JOURNAL OF BACTERIOLOGY, Feb. 1970, p. 350-354
Vol. 101, No. 2

Tryptophanless Death in *Bacillus subtilis*

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Received for publication 10 November 1969

A decline in colony-forming ability is observed in actively growing cultures of a tryptophan arginine auxotroph of *Bacillus subtilis* after removal of tryptophan (tryptophanless death). This phenomenon can be prevented by simultaneous starvation of the other required amino acid or by chloramphenicol administered in bacteriostatic concentration but not by actinomycin. Addition of tryptophan analogues not only prevents the death but also allows recovery of the cells that have lost the ability to form colonies on solid media. The term tryptophanless death is therefore inappropriate. Chloramphenicol but not actinomycin inhibits the recovery brought about by tryptophan analogues.

A decline in colony-forming ability in a tryptophan auxotroph of *Bacillus subtilis* as a function of the time of incubation in a liquid medium deprived of tryptophan has been reported (1, 4).

We observed the same phenomenon in a double auxotroph of *B. subtilis* when starvation of tryptophan alone was performed.

The experiments reported in this paper were designed to determine whether tryptophanless death (Trp-LD) is a permanent or a transient state of the cells. Furthermore, we studied the conditions that can prevent it. It is concluded that Trp-LD is a transient state from which the cells, appropriately treated, can fully recover.

MATERIALS AND METHODS

Strains. Indole-requiring *B. subtilis* strain 168M and a tryptophan-arginine derivative of it, *B. subtilis* SB57, were used.

Media. Spizizen minimal medium (7) was used as the basal medium in all the experiments, and it was supplemented with 0.5% glucose, 10^{-2} M glutamic acid (S medium), and 20 μg of the required amino acid per ml. Viable counts were determined on plates of Nutrient Agar (Difco).

Growth of cultures and amino acid starvation. An overnight culture of SB57 grown in supplemented S medium was diluted 1:10 in the same medium and incubated with shaking for 4 hr at 37 C (about 10^8 cells/ml). Glycerol was added to a final concentration of 5%, and 1-ml portions were frozen and stored in liquid nitrogen. These cells were used as inocula for growth of the cultures and gave very reproducible results.

For starvation experiments, the frozen cells were thawed and diluted 10 times in the usual supplemented S medium. After 3 hr of incubation at 37 C, the culture, in the logarithmic phase, was filtered through membrane filters (Millipore Corp., Bedford, Mass.) or centrifuged. The cells were washed twice with the medium to be used for starvation and resuspended in twice the volume of the filtered culture. Viable counts were determined as a function of the time of starvation at 37 C.

Isotope incorporation. To determine the incorporation of radioactive precursors into macromolecular cell components, isotopic compounds were added to the culture, and 0.2-ml samples were taken at different time intervals and diluted into 0.5 ml of 10% ice-cold trichloroacetic acid. After standing for at least 30 min at 0 C, the samples were filtered through Millipore filters and washed with 10 ml 5% trichloroacetic acid. The filters were dried, and the radioactivity was determined on a Tri-Carb liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.).

Chemicals. DL-7-Azatryptophan (7-Atrp) was from Cyclo Chemical Corp.; 6-methyl-DL-tryptophan (6-Mtrp) and chloramphenicol were purchased from Sigma Chemical Co., St. Louis, Mo. 5-Hydroxy-DL-tryptophan (5-Htrp), ^{3}H-leucine (5 c/mmole), and ^{14}C-uracil (31 mc/mmole) were from Calbiochem, Los Angeles, Calif. 5-Methyl-DL-tryptophan (5-Mtrp) and actinomycin C were from Mann Research Laboratories. ^{3}H-thymidine (0.24 μc/μmole) was from Schwarz BioResearch, Inc., Orangeburg, New York.

RESULTS

Effect on cell viability of starvation for different amino acids. Tryptophanless death takes place when certain strains of *B. subtilis* are deprived of tryptophan. However, in double auxotrophs, simultaneous deprivation of the other required amino acid (arginine) prevents the decrease in viable counts (Fig. 1).

The decrease in viable counts was accompanied

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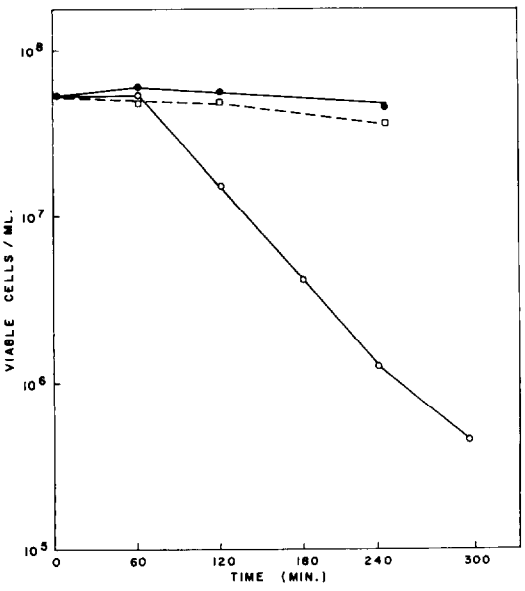

FIG. 1. *Effect of amino acid starvation on viability of B. subtilis SB57 cells. Cells were starved for arginine* (□), *arginine and tryptophan* (●), *and tryptophan* (○). *Viable counts were taken as a function of the time of incubation at 37 C.*

by an increase in turbidity. The number of cells seen under the microscope doubled in the first 60 min of starvation and then remained constant. No lysis of cells, filament formation, or other anomalous morphology was observed. After 6 hr of starvation, the viable count leveled off at a value that is 0.5 to 5% the original number of cells.

Trp-LD is sensitive to temperature, the death rate being higher at 42 than at 37 C, and no Trp-LD was observed when tryptophan starvation was carried out at 30 C.

Trp-LD is not related to the particular mutational site of SB57, since other tryptophan auxotrophs, blocked at different steps of the tryptophan pathway, are also susceptible to Trp-LD.

In transformation experiments, the deoxyribonucleic acid (DNA) extracted from cells after tryptophan starvation had the same biological activity as the DNA from arginine-starved cells or from growing cells (Table 1).

Although the decrease in viability was somewhat reminiscent of that obtained in the case of thymineless death (3, 5), morphological evidence indicates that the two phenomena are not related. No cell filaments are formed during tryptophan starvation, but they have been observed during thymineless death. Actinomycin prevents thymineless death when added early during starvation (5). As will be shown, Trp-LD is insensitive to this antibiotic.

It has been hypothesized that thymineless death is a consequence of defective phage induction (5). This is consistent with a requirement for ribonucleic acid (RNA) and protein synthesis for thymineless death to occur. Phage induction does not seem to be the cause of Trp-LD. We did not detect phage particles by analyzing, by electron microscopy, the supernatant fluid of cells undergoing Trp-LD, or on extract of a tryptophan-starved culture. Furthermore, after removal of tryptophan, polysomes were degraded at the same rate as during arginine starvation, and no polysome reassociation was observed up to 3 hr after starvation (S. Barlati, *in preparation*). Reaggregation would be expected during phage induction.

No decrease in viable count occurred when the indole-requiring strain 168M was starved of tryptophan. Since SB57 is an arginine derivative of 168M, and since we determined that the Trp-LD phenotype is independent of the arginine markers (arg^+ transformants or revertants of SB57 undergo Trp-LD), we interpret that this strain has lost, in our laboratory, the character in study. In fact, Trp-LD was originally demonstrated in strain 168M by Pritikin and Romig (4).

Effect of chloramphenicol and actinomycin on Trp-LD. During tryptophan starvation, the inhibition of DNA synthesis, as measured by the decrease in the incorporation of radioactive thymidine, was not different from that observed during depletion of arginine only, a condition that does not lead to a decrease in viable counts. RNA and protein synthesis appear to be inhibited more during arginine than during tryptophan starvation. These results suggest that Trp-LD could be related to an alteration of RNA or protein metabolism (or both), rather than to

TABLE 1. *Transforming activity of SB57 DNA after 6 hr of amino acid starvation*[a]

DNA from SB57 cells incubated in	$Shik^+$ transformants/ arg^+ transformants
+arg +trp	1.20
+arg −trp	1.05
−arg +trp	1.12
−arg −trp	1.15

[a] After incubation of SB57 (arg^- trp^-) in the media shown, approximately the same number of cells of strain SB532 (arg^+, $shik^-$, tyr^-) was added, and the DNA was extracted from the mixture of the two cultures. Transformation was performed with limiting amounts of DNA (approximately 0.1 μg/ml) on competent cells of strain SB883 (arg^-, $shik^-$, tyr^-). The result is substantially the same when 10 times higher or lower concentrations of DNA are used.

that of DNA. Addition of chloramphenicol at a concentration of 20 μg/ml strongly inhibits Trp-LD when the antibiotic is added at any time during tryptophan starvation (Fig. 2a). The inhibition of protein synthesis and the stimulation of RNA synthesis were also observed (Fig. 2b, 2c).

These results indicate that repression of Trp-LD may be correlated with inhibition of protein synthesis or, less likely, with the stimulation of RNA synthesis by chloramphenicol. RNA production during amino acid starvation is not inhibited by actinomycin C, but the RNA made has a much lower molecular weight and is not utilized as messenger (S. Barlati, *in preparation*).

Actinomycin C, present in the starvation medium in bacteriostatic concentration, did not prevent Trp-LD (Fig. 3). The effect of the simultaneous administration of chloramphenicol and actinomycin C during amino acid starvation on viable counts, RNA, and protein synthesis is also shown in Fig. 3. Even when the synthesis of RNA, stimulated by chloramphenicol, was prevented, Trp-LD did not take place. Therefore, we conclude that protection from Trp-LD is correlated with inhibition of protein synthesis and that the decrease of viability is not related to synthesis of new species of RNA induced by tryptophan starvation.

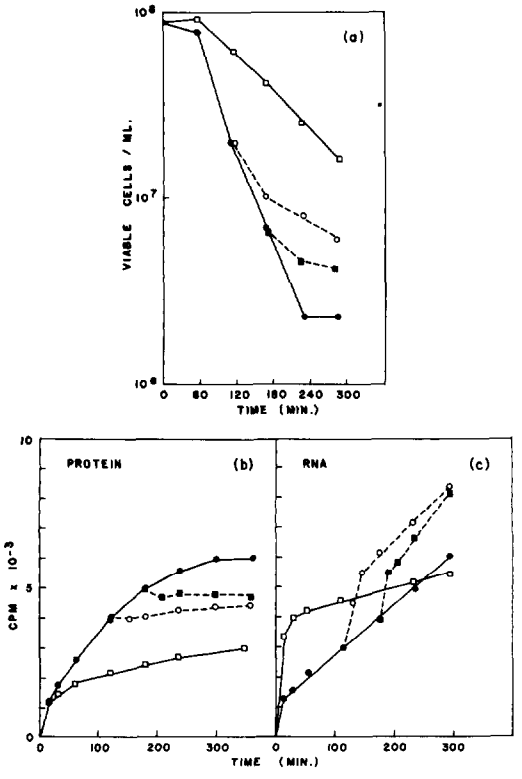

FIG. 2. *Effect of chloramphenicol on Trp-LD. Chloramphenicol (20 μg/ml) was added at 0 min (□), 120 min (○), and 180 min (■) after tryptophan starvation. Viable count (a); ^{3}H-leucine, 15 μg (0.5 μc)/ml incorporation (b); and ^{14}C-uracil, 15 μg (0.5 μc)/ml incorporation (c). Incorporation into trichloroacetic acid-insoluble material was measured as described in the methods section. Trp-LD (●).*

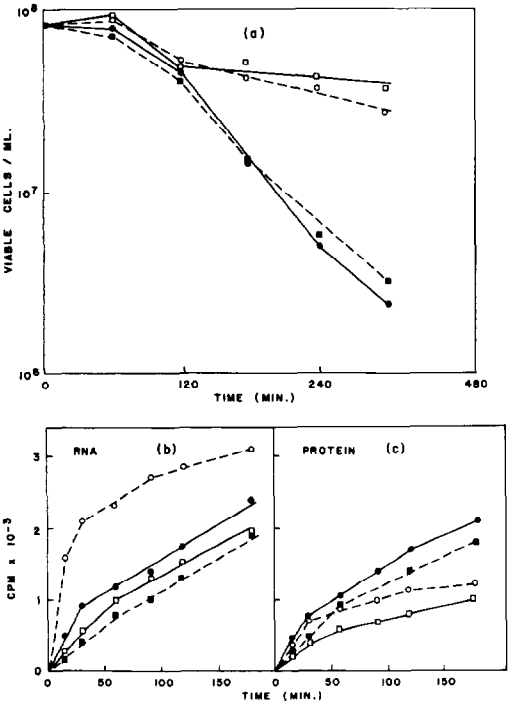

FIG. 3. *Effect of actinomycin on Trp-LD. Actinomycin C (2 μg/ml) was added in the absence (■) or in the presence (□) of 20 μg of chloramphenicol/ml at the time of tryptophan starvation. As controls, Trp-LD (●) and treatment with 20 μg of chloramphenicol/ml (○) are also shown. Viable count (a), incorporation into trichloroacetic acid-insoluble material of ^{14}C-uracil, 7 μg (0.2 μc)/ml (b), and ^{3}H-leucine, 7 μg (0.2 μc)/ml (c) are reported.*

Effect of tryptophan analogues on Trp-LD. Several structural analogues of tryptophan have been tested for their effect on Trp-LD. All of them prevent Trp-LD, though to different extents (Fig. 4). The effect is displayed regardless of the time of addition of the analogue. All the tested compounds appear not only to inhibit but also to stimulate recovery or growth (or both) of cells that underwent Trp-LD. These effects were also evident by adding indole, as would be expected from the fact that SB57 can utilize indole for growth. On the other hand, tryptamine, a compound that cannot be transformed into tryptophan nor be incorporated into protein, does not prevent Trp-LD or support recovery.

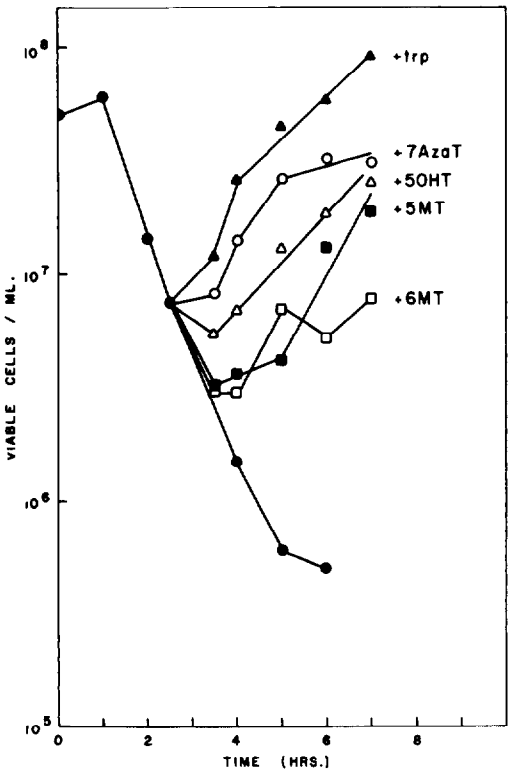

FIG. 4. *Effect of tryptophan analogues on Trp-LD. After 2.5 hr of tryptophan starvation, to portions of the culture 5 μg of various tryptophan analogues per ml was added. Trp-LD (●); tryptophan (▲); 7-Atrp (○); 5-Htrp (△); 5-Mtrp (■); 6-Mtrp (□). Viable counts are shown.*

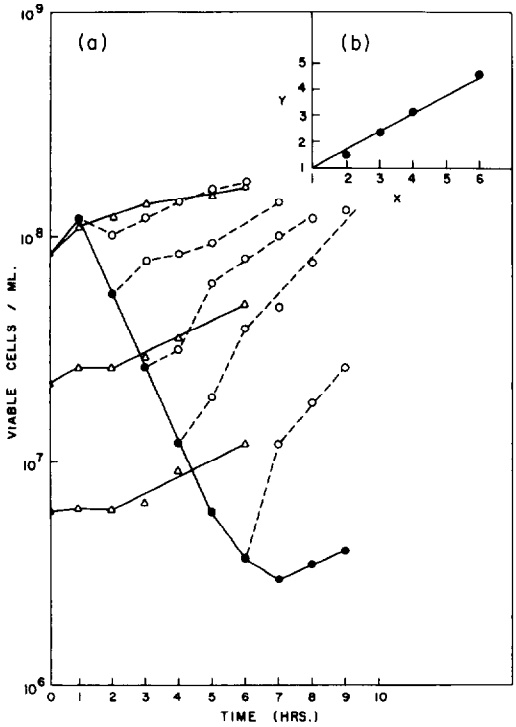

FIG. 5. *Recovery from Trp-LD in the presence of 5-Mtrp. Viable counts are shown after addition of 5-Mtrp at different times during tryptophan starvation. (a) Trp-LD (●); 20 μg of 5-Mtrp per ml during Trp-LD (○); + 20 μg of 5-Mtrp per ml to different cell dilutions (△). (b) Y = (viable count after 2 hr with 5-Mtrp)/(viable count at the time of addition of 5-Mtrp during Trp-LD); X = hours of tryptophan starvation at the time of addition of 5-Mtrp. When 5-Mtrp is added at the time of tryptophan starvation to different cell dilutions, the value of Y is 1.4 and is independent from the initial viable count.*

In a previous report (2), we showed that the analogues 5-Mtrp and 5-Htrp are incorporated into protein of *B. subtilis* during tryptophan starvation and that they stimulate amino acid incorporation but do not allow growth. So it would seem that the analogues are not preventing Trp-LD by inhibiting protein synthesis in the same way in which chloramphenicol does.

Figure 5 shows the effect of the addition of 5-Mtrp at different time intervals after tryptophan starvation. The number of viable cells after addition of the analogue increases at a rate that is higher when the addition of the analogue is made at a later time. The addition of 5-Mtrp at different cell dilutions, on the other hand, results in a constant and very low increase in viable cells, indicating that the rise in viable counts upon addition of 5-Mtrp during Trp-LD is not due to growth of the surviving cells.

Such results suggest that the cells that underwent Trp-LD have lost the capability of forming colonies on solid media after tryptophan starvation, and that this is not a permanent state but a transient one from which cells can fully recover.

Effect of chloramphenicol and actinomycin C on recovery. Since chloramphenicol stops Trp-LD but does not allow for recovery, it would seem that recovery requires protein synthesis. In fact, no recovery of cell viability takes place during incubation in the presence of both 5-Htrp and chloramphenicol (Fig. 6). This observation confirms that the recovery brought about by tryptophan analogues requires protein synthesis.

The presence of actinomycin C at concentrations that completely inhibit RNA synthesis does not suppress recovery. As can be seen from Fig. 6b, this is true whether actinomycin C is added at the beginning of starvation or together with the analogue at a later time during the logarithmic phase of killing.

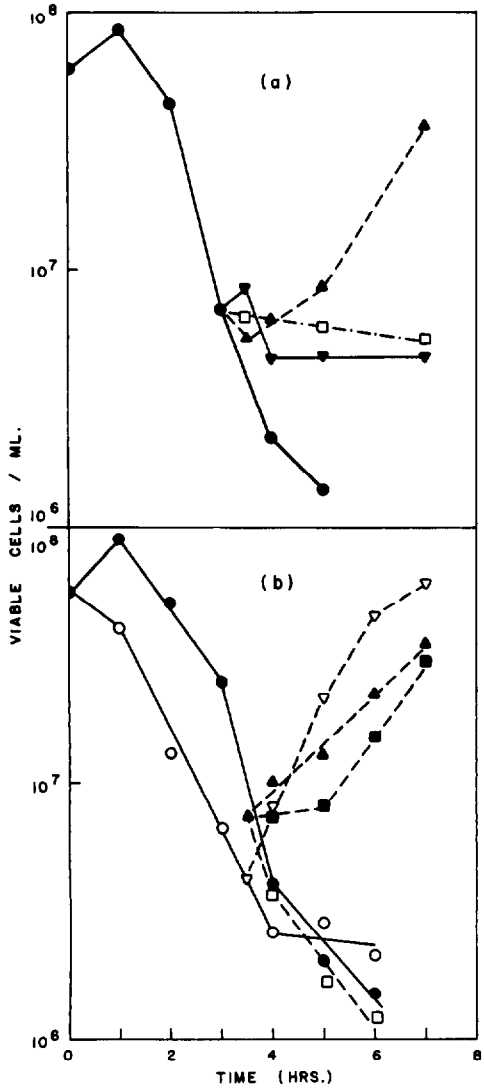

FIG. 6. *Effect of chloramphenicol and actinomycin C on recovery from Trp-LD. (a) After 180 min of Trp-LD (●), the culture was divided into portions containing: 20 μg of chloramphenicol/ml (□); 20 μg of 5-Htrp/ml (▲); and 20 μg of chloramphenicol plus 20 μg of 5-Htrp/ml (▼). Viable counts are shown. (b) As in a, but portions of culture contained: 2.5 μg of actinomycin C/ml (□); 20 μg of 5-Htrp/ml (▲); and 2.5 μg of actinomycin C plus 20 μg of 5-Htrp/ml (■). After 180 min of Trp-LD in the presence of 2.5 μg of actinomycin C/ml (○), 20 μg of 5-Htrp per ml was added to a portion of this culture (▽). Viable counts are shown.*

DISCUSSION

From the above results, it appears that the term Trp-LD as applied to the phenomenon described in this and other papers (1, 4) is a misnomer, since there is no real death of cells during starvation of tryptophan. Indeed, the cells appear to be in a transient state, which is evidenced by the loss of colony-forming ability on solid media, but they can recover quantitatively from such a condition upon addition of either tryptophan or tryptophan analogues.

No complete or defective phage particles can be seen in the supernatant fluid or extract of a dying culture. Furthermore, no lysis is observed at any time during Trp-LD as it occurs during induction of a defective phage (6, 8). These differences lead us to believe that induction of a phage, caused by arrest of DNA synthesis after amino acid starvation, is not likely to be the cause of Trp-LD.

The inhibition by chloramphenicol of the tryptophan and tryptophan analogue-induced recovery indicates that active protein synthesis is a prerequisite for the recovery to occur.

Since actinomycin C fails to prevent recovery in the presence of analogues, any protein synthesis involved in this process has to take place on stable messenger molecules that were already present at the time of starvation.

ACKNOWLEDGMENTS

We are grateful to Joshua Lederberg for support and discussion throughout this study.

This investigation was supported by Public Health Service research grant AI-05160 from the National Institute of Allergy and Infectious Diseases, and by the cooperative U.S.-Italy Science Program (GB 7785 of the National Science Foundation and 115 0308 04633 of the Consiglio Nazionale delle Ricerche).

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