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Deoxyribonucleic Acid Synthesis After Inhibition of Initiation of Rounds of Replication in *Escherichia coli* B/r[1]

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The theory describing the effect of inhibition of initiation of rounds of deoxyribonucleic acid (DNA) replication on the accumulation of DNA is derived, and an analysis is presented which allows the determination of the time *C* taken to replicate the bacterial chromosome from the kinetic changes in the accumulation of DNA. This analysis is applied to experiments in which inhibition of initiation was achieved by inhibiting protein or protein and ribonucleic acid synthesis with chloramphenicol or rifampin. The results for both antibiotics are identical and indicate that there is a delay of 6 to 11 min in the effect of the antibiotics on initiation of rounds of replication. If this delay is taken into account, then the value of the *C* period estimated from such experiments agrees with values obtained by other methods, whereas by conventional data evaluation of such experiments the C period would be overestimated. In the low thymine-requiring derivative of *Escherichia coli* B/r ATCC 12407 used here, the C period was found to be between 38 and 41 min for cultures growing with a mass doubling time of 29 min in glucose-amino acids medium, supplemented with 20 μg of thymine/ml.

The increment in the amount of deoxyribonucleic acid (DNA) in a bacterial culture after inhibition of protein synthesis has been used in several studies to estimate the time *C* taken to replicate the bacterial chromosome (4, 7, 12, 14). These studies have all been based on the assumption that initiation of rounds of replication ceases concomitantly with inhibition of protein synthesis. However, the values of the *C* period obtained in this manner are 20 to 30% longer than the values obtained from measurements on synchronous cultures (see Discussion). This discrepancy might be due to a delay in the cessation of replication initiation after inhibition of protein synthesis. There is some evidence that such a delay occurs (7).

Here we have derived a method of data evaluation which enables one to determine this delay quantitatively from the kinetics of residual DNA accumulation after inhibition of initiation. When this method was applied to experiments in which protein synthesis was inhibited by high concentrations of chloramphenicol or rifampin, it was found that (taking this delay into account) the previous discrepancy disappeared.

MATERIALS AND METHODS

Bacterial strain and growth conditions. The bacterial strain used in this study was *Escherichia coli* B/r TJK16 (1), which is an F^- *thyA drm* derivative of *E. coli* B/r ATCC 12407. Cultures were grown at 37°C in C medium (3) supplemented with 0.2% (wt/vol) glucose, an artificial mixture of all 20 amino acids, each to a final concentration of 0.05%, wt/vol (50 μg/ml), and 20 μg of thymine/ml. Aeration was performed by vigorous shaking in a New Brunswick gyratory water bath. All cultures were inoculated with a 1:1,000 dilution of a fresh overnight culture. These conditions maintained a doubling time of 29 min.

Measurement of DNA synthesis after addition of antibiotics. Medium containing 20 μg of [2-^{14}C]thymine/ml (Schwarz/Mann; final specific activity, 0.01 μCi/μg) was inoculated and the absorbancy at 460 nm (A_{460}) and accumulation of DNA were monitored. Optical density was followed by use of a Zeiss PMQII spectrophotometer. For estimation of DNA, samples (0.5 ml) were removed at intervals into 1.0 ml of 1 M trichloroacetic acid (containing 2 M NaCl) and kept on ice. After at least 30 min, the precipitates were collected on Schleicher & Schuell membrane filters (0.45-μm pore size), washed with cold 0.01 M trichloroacetic acid, dried, and placed in vials with 5 ml of toluene-based scintillation fluid. Radioactivity was determined with a Beckman LS-100 scintillation counter. At an A_{460} of 0.6, chloramphenicol or rifampin (Schwarz/Mann) was added to a final concentration of 200 μg/ml. In the experiments described in Fig. 6, an equivalent amount of rifampin was added to all samples from the chloramphenicol-treated portion of the cultures along with trichloroacetic acid, to equalize quenching of radioactivity by the rifampin.

[1] No reprints of this paper will be available.

THEORY

Definitions. I = Number of chromosome origins (i.e., initiation sites for DNA replication) per milliliter of culture. T = Number of chromosome termini per milliliter of culture. F = Number of replication forks per milliliter of culture. G = Amount of DNA in genome equivalents ($8.2 \cdot 10^6$ nucleotides for *E. coli*) per milliliter of culture. C = Time (minutes) to replicate a chromosome. τ = Doubling time (minutes) of cell mass, protein, and ribonucleic acid. I_0, T_0, F_0, and G_0 = I, T, F, and G, respectively, at $t = 0$ (time of inhibition of protein synthesis).

For a culture with one cell per unit of culture volume where each cell has one complete chromosome (no replication forks), we define $I = T = 1$ origin or terminus respectively per unit of volume. If all chromosomes begin to replicate, we have one additional initiation (or origin) per unit of volume, independently of whether replication is unidirectional or bidirectional, i.e., $I = 2$, $T = 1$. After C minutes when replication is complete, we define $I = 2$, $T = 2$. Since cell divisions can be ignored, all numbers are given per unit of volume rather than per cell.

Accumulation of DNA after cessation of initiation of DNA replication. We consider an exponentially growing culture in which all initiations of DNA replication cease at zero time. Thus, for $t \geq 0$

$$I = I_0 \tag{1}$$

We further assume that the replication forks continue with unchanged speed until completion of replication; hence, for $0 \leq t \leq C$

$$T = I_0 2^{(t-C)/\tau} \tag{2a}$$

and for $t \geq C$

$$T = I = I_0 \tag{2b}$$

These two relations (equations 1 and 2) are illustrated in Fig. 1 (top two curves).

The number of replication forks is twice the difference $(I - T)$, assuming that two forks are formed at every initiation (in bidirectional replication) and disappear again at every termination:

$$F = 2(I - T) \tag{3a}$$

or after substitution of equations 1 and 2:

$$F = 2I_0(1 - 2^{(t-C)/\tau}) \text{ for } 0 \leq t \leq C \tag{3b}$$

$$F = 0 \qquad \text{for} \qquad t \geq C \tag{3c}$$

The rate of DNA synthesis (per unit of culture volume) is proportional to the number of forks; the factor is $^1/_2C$ (i.e., every fork produces DNA at a rate of $^1/_2$ genome equivalent per replication time C):

$$\frac{dG}{dt} = \frac{F}{2C} \tag{4}$$

If C is constant (independent of time), then the rate of DNA accumulation is also given by equation 3, as illustrated in Fig. 1 (third curve from top).

Finally, the accumulation of DNA is given by the integral over equation 4:

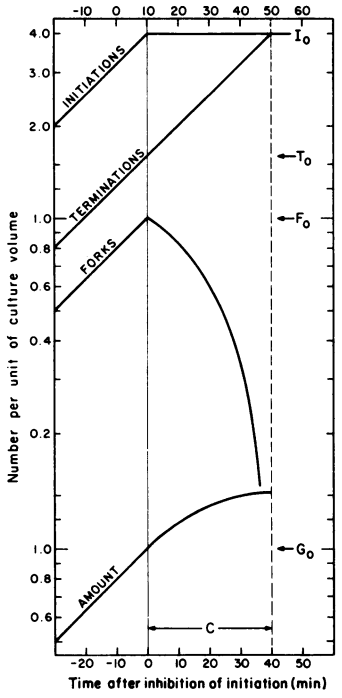

FIG. 1. *Theoretical relation of the number of chromosome origins (equation 1), the number of termini (equations 2a and 2b), the number of replication forks (equations 3b and 3c), and the amount of DNA (equation 5b) before and after addition of chloramphenicol (C period = 40 min; doubling time of culture τ = 30 min). The number of forks and the amount of DNA are normalized to a value of 1.0 per unit of culture volume at zero time (lower abscissa). The bottom abscissa represents an immediate effect of chloramphenicol on initiation. The top abscissa represents a delay of 10 min (see Fig. 3 and 4).*

$$G_t = G_0 + \int_0^t \frac{F}{2C}\,dt \tag{5a}$$

or after substitution of equation 3 for F and equation 8 of reference 1 for I_0:

$$G_t = G_0 \left\{ \frac{\ln 2}{\tau(1 - 2^{-C/\tau})}\, t - \frac{2^{t/\tau} - 1}{2^{C/\tau} - 1} + 1 \right\} \cdot \text{for } 0 \leq t \leq C \tag{5b}$$

as illustrated in Fig. 1 (bottom curve).

The final increase in the amount of DNA between $t = 0$ and ∞

$$\frac{G(t \to \infty)}{G_0} = \frac{G(t = C)}{G_0} = \frac{C(\ln 2) \cdot 2^{C/\tau}}{\tau(2^{C/\tau} - 1)} = \frac{C(\ln 2)}{\tau}(1 - 2^{-C/\tau})^{-1} \tag{5c}$$

is only a function of C and τ. The relation 5c has been derived previously in a different form (10, 12) but is here obtained as a special case from the more general relation 5b.

Analysis of the theoretical kinetics of DNA accumulation. For the further analysis we define (in analogy to a previous definition, reference 1):

$$\Delta G_t = \frac{G_t}{G_0}\ ;\ \Delta G_\infty = \frac{G_{t\to\infty}}{G_0} \tag{6}$$

and

$$\Gamma_t = \Delta G_\infty - \Delta G_t \tag{7}$$

$$\gamma_t = 2^{t/\tau} - \Delta G_t \tag{8}$$

These definitions are illustrated in Fig. 2a. The functions Γ_t and γ_t are nearly parabolic (they are exactly parabolic for $\Gamma \to 0, \gamma \to 0$), and therefore the plot $\sqrt{\Gamma}$ and $\sqrt{\gamma}$ versus t gives nearly straight lines.

The intersection value for the $\sqrt{\gamma}$ curve with the time axis is the time at which the rate of initiation of replication begins to decrease in comparison to exponential growth; hence, the $\sqrt{\gamma}$ curve shows whether there is any delay in inhibition of initiation; if initiation stops immediately (at $t = 0$), then the curve extrapolates to $t = 0$ (Fig. 2b); if initiation stops only after some delay (t_d), then the curve extrapolates to a positive time equal to t_d. The intersection value for the $\sqrt{\Gamma}$ curve with the time axis is at $t = C$ (Fig. 2b).

Alternately, the C period can be obtained from ΔG_∞, by use of equation 5c. The increase in DNA after cessation of initiation (ΔG_∞) is independent of any change in the speed of replication occurring after zero time; hence, the ΔG_∞ method determines the C period before zero time (C_1). The $\sqrt{\Gamma}$ curve determines the C period after zero time (C_2). The comparison of C_1 and C_2 shows whether the replication forks slow down after cessation of initiations.

Whether replication goes to completion can be checked within the limits of the accuracy of the determination of G (e.g., if the limiting accuracy for G is 1%, then it cannot be determined whether or not the last 10^5 base pairs of the *E. coli* genome are replicated). Any incomplete replication would show up as a deviation of the observed kinetics from the calculated kinetics (equation 5b). The type of deviation depends on the manner in which completion of termination is prevented. In general, the deviations would show up as deviations from the (approximate) linearity of the $\sqrt{\gamma}$ and $\sqrt{\Gamma}$ curves. A simpler and more direct method to check the completion of replication is possible if the C period (C_1) is independently known from some other type of experiment. In that case it is only necessary to calculate ΔG_∞ from equation 5b, using the known value for C_1: if this calculated value agrees with the observed value, then replication goes to completion (within the limits of experimental accuracy); if the observed value of ΔG_∞ is smaller than the calculated value, then the replication ceases prematurely.

Delay in inhibition of initiation. If replication initiations continue for some time after cessation of protein synthesis as illustrated in Fig. 3a, then the two gamma curves become shifted to the right by a length of time equal to the delay, t_d, between cessation of protein synthesis and cessation of initiations, as illustrated in Fig. 3b. Hence, t_d can be determined from the $\sqrt{\gamma}$ curve; C_2 is found from the $\sqrt{\Gamma}$ curve by subtracting t_d from the apparent C_2 value; C_1 is obtained from ΔG_∞ and equation 5c after dividing ΔG_∞ by $2^{t_d/\lambda}$ (Fig. 3a).

RESULTS

The effect of 200 μg of chloramphenicol/ml on the increase in A_{460} and accumulation of DNA

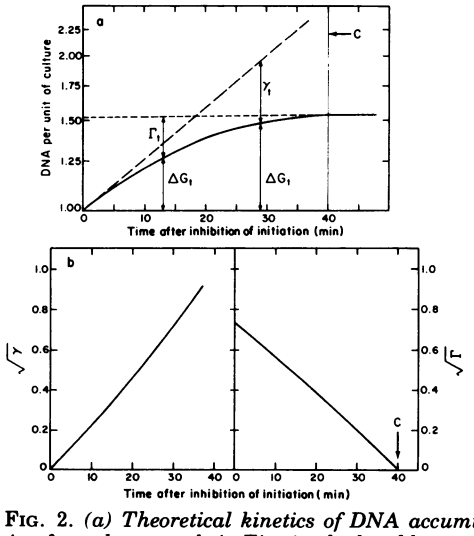

FIG. 2. *(a) Theoretical kinetics of DNA accumulation from the example in Fig. 1 calculated by use of equation 5b. The horizontal dashed line represents G_∞. The dashed exponential represents $2^{t/\tau}$. The functions ΔG_t, Γ_t, and γ_t are defined as indicated by the arrowheads (equations 6, 7, and 8). (b) The functions $\sqrt{\gamma_t}$ and $\sqrt{\Gamma_t}$ for the example shown in Fig. 1; $\sqrt{\gamma_t}$ extrapolates to 0 min and $\sqrt{\Gamma_t}$ extrapolates to C min on the abscissa.*

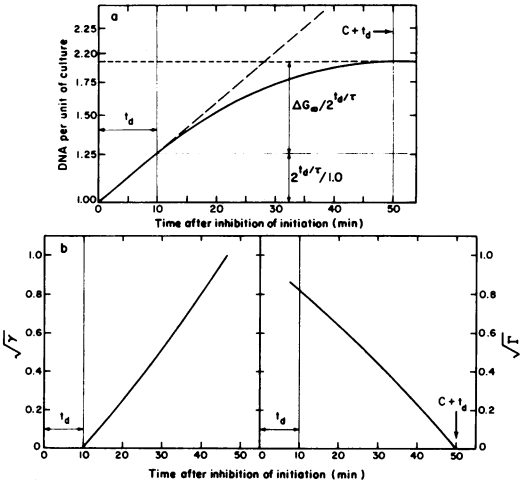

FIG. 3. *Same examples as in Fig. 2 except that a delay (t_d = 10 min) in the inhibition of initiation of round of replication is assumed. The function $\sqrt{\gamma_t}$ extrapolates to t_d on the abscissa.*

is shown in Fig. 4a. The data are not corrected for the dilution caused by the addition of chloramphenicol. The DNA accumulation data are averages of duplicate samples, which differed by less than 1.5%. It can be seen that for a short period DNA accumulates at the same rate as before chloramphenicol treatment; then the rate gradually slows until the final amount of DNA is reached between 45 and 60 min after addition of chloramphenicol. The analysis of these data is shown in Fig. 4b. The functions $\sqrt{\Gamma_t}$ and $\sqrt{\gamma_t}$ were plotted after normalization to $G_0 = 1.0$. The $\sqrt{\gamma_t}$ curve extrapolates to a value of 7 min on the abscissa, indicating that there is a period (t_{d1}) of 7 min after the addition of chloramphenicol before there is any effect on the rate of accumulation of DNA. Because of the nature of the plot, a small deviation (1%) in the observed value of G_t from the reference value is expanded (10%) by taking the square root (Fig. 4b and c, horizontal lines at ±0.1). Therefore, there is considerable scatter of the points when the values approach zero. However, as the difference (γ or Γ, respectively) increases, the scatter decreases.

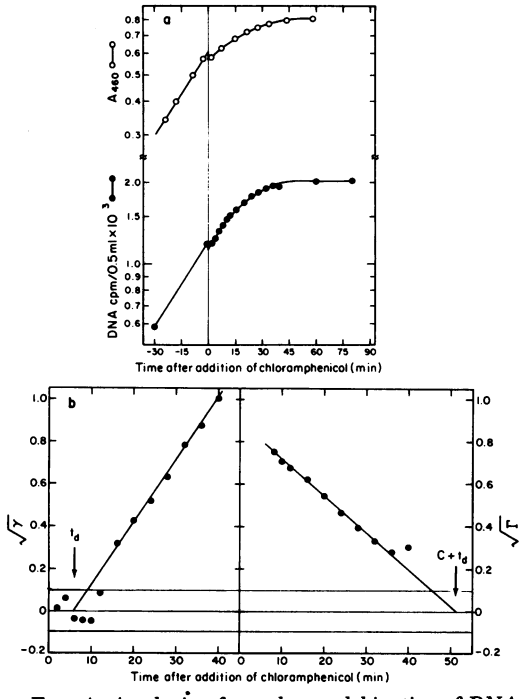

FIG. 4. *Analysis of an observed kinetics of DNA accumulation after the addition of chloramphenicol. (a) Accumulation of DNA and culture growth. At time zero, chloramphenicol was added, to a final concentration of 200 μg/ml, to a culture supplemented with 20 μg of [2-^{14}C]thymine/ml (specific activity, 0.01 μCi/μg). (b) Functions $\sqrt{\gamma_t}$ and $\sqrt{\Gamma_t}$ calculated from the data in Fig. 4a. $\sqrt{\gamma_t}$ extrapolates to a value of 6 min (= t_d) on the abscissa. The horizontal lines at ±0.1 represent 1% deviation of the observed value (G_t) from the reference value ($2^{t/\tau}$ for γ, G_∞ for Γ) in the differences γ or Γ, respectively. The negative values are taken from the modulus of γ. $\sqrt{\Gamma_t}$ extrapolates to a value of 51 min (= $C + t_{d1}$).*

The plot of $\sqrt{\Gamma_t}$ extrapolates to a value of 51 min, indicating a value of 44 min for the C_2 period ($C_2 = 51 - t_{d1}$) under these conditions. The C_1 period is calculated from the increment in DNA between t_{d1} and $t > C + t_{d1}$. The value thus obtained is 39 min. The 10% difference between the two values (39 min versus 44 min) is presumably due to an effect of the antibiotic on the DNA chain growth rate (see Discussion). This experiment has been repeated three times, and the results obtained for C_1 were 39, 41, and 38 min (Table 1).

To demonstrate the delay in the action of chloramphenicol more directly, the observed kinetics of DNA replication were compared with three possible theoretical kinetics (Fig. 5). As can be seen, initially an exponential increase provides the closest fit to the observed data points. This indicates that DNA continues to accumulate exponentially for several minutes after addition of chloramphenicol, which suggests that initiations, terminations, and chain elongations continue normally during this time.

Pato found that in certain *E. coli* strains (K and 15) chloramphenicol causes an immediate reduction in the rate of DNA accumulation; this effect was not observed with rifampin and was not observed with chloramphenicol in *E. coli* B/r (11). According to Fig. 4a and 5, this effect did not occur in the B/r strain used here (which was different from the one used by Pato). As in Pato's experiment with B/r, we found the kinetics of DNA accumulation after rifampin indistinguishable from the kinetics observed after chloramphenicol (Fig. 6). We

TABLE 1. *C period for bacteria growing in glucose plus amino acids medium supplemented with 20 μg of thymine/ml*[a]

Expt	τ (min)	t_d (min)	C_2 (min)	ΔG_∞	C_i (min, uncorrected)	$G_\infty/2^{t_d/\tau}$	C_1 (min)
1	29	6	44	1.79	55	1.54	39
2	29	7	50	1.80	56	1.57	41
3	33	11	44	1.80	63	1.44	38

[a] Results of three experiments. For definitions, see Theory. The values of C_1 were calculated from $\Delta G_\infty/2^{t_d/\tau}$ using equation 5c, i.e., after allowance was made for the delay time t_d, determined from $\sqrt{\gamma_t}$. Experiment 1 is the experiment in Fig. 4.

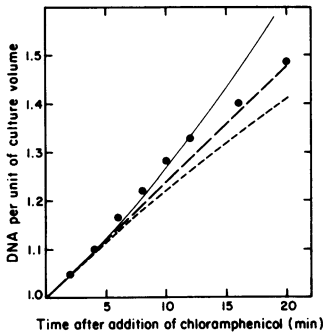

FIG. 5. *Delay in the action of chloramphenicol. Data from Fig. 4 plotted on a linear scale: ——, exponential increase; - - -, linear increase; ----, expected increase, assuming immediate cessation of replication initiations after chloramphenicol (equation 5b).*

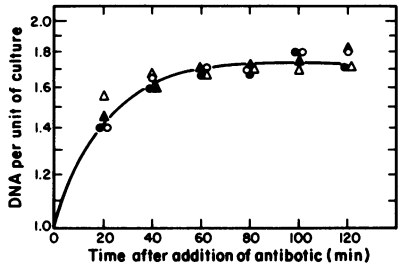

FIG. 6. *Comparison of the kinetics of DNA accumulation after addition of chloramphenicol or rifampin. At time zero chloramphenicol or rifampin was added, to a final concentration of 200 μg/ml, to portions of a culture supplemented with 20 μg of [2-^{14}C]thymine/ml (specific activity, 0.01 μCi/μg). The open and closed symbols indicate two separate experiments: ●, ○ = chloramphenicol; ▲, △ = rifampin.*

conclude, therefore, that rifampin produces the same delay in the inhibition of initiation of replication as chloramphenicol.

DISCUSSION

The analysis presented in this paper indicates that there is a delay in the effect of chloramphenicol, at a concentration of 200 μg/ml, on initiation of replication. At this concentration the drug causes a rapid reduction in the rate of protein synthesis in *E. coli* B/r to less than 0.25% of that immediately prior to addition of chloramphenicol (V. Shen, personal communication). However, the rate of initiation continues to increase exponentially for several minutes (Fig. 5). We interpret this to reflect the maturation of some protein or protein complex required for initiation. Whatever the basis for this effect, it leads to an overestimate of the *C* period if it is not taken into account (Table 1). In their experiments with *E. coli* 15T$^-$ and CR34, Pritchard and Zaritsky (12, 14) measured relative values of the *C* period by different methods and calibrated them with a value obtained by measurement of ΔG_∞ after amino acid starvation. The value thus obtained for both strains was 57 min at infinite thymine concentration, much longer than other reported values for strains of *E. coli* (3), but in close agreement to the values calculated from our uncorrected ΔG_∞ (Table 1) measurements. This could be due to a similar delay in the effect of amino acid starvation on the initiation of rounds of replication, although 15T$^-$ is unusual even among low thymine-requiring strains in its ability to grow on extremely low concentrations of thymine (14).

As stated above (Results), values of the *C* period calculated from ΔG_∞ after correction for the delay give values (of C_1) between 38 and 41 min under these conditions, i.e., close to the value reported previously for the wild-type parent of this strain (3), but about 15% longer than our own value (33 to 35 min) obtained from analysis of a "step up" (1).

The value of C_1 obtained from $\sqrt{\Gamma_t}$, 44 min (Fig. 4b), is 10 to 15% longer than C_1, and we interpret this as a late effect of the antibiotic on the speed of replication forks.

The question of whether all forks go to completion is more difficult to answer. The observed kinetics of accumulation of DNA are in close agreement with the theory apart from the time taken to reach the final value, which is 10% longer than predicted. If all forks traveled less than a half chromosome (assuming bidirectional replication) at undiminished speed, the time taken to reach the final value should be shorter and the final value (ΔG_∞) should be less, leading to a lower value for *C*. Since the value of *C* is greater than that observed by an independent method (1), we believe that all forks do approach closely to the chromosome terminus. An abrupt cessation of movement of forks at any time after addition of chloramphenicol would result in a sharp deviation from pseudo-linearity of the functions $\sqrt{\Gamma_t}$ and $\sqrt{\gamma_t}$, which is not observed.

As mentioned, our results with high concentrations of chloramphenicol or rifampin are essentially identical to those obtained by amino acid starvation. There have been reports of incomplete replication (i.e., premature termination) under such conditions (8). However, the unreplicated portion of the chromosome was estimated to be only 0.5%, which would not significantly affect our results.

There have been several reports of the effect

of chloramphenicol on the initiation of DNA replication which apparently demonstrate the occurrence of processes with different sensitivities to the antibiotic (5, 6, 9, 13). We were not concerned with such effects here but with the use of chloramphenicol to completely inhibit initiation of rounds of replication. Besides, as shown by Cooper's theoretical analysis (2), there is no firm evidence for such different sensitivities.

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