*ON THE MECHANISM OF THYMINELESS DEATH IN BACILLUS SUBTILIS**

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Thymineless death (TD), the loss by a thymine-requiring auxotroph of the ability to multiply after a period of thymine starvation, was first described[1] in *Escherichia coli* 15T$^-$, and was attributed to "unbalanced growth," the synthesis of RNA and protein in the absence of DNA synthesis. This phenomenon has been investigated in thymineless auxotrophs of a number of bacterial species: *E. coli* 15T$^-$ and various derivatives;[2, 3] *E. coli* B3;[4] *E. coli* K12 thy$^-$;[5] *B. megaterium* thy$^-$;[6] and *B. subtilis* thy$^-$.[7, 8] A number of different hypotheses have been advanced to explain TD, including "unbalanced growth,"[1] "nuclear damage,"[4] single-stranded nucleolytic scissions in DNA,[9, 10] colicin induction,[5, 11, 12] and prophage induction.[5, 13] The involvement of macromolecular synthesis in TD has been examined in *E. coli* 15T$^-$ and other strains with conflicting results. Although several investigators have claimed an absolute requirement for protein synthesis,[14, 3, 5, 6] others have found that TD proceeds in the apparent absence of protein synthesis.[2, 15] It has been suggested that the synthesis of RNA rather than protein is essential for TD,[4] and there have been reports suggesting that messenger-RNA synthesis may be involved.[15, 16]

Perhaps complicating the interpretation of many of these studies is the fact that the course of TD may be influenced by conditions prevailing before or after the period of thymine starvation. Full immunity to TD in *E. coli* 15 TAU is attained by chromosome completion prior to thymine starvation.[2] Also, the level of survival of a thymine-starved culture of this strain depends upon the conditions in which thymine is restored to the cells: in cells starved for thymine, arginine, and uracil, there is a higher proportion of survivors if starvation for arginine and uracil persists for a time after thymine is restored.[17]

We have utilized exponentially growing cultures of thymineless strains of *Bacillus subtilis* to show that actinomycin blocks TD. This effect ensues at concentrations of actinomycin just sufficient to inhibit RNA synthesis, with little inhibition of DNA synthesis. It is known that actinomycin blocks protein synthesis by preventing the synthesis of messenger RNA,[18] and we have found that other treatments which inhibit protein synthesis also stop TD in *B. subtilis*. Studies in which actinomycin is administered at different times after removal of thymine suggest that TD involves two distinct steps, only the first of which is blocked by actinomycin.

Materials and Methods.—Strains: *B. subtilis* S (S22, thy$^-$1, arg$^-$) derived from ATCC 6633 was obtained from R. Romig.[7] *B. subtilis* strains R (R168, thy$^-$, tryp$^-$) and W (W23, thy$^-$, his$^-$) were obtained from F. Rothman.[19]

Media: Luria broth[20] (LB) or tris-salts, minimal medium[21] (TS), were used with the following supplements: amino acids, 20 μg/ml; thymine, 2 μg/ml (S), or 20 μg/ml (R, W); glucose, 5 mg/ml; P as phosphate, 10 μg/ml. Glutamate (10 mg/ml) reduces the lag before growth of strains R and W in TS, and was used in a number of experiments.

Conditions of growth: Overnight cultures were made in thymine-supplemented LB from thymine-supplemented LB agar slants. Dilutions from overnight cultures were 1:1000 into fully supplemented TS. These were stored at ambient temperature for one to two days until slightly turbid, and then were aerated at 37 or 30°C by shaking. Growth was monitored by measurement of turbidity at 450 mμ and by microscopic bacterial count. Before the start of each experiment, bacteria were maintained for several generations in exponential growth below $OD_{450} = 0.5$ by dilution into prewarmed media. Membrane or Millipore filters were used for rapid washing of cells and media shifts.[2] Viable count was determined by plating upon LB agar, dilutions for plating being carried out in LB.

Nucleic acid synthesis: Rates of RNA and DNA synthesis were measured by incorporation of radioactive uracil or thymine, respectively (New England Nuclear Corp.). For studies of uracil incorporation, bacteria were grown in fully supplemented TS medium with cold uracil (5 μg/ml) for several generations before radioactive uracil was added. Uracil incorporation into RNA was taken as total uracil incorporation minus uracil incorporation into DNA. Incorporation of uracil into DNA was estimated as alkali-resistant (*N* KOH, 60°C, 2 hr), acid-precipitable radioactivity. Culture aliquots were pipetted into ice-cold 5 per cent trichloroacetic acid, collected and washed on Millipores, and counted with a gas-flow or scintillation system.

Actinomycin: A gift of Merck, Sharpe and Dohme Laboratories, actinomycin D was used as a 0.1 per cent stock solution in 70 per cent ethanol, protected from light and refrigerated.

Results.—The course of thymineless death (TD) in *B. subtilis* S at 37°C and the effects of various levels of actinomycin are depicted in Figure 1. After about 30 min of thymine starvation (lag), cell death commences (shoulder), and shortly thereafter becomes exponential. A small fraction of the cell population, variable in different experiments between 0.1 and 10 per cent, displays an increased resistance to TD, as indicated by the break in the exponential killing curve. The shape of the curve in the experiment of Figure 1 suggests that for about 1 per cent of the cells there is a considerably longer lag period than for the bulk of the population. In considering the effects of actinomycin upon the course of TD, it is convenient to distinguish the behavior at low levels (0.01–0.1 μg/ml) from that at higher levels (0.2–10 μg/ml), as well as the differences between the behavior of the "resistant fraction" and that of the bulk of the population.

Below 0.1 μg/ml, the effect of actinomycin is primarily upon the "resistant fraction." Actinomycin concentrations as low as 0.001 μg/ml increase the lag period for the resistant fraction. For the bulk of the population, the lag is slightly decreased at 0.05 μg/ml and slightly increased at 0.01 μg/ml.

There is a striking inhibition of TD in the bulk of the population as the actinomycin concentration is increased from 0.1 to 0.2 μg/ml. This is expressed in Figure 1 as a large decrease in the initial killing rate. The inhibitory effect of 0.2 μg/ml actinomycin upon TD is nearly maximal, for the killing rate is not much more reduced by 1 μg/ml than by 0.2 μg/ml. Even at 10 μg/ml, there is a slow loss of viability at 37°C. Other experiments show that there is a slowly lethal effect of actinomycin at 37°C, occurring in the presence or absence of thymine. This effect is minimized in experiments at 30°C, and protection against TD is essentially complete at 1 μg/ml actinomycin.

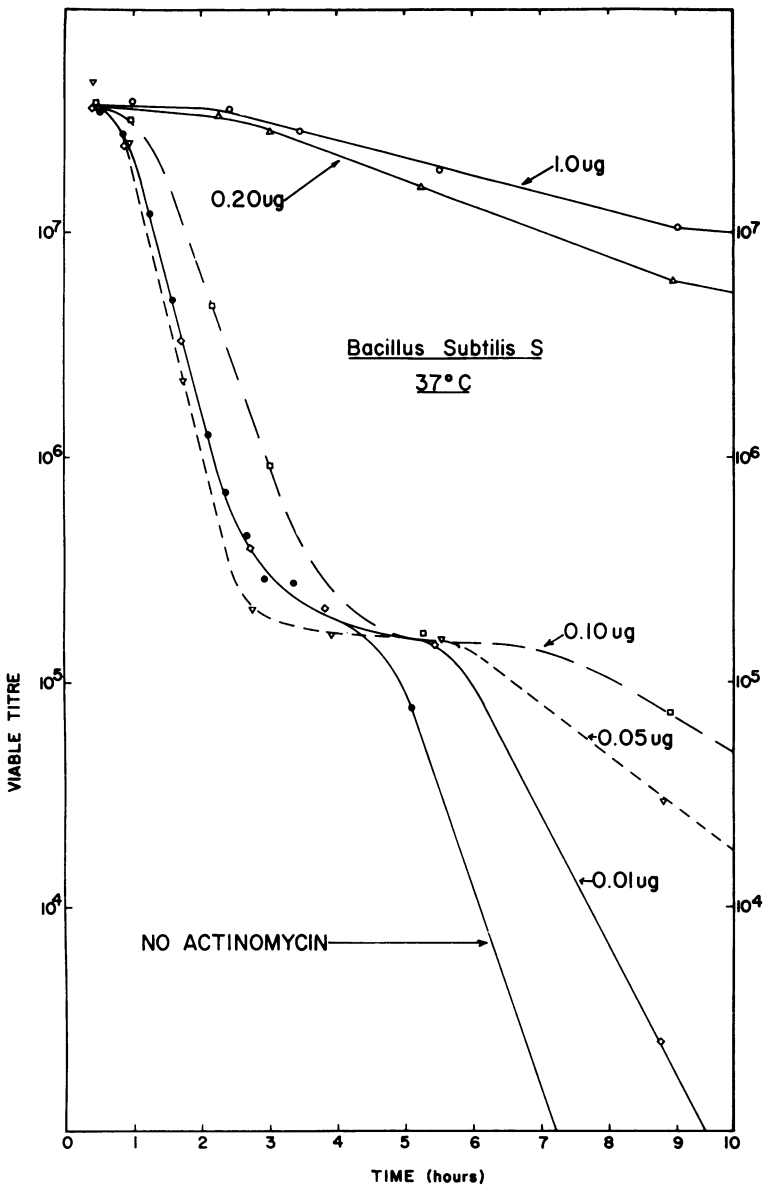

FIG. 1.—Thymineless death in *Bacillus subtilis* S and the effect of actinomycin D at different concentrations. Cells growing exponentially at 37°C were filtered, washed, and resuspended in thymineless media containing actinomycin at the following concentrations: 1.0 μg/ml (○), 0.2 μg/ml (△), 0.1 μg/ml (□), 0.05 μg/ml (▽), 0.01 μg/ml (◇), no actinomycin (●). Viable titers were measured at intervals as described. The following experimental points are not shown in the figure.

Time	Actinomycin (μg/ml)	Viable titer
8 hr, 20 min	None	10^2
16 hr	0.05	10^3
	0.10	6×10^3
	0.20	3×10^6
	1.00	8×10^6

If we compare the effect of actinomycin upon TD with its effect upon RNA synthesis in exponentially growing cultures of the same organism, it is clear that the inhibition of TD coincides with the inhibition of RNA synthesis. Figure 2 shows a

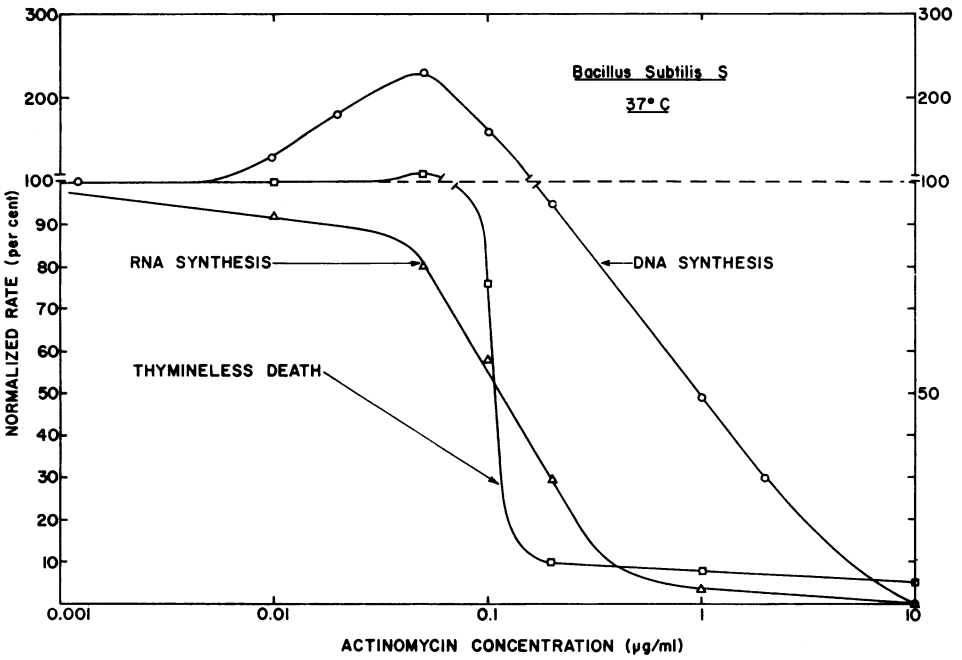

FIG. 2.—Effect of actinomycin D upon the rate of synthesis of RNA (△) and DNA (○), and upon the rate of thymineless death (□) in *Bacillus subtilis* S. The rate of thymineless death, taken as $-(d \ln S)/dt$, where S is the viable titer at time t in the exponential phase of killing, was obtained from the data of Fig. 1. The rates of RNA and DNA synthesis were measured as described in *Methods*, after adding different concentrations of actinomycin to cells growing exponentially at 37°C. For DNA synthesis the rate is a function of the time after addition of actinomycin, and this figure shows the rate at 35 min. All rates are normalized with respect to control cultures without actinomycin.

plot of the rates of thymineless death, RNA synthesis, and DNA synthesis as a function of actinomycin concentration over a range of 0.01–10 μg/ml. The rates are expressed relative to those in a culture without the drug taken as 100 per cent. It may be seen that the sharp reduction in the rate of TD occurs at levels of actinomycin just sufficient to inhibit RNA synthesis. The rate of DNA synthesis is only slightly reduced below control values at these levels, but it is of interest that lower levels markedly stimulate the synthesis of DNA.

These results have been extended to *B. subtilis* strains R and W (unpublished experiments). For these strains the inhibition of TD is obscured by a strongly lethal effect of actinomycin at higher concentrations. Killing of R and W by high levels of actinomycin occurs at essentially the same rate in the presence or absence of thymine.

Inhibition of protein synthesis in strain S prevents TD. Figure 3 shows the effect of varying levels of chloramphenicol upon the rate of thymineless killing of strain S. The protection by chloramphenicol is nearly maximal at 50 μg/ml, as levels above 100 μg/ml often result in a very rapid loss of viability accompanied by cell lysis, even

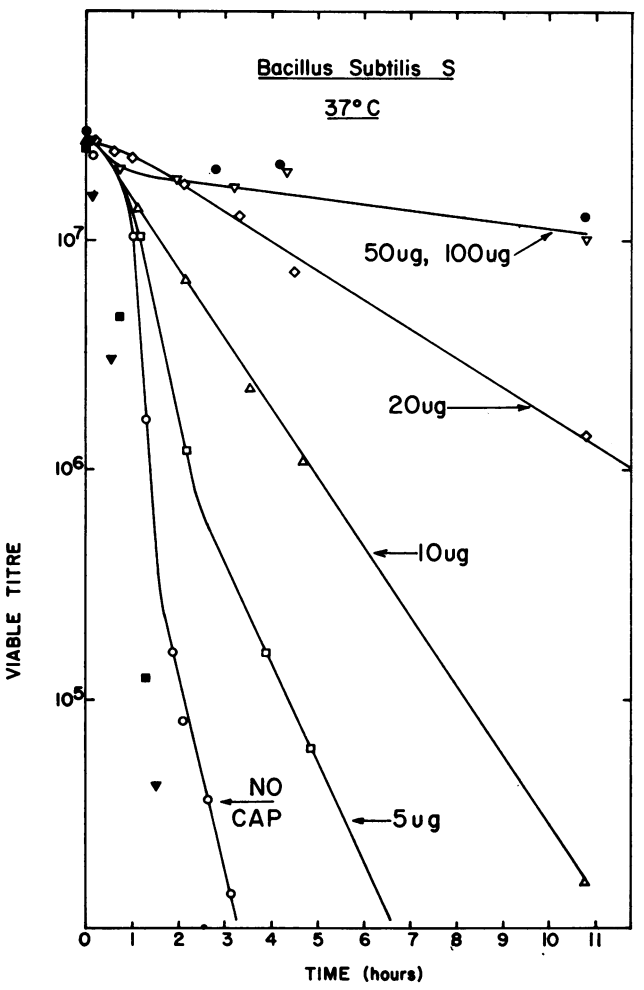

FIG. 3.—Effect of chloramphenicol upon thymineless death in *Bacillus subtilis* S. Cells growing exponentially at 37°C were filtered, washed, and resuspended in thymineless media containing chloramphenicol at the following concentrations: 100 μg/ml (●), 50 μg/ml (▽), 20 μg/ml (◇), 10 μg/ml (△), 5 μg/ml (□), 400 μg/ml (■), 400 μg/ml + thymine (▼), no chloramphenicol (○). Viable titers were measured at intervals as described.

in the presence of thymine. Other treatments which prevent protein synthesis also inhibit TD in strain S; these include puromycin administration (200 μg/ml) and arginine starvation.

We have examined the ability of actinomycin to stop TD after various times of thymine starvation. Figure 4 shows such an experiment carried out at 30°C. When actinomycin was added at any time before there was any loss of cell viability, essentially the entire population was protected from TD. At 140 minutes of thymine starvation, the cells were in the exponential phase of death and 58 per cent were still viable. Addition of actinomycin at this time resulted in a cessation of killing, but this was not immediate. The final level of survival after actinomycin indicates that TD continued for a short period after inhibition of RNA synthesis. This period was less than that necessary for the decay of any messenger RNA present at the time of addition of actinomycin.[18] Hence the result is consistent with the notion that inhibition of mRNA synthesis alone is not sufficient to stop TD, but that TD proceeds as long as existing mRNA may be utilized in protein synthesis.

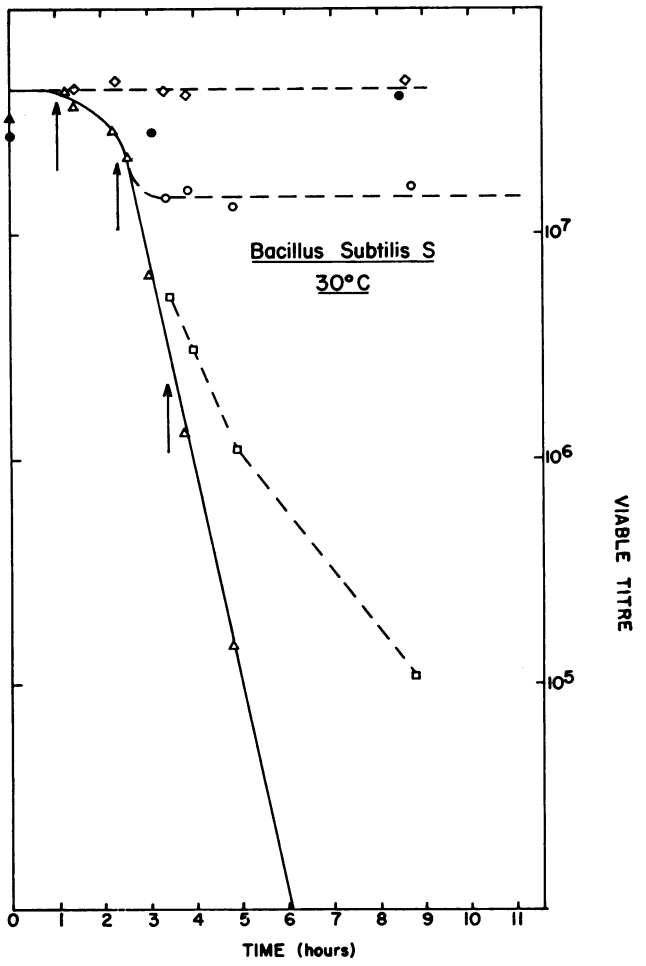

FIG. 4.—Effect of actinomycin D added at different times during the course of thymineless death in *Bacillus subtilis* S. Cells growing exponentially at 30°C were filtered, washed, and resuspended in thymineless medium. Actinomycin D (10 μg/ml) was added to different aliquots at the following times: 0 min (●), 65 min (◇), 140 min (○), 205 min (□), no actinomycin (△). Viable titers were measured at intervals as described.

When actinomycin is added at later times of thymine starvation, the result is strikingly different. Actinomycin was not able to protect the 7 per cent of cells surviving at 205 minutes of thymine starvation from continuing TD. Other experiments confirm the notion that there is a critical time of addition of actinomycin, corresponding to 60–70 per cent survival, after which the surviving fraction continues to lose viability in the presence of actinomycin. Evidently, the fraction of cells surviving at these late times has carried out an essential step in thymineless death, which would have been prevented by prior actinomycin administration. We suggest that this step may be the synthesis of messenger RNA coding for a specific lethal protein. The loss of viability of the surviving fraction when actinomycin is administered at late times also demonstrates that there is a second step in TD, requiring continued starvation for thymine, but independent of continuing RNA and protein synthesis.

Discussion.—We have shown that TD in *Bacillus subtilis* involves at least two distinct steps: (1) a step involving RNA and protein synthesis during thymine starvation, and (2) a subsequent step requiring continued thymine starvation but

independent of RNA and protein synthesis. Similar observations, implicating protein synthesis in an early step in TD, have been reported for *E. coli* 15T$^-$ [14, 22] and for TD in *E. coli* B3 lysogenic for P1 prophage.[23] In these experiments, chloramphenicol was found to be effective in stopping TD only if added at the beginning of the starvation period. Addition of chloramphenicol at later times, even before any cell death has taken place, allows TD to proceed, although at a somewhat reduced rate.

The notion that TD is caused by the induction of defective prophage has received considerable support in recent investigations. Sicard and Devoret[5] showed that thymine starvation in *E. coli* 15T$^-$ induces the formation of colicin 15, and Endo *et al.*[13] showed that colicin 15 activity is associated with defective phage particles. Sicard and Devoret found that the rapid rate of killing characteristic of TD was dependent upon the presence of inducible lambda prophage in thymineless *E. coli* K12, and upon the colicinogenic factor in *E. coli* 15T$^-$. The *Bacillus subtilis* strains which we have studied have been shown to contain the defective prophage SP alpha (PBS X), inducible by thymine starvation.[24, 25, 11]

Melechen and Skaar[23] compared the effects of chloramphenicol upon TD and upon P1 prophage induction in lysogenic *E. coli* B3. They found that the formation of "blocked induced complexes" was halted abruptly by chloramphenicol, even when chloramphenicol was administered at a time when it was no longer effective in halting TD. From this observation they concluded that TD could *not* result from a cryptic form of prophage induction. However, these results are also consistent with the possibility that the formation of a blocked induced complex involves a series of steps *beyond* those leading to cell death, at least one of which requires the synthesis of a protein.

The finding that TD requires RNA and protein synthesis during thymine starvation is consistent with the hypothesis that TD is a consequence of the induction of defective prophage. Recent studies of the heat induction of lambda prophage show that both lambda mRNA and concomitant protein synthesis are necessary for host cell killing. For one class of temperature-sensitive lambda C_I mutants, the presence of chloramphenicol prevents the loss of host cell viability during heat inactivation of the lambda repressor, although the lambda prophage is fully derepressed under these conditions, as indicated by the formation of lambda mRNA.[26] For a second class of C_I temperature-sensitive mutants the presence of chloramphenicol does not prevent loss of host cell viability during heating.[27] Naono and Gros[28] have shown that these two classes of lambda C_I mutants are distinguished by the behavior of the repressor during heating and subsequent cooling of the cells. For class 1 mutants the repressor is reversibly inactivated by heating, and upon cooling, repression is restored, while for class 2 mutants the repressor is irreversibly denatured by heating.

From these results it would appear that (1) prophage induction involves a prophage-directed protein which must be synthesized during prophage derepression; (2) reversal of derepression of the prophage may prevent induction, even after considerable synthesis of prophage mRNA has taken place. It has been postulated that thymine starvation causes the accumulation of an internal "inducer" substance.[5, 29] If the derepression produced by increased levels of "inducer" is reversible, thymineless induction would require (1) RNA and protein synthesis during thymine starvation, and (2) accumulation of inducer sufficient to prevent rapid reversal

of derepression upon readdition of thymine. We suggest that these two postulated requirements may correspond to the two steps in TD which have been described.

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