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Rate Stimulation of Deoxyribonucleic Acid Synthesis After Inhibition

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The degree to which the rate of deoxyribonucleic acid synthesis in *thy*$^-$ cultures of *Escherichia coli* is stimulated after a period of thymine starvation is shown to be a function of the concentration of thymine present as well as of the culture doubling time. Inhibition of deoxyribonucleic acid synthesis by nalidixic acid yields comparable results. Periods of thymine starvation exceeding one doubling time appear to cause an irreversible inactivation of a fraction of the replication forks in the culture.

Specific inhibition of deoxyribonucleic acid (DNA) synthesis by thymine starvation or by addition of nalidixic acid (NAL) in a bacterial culture results in an "unbalanced growth," during which the DNA/mass ratio decreases progressively (7, 11). When DNA synthesis is restored, its rate of synthesis is faster than it was before starvation (1, 4, 10, 18). The degree of this rate stimulation is proportional to the length of the inhibition period up to a maximum value, which is reached when the culture has doubled its mass (17). The two- to threefold increase in rate was originally interpreted by Pritchard and Lark (18) as a consequence of reinitiation at only one of the two available chromosome origins of each replicating chromosome. As previously discussed (3, 17), this interpretation is incompatible with current models describing the mechanisms governing initiation of chromosome replication (8, 13, 17; R. H. Pritchard, Heredity 23:472, 1968).

Most of the results mentioned above were obtained with glucose-grown cultures of thymine-requiring strains of *Escherichia coli*. It has recently been demonstrated (19, 21; A. Zaritsky, Ph.D. thesis, Univ. of Leicester, Leicester, England, 1971) that in several such strains studied the replication time (C) of the chromosome is longer than it is in their *thy*$^+$ counterparts irrespective of the concentration of thymine supplied. The average number of replication forks per chromosome in *thy*$^-$ cultures is thus greater than it is in wild-type cells under the same conditions. It has been shown that the rate stimulation of DNA synthesis is inversely proportional to the number of forks replicating in the culture before inhibition (3, 17; P. T. Barth, Ph.D. thesis, Univ. of Leicester, Leicester, England, 1968). Therefore, the observed lesser stimulation of DNA synthesis after its inhibition in *thy*$^-$ strains compared to that expected can be explained by the slower rate of chromosome replication in these strains compared to that in their *thy*$^+$ counterparts, as will be shown further on in this paper.

The results described herein will be divided into four sections, namely: (i) variation of rate stimulation factor with the thymine concentration, (ii) NAL treatment and thymine starvation, (iii) long-term rates of DNA synthesis, and (iv) rate stimulation after longer starvation periods.

For the sake of simplicity the results are described in terms of an unidirectional replication chromosome. The only modification necessary for transforming them into the bidirectional replication model (2, 14) is to consider twice as many forks replicating at half the rate described here.

MATERIALS AND METHODS

Bacterial strains. Two strains were employed during this work: *E. coli* 15T$^-$ strain (*thy*$^-$, *drm*$^-$, *arg*$^-$, *met*$^-$, *trp*$^-$) (18, 19) and *E. coli* K-12 strain CR34 (*thy*$^-$, *drm*$^-$, *dra*$^-$, *thi*$^-$, *leu*$^-$, *thr*$^-$) (21).

Growth conditions and measurements. M9 synthetic medium (18) was used in all experiments with either glucose (0.4%) or glycerol (0.4%) as the sole carbon source. Required amino acids (50 μg/ml) were added when necessary. Thymine was supplemented at the concentration indicated in each experiment.

Medium inoculated with a single colony of the appropriate strain was aerated vigorously at 37 C in a New Brunswick gyratory shaker, and growth was monitored by measuring absorbance at 450 nm, with a Gilford microsample spectrophotometer. An experiment was never started before at least three mass doublings with a constant growth rate were followed.

When the absorbancy at 450 nm reached a value between 0.2 and 0.4 the cells were pulled onto a

membrane filter (0.45 μm, 47 mm; Millipore Corp.), washed on the filter with approximately the same volume of prewarmed unsupplemented M9 medium, and suspended in the growth medium lacking only thymine. At zero time and after one or two doubling times a portion was diluted into the same concentration of [^{14}C]thymine (0.02 μCi/μg). Incorporation of the radioactivity into DNA was determined as described previously (19).

RESULTS

Variation of RSF with the thymine concentration. The ratio between (i) the rate of DNA synthesis in a bacterial culture after specific inhibition of DNA synthesis for one mass doubling time and (ii) the rate of DNA synthesis in the same culture before the start of the inhibition (rate stimulation factor [RSF]) has been shown to be equal to:

$$\mathrm{RSF} = (2^{C/\tau+1} - 1)/(2^{C/\tau} - 1) \qquad (1)$$

where τ is the mean doubling time of the culture and C is the replication time defined above (3; P. T. Barth, Ph.D. thesis; A. Zaritsky, Ph.D. thesis).

The following four assumptions underlie this relationship. (i) Initiation of a cycle of replication occurs when the cell mass per chromosome origin reaches a fixed value (8, 17; Pritchard, Heredity **23**:472), (ii) Replication forks traverse the chromosome with uniform velocity at steady states of growth (5, 6), (iii) Powell's age distribution function for cells, $f(x) = (\ln 2) \cdot 2^{1-x}$ (where $0 \leq x \leq 1$ is cell age in fractions of generations), is valid and also describes the age distribution of replication forks in steady-state exponentially growing cultures (16, 20). (iv) Inhibition of DNA synthesis in a steady-state culture for a period sufficient to give one mass doubling does not alter the replication velocity when the cause of inhibition is removed. A graphical description of the variation of RSF with C (and with τ) is shown in Fig. 1.

Figure 2 describes an experiment in which a glucose-grown culture of *E. coli* 15T$^-$ (555-7) was thymine starved for 40 min. During this period its absorbance doubled. The incorporation of [^{14}C]thymine into the trichloroacetic acid-insoluble fraction was monitored afterwards. The factor by which the rate of DNA synthesis was stimulated as result of the starvation was 2.59, giving a value of 57 min for C (equation 1).

Table 1 summarizes three similar experiments. As the thymine concentration in the medium was reduced, the RSF decreased; hence, the calculated replication time (C) is longer. This is consistent with previous data

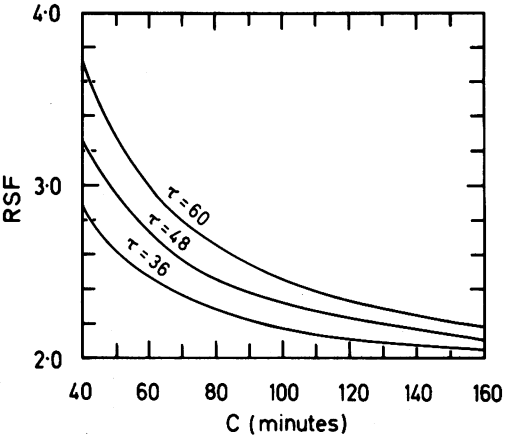

FIG. 1. *RSF as a function of C and the doubling time (τ), calculated from equation 1.*

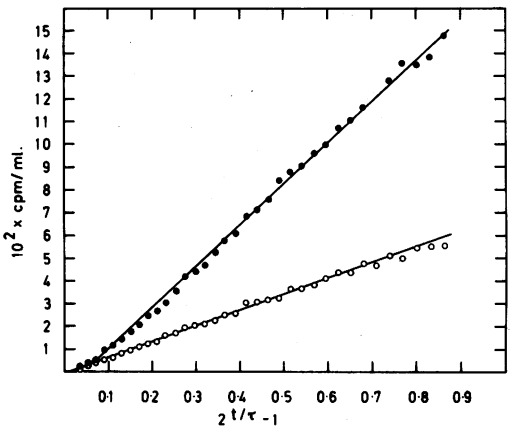

FIG. 2. *Rate of [^{14}C]thymine incorporation in a control glucose-grown subculture of E. coli 15T$^-$ (○) and in a similar subculture deprived of thymine for 40 min (●). The culture was pregrown and kept throughout the experiment in the presence of 2.0 μg of thymine per ml. The horizontal axis gives the theoretical increment in DNA in arbitrary units at the time of sampling (t) assuming DNA synthesis to be exponential (see references 3, 17).*

which derived the change of C with the concentration of thymine supplied to this strain through other methods (19).

Changes in RSF are more sensitive to variation in C at longer doubling times (slower-growing cultures). Therefore, glycerol was used as the carbon source when less than 1.0 μg of thymine per ml was supplied to the growth medium (Table 1b).

The addition of deoxyguanosine to the medium of *E. coli* 15T$^-$ (Table 1c) reduced the replication time to a value similar to that found

TABLE 1. *Variation of RSF with the thymine concentration in E. coli 15T⁻*

Carbon source	Thymine concn (μg/ml)	τ (min)	RSF	C (min)
(a)				
Glucose	10.0	40	2.82	46
Glucose	2.0	40	2.60	57
Glucose	1.0	40	2.47	66
(b)				
Glycerol	0.7	58	2.64	79
Glycerol	0.5	61	2.53	93
(c)				
Glucose	5.0 + dG[a]	64	3.90	40
Glucose	5.0 + dG[a]	65	3.40	50

[a] dG means that the cultures were supplemented with 200 μg of deoxyguanosine per ml.

with *E. coli* B/r (12). This had previously been determined by different methods (21). (The mechanism of growth inhibition due to this nucleoside is not known.)

Table 2 summarizes the data from a series of similar experiments with *E. coli* K-12 (CR34) resulting in estimates of *C* values comparable to those obtained previously for this strain (21).

Figures 3 and 4 show graphically the data of Tables 1 and 2, respectively, and a comparison with the existing results of different types of experiments (21).

NAL treatment and thymine starvation. Pritchard et al. (17) noted that the rate stimulation after NAL treatment was 1.4 times greater than that achieved after an equivalent period of thymine starvation. The fact that the maximum rate of DNA synthesis was reached very quickly after thymine was restored following 40 min of deprivation (Fig. 2) makes any explanation (17; P. T. Barth, Ph.D thesis) that is based on precursor limitation unlikely.

Since a period of inhibition of DNA synthesis might result in a changed resistance of the average cell to physical treatments (9), it is conceivable that the different recoveries of the cells in the two cultures are a consequence of the different experimental procedures. This is conceivable because a thymine-starved culture is washed free of thymine at time zero only, whereas a NAL-treated culture is washed free of the drug only before labeling. Table 3 describes the protocol of an experiment designed especially to test this possibility. The cells were washed twice, at time zero and at τ minutes, irrespective of the treatment to which they were subjected. Relative rates of DNA synthesis for the three portions are shown in Fig. 5. No significant difference in RSF between the thymine-starved and the NAL-treated fractions was observed. Hence, it may be concluded that a difference between thymine starvation and exposure to NAL, if any exists, cannot be demonstrated by experiments like those described here.

Long-term rates of DNA synthesis. The stimulated rate of [^{14}C]thymine incorporation (Fig. 2, 5) did not change for a 40-min period, at which time it started to decline (see also 17). It has been proven (P. T. Barth, Ph.D thesis) that this stimulated rate must drop to the level of the control culture when all newly initiated forks terminate a round of replication. This occurs *C* minutes after the addition of thymine (19). Since *C* has been shown to vary with the thymine concentration (19), it follows that the stimulated rate should be reduced to the level of

TABLE 2. *Variation of RSF with the thymine concentration in glucose-grown E. coli K-12*

Thymine concn (μg/ml)	τ (min)	RSF	C (min)
20.0	52	2.95	54
10.0	57	2.77	69
5.0	56	2.54	85
5.0	60	2.88	66
3.0 + dG[a]	56	3.26	47
4.0 + dG	57	3.27	48

[a] dG means that the cultures were supplemented with 200 μg of deoxyguanosine per ml.

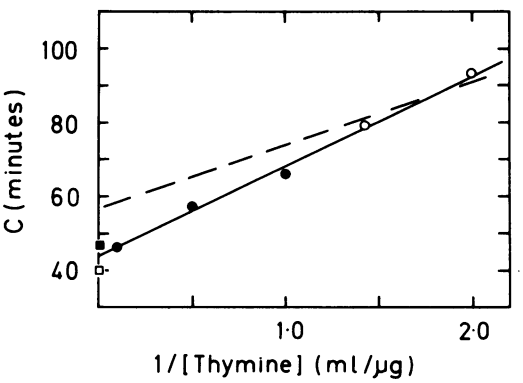

FIG. 3. *Values of C, calculated from RSF measurements (Table 1), plotted against the reciprocal of the thymine concentration that was supplied to E. coli 15T⁻ grown in glucose (●) or glycerol (○) minimal medium. (■) Glucose-grown cultures supplemented also with 200 μg of deoxyguanosine per ml. The dashed line is reproduced from Fig. 2 in reference 21.*

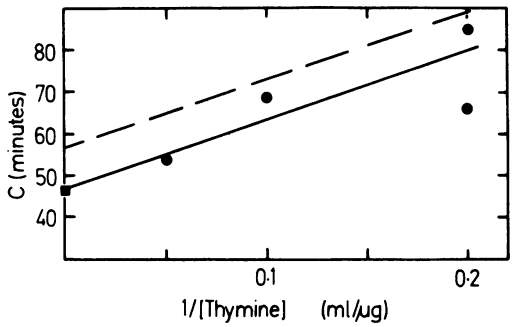

FIG. 4. *Values of C, calculated from RSF measurements (Table 2), against the reciprocal of the thymine concentration that was supplemented with glucose-grown E. coli K-12 (CR34) with (■) or without (●) 200 μg of deoxyguanosine per ml. The dashed line is reproduced from Fig. 2 in reference 21.*

TABLE 3. *Protocol of experiment*[a]

Culture	0 min	τ min
a	+[^{14}C]thymine	
b	−Thymine	+[^{14}C]thymine
c	+Nal	−Nal; +[^{14}C]thymine

[a] Results are described in Fig. 5.

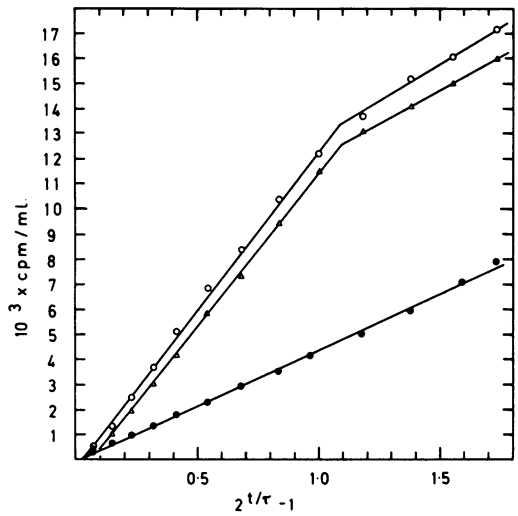

FIG. 5. *Rates of [^{14}C]thymine incorporation in a control culture of E. coli 15T^{-} (●), after 40 min of thymine starvation (Δ) and after 40 min of exposure to 50 μg of NAL per ml (○). The horizontal axis is as in Fig. 2.*

the control about 80 min after 0.7 μg of thymine per ml is restored to a starved culture of *E. coli* 15T^{-} (broken line in Fig. 6). This was not found to be the case (Fig. 6).

This discrepancy could be due to the lethal effects of thymine starvation (thymineless death; e.g., 1, 9, 10). The rate of increase in absorbance of a thymine-starved glucose culture remained constant during the entire starvation period but declined irrespective of whether thymine was restored at 40 min (unpublished data), thus indicating the inability of a fraction of the cells in the culture to recover from that treatment. The earlier-than-expected decrease in rate of DNA synthesis might be explained by an irreversible inactivation of part of part of the replicating forks in the culture (9; probably those existing in the older cells). More experimental work is needed to evaluate this interpretation.

Rate stimulation after longer starvation periods. It has already been noted (3, 17) that the capacity of a starved culture to initiate chromosome replication should be permanently increased during the inhibition period and, consequently, no limiting value for RSF is to be expected. The fact that RSF did reach a maximal value (17) was, therefore, puzzling.

In a preliminary attempt to resolve the above inconsistency without introducing more, and perhaps unnecessary, assumptions, the rate of DNA synthesis was followed after 80 min of thymine starvation (Fig. 7). The rate stimulation after that long treatment was found to be lower than RSF, although the absorbance reached a value threefold higher than it was at time zero, indicating again a possible inactivation of some forks in the culture. However, this lower rate started to accelerate about 12 min

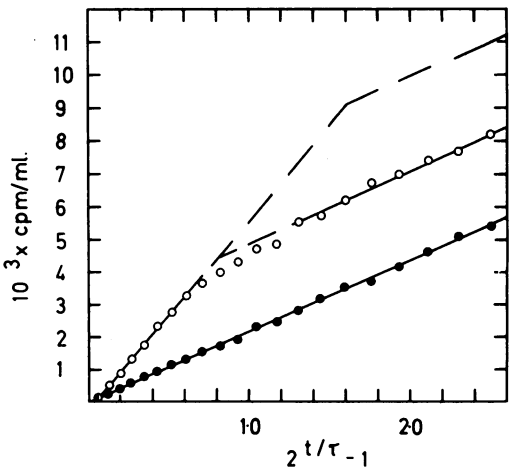

FIG. 6. *Rate of [^{14}C]thymine incorporation before (●) and after (○) 40 min of thymine starvation of E. coli 15T^{-}. The dashed line represents the rate predicted if C = 80 min (0.7 μg of thymine per ml) and if no fork is inactivated. The horizontal axis is as in Fig. 2.*

after thymine was added. The possible reasons for this acceleration will be discussed later.

DISCUSSION

The experiments described above were performed to analyze some observations that were not concordant with current concepts on the mechanism of initiation of chromosome replication. They took advantage of the quantitation of C in some *thy*$^-$ strains of *E. coli* (19, 21), and the results resolved some of these previously puzzling phenomena.

The factor by which the rate of DNA synthesis in a culture is stimulated as a consequence of a specific inhibition of DNA synthesis is known to be a function of the length of the inhibition period (17). It was shown here that this factor also depends on the doubling time of the culture and on the concentration of thymine supplied to the thymine-requiring cells. This can simply be explained by the effect of thymine concentration on the chromosome replication time (e.g., 19). The calculated changes of C in two strains (Fig. 3 and 4) were found to be similar to those estimated by other indirect methods (19, 21) that are independent of all or part of the assumptions made here. This not only confirms these observations but also explains why it was not possible to achieve a threefold stimulation by one doubling time period of thymine starvation of *thy*$^-$ strains of *E. coli* without assuming an abnormal act of initiation (19). The data also demonstrate (Table 1c and Table 2) that the value of 3 for RSF is not the upper limit, but that it depends on the ratio C/τ (see equation 1 and Fig. 1).

Evidence was presented that indicated no difference between the action of NAL and the effects of thymine starvation. Obviously, this observation is not sufficient to prove a similar mechanism of action for both treatments, but it is lack of evidence against a similar mechanism.

The constant rate of DNA synthesis after a relatively long starvation period (Fig. 2), which was achieved very soon after the restoration of thymine, may suggest that the balance of intracellular concentrations of the DNA precursors, which is disturbed during the starvation period (15), is restored fairly quickly. However, in both strains studied here, starvation for a period of two doubling times seemed to cause irreversible effects which were indicated either by the initial slower rate of DNA synthesis (Fig. 7) or by a smaller yield of mass during that period (280% in *E. coli* 15T$^-$ and 330% in *E. coli* K-12 in contrast to 400% in the respective unstarved controls) which never caught-up again with that of

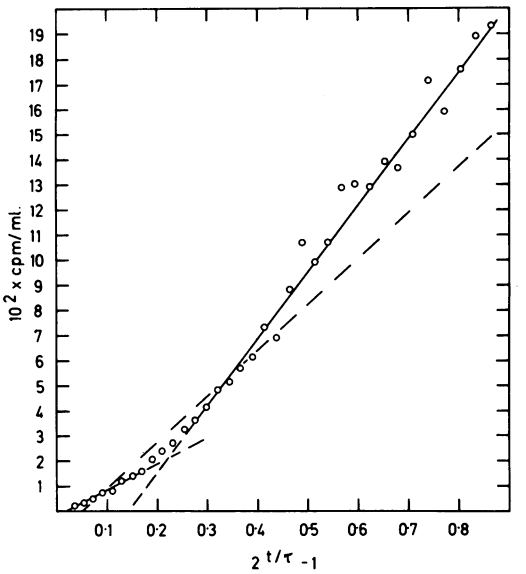

FIG. 7. *Rate of [^{14}C]thymine incorporation in a glucose-grown culture of E. coli 15T$^-$ after 80 min of thymine starvation (extension of the experiment described in Fig. 2). The dashed line is reproduced from Fig. 2 and describes the incorporation after 40 min of thymine starvation. The horizontal axis is as in Fig. 2.*

the unstarved cultures (unpublished data). Any argument based on the results of a long starvation experiment is, therefore, subjected to very strong and valid criticism. Nevertheless, an hypothesis is presented that suggests the existence of a minimal possible distance between two successive active forks along the chromosome. Thus, the inflexion in the rate of DNA synthesis found (Fig. 7) about 12 min after the readdition of thymine is interpreted as the reflection of a second synchronous act of initiation on all the arms of those chromosomes that were still able to recover. It is postulated that this second replication position (20), which was ready to initiate when thymine was restored, remained "stacked" until the previous one had traversed the presumed minimal distance away from the origin of replication. This hypothesis should not be elaborated further, but should serve as a working hypothesis to be tested by direct means.

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