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THE PITFALLS OF MEASURING DNA SYNTHESIS KINETICS AS EXEMPLIFIED IN ULTRAVIOLET RADIATION STUDIES

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SUMMARY

1. Previous reports on the effect of ultraviolet irradiation on the incorporation of radioactive precursors of DNA by bacteria have presented the data as linear plots of counts/min *vs.* time. Using this type of plot one only obtains the apparent rate of DNA synthesis in bacterial cultures because the slope of these curves depends in part upon trivial parameters such as the initial viable cell number and the specific activity of the radioactive precursor. The true rate of DNA synthesis only depends upon the DNA content per cell and their generation time. Examples are given to demonstrate that linear plots can lead to erroneous conclusions concerning DNA synthesis kinetics.

2. We recommend a new protocol for studying the effect of radiation (and other agents) on macromolecular synthesis kinetics which circumvents the pitfalls inherent in previous methods: (1) use mutants requiring the labeled precursor (*e.g.*, thymine), prelabel the cells and keep the radioactivity present after irradiation (equivalent to the direct chemical determination of DNA); (2) plot the data as log counts/min *vs.* time (DNA synthesis is an exponential function in bacteria); and (3) determine the kinetics of cell growth at the same time aliquots are taken for the determination of incorporated radioactivity (one may then hope to deduce the biological relevance of the observed synthesis). The advantages of this protocol over previous methods are documented and discussed with special emphasis on the measurement of the extent of delay in DNA synthesis in radiation-resistant bacteria as a function of dose of ultraviolet radiation.

INTRODUCTION

One of the more common experiments reported in the literature is the effect of radiation, drugs, *etc.* on the incorporation of radioactive precursors into the DNA of bacteria, yet the pitfalls inherent in the methods being used are not generally appreciated. As a specific example, knowledge of the kinetics of DNA synthesis in bacteria after ultraviolet irradiation is important to our understanding of the biological effects of the ultraviolet-induced lesions produced in DNA and to our understanding of the ability of some strains of bacteria to circumvent or repair radiation damage to their DNA. Toward these goals there have been numerous publications[1-6] showing graphs

of uptake of radioactive thymine (thymidine or ^{32}P) by cultures of bacteria as a function of the time of growth after ultraviolet irradiation. The radioactive precursor was added immediately after irradiation and the data for the incorporation of radioactive thymine, for example, into acid-insoluble material per aliquot of culture *vs.* time of growth after irradiation were plotted as a linear function. Since by this type of graph the irradiated cultures appeared to show a slower rate of uptake of thymine relative to the unirradiated control, the conclusion was offered that ultraviolet light inhibits DNA synthesis and the time of inhibition is dependent upon the dose of radiation.

We have found that thymine uptake curves (on linear plots) whose shapes are similar to those obtained after ultraviolet irradiation can be generated simply by diluting unirradiated bacteria to different cell densities prior to adding the radioactive thymine. Similar curves can also be generated by keeping the number of unirradiated cells constant and using radioactive thymine with different specific activities. These results emphasize that when using linear plots of thymine incorporation data one only obtains the apparent rate of DNA synthesis in bacterial cells because the slope of these curves depends in part upon such trivial parameters as the initial viable cell number and the specific activity of the radioactive precursor. The true rate of DNA synthesis, of course, only depends upon the DNA content of the cells and their generation time. Therefore, since cells are killed by radiation, the use of graphical procedures that generate curves whose slopes are in part dependent upon cell number may lead to misinterpretations of the true kinetics of DNA synthesis after irradiation. Certainly previous authors have overestimated the duration of DNA synthesis delay in ultraviolet-irradiated bacteria by these methods. We document herein several pitfalls of measuring DNA-synthesis kinetics and offer a new protocol for measuring DNA synthesis kinetics to circumvent these pitfalls. Although we emphasize the use of this new protocol in radiation studies, the arguments are equally meaningful to the measurement of DNA synthesis after treatment of cells with drugs or for the measurement of the kinetics of RNA and protein synthesis or of enzyme induction.

MATERIALS AND METHODS

Escherichia coli B/r, T^- (obtained from D. FRIEFELDER, Brandeis University) were grown on a salts-glucose medium[7] supplemented with [2-^{14}C]thymine (2 μg/ml; 0.169 μC/μg, Calbiochem). When they had entered log phase growth and reached a density of about $8 \cdot 10^7$ cells/ml they were harvested by centrifugation, suspended at the appropriate cell density in minimal medium *minus* glucose and thymine and irradiated at 2537 Å as previously described[8]. The irradiated cells were then returned to a flask at 37° containing glucose and [2-^{14}C]thymine. Aliquots of this culture, in a Gyrotory water bath (New Brunswick Scientific Co.) at 37°, were taken at various times for the determination of viable counts and for the measurement of [2-^{14}C]-thymine incorporation.

For determining the number of colony-forming units present, aliquots were spread on agar plates made with the same medium used for the incorporation studies except for the substitution of non-radioactive thymine. The aliquots taken for meas-

urement of radioactivity were diluted 1:2 with 4 % formaldehyde to stop metabolism. Aliquots (0. 1 ml) of the formaldehyde-killed cells were placed on 2.3-cm Whatman 3MM discs and dried under heat lamps. The discs were then washed in bulk three times in 5 % trichloroacetic acid and two times in water and once in ethanol and dried. Their radioactivity content was determined in a liquid-scintillation counter as previously described[9]. The results are plotted as counts/min per 0.05 ml of culture.

RESULTS AND DISCUSSION

Fig. 1 shows typical results for the uptake of [2-^{14}C]thymine by *E. coli* B/r, T$^-$ after various doses of ultraviolet light. The data appear consistent with the hypothesis that ultraviolet light causes a delay in DNA synthesis and that this delay is dependent upon the dose of ultraviolet light. It should be emphasized, however, that the number of colony-forming cells remaining is also dependent upon the dose of ultraviolet light.

Fig. 2 demonstrates that curves similar in shape to those obtained after ultraviolet irradiation (Fig. 1) can be obtained with unirradiated cells simply by starting the thymine incorporation experiment with different numbers of cells per ml. These data appear to suggest that DNA synthesis has been delayed, but by measuring the

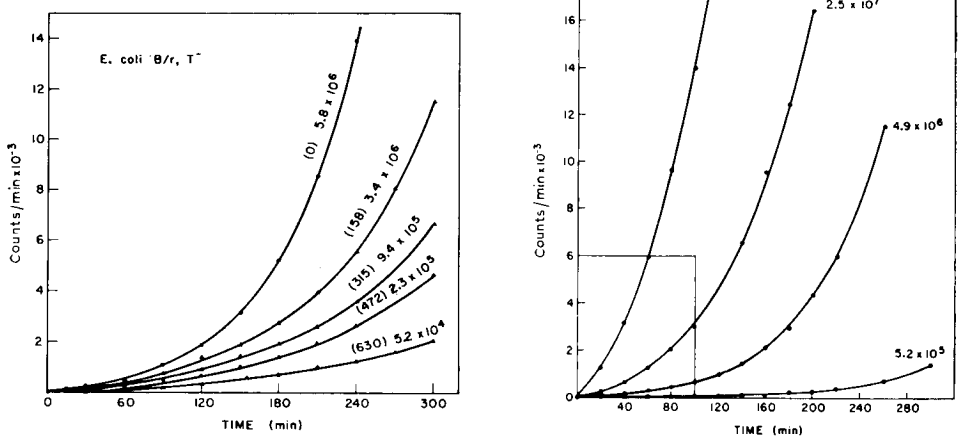
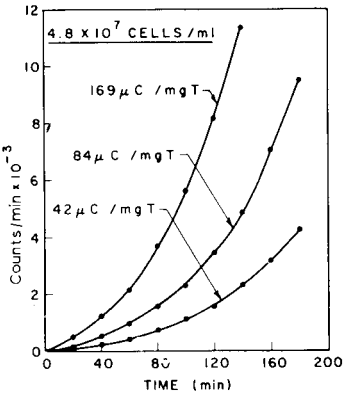


Fig. 1. The uptake of [^{14}C]thymine by cultures of *E. coli* B/r, T$^-$ after various doses of ultraviolet light (2537 Å). The experimental procedures are described in the text. The exposure doses of ultraviolet light in ergs/mm^2 are shown in parentheses for each curve. The numbers following each parenthesis indicate the number of colony-forming units (CFU) present at time zero after irradiation. The results are the average of two experiments.

Fig. 2. The uptake of [^{14}C]thymine by unirradiated cultures of *E. coli* B/r, T$^-$ having different initial numbers of colony-forming units. The faint box in the lower left-hand corner is to call attention to the fact that most experiments in the literature are only run for about 100 min and that different interpretations of the results are possible for short experiments *vs.* longer experiments. This point is further discussed in the text.

counts/min of [^{14}C]thymine incorporated per viable cell per unit time, it can be shown that the rates of DNA synthesis for all the cultures in Fig. 2 are identical. (This point will be further discussed in conjunction with Fig. 6.) The apparent differences in the DNA synthesis kinetics in Fig. 2 are an artifact of plotting exponential functions on linear graph paper. Since this artifact can be demonstrated using unirradiated cells (as shown in Fig. 2) it argues strongly against the continued use of this method for plotting the data for irradiated cells.

Confining our attention to the first 100 min of the experiments shown in Fig. 2 (the maximum time used for many experiments on DNA synthesis kinetics in the literature) it is apparent that quite different conclusions about the kinetics can be made after 100 min and after 300 min. One might conclude for example, that for the first 100 min the kinetics of synthesis for the cells at $4.9 \cdot 10^6$ per ml are essentially linear, while they are exponential for the cells at 10^7 and 10^8 per ml. Such conclusions appear in the literature relevant to RNA and protein synthesis in ultraviolet-irradiated populations[1,4] but it is well known[10] and the results in Fig. 2 also demonstrate that the early part of an exponential function plotted on linear paper appears linear. The less steep the exponential function, the longer is the apparent linear portion of the curve (on linear plots). It would be significant if the kinetics for macromolecular synthesis truly changed from exponential to linear, but this appears to be another pitfall in the use of short-term experiments and linear plots. The results for cells at 10^5 per ml (Fig. 2) suggest that DNA synthesis has been completely stopped for 100 min, yet by using the new experimental protocol and plotting methods to be



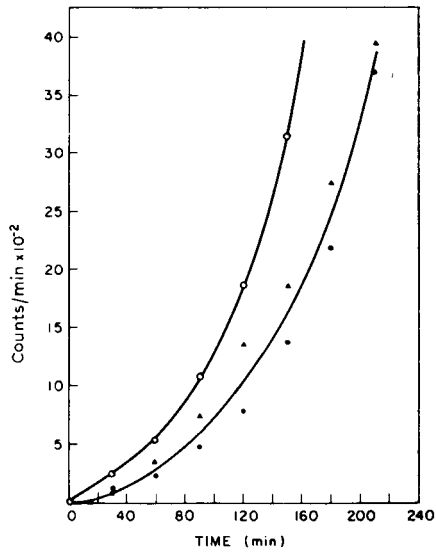


Fig. 3. The uptake of [^{14}C]thymine of different specific activities by cultures of unirradiated *E. coli* B/r, T$^-$ having the same initial number of colony-forming units.

Fig. 4. The uptake of [^{14}C]thymine by cultures of *E. coli* B/r, T$^-$. A culture at $5.8 \cdot 10^6$ colony-forming units per ml was irradiated with 158 ergs/mm^2 (2537 Å) to yield $3.4 \cdot 10^6$ colony-forming units per ml (▲–▲) (minimal medium agar plates). A third culture (unirradiated) was diluted to give $3.5 \cdot 10^6$ colony-forming units per ml (●–●). ○–○, $5.8 \cdot 10^6$ colony-forming units (unirradiated).

described below, it can be demonstrated that this culture is synthesizing DNA at the same rate per cell as the culture at $1.2 \cdot 10^8$ cells per ml.

Another pitfall in the use of linear plots for determining the rate of DNA synthesis is seen in Fig. 3 where an apparent delay of DNA synthesis can be demonstrated with unirradiated cells by keeping the initial cell number per ml constant and by varying the specific activity of the thymine. This artifact of differing specific activities is of particular importance to experiments in which one hopes to compare the apparent rates of uptake of thymine, uridine and leucine (all at different specific activities).

Fig. 4 demonstrates that after doses of ultraviolet light that leave about 50 % survival, the thymine incorporation curve (on a linear plot) appears similar to that for unirradiated cells at the same number of colony formers per ml at time zero. At higher doses of ultraviolet light this correlation fails because for a considerable time after irradiation these cultures take up more thymine than would be expected from the number of colony-forming cells present, *i.e.*, many 'dead' cells are synthesizing DNA.

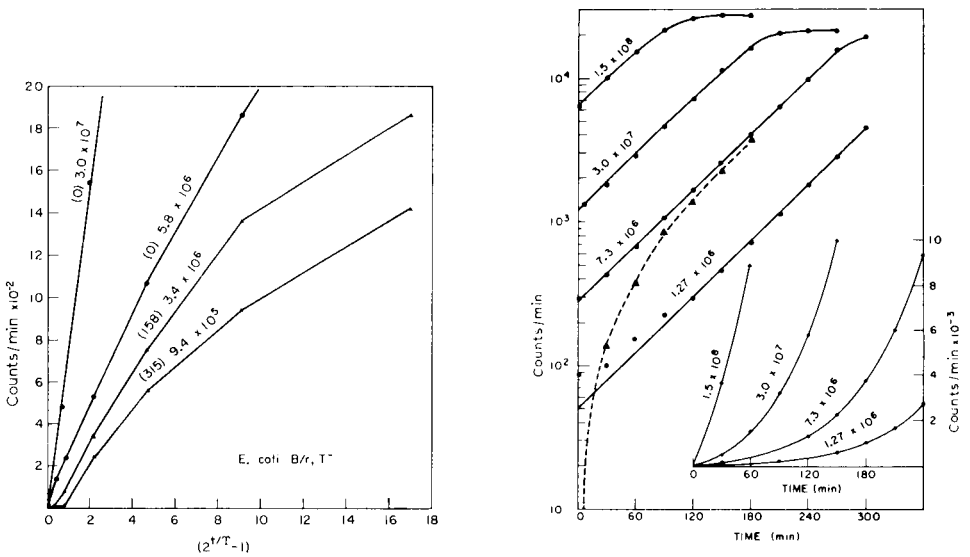


Fig. 5. The uptake of [^{14}C]thymine by control and irradiated cultures of *E. coli* B/r, T$^-$ *vs.* $(2^{t/T}-1)$ where t is the time and T is the generation time of the unirradiated culture (see ref. 4 for the derivation of this expression). The data for the control ($5.8 \cdot 10^6$ colony-forming units) and the two irradiated curves (158 and 315 ergs/mm^2, respectively) are taken from the experiment described in Fig. 1 (T = 36 min). The control curve for $3.0 \cdot 10^7$ colony-forming units/ml is taken from a separate experiment (T = 39 min).

Fig. 6. The uptake of [^{14}C]thymine by unirradiated prelabeled cultures of *E. coli* B/r, T$^-$, having different initial numbers of colony-forming units. In contrast to the linear plots (corrected to zero counts at zero time) shown in the lower right-hand corner where the rates of thymine uptake appear different for cultures containing different initial numbers of colony-forming units, the semi-log plot shows that the rates are independent of cell number but the amount of radioactivity present at time zero (prelabeled cells) is of course dependent upon the initial cell density. The dotted curve (log scale) indicates the type of results obtained if one attempts a semi-log plot without using prelabeled cells (mimicked here by subtraction of the zero time counts for the culture at $7.3 \cdot 10^6$ colony forming units/ml from all subsequent samples). This curved line is an artifact produced because there is no zero on log graph paper.

The results thus far presented indicate that the rate of DNA synthesis as determined by using a linear plot of counts/min *vs.* time gives only an apparent rate because the slope of this type of plot is in part dependent upon the number of colony-forming cells present. It is perhaps superfluous, therefore, to add that attempts to quantitate these results in terms of rate of synthesis or the time of synthesis delay are futile. The techniques that have been used, however, are to measure the horizontal distance between the control curve and the subsequent curve for the irradiated aliquots or to measure the distance between the points of intersection on the abscissa of tangents to the curves. Neither technique would be expected to give rise to meaningful data because of the uncertainty of where on the curves the comparisons should be made. The results would depend, for example, on whether a 100-min or a 300-min experiment had been run (Fig. 2).

There has been one attempt to transform [^{3}H]thymidine incorporation data into a more easily interpretable graphical plot[4]. This involves a plot of counts/min *vs.* $(2^{t/T}-1)$ where t is the time at which the sample is taken and T is the generation time of the unirradiated culture. Although this transformation may be useful for estimating the duration of DNA synthesis delay, the apparent DNA synthesis kinetics derived from this type of plot are also proportional to the number of cells synthesizing DNA (Fig. 5). This dependence of slope on cell number can also be confirmed from theoretical calculations of counts/min *vs.* $(2^{t/T}-1)$. An assumption made in this transformation is that the generation time of the irradiated cells is the same as that for unirradiated cells. This is inconsistent with the known inhibiting effects of ultraviolet radiation upon cellular division[11] (see also Fig. 8). Although from theoretical considerations a plot of counts/min *vs.* $(2^{t/T}-1)$ should be linear, experimentally this is found to be true only over short time periods and then the curves become progressively non-linear (especially for irradiated cultures) (Fig. 5). Although there are certain advantages of this method over a linear plot of counts/min *vs.* time, the pitfalls mentioned above would appear sufficiently serious to counteract its advantages.

We offer the following protocol to circumvent the ambiguities created by the methods presently in the literature for the determination of DNA synthesis kinetics. The current practice is to add the radioactive precursor immediately after irradiation of the cells (time zero). We suggest that prelabeled cells should be used and the label should continue to be present after irradiation. This will eliminate the problems of precursor pool dilutions[1] and will allow the use of a graphical presentation of the data that is more easily and directly interpretable (see below).

Rather than a linear plot, a semi-log plot of counts/min *vs.* time should be used. With a semi-log plot the slopes of DNA-synthesis curves are independent of the initial number of cells present (Fig. 6). If prelabeled cells are not used, the experiment will start with zero counts at zero time. Since there is no zero on logarithmic graph paper, instead of straight lines from time zero, the curves will sweep up from infinity and curve over finally to yield the straight line function after the counts/min incorporated per unit time achieves the equilibrium situation for the number of cells present in the culture (dotted line in Fig. 6). However, semi-log plots can be used for non-prelabeled cultures if cell numbers are determined during the experiment until the counts/min per cell in the control culture becomes constant. This amount of counts/min can then be added (appropriate to the number of cells present at time zero in the control) to all experimental values (including time zero).

A third requirement is that these experiments be performed with cultures that are sufficiently dilute at time zero so that the controls maintain exponential growth for several hours. When this is done, sufficient time-course data points can be gathered to establish the true rates of DNA synthesis and of growth. DNA synthesis stops prior to the cessation of cell division at the end of exponential growth and irradiated cultures saturate at considerably fewer colony-forming cells per ml than do unirradiated cultures. Unless properly monitored, these events could lead to incorrect conclusions about DNA synthesis inhibition.

A fourth requirement is that at the same time aliquots are taken for radioactivity determinations aliquots should also be taken to measure colony-forming ability. Since some strains of bacteria show no division delay after ultraviolet irradiation (*E. coli* B_s, T^-) while others do (*E. coli* B/r, T^- and B, T^-)[12] a meaningful interpretation of the DNA synthesis kinetics for these strains is not possible without additional data on growth and division delay.

A fifth requirement is that in determining the colony-forming ability of the irradiated cultures the agar plates contain the same media used in the thymine uptake part of the experiments, except for the substitution of non-radioactive thymine. This precaution is essential since the survival of irradiated *E. coli* B/r (and other

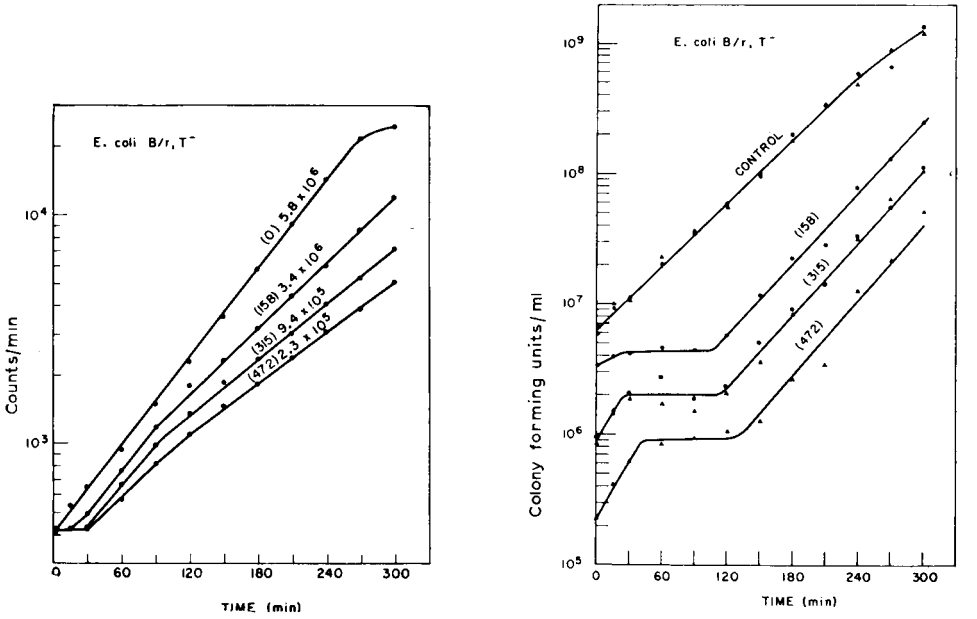


Fig. 7. The uptake of [^{14}C]thymine by cultures of *E. coli* B/r, T^- after various doses of ultraviolet light (2537 Å). These data are taken from the experiment shown in Fig. 1 (linear plot) but are presented here in the more appropriate semi-log plot. Note that the absolute delay in DNA synthesis can be read directly from the graph and the rates of synthesis after resumption can be easily compared with that for the unirradiated control (since the rates are not dependent upon cell number for this method of plotting).

Fig. 8. The growth rate of control and irradiated cultures of *E. coli* B/r, T^-. At the time aliquots were taken for the determination of [^{14}C]thymine incorporation (Fig. 7), aliquots were taken for plating on minimal medium agar plates. These latter results are plotted here as colony-forming units/ml *vs.* time of incubation.

strains) is significantly different on minimal medium plates as compared to enriched medium plates[13]. This precaution has not usually been followed by previous authors studying DNA synthesis kinetics in the few instances where they have recorded the initial viability of the cultures after ultraviolet irradiation.

Typical plots obtained using our protocol for determining the true kinetics of thymine incorporation into *E. coli* B/r, T^- as a function of dose of ultraviolet radiation are shown in Fig. 7 (*cf.* the same data in a linear plot in Fig. 1). If in Fig. 1 one measures the time it took for each of the curves to reach 4000 counts/min and subtracts from each value the time it took the unirradiated control sample to reach 4000 counts/min one may conclude that 158 ergs/mm^2 caused a delay in DNA synthesis of 50 min, 315 ergs/mm^2 a delay of 88 min and 472 ergs/mm^2 a delay of 128 min. Since the generation time of the control cells is 36 min, the above data indicate that 472 ergs/mm^2, which leaves a surviving fraction of $0.4 \cdot 10^{-1}$, causes a delay in DNA synthesis of a little over 3.5 generation times. Information of this type on the ability of cells to stop and resume DNA synthesis would be of great importance to the understanding of cellular control mechanisms if the data were correct. We have already discussed the pitfalls of using linear plots and the incorrect conclusions that may result from using these plots.

In Fig. 7 there is no ambiguity about the time that DNA synthesis is stopped as a function of ultraviolet light. The control culture shows no lag in DNA synthesis (as it appears to show in Fig. 1) and the time of resumption of DNA synthesis in the irradiated cultures can be read directly from the graph. A very important observation is that at none of the ultraviolet doses used (including higher doses not shown) was DNA synthesis stopped for longer than about one generation time. Clearly any repair of ultraviolet damage that requires that DNA synthesis be stopped must be accomplished within about one generation time. These results on the extent of DNA synthesis delay are in agreement with earlier data of DOUDNEY AND YOUNG[14] using a direct chemical method for the analysis of DNA synthesis, but are in complete disagreement with published data using isotope incorporation as the criterion for DNA synthesis. The direct chemical analysis of DNA synthesis is analogous to using cells prelabeled with thymine but semi-log plots should also be used. DOUDNEY AND YOUNG[14] only followed DNA synthesis for 120 min so that they did not see the secondary changes in the rate of DNA synthesis subsequent to this time, as shown in Fig. 7.

Data for the growth and division delay of these cultures are presented in Fig. 8. Note a rapid increase in colony-forming units during the first 30 min of growth after ultraviolet radiation, followed by a delay in cell division for about 90 min and then a resumption in growth at about the normal rate. It will be noted by comparing with Fig. 7 that DNA synthesis is stopped during the time of the large increases in viable cells (the first 30 min after ultraviolet irradiation) and that DNA synthesis resumes during the time when the cells are undergoing no division and that the rate of DNA synthesis changes upon the resumption of growth of the surviving cells, but not at a rate that is equal to the growth rate. Clearly at times longer than 300 min either the rate of DNA synthesis must increase or the rate of growth must decrease for the two rates to again become in step. We will discuss in a subsequent publication that much of the synthesis observed at these early times is due to cells which are not capable of forming colonies. Experiments must, therefore, be carried out for a long time (15–20

h) so that the contribution of the surviving cells to DNA synthesis can finally be ascertained and the contribution of the non-viable cells to early synthesis can then be determined.

Data of the type shown in Figs. 7 and 8 for *E. coli* B/r will be interpreted in relation to similar data for cultures of *E. coli* B_s and *E. coli* B in subsequent publications.

We have seen that the apparent kinetics (but not the true kinetics) of DNA synthesis in an unirradiated bacterial culture depend upon numerous experimental and graphical variables. The problem of understanding DNA synthesis kinetics, however, is even more complex in a heterogeneous population of cells produced as a consequence of ultraviolet irradiation. One can postulate the presence of three major classes of cells after irradiation: (1) colony formers synthesizing DNA; (2) non-colony formers synthesizing DNA for variable amounts of time; and (3) non-colony formers not synthesizing DNA. Classes (1) and (2) can be subdivided according to their rate of DNA synthesis (delayed, normal, or reduced). Therefore, the interpretation of DNA synthesis kinetics and its relation to growth kinetics would appear to be complicated at best, but if the experimental design or subsequent graphing of the data produces artifacts then a meaningful interpretation of the results becomes impossible.

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