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# THYMINELESS DEATH IN *BACILLUS MEGATERIUM*

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ABSTRACT

WACHSMAN, J. T. (University of Illinois, Urbana), S. KEMP, AND L. HOGG. Thymineless death in *Bacillus megaterium*. J. Bacteriol. **87**:1079-1086. 1964.—Strain KM:T$^-$, a thymine auxotroph of *Bacillus megaterium* strain KM, rapidly loses the ability to multiply when incubated in the absence of thymine, on an otherwise sufficient medium. At 37 C, there is a lag of approximately 60 min, prior to the onset of exponential death (decrease of 1 decade per 50 min). The extent of the decrease in viable count varies from 4 to 5 decades after 5 hr of starvation. The cells die more slowly at 30 C (decrease of 1 decade per 120 min) after a lag of approximately 90 min. Thymine starvation permits substantial net ribonucleic acid (RNA) and protein synthesis, but only slight deoxyribonucleic acid synthesis. In contrast with the changes occurring at 30 C, thymineless death at 37 C is eventually accompanied by a rapid hydrolysis of RNA and by cell lysis. Chloramphenicol inhibits thymineless death at 37 C. Strain T$^-$R$_1$, a derivative of strain KM:T$^-$, undergoes a very low rate of thymineless death at 37 C (decrease of 1 decade per 240 min). Neither hydrolysis of RNA nor cell lysis occurs during 8 hr of thymine starvation. Strain KM:T$^-$H$^-$ (doubly auxotrophic for thymidine and histidine) requires histidine for maximal thymineless death at 37 C. Preincubation of this strain on the basal medium supplemented with thymidine alone enables the population to become increasingly immune to subsequent thymineless death.

The phenomenon of thymineless death was discovered in *Escherichia coli* 15T$^-$ (Cohen and Barner, 1954) and was subsequently described for other thymineless strains of *E. coli:* strain B$_3$ (Gallant and Suskind, 1962; Melechen and Skaar, 1962), and strain K$_{12}$ (λ) (Korn and Weissbach, 1962). When thymine auxotrophs are deprived of thymine on an otherwise sufficient medium, the cells rapidly die. During the initial stages of starvation, there is substantial protein and ribonucleic acid (RNA) synthesis, but little, if any, deoxyribonucleic acid (DNA) synthesis. The bactericidal effect is sharply curtailed by the omission of a carbon and energy source (Barner and Cohen, 1954), the presence of 5-methyltryptophan (Cohen and Barner, 1954), omission of a required amino acid (Barner and Cohen, 1957), or the addition of chloramphenicol (Billen, 1959; Nakada, 1960; Okagaki, Tsubota, and Sibatani, 1960). The experiments of Maaløe and Hanawalt (1961) showed that cells which were allowed to complete a DNA replication cycle in the absence of appreciable RNA and protein synthesis became immune to thymineless death. They proposed that thymineless death is due to irreparable DNA damage, resulting from an abortive attempt at DNA synthesis in the absence of thymine. Ion-exchange chromatography failed to reveal any differences in the profile of soluble proteins from thymine-starved cells, as compared to normal cells (Aronson et al., 1959). In addition, the RNA synthesized during thymine starvation was found to have the same nucleotide composition as normal RNA (Gallant and Suskind, 1962) and to be equally capable of supporting β-galactosidase synthesis (McFall and Magasanik, 1962). Both thymine-starved and normal cells synthesized the same classes of ribosomes (Norcross, Comly, and Roberts, 1959; Melechen and Skaar, 1962). The DNA of cells starved for several hours remained double-stranded (Nakada, 1962; Luzzati and Revel, 1962), and had the same molecular weight and melting profile as normal DNA (Luzzati and Revel, 1962). Mennigmann and Szybalski (1962), however, found a decrease in DNA viscosity after starvation of *E. coli* 15T$^-$ for several hours. They also reported a decrease in both viscosity and transforming activity of *Bacillus subtilis* DNA, after exposure of intact cells to 5-fluoro-2′-deoxyuridine. In addition, the DNA of *E. coli* 15T$^-$ grown on limiting thymine contained much more 6-methyl aminopurine than normal DNA (Dunn and Smith, 1958). In contrast to other strains, the DNA methylating

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enzyme from *E. coli* 15T$^-$ was found to methylate its own DNA and the DNA from other strains of *E. coli* (Gold, Hurwitz, and Anders, 1963).

The present studies stem from the observation that exposure of *B. megaterium* strain KM to both 5-fluorouracil and uridine results in changes in cell chemistry and viability which resemble thymineless death in *E. coli* (Wachsman, Kemp, and Hogg, 1964). A thymine auxotroph of *B. megaterium* KM was isolated (KM:T$^-$), and the changes accompanying thymineless death were studied. Data are also presented on the effect of thymine starvation on two substrains of KM:T$^-$, a double auxotroph and a strain with a reduced rate of thymineless death.

## MATERIALS AND METHODS

*B. megaterium* was grown on the previously described basal medium (Mangalo and Wachsman, 1962). Thymine auxotrophs were grown on the basal medium supplemented with thymidine (0.5 μmole/ml). In experiments with strain KM:T$^-$H$^-$, both thymidine and histidine (0.5 μmole of each per ml) were added. The components of the basal medium were sterilized by autoclaving. All supplements to the basal medium were sterilized by Millipore filtration.

Strain KM:T$^-$, a thymine auxotroph of *B. megaterium* strain KM, was isolated by a modification of the method of Okada, Homma, and Sonohara (1962). The basal medium was supplemented with aminopterin (400 μg/ml) and thymine (1.0 μmole/ml), and was inoculated with strain KM at a population density of $4 \times 10^7$ cells per ml. As measured turbidimetrically, growth was observed after a lag of up to 7 days at 37 C. After an additional subculture on the same selective medium, the population consisted almost exclusively of thymine auxotrophs. When the selective medium was supplemented with 12 compounds whose biosynthesis is thought to be inhibited by aminopterin (Okada, Yanagisawa, and Ryan, 1961), identical high mutant yields were obtained. However, the lag was shortened to 2 days. Strain T$^-$R$_1$ is a thymine auxotroph that has a very low rate of thymineless death. It was obtained by allowing strain KM:T$^-$ to undergo extensive thymineless death on basal agar (basal medium containing 2% agar) and then selecting for survivors in the presence of thymidine. Strain KM:T$^-$H$^-$ (thymidine and histidine double auxotroph) was isolated from strain KM:T$^-$ by repeated cycles of thymine starvation in the absence of histidine, followed by growth in the presence of both thymidine and histidine.

The growth conditions and methods for the assay of RNA, DNA, protein, and turbidity have been described (Wachsman et al., 1964). The growth temperature used for the preparation of the inoculum was the same as the temperature employed during thymine starvation.

For the determination of viable cells, samples were diluted in the basal medium with or without thymidine (0.1 μmole/ml). Samples (0.1 ml) were spread on the surface of plates containing basal agar and thymidine (0.1 μmole/ml) or on plates of AC$_3$ agar (1.0% Tryptone, 1.0% yeast extract, 0.5% $K_2HPO_4$, 0.3% glucose, and 2% agar) supplemented with thymidine (0.1 μmole/ml). Similar counts were obtained with both plating media. Colonies were usually counted after incubation for at least 48 hr at 35 C. In the experiment on thymineless death at 30 C, the plates were incubated for 72 hr at 30 C.

Thymine, thymidine, L-histidine, and aminopterin were obtained from Mann Research Laboratories, Inc., New York, N.Y. Chloramphenicol (Chloromycetin) was a product of Parke, Davis & Co., Ann Arbor, Mich.

## RESULTS

Of the compounds tested, only thymine and thymidine support the growth of strain KM:T$^-$ on basal medium. Both are utilized with equal efficiency. Although maximal growth is obtained with thymidine at a concentration of 0.1 μmole/ml, in most experiments, a fivefold excess was employed (0.5 μmole/ml). The following groups of compounds do not support growth on the basal medium in the absence of thymidine: complete amino acid mixture; folic acid and vitamin $B_{12}$; adenosine and guanosine; uridine and cytidine; and a combination of L-methionine, folic acid, vitamin $B_{12}$, and a complete amino acid mixture. The mutant is relatively stable with respect to its thymidine requirement. No revertants to prototrophy were found when as many as $1.2 \times 10^9$ cells were plated on basal agar in the absence of thymidine.

When strain KM:T$^-$ is deprived of thymidine on an otherwise sufficient medium, the cells rapidly lose their ability to multiply. The results of an experiment on starvation at 37 C are shown

in Fig. 1. There is a slight increase in the viable count (35 to 50%) for the first 30 to 40 min. This is probably due to residual thymidine. After approximately 1 hr, the cells begin to die exponentially, with a decrease in viable count of 1 decade per 50 min. At 37 C, this strain has a generation time of about 50 min on the thymidine-supplemented basal medium. In several experiments, 5 hr of thymidine starvation at 37 C resulted in a decrease in viable count of from 4 to 5 decades.

There is only a slight increase in total DNA (20 to 30%) during starvation at 37 C, but substantial RNA and protein synthesis (Fig. 2). The ratio of RNA to DNA increases from a value of 13 to 1 at zero time to a value of 34 to 1 at 2 hr. This is followed by a rapid decline in acid-precipitable RNA and, subsequently, by cell lysis. It should be emphasized that exponential death commences at least 1 hr prior to the hydrolysis of RNA (Fig. 1). After 5 hr of starvation, the population consists of a mixture of very long cells (two to eight times normal length), lysed cells, and a few intact and lysed protoplast-like bodies.

The course of thymineless death at 30 C is shown in Fig. 3 (curve KM:T$^-$; 30 C). After a lag of approximately 90 min, the cells begin to die exponentially, with a decrease in the viable count of 1 decade per 120 min. Thus, the exponential death rate at 30 C is about two and one-half times lower than that at 37 C; the generation time at 30 C is 73 min. After 5 hr of starvation, total DNA increases by about 15%, while RNA and protein increase 2.8-fold and 5.4-fold, respectively (Fig. 4). In contrast to the changes occurring at 37 C, the elevated RNA to DNA ratio (37 to 1) persists throughout the 2- to 5-hr starvation period. In addition, there is little or no lysis at 5 hr. The population consists primarily of thin cells, two to eight times their normal length.

The exponential death rate of strain T$^-$R$_1$ at 37 C (one-decade kill per 240 min) is approximately one-fifth that of strain KM:T$^-$ at the same temperature (Fig. 3). Both the lag prior to exponential death and the generation times at 37 C are the same in both strains. Total DNA increases by 20 to 30% during the first 6 hr of starvation, and then declines slightly (Table 1). In contrast to strain KM:T$^-$, neither hydrolysis of RNA nor cell lysis occurs over an 8-hr starvation period at 37 C. The ratio of RNA to DNA is approximately 40 to 1 at 8 hr.

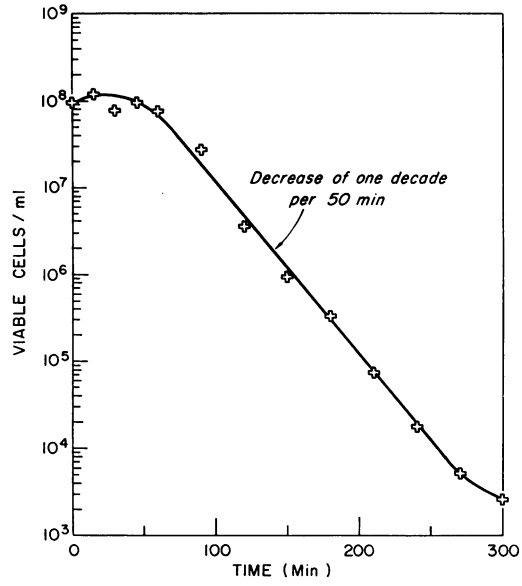


FIG. 1. *Effect of thymine starvation at 37 C on the viability of strain KM:T$^-$. Cells growing exponentially on the basal medium supplemented with thymidine (0.5 μmole/ml) were harvested, washed once, and resuspended in the basal medium alone. Samples were removed and plated for viable count on AC$_3$ agar supplemented with thymidine (0.1 μmole/ml).*

Strain KM:T$^-$H$^-$ (thymidine-histidine double auxotroph) was used to study the effect of amino acid deprivation on thymineless death. Histidine is required for maximal thymineless death at 37 C (Table 2). However, there is some killing between 2 and 5 hr on the basal medium, with or without thymidine. The addition of thymidine alone stimulates DNA synthesis twofold by 5 hr, but has little effect on changes in RNA, protein, and turbidity. The addition of histidine alone stimulates increases in RNA, protein, and turbidity for the first 2 hr; there is only a very slight stimulation of DNA synthesis. Histidine is required for RNA hydrolysis and cell lysis in the absence of thymidine. Incubation of this strain on a thymidine-supplemented basal medium permits the population to slowly develop increasing degrees of immunity to subsequent thymineless death (Table 3). It is apparent that preincubation for 2 hr in the presence of thymidine enables the population to become approximately 200-fold more resistant to thymineless death than are cells which had not been preincubated.

Chloramphenicol inhibits thymineless death in

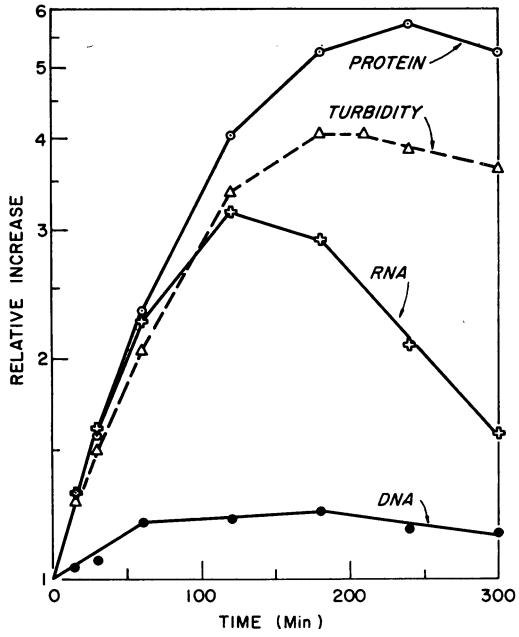


FIG. 2. *Effect of thymine starvation at 37 C on turbidimetric and chemical changes in strain KM:T⁻. The above data were obtained in the same experiment described in Fig. 1. At zero time, the culture contained the following amounts per ml: 83.7 μg of protein, 24.6 μg of RNA, and 1.88 μg of DNA.*

strain KM:T⁻. When chloramphenicol is added at 2 and at 100 μg/ml, the number of survivors after 5 hr of thymidine starvation at 37 C is 50-fold and 500-fold greater, respectively, than is the number of survivors in the absence of the antibiotic. Low levels of the antibiotic (2 μg/ml) inhibit net protein synthesis by about 40%, but stimulate RNA synthesis by 30 to 40%. With high levels of chloramphenicol, net protein synthesis is completely inhibited, whereas RNA synthesis is inhibited by about 30%.

## DISCUSSION

After a lag of 60 min at 37 C, strain KM:T⁻ undergoes rapid thymineless death, culminating in a 4- to 5-decade kill in 5 hr. For the first 2 hr of thymine starvation, the changes in viability and chemical composition of the population resemble those described for thymineless strains of *E. coli* (Cohen and Barner, 1954; Gallant and Suskind, 1961; Nakada, 1962). However, the subsequent decrease in acid-precipitable RNA, followed by cell lysis and the formation of some protoplast-like bodies, has not been described for *E. coli* (Gallant and Suskind, 1961; Korn and Weissbach, 1962). The events accompanying thymine starvation of strain KM:T⁻ at 37 C are similar to those induced by exposure of *B. megaterium* KM to a combination of 5-fluorouracil and uridine (Wachsman et al., 1964). In the latter case, there is a longer lag period prior to exponential death, and DNA synthesis is somewhat more extensive.

An eventual decrease in acid-precipitable RNA is observed when strain KM:T⁻ is starved for thymine at 37 C, but not at 30 C. In addition, there is no hydrolysis of RNA when the partially resistant strain ($T^-R_1$) is starved for as long as 8 hr at 37 C. This hydrolysis could be due to ribosomal breakdown and release of ribonuclease. Because exponential death precedes RNA hy-

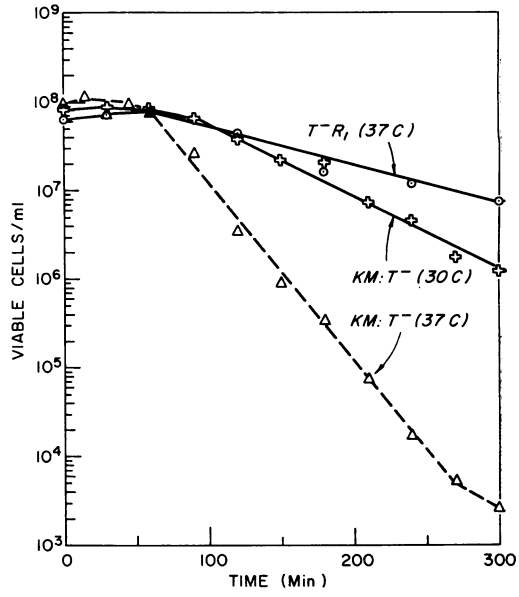


FIG. 3. *Comparative effects of thymine starvation on viability. With all strains, cells growing exponentially on the basal medium with thymidine (0.5 μmole/ml) were harvested, washed once, and resuspended in the basal medium alone. The growth temperature was the same as the starvation temperature. The data for KM:T⁻ (37 C) are from Fig. 1. Strain KM:T⁻ was plated for viable count on $AC_3$ agar supplemented with thymidine (0.1 μmole/ml). Strain $T^-R_1$ was plated on basal agar supplemented with thymidine (0.1 μmole/ml). The following rates of exponential death are presented as the time required for a one-decade kill: KM:T⁻ (37 C) = 50 min; KM:T⁻ (30 C) = 120 min; and $T^-R_1$ (37 C) = 240 min.*

drolysis by at least 60 min, it would appear to be a consequence, rather than a cause, of rapid thymineless death.

Within the past few years, the following sugar derivatives of thymidine diphosphate have been identified in microbial systems: mannose (Blumsom and Baddiley, 1961), glucose (Kornfield and Glaser, 1961), L-rhamnose (Glaser and Kornfield, 1961), D-galactose (Tinelli, Michelson, and Strominger, 1963), and both *N*-acetylglucosamine and *N*-acetylgalactosamine (Kornfield and Glaser, 1962). *B. megaterium* KM cell walls were reported to contain glucose, muramic acid, and glucosamine as the only carbohydrate constituents (Salton and Pavlik, 1960). The involvement of thymidine derivatives in *B. megaterium* cell-wall biosynthesis could explain the lysis and formation of protoplast-like bodies, induced by deprivation of exogenous thymine or by exposure to 5-fluorouracil and uridine (Wachsman et al., 1964).

When strain KM:T$^-$ is starved for thymine at 30 C, there is an increase in the lag period and a decrease in the rate of exponential death, as compared to starvation at 37 C. A similar effect of decreasing temperature was reported for *E. coli* 15T$^-$ (Maaløe and Hanawalt, 1961). Although *E. coli* B$_3$ undergoes rapid thymineless death at 37 C, thymine starvation at 25 C for as long as 4 to 5 hr does not result in death (Gallant, 1962). There is, however, extensive unbalanced RNA and protein synthesis.

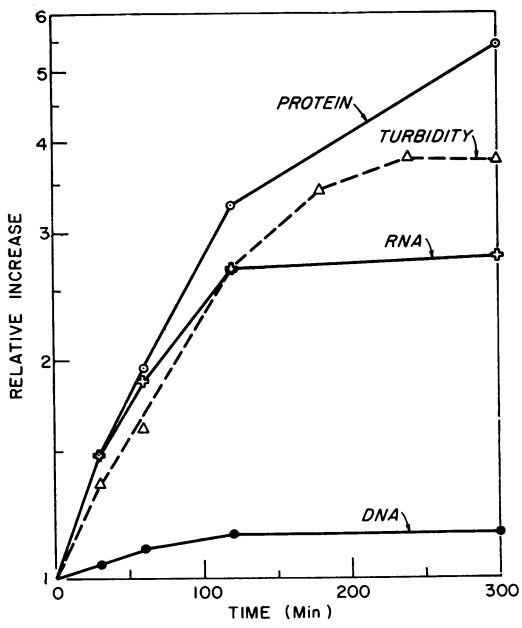


FIG. 4. *Effect of thymine starvation at 30 C on turbidimetric and chemical changes in strain KM:T$^-$. Cells growing exponentially on the basal medium with thymidine (0.5 μmole/ml) were harvested, washed once, and resuspended in the basal medium alone. At zero time, the culture contained the following amounts per ml: 84.7 μg of protein, 26.5 μg of RNA, and 1.72 μg of DNA.*

TABLE 1. *Effect of thymine starvation on strain T$^-$R$_1$**

| Time | Relative increase | | | |
|---|---|---|---|---|
| | Turbidity | Protein | RNA | DNA |
| *hr* | | | | |
| 0 | 1.00 | 1.00 | 1.00 | 1.00 |
| 2 | 3.60 | 3.18 | 2.79 | 1.31 |
| 4 | 5.74 | 6.78 | 2.88 | 1.27 |
| 6 | 6.84 | 8.87 | 2.96 | 1.28 |
| 8 | 7.56 | 9.85 | 3.10 | 1.13 |

* Cells growing exponentially on the basal medium with thymidine (0.5 μmole/ml) were harvested, washed once, and resuspended in the basal medium alone. Incubation was at 37 C. At zero time, the culture contained the following amounts per ml: 68.5 μg of protein, 26.4 μg of RNA, and 1.82 μg of DNA.

Chloramphenicol inhibits thymineless death in strain KM:T$^-$ and in strains of *E. coli* (Billen, 1959; Nakada, 1960; Okagaki et al., 1960). On the basis of experiments with *E. coli* B$_3$, Gallant and Suskind (1962) concluded that the extent of thymineless death was closely correlated with net RNA synthesis. They found that 5-methyltryptophan severely inhibits thymineless death, RNA, and protein synthesis. The simultaneous addition of chloramphenicol (2 μg/ml) had little effect on protein synthesis, but stimulated both RNA synthesis and thymineless death to approximately the same extent. The experiments with strain KM:T$^-$ suggest that thymineless death is independent of RNA synthesis, but can be correlated with net protein synthesis. Low levels of chloramphenicol (2 μg/ml) stimulate RNA synthesis by 30 to 40%, whereas higher levels (100 μg/ml) inhibit RNA synthesis by about 30%. Correspondingly, survival is increased 50-fold and 500-fold, respectively, whereas net protein synthesis is inhibited 40% and 100%, respectively.

Strain KM:T$^-$H$^-$ becomes partially immune to subsequent thymineless death, if preincubated

TABLE 2. *Effect of thymine starvation on strain KM:T⁻H⁻**

| Additions to basal medium | Time | Relative increase | | | | No. of viable cells per ml |
|---|---|---|---|---|---|---|
| | | Turbidity | Protein | RNA | DNA | |
| | *hr* | | | | | |
| None | 0 | 1.00 | 1.00 | 1.00 | 1.00 | $8.0 \times 10^7$ |
| | 1 | 1.43 | 1.02 | 1.22 | 1.13 | |
| | 2 | 1.62 | 1.13 | 1.29 | 1.04 | $8.2 \times 10^7$ |
| | 5 | 1.84 | 1.35 | 1.38 | 1.01 | $4.0 \times 10^6$ |
| Thymidine | 1 | 1.37 | 0.95 | 1.15 | 1.45 | |
| | 2 | 1.56 | 1.05 | 1.32 | 1.77 | $1.2 \times 10^8$ |
| | 5 | 1.77 | 1.17 | 1.44 | 2.02 | $1.0 \times 10^6$ |
| Histidine | 1 | 2.41 | 2.52 | 2.22 | 1.21 | |
| | 2 | 3.41 | 4.19 | 2.45 | 1.21 | $1.4 \times 10^6$ |
| | 5 | 1.92 | 4.38 | 0.90 | 1.02 | $1.2 \times 10^4$ |
| Thymidine + histidine | 1 | 2.21 | 2.23 | 2.25 | 2.38 | |
| | 2 | 4.45 | 5.23 | 5.28 | 5.56 | $5.2 \times 10^8$ |
| | 5 | 10.4 | 13.7 | 7.98 | 14.3 | $1.3 \times 10^9$ |

* Cells growing exponentially on the basal medium supplemented with thymidine (0.5 μmole/ml) and L-histidine (0.5 μmole/ml) were harvested, washed twice, and resuspended in the basal medium alone. Portions were distributed in growth flasks with the above additions at 0.5 μmole of each per ml. Incubation was at 37 C. At zero time, the cultures contained the following amounts per ml: 72.3 μg of protein, 26.8 μg of RNA, and 1.72 μg of DNA. Samples were plated for viable count on $AC_3$ agar supplemented with thymidine (0.1 μmole/ml).

TABLE 3. *Immunity to thymineless death in strain KM:T⁻H⁻**

| Preincubation with thymidine in absence of histidine | No. of viable cells per ml after subsequent exposure to histidine in absence of thymidine for 5 hr |
|---|---|
| *min* | |
| 0 | $6.6 \times 10^2$ |
| 30 | $1.2 \times 10^3$ |
| 60 | $9.4 \times 10^3$ |
| 120 | $1.2 \times 10^5$ |

* Cells were grown, harvested, and washed as described in Table 2. Cells were resuspended in basal medium containing thymidine (0.5 μmole/ml) at approximately $4 \times 10^8$ cells per ml. Samples were removed after preincubation at 37 C for the indicated time intervals; cells were washed twice with basal medium and were resuspended in basal medium containing histidine (0.5 μmole/ml) at approximately $8 \times 10^7$ cells per ml. After incubation for 5 hr at 37 C, samples were plated for viable count on $AC_3$ agar supplemented with thymidine (0.1 μmole/ml).

with thymidine, under conditions (absence of histidine) which severely inhibit RNA and protein synthesis. In the experiments of Maaløe and Hanawalt (1961) with a polyauxotrophic mutant of *E. coli* 15T⁻, 100% immunity was achieved after preincubation for 90 min. The latter investigators assayed for immunity to thymineless death under conditions which maintained the restriction of RNA and protein synthesis. In the experiment with strain KM:T⁻H⁻, immunity was determined under conditions (presence of histidine) which did not maintain this restriction. Consequently, a large portion of the population was probably able to initiate a new round of abortive DNA replication. Maaløe and Hanawalt (1961) reported that immunity is only gradually lost under conditions where RNA or protein synthesis (or both) can occur.

Strain KM:T⁻H⁻ requires histidine for maximal thymineless death, although there is some killing after prolonged incubation on the basal medium alone, even when thymidine is present. Similar results were obtained with other doubly auxotrophic mutants of *B. megaterium* (thymine⁻ arginine⁻; thymine⁻ tryptophan⁻; Wachsman and Farhi, *unpublished data*). On the basis of experiments with *E. coli* 15T⁻PA⁻ (thymine⁻ and phenylalanine⁻), Barner and Cohen (1957) reported than an amino acid deficiency sharply increased survival in the absence of thymine.

The effect of the addition of thymidine or histidine (or both) on the cell chemistry of strain KM:$T^-H^-$ is in reasonably good agreement with experiments on polyauxotrophic mutants of *E. coli* (Barner and Cohen, 1957; Nakada, 1960). The experiments on amino acid deprivation and the effect of chloramphenicol are consistent with the concept that protein synthesis is required for thymineless death. However, it should be borne in mind that, in stringent bacterial strains, amino acids exert a controlling effect on DNA synthesis, as well as on protein and RNA synthesis (Kellenberger, Lark, and Bolle, 1962).

Strain $T^-R_1$ is under present investigation in an attempt to gain some insight into the mechanisms of both thymineless death and partial resistance.

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