient centrifugation technique of McGrath and Williams (1966). Cell suspensions were kept at 4°C from the end of washings (to remove excess radioactive labels) through irradiation until the start of lysis, thus minimizing repair of single-strand breaks prior to sedimentation. Less than 5×10^7 cells, with a total of <0.1 μg of DNA, were lysed in 0.1 ml of 0.5 N NaOH on the top of 5–20% sucrose gradients with 0.1 M NaCl at pH 12. Sedimentation at about 20°C was achieved using Beckman Model L Ultracentrifuges (Beckman Instruments, Inc., Fullerton, Calif.) and a SW-39 or SW-50.1 rotor spun at ω = 30,000 or 40,000 rpm for appropriate times (t = 50–200 min). Sampling after centrifugation was done by punching a hole in the bottom of the tube and collecting drops either on filter discs or on paper strips as described by Carrier and Setlow (1971). Calibrations for determination of molecular weights from distance sedimented were done as described previously (Randolph and Setlow, 1972). Weight-average and number-average molecular weights were computed on a time-sharing computer with a program which also made spillover and background corrections as necessary. The averaging was limited to the middle 80–90% of the gradient length in order to avoid artifacts at the tops and/or bottoms of gradients. Unirradiated ^{14}C-labeled cells were lysed on the top of most gradients simultaneously with irradiated, ^3H-labeled cells, thus providing an internal measure of breaks

occurring during lysis. If breaks are additive and random, the number of radiation-induced breaks per initial single-strand molecule is given by:

$$B(D) = M_O\left[\frac{1}{M_N(D)} - \frac{1}{M(O)}\right] = \beta D, \quad (1)$$

where D is the radiation dose, M_O the number-average molecular weight of the full single strand, $M_N(D)$ the number-average molecular weight of the irradiated ^{3}H-labeled DNA, $M(O)$ the number-average molecular weight of unirradiated ^{14}C-labeled DNA, and β a constant determined by least-squares analysis. We have approximated the number-average molecular weight as half the weight-average value, M_W which is generally (e.g., Charlesby, 1954) considered acceptable for more than about five random breaks. We discuss this assumption in more detail later. Here we take M_O as 830×10^6, as reported by Gillis et al., (1970). When there are many radiation-induced breaks one cannot set the rotor speed to measure $M_N(D)$ and $M(O)$ simultaneously. In these cases we have determined the total number of breaks by a least-squares fit to

$$B_T(D) = 2\,M_O/M_w(D) = \alpha + \beta D \quad (2)$$

where α is the number of breaks caused by factors other than radiation. Then using Eq. 1 and 2, the number of radiation-induced breaks per initial single strand becomes:

$$B(D) = \beta D. \quad (3)$$

RESULTS

Typical kinetics of DNA degradation in log-phase *Haemophilus* cells during incubation at 37°C after X-ray irradiation are shown in Fig. 1. (All figures are for wild-type strain Rd.) Degradation or turnover is relatively slow in unirradiated cells. In irradiated cells there is a rapid initial degradation of DNA but very little degradation after 40 min. The initial rate of degradation increases with radiation exposure. The fraction of the initial DNA remaining after long incubation (the plateau) decreases with radiation exposure, but the plateaus are neither linearly nor exponentially dependent on exposure (Fig. 2). The slopes of plateaus in curves such as Fig. 1 very nearly parallel turnover rates for unexposed cells. The initial degradation rate in irradiated cells decreases markedly with decreasing temperature (Fig. 3). By use of the technique for simultaneous measurement of degradation and CFA described in Methods we find similar kinetics for stationary-phase cells (Fig. 4), although the effects for equal doses are somewhat greater for stationary cells. Degradation kinetics for the wild-type strain Rd, and ultraviolet-sensitive mutant stains DB112, DB116, and DB117 were all similar, although there was perhaps more degradation of DB117 DNA, which alone has greater X-ray sensitivity for CFA (Setlow et al., 1968 *a*).

Using the same technique for simultaneous measurements, we found the CFA of log- and stationary-phase *Haemophilus* cells to be of equal X-ray sensitivity (Fig. 5).

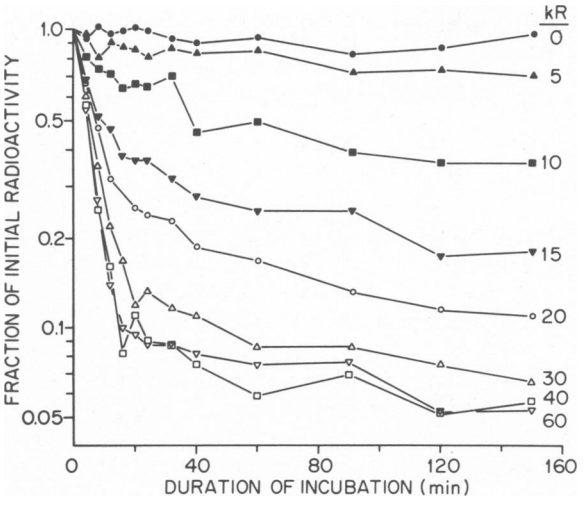

FIGURE 1

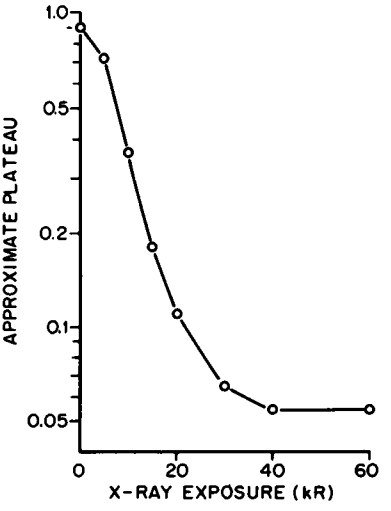

FIGURE 2

FIGURE 1 Semilog plot of degradation of DNA in log phase *Haemophilus influenzae* cells, strain Rd, during incubation at 37°C after various X-ray exposures. To within ± 10%, the measured initial DNA was independent of exposure.

FIGURE 2 Semilog plot of approximate plateau values from Fig. 1 versus X-ray exposure. (Other plots of similar data are not so nearly exponential at low exposures.)

Typical alkaline-sucrose gradient curves for *Haemophilus influenzae* before and after irradiation are shown in Figs. 6 and 7. The gradients for unirradiated DNA from stationary-phase cells are consistently somewhat sharper than for log-phase cells. In neither case, however, is the observed distribution of molecular weights nearly monodispersive, nor is it random even after irradiation, as judged by the nonlinearity of plots (Fig. 8) or ln (counts/$M\Delta M$) vs. molecular weight, M. This test of randomness for fully labeled cells has been introduced by Dean et al. (1969) and fully discussed by

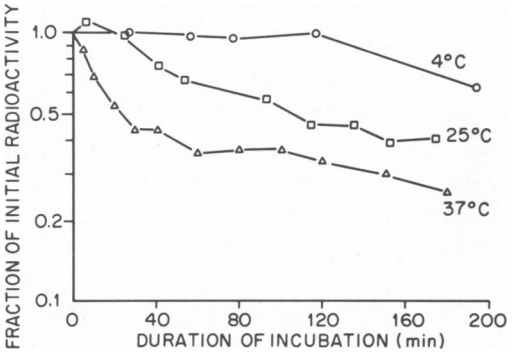

FIGURE 3 Degradation of DNA in log-phase *Haemophilus* cells after 20 kR exposure during incubation at different temperatures.

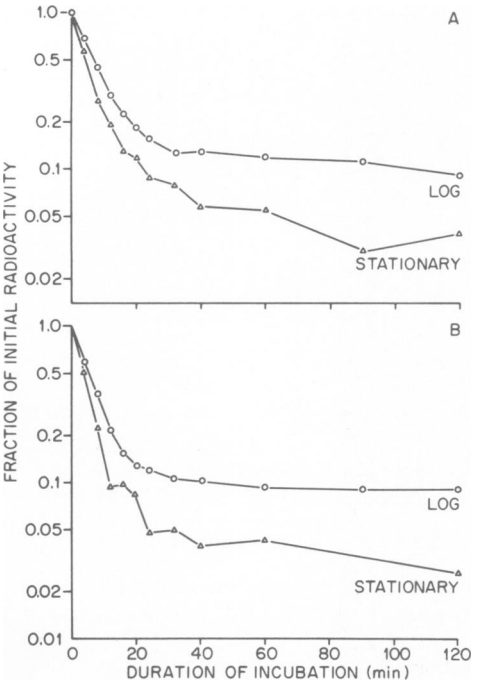

FIGURE 4

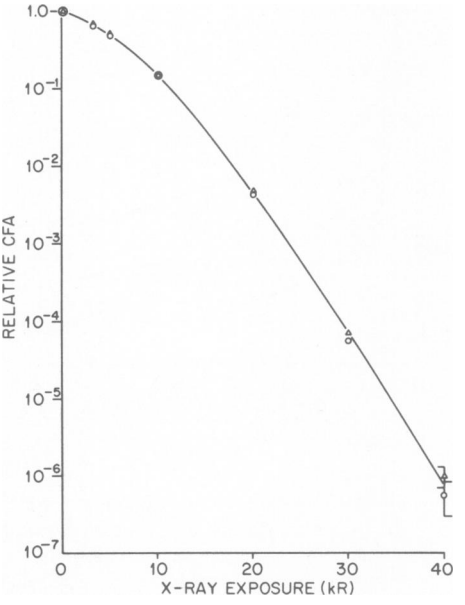

FIGURE 5

FIGURE 4 Degradation of DNA in stationary- and log-phase cells during incubation at 37°C after X-ray exposure. To within ± 10%, the measured initial DNA was independent of exposure. *A*, 30-kR exposure; *B*, 40-kR exposure.

FIGURE 5 Relative colony forming ability of log- and stationary-phase *Haemophilus* cells versus X-ray exposure. Log phase cells with streptomycin resistance, ○; stationary phase cells with novabiocin resistance, △.

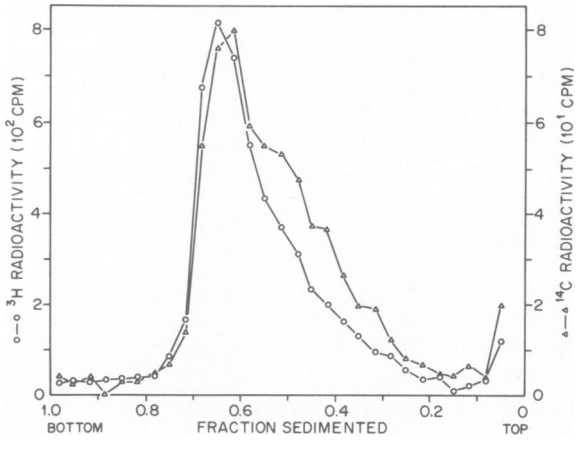

FIGURE 6 Double-label alkaline sucrose gradient with unirradiated ^{3}H-labeled DNA from stationary phase *Haemophilus* cells and with unirradiated ^{14}C-labeled DNA from log phase cells. Sedimentation was during 50 min at 40,000 rpm in a SW-50.1 rotor.

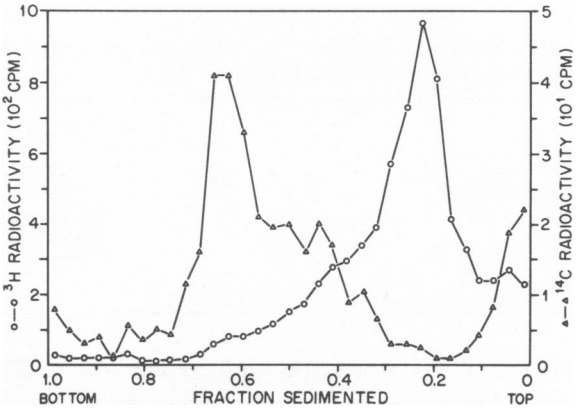

FIGURE 7 Double-label alkaline sucrose gradient with ^{3}H-labeled DNA in irradiated (15 kR) log-phase *Haemophilus* cells and ^{14}C-labeled DNA in unirradiated cells. Sedimentation was during 90 min at 30,000 rpm in SW39 rotor. Cells were kept at 4°C between the end of washings after growth and lysis on the gradient.

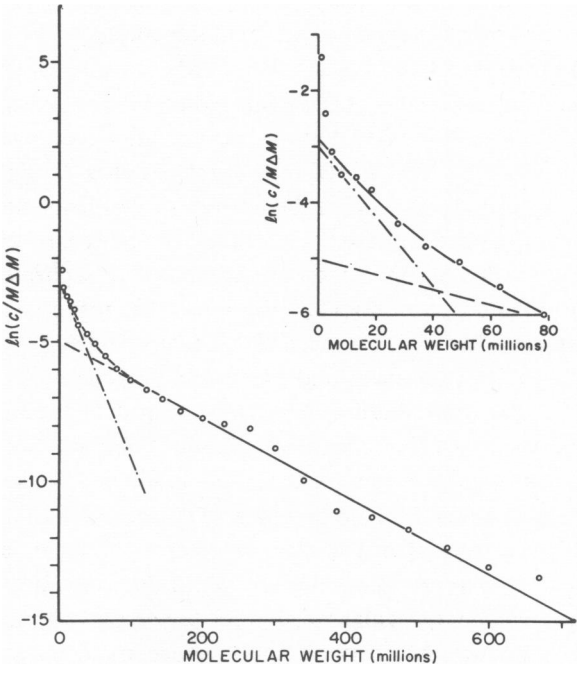

FIGURE 8 Test for random distribution of observed molecular weights from alkaline sucrose gradient data. (5-kR exposure, 50 min at 40,000 rpm in SW50.1 rotor). The ordinate is ln [mass of DNA measured as radioactive counts per minute per sample divided by (average molecular weight times difference between maximum and minimum molecular weights in the sample)]. A random distribution of molecular weights should fit an equation of the form $\ln(c/M\Delta M) = A + BM$, where A is a constant and $-1/B$ is the number-average molecular weight. (See Dean et al., 1969.) An empirical attempt to approximate the data as the sum of two random distributions is shown. The dashed line represents $\ln(c_1/M\Delta M) = -5 - M/72$; the dotted line represents $\ln(c_2/M\Delta M) = -3 - M/16$; and the solid line represents $\ln[(c_1 + c_2)/M\Delta M]$. The weight-average molecular weight for the entire distribution was about 120 million.

TABLE I

AVERAGE SINGLE-STRAND BREAK FREQUENCIES INDUCED IN THE DNA OF LOG PHASE *HAEMOPHILUS INFLUENZAE* BY X-RAYS

Observed breaks per initial strand per kiloroentgen	Break frequencies	
	Total	Assuming 20% alkaline labile
	eV/break	
2.0	40	50

Lehmann and Omerod (1970). Calculations of average molecular weights, using the computer program, yielded number-average values much less than the expected (Charlesby, 1954) half the weight-average values. Calculations of numbers of radiation-induced single-strand breaks from Eq. 1 and 3 and weight-average molecular weights yielded the radiosensitivities listed in Table I.

DISCUSSION AND CONCLUSIONS

The nonrandom distributions of molecular weights which we consistently find (Fig. 8) for both unirradiated and irradiated DNA were unexpected. We do not believe that artifacts reported by others (e.g., Elkind, 1971; Lehman and Omerod, 1970; van der Schans et al., 1969) for large molecular weights sedimented at high speeds are the cause of our apparently nonrandom distribution because (*a*) we do not observe the characteristically sharp leading edge in sedimentation patterns (Lehmann and Omerod, 1970) nor large amounts of material at the bottom of gradients, and (*b*) our observed distributions remain nonrandom even when many strand breaks are present (Fig. 8). In a few cases, also shown in Fig. 8, we have attempted to separate the observed nonrandom distribution into two random distributions. The result of such manipulations of the data for unirradiated cells has been that one distribution involves perhaps 60% of the molecules, 30% of the mass, and has a number-average molecular weight of 10–15 million; the other distribution, involving the rest of the molecules and mass, has a number-average molecular weight of perhaps 70 million, which is roughly half the weight-average molecular weight for the whole gradient. We suggest that the first, low molecular weight distribution might represent episomes or an artifact and that the second, high molecular weight distribution represents the genome. Rather than attempt many difficult and imprecise resolutions of our data into two exponential curves to get the number-average molecular weights for the second distribution, we base our estimates of break frequencies on the weight-average molecular weights, which are influenced little by the low molecular weight distribution. We calculate roughly 12 single-strand breaks per molecule for DNA from unirradiated cells. Our value for electron volts per single strand break for log-phase cells is well within the range of values commonly observed (e.g., see Setlow and Setlow, 1972, p. 302, or Town et al., 1972). If there is Type I or Type II repair (see Johansen et al., 1971; Town et al., 1972,

1973; Paterson et al., 1973, and Johansen et al., 1974) during the storage at 4°C between irradiation and lysis, we underestimate the number of strand breaks. Johansen et al. (1971) and Paterson et al. (1973) found a yield of 18–29 eV/break for DNA in alkaline gradients after cellular irradiation in air. Although our cells were kept at 4°C for a period of perhaps 30 min from the end of washings after growth through irradiation and up to the start of lysis on the tops of gradients, some Type I, if it exists, or Type II repair may occur even at low temperatures (Town et al., 1972) and in the brief period—10 min or less—at room temperature while the cells are lysing. Previous work (Setlow et al., 1968 *b*) has shown marked changes in sedimentation patterns for DNA from wild-type *H. influenzae* after 20 min incubation at 37°C. Other workers (e.g., Ginsberg and Webster, 1969; Lett and Sun, 1970; Town et al., 1971), using various other cells, have reported radiosensitivity for strand breaking to be independent of cell cycle.

Our results for untreated and irradiated log- and stationary-phase *Haemophilus influenzae* cells yield the following comparisons: (*1*) the molecular-weight distribution of DNA for unirradiated cells is probably somewhat sharper for stationary phase (Fig. 6). This would be expected if, in stationary phase, DNA synthesis has ceased immediately after division. (*2*) Cellular degradation of DNA after irradiation of cells in the two phases is similar, but somewhat greater in stationary phase (Fig. 4). Town et al. (1971) have found degradation in stationary *Escherichia coli* B/r is delayed and reduced, and Trgovčević and Kućan (1968, 1969) have found less degradation in stationary-phase *E. coli* B cells than in log-phase. We suppose these differences in degradation kinetics for different strains reflect differences in enzyme systems resulting from the marginal nutritional state of stationary phase cells. (*3*) The radiosensitivity for CFA is equal in the two phases (Fig. 5). Town et al. (1971) find the stationary phase in *E. coli* B/r to be less radiosensitive.

The values of DNA remaining after long incubation times reflect, we believe, the fate of DNA strands present at the time of irradiation regardless of subsequent DNA synthesis or resynthesis.

Qualitatively, the results of our DNA degradation measurements for *Haemophilus* are similar to those of Stuy (1960), Pollard and Achey (1964), Pollard et al. (1966), Frampton and Billen (1966), Grady and Pollard (1967), Achey and Pollard (1967), Stavrić et al. (1968), Chapman et al. (1968), Chapman and Pollard (1969), Trgovčević and Kućan (1969), Town et al. (1971), Paterson et al. (1971), Pollard and Tilberg (1972), and Pollard and Krauss (1973). The similarities that these workers and we find for various bacteria suggest that similar mechanisms may operate generally in bacterial cells.

As others have found with *E. coli* $15T^{-}L^{-}$ (Achey and Pollard, 1967; Chapman and Pollard, 1969; and Pollard, 1970) our measurements show increasing degradation rates in *Haemophilus* as the incubation temperature is increased. However, perhaps because of the limited range of temperatures 4–37°C) we used, we, unlike them, have no measurements which suggest thermal inactivation of the degrading enzyme mech-

anism above 40°C, nor have we sufficient data to attempt an Arrhenius plot of the initial degradation rate coefficients as Chapman and Pollard (1969) did.

The promptness of DNA degradation that we find with *H. influenzae* supports the notion (which Pollard and Tilberg [1972] have most carefully studied with *E. coli* B) that degradation starts immediately after irradiation from a preexisting enzyme system rather than that the irradiation induces the enzyme system.

Up to an exposure of at least 40 kR, the plateaus for *Haemophilus* decrease with increasing exposure, although others find a maximum degradation at exposures 20–50 kR depending on conditions (e.g., nutrients) during incubation and bacterial strain (Frampton and Billen, 1966; McGrath et al., 1966; Stavrić et al., 1968; Trgovčević and Kućan, 1969; Chapman and Pollard, 1969; and Pollard and Tilberg, 1972). The data of Grady and Pollard (1967) for *Bacillus subtilis* suggest a maximum degradation at an exposure of about 200 kR. Such maximum effects might reflect radiation damage to the degrading enzyme, exonuclease V. However, for exposures up to 60 kR, neither we nor Stuy (1960), who also measured degradation of DNA in *H. influenzae* in the presence of oxygen and up to an exposure of 144 kR, have observed a clear maximum in the radiation effect.

After prolonged incubation, we find plateau values of DNA remaining (Fig. 2) that are much greater than expected by direct relation to either cell survival or the number of strands having no breaks. In the case of cell survival, both previous studies (e.g., Setlow et al., 1968 *a*) and our Fig. 5 show $< 1\%$ survival after 20 kR exposure, whereas we find that $> 10\%$ of the DNA remains after 20 kR. In the case of strands having no breaks we estimate from the data given in Table I and from the Poisson distribution that $\ll 1\%$ of the initial single strands are free of breaks after a 20 kR exposure. Pollard and Tilberg (1972) have reported that plateaus for degradation and CFA have similar dose-response curves for a radiosensitive bacterial strain, *E. coli* $B_{s\text{-}11}$, which also markedly degrades its DNA. Their conclusion is based on similar initial slopes of semilogarithmic plots of CFA and degradation plateaus vs. dose, but the plateaus deviate markedly from linearity (on this semi-log plot) at high dose and they give no data (nor do we know of data) showing a similar deviation for CFA. Comparison of our Figs. 2 and 5 reveals that for *H. influenzae* neither the initial slopes nor the overall curve shapes of plateau and CFA versus dose are similar.

Some of what we measure and call radiation-induced, single-strand breaks are really radiation-induced, alkaline-labile bonds, but the results of Paterson et al. (1973) suggest that perhaps 75% of the breaks observed under these oxygenated conditions are directly induced by the radiation treatment and do not represent other damage to the DNA. Thus we cannot explain our degradation plateaus as being simply related to either colony-forming ability or single-strand breaks. We have not measured radiation-induced, double-strand breaks in vivo. From the commonly observed ratio of about 10 single-strand breaks to one double-strand break (e.g., see Setlow and Setlow, 1972, p. 304), the probability that a cell has unbroken double strands at some exposure may equal the DNA plateau value of Fig. 2. However, the dose curve for

double strand breaks is expected to increase at least linearly with dose, whereas the observed degradation, as judged by plateaus, does not.

Our degradation kinetics might be explained, at least qualitatively, by assuming the presence of an X-ray inducible inhibitor of degradation, similar to the ultraviolet nalidixic acid or mitomycin C induced inhibitor discussed by others (Pollard and Randall, 1973; Marsden et al., 1974; and Tolun et al., 1974), with events occurring in the order: (*a*) during irradiation of cells, production of lesions and "birth" of inhibitor; (*b*) during storage at 4°, neither degradation nor growth of inhibitor; (*c*) during initial incubation at 37°, degradation at rates increasing with exposure and decreasing with growth of inhibitor; and (*d*) after about 40 min incubation cessation of degradation because of inhibition. The split dose experiments of Grady and Pollard (1968) with *E. coli* 15 fit in well with this hypothesis. A more detailed study of the mechanism of cellular degradation of DNA following X-ray exposure and quantitative evaluation of rate constants will be given elsewhere.[1]

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