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Thymineless Mutagenesis in *Escherichia coli*

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To clarify the relationship between thymineless death and thymineless mutagenesis, the induction of arginine revertants of *Escherichia coli* TAU-bar by thymine starvation was examined in physiological terms. Induced revertants were detectable both on minimal medium lacking arginine and minimal medium supplemented with 1 μg of arginine per ml. Substantial thymineless mutagenesis occurred during the period before the onset of thymineless death. Mutagenesis and loss of viability were observed upon incubation in medium lacking thymine and arginine, and both were inhibited upon incubation in medium lacking thymine and uracil. Mutagenesis also occurred during thymine starvation at 25 C, where there was relatively little loss of viability. At 37 C thymineless mutagenesis did not require complete thymine starvation, and the induction of revertants appeared to be initiated at the same suboptimal thymine concentration at which lethality was first detectable. Mutagenesis was found not to occur preferentially at the growing point of deoxyribonucleic acid replication. These results suggest that thymineless mutagenesis does not involve simply errors in base pairing due to the absence of thymine. The data also suggest that the induction of mutations and thymineless death are due to the same primary event but that mutagenesis is the more sensitive response.

Thymine auxotrophs of many species of bacteria have been found to lose viability when incubated in medium lacking thymine. This "thymineless death" has been the subject of numerous sudies, but its underlying mechanism is still not understood. The analysis of thymineless death has been complicated by the fact that loss of viability is dependent on the assay conditions used and shows considerable variation even within related strains of the same organism (17).

In addition to causing loss of viability, thymine starvation has been observed to induce mutations in *Escherichia coli* (7), *Bacillus subtilis* (3), and *Salmonella typhimurium* (15). Thymineless mutagenesis has also been demonstrated in cells infected with bacteriophage T4 (1, 23). Experiments on thymineless mutagenesis have been directed primarily towards understanding its specificity. Pauling (18) demonstrated that the efficiency of thymineless mutagenesis was substantially increased by allowing the cells to incorporate the base analogue 2-aminopurine prior to thymine starvation. This synergism did not occur with 5-bromouracil, and it was concluded that thymine starvation preferentially induces transition mutations in the AT to GC direction. Recently the same conclusion was reached based on the thymineless reversion of well-characterized rII mutants of bacteriophage T4 (23). However, Holmes and Eisenstark (15) determined the response to different mutagens of a series of auxotrophs of *S. typhimurium* induced by thymine starvation. They found that some mutants were reverted by base analogues, others were reverted by alkylating agents, and still others were nonrevertible. Thymineless mutagenesis may therefore induce more than one class of mutations.

In most cases thymineless death and thymineless mutagenesis have been studied independent of each other. In this paper, we describe some physiological experiments aimed at clarifying the relationship between these two effects of thymine starvation. We also report experiments designed to test one possible hypothesis for the mechanism of thymineless mutagenesis.

MATERIALS AND METHODS

E. coli strain TAU-bar (Thy^-, Ura^-, Arg^-, Met^-, Pro^-, Try^-) was grown in glucose-salts minimal medium (8) supplemented with thymine (2 μg/ml), uracil (10 μg/ml), and amino acids (20 μg/ml) as

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required. Viable cell counts were performed by plating on enriched medium as previously described (8), and revertants to arginine prototrophy were determined by plating on glucose-salts selective media. Plates were incubated at 37 C and scored after 3 days. In experiments involving mutagenesis with nitrosoguanidine, concentrated suspensions of cells were incubated at 37 C in 0.15 M acetate buffer, pH 5.0, containing 1 mg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Mann Research Laboratories, Inc., reference 5) per ml. After 30 min of treatment, the cells were harvested by centrifugation and washed twice with minimal medium before plating.

RESULTS

Detection of thymineless mutagenesis. The spontaneous frequency of Arg$^+$ revertants of *E. coli* TAU-bar was determined by preparing a series of dilutions of a concentrated suspension of exponential-phase cells; each dilution was assayed for viable count on enriched medium and for revertant counts on minimal medium lacking arginine or minimal medium supplemented with 1 μg of arginine per ml. The amount of growth occurring on the selective media after plating was determined by washing replicate plates after 6 h of incubation at 37 C and assaying the cell suspensions for viable count on enriched medium. Essentially no increase in cell count was observed for the unsupplemented minimal medium. However, a substantial amount of growth did occur on the minimal medium containing 1 μg of arginine per ml, and the amount of growth was inversely related to the number of cells initially plated. Since additional mutations may arise during this postplating growth, the reversion frequencies for the supplemented minimal plates were expressed in terms of the final cell count rather than the number of cells initially plated.

Figure 1 shows the spontaneous frequencies of Arg$^+$ revertants on the two minimal media, plotted as a function of the number of cells initially plated. The frequency ranged between 10^{-8} and 10^{-10}, and was higher on the unsupplemented medium than on the medium containing 1 μg of arginine per ml. The frequency of revertants was essentially constant if 10^8 to 10^9 viable cells were plated, but it decreased if more than 2×10^9 cells were used. Since all the samples in each experiment were from the same initial cell suspension, this decrease in frequency must be related to events occurring on the selective plates.

To demonstrate thymineless mutagenesis of this arginine marker, exponential-phase cells were starved of thymine for 60 min at 37 C; dilutions of a concentrated cell suspension were then assayed as with the untreated cells. Figure 1 shows that thymine starvation increased the mutant frequency 10- to 15-fold. Somewhat higher frequencies were again observed on minimal medium lacking arginine, but a comparison of the curves for spontaneous and induced mutations indicates that the degree of mutagenesis was the same under both plating conditions. In some cases it has been found that plating on supplemented minimal medium is necessary to obtain complete recovery of induced mutations (25). This does not seem to be true for mutagenesis of the arginine marker of *E. coli* TAU-bar by thymine starvation.

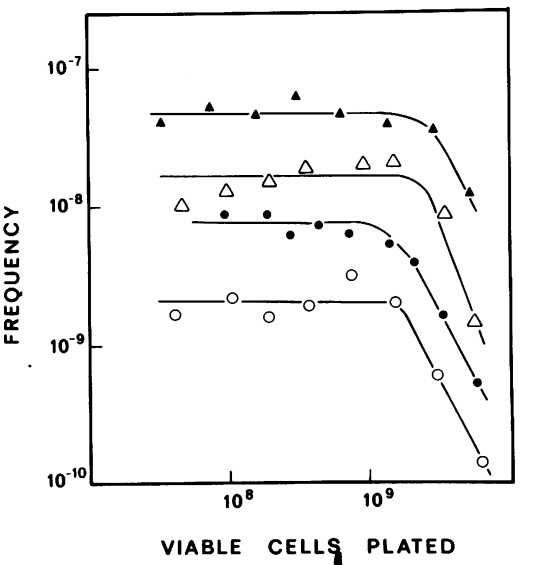

FIG. 1. *Frequency of Arg$^+$ revertants on different selective media. Untreated (●) or thymine-starved (▲) cells plated on unsupplemented minimal medium; untreated (○) or thymine-starved (△) cells plated on minimal medium containing 1 μg of arginine per ml.*

The frequency of Arg$^+$ revertants recovered was found to decrease if more than 2×10^9 untreated or thymine-starved cells were plated on the selective media. Auxotrophic cells have been found to inhibit the growth of revertants by metabolizing components of the selective medium (12, 22). To test whether this was happening in our experiments, exponential-phase arginine auxotrophs were suspended in liquid minimal medium lacking arginine and incubated for 8 h at 37 C. No growth occurred during this time, as measured by an increase in turbidity. The cells were removed by centrifugation and the "conditioned medium" was tested for its ability to support the growth of previously isolated Arg$^+$ revertants. No growth was found to occur unless the medium was supplemented with additional amounts of pro-

line. It seems likely, therefore, that the decreasing frequencies shown in Fig. 1 were the result of the consumption of proline needed by the revertants when large numbers of auxotrophs were plated.

The procedure that was adopted for the remaining experiments was to concentrate the cells to be assayed and plate approximately 10^9 viable cells on minimal medium lacking arginine. This procedure allowed maximal revertant colony counts and avoided the decrease in frequency associated with proline utilization. To demonstrate that thymineless mutagenesis could be detected quantitatively under these conditions, the following reconstruction experiment was performed. Varying proportions of untreated exponential-phase cells and cells which had been starved of thymine for 60 min at 37 C were plated together on unsupplemented minimal medium at a total concentration of 10^9 viable cells per plate. The results (Fig. 2) indicate that the frequency of Arg^+ revertants was directly related to the proportion of thymine-starved cells present.

Mutagenesis during thymine starvation. To determine the relationship between thymineless death and thymineless mutagenesis, the survival and frequency of Arg^+ revertants were measured during several growth conditions. Figure 3 shows that, in the presence of thymine and other required supplements, the cells grew exponentially and the frequency of revertants remained essentially constant. In the absence of thymine, a typical thymineless death curve was obtained. At 37 C, this consisted of an exponential loss of viability after a lag of about 45 min. During thymine starvation, there was a rapid increase in Arg^+ revertants. This increase began immediately upon starvation and reached a plateau in about 60 min. Although both loss of viability and mutagenesis required the absence of thymine, the induction of mutants occurred primarily during the lag period before the onset of thymineless death. These results are in general agreement with earlier descriptions of thymineless mutagenesis (16, 18). However, that the increase in the frequency of mutants leveled off at 60 min rather than continuing exponentially was somewhat unexpected. This response was consistently seen during thymine starvation at 37 C, but was not found during thymineless mutagenesis under other growth conditions (see Fig. 3 and 4).

During thymine starvation, deoxyribonucleic acid (DNA) synthesis stops while ribonucleic acid (RNA) and protein synthesis continue for some time (6). To determine the effect of inhibiting each of these other processes as well, exponential-phase cells were starved for thy-

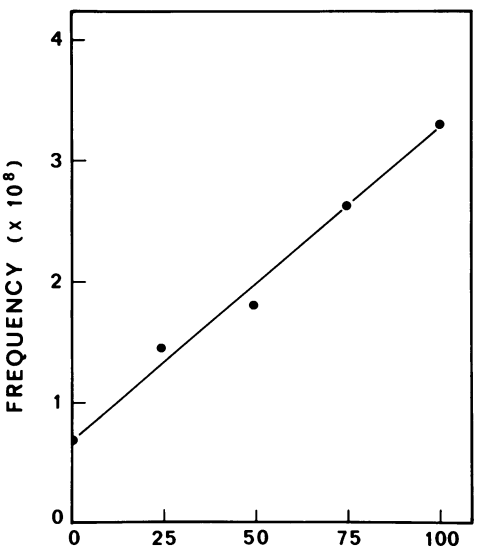

FIG. 2. *Frequency of Arg^+ revertants on plating mixtures of exponential and thymine-starved cells. Viable cells ($1.04 \pm 0.02 \times 10^9$) in mixtures of varying proportions of exponential and thymine-starved (60 min at 37 C, survival = 0.5) cells were plated on unsupplemented minimal medium.*

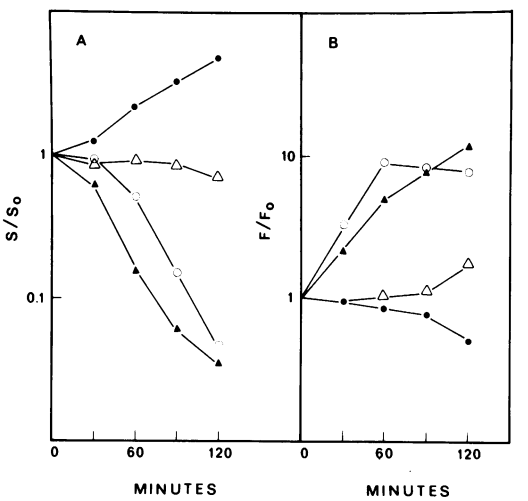

FIG. 3. *Survival and relative frequency of Arg^+ revertants during various starvation treatments at 37 C. Survival (S/S_0) indicates the viable count at time t relative to that at time 0; relative frequency (F/F_0) of Arg^+ revertants indicates the frequency of Arg^+ revertants at time t relative to that at time 0. Symbols: + thymine (●); − thymine (○); − thymine, − arginine (▲); − thymine, − uracil (△).*

mine in media also lacking exogenous uracil or exogenous arginine (Fig. 3). In the first case, loss of viability was inhibited (at least initially) and no increase in Arg^+ revertants occurred. In the second case, thymineless death did occur (with a somewhat shorter lag than usual) and there was an increase in mutations. Thymineless death and mutagenesis are therefore correlated to the extent that conditions which permit or inhibit one also permit or inhibit the other. Amino acid starvation blocks ribosomal RNA and transfer RNA synthesis in *rel*$^+$ (RC-stringent) strains such as *E. coli* TAU-bar, but it does not necessarily block messenger RNA synthesis (10). These results, therefore, are consistent with a role of messenger RNA synthesis in the process of death and mutagenesis, in agreement with the proposal of Hanawalt (14).

Mutagenesis and sublethal thymineless damage. Gallant (12) observed that thymine starvation at 25 C causes little loss of viability, but that subsequent incubation at 37 C results in thymineless death with a reduction in the length of the normal lag. To determine if this sublethal thymineless damage occurring at 25 C is mutagenic, the survival and frequency of Arg^+ revertants were compared during thymine starvation at 25 and 37 C. Figure 4 shows that sublethal thymineless damage was mutagenic. The increase in revertants at 25 C was similar to that observed at 37 C, although there was essentially no loss of viability at the lower temperature. These results, together with the observation that mutagenesis at 37 C occurs during the lag before the onset of thymineless death, suggest that the induction of mutations is a more sensitive indicator of the changes occurring during thymine starvation than loss of viability.

Mutagenesis in limiting thymine. The inhibition of DNA synthesis caused by thymine starvation has been considered an important factor in thymineless mutagenesis. It has been suggested that mutations may arise by errors in base pairing during attempted DNA synthesis in the absence of thymine (18). To test this hypothesis, the survival and frequency of Arg^+ revertants were measured during incubation of *E. coli* TAU-bar in a series of media containing suboptimal amounts of thymine. Figure 5 shows that, with decreasing concentrations of thymine, there was a progressive increase in the rate of loss of viability. The effects of limiting thymine on viability were discussed in detail in an earlier paper (8). With thymine concentrations below 0.2 μg/ml, the frequency of Arg^+ revertants began to increase immediately upon suspension in limiting thymine. Essentially the same number of revertants resulted as during complete thymine starvation. With thymine concentrations above 0.2 μg/ml, mutagenesis occurred only after a lag period which was related to the initial thymine concentration. With 0.2 μg/ml, induced mutations appeared after 30 min; with 0.3 μg/ml, these mutations appeared after 60 min; and with 0.5 μg/ml, they appeared after 90 min. These results indicate

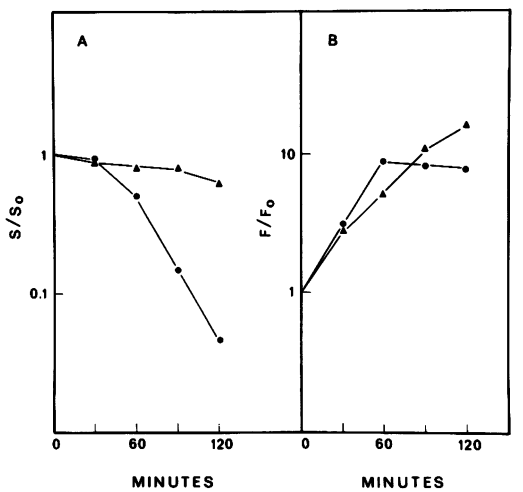

FIG. 4. *Survival and relative frequency of Arg^+ revertants during thymine starvation at 37 C (●) and 25 C (▲).*

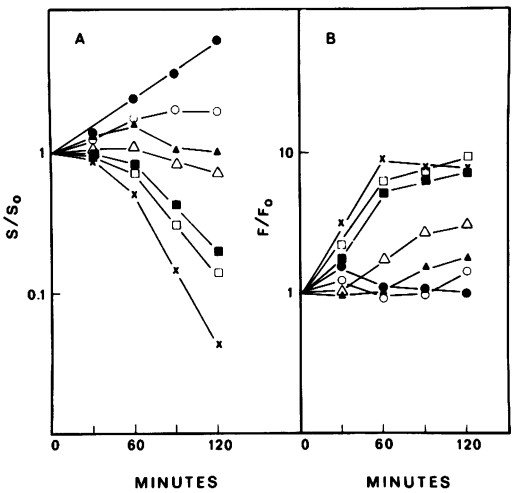

FIG. 5. *Survival and relative frequency of Arg^+ revertants during incubation in limiting thymine. Symbols: 2 μg/ml (●), 0.5 μg/ml (○), 0.3 μg/ml (▲), 0.2 μg/ml (△), 0.1 μg/ml (■), 0.06 μg/ml (□), – thymine (×).*

that thymineless mutagenesis does not require complete thymine deprivation.

DNA synthesis under these conditions was measured by the incorporation of [^{3}H]uracil into alkaline-resistant, acid-precipitable material. Both the rate and total amount of incorporation decreased in direct proportion to the thymine concentration (8). A significant amount of DNA synthesis still occurred at thymine concentrations which yielded the maximum number of induced mutations, e.g., 0.1 μg/ml. The rate of [^{3}H]uracil incorporation was even greater at the higher concentrations of thymine, which also permitted mutagenesis after some lag. Thymineless mutagenesis, therefore, does not require the complete inhibition of DNA synthesis.

With thymine concentrations above 0.2 μg/ml, mutagenesis began only after a short lag period. This suggests that, as the thymine was used up, a critical concentration was reached at which mutagenesis commenced. The data in Fig. 5 indicate that this concentration was between 0.1 and 0.2 μg/ml; this was also the concentration at which loss of viability began to occur.

Mutagenesis at the growing point. A prediction of the hypothesis that thymineless mutagenesis involves errors in base pairing is that mutations might be induced preferentially at the growing points of DNA replication. To test this prediction, the induction of Arg$^+$ revertants by thymine starvation was compared for exponential-phase cultures and for cultures in which chromosome replication had been aligned by successive starvation treatments. This type of experiment was performed successfully with nitrosoguanidine (NTG) as the mutagen, and it was shown that NTG causes a maximal number of induced Arg$^+$ revertants in aligned cultures of *E. coli* TAU-bar at the beginning of the replication cycle (5). For the experiments shown in Fig. 6, chromosome alignment was accomplished by starvation for amino acids in the presence of thymine for 90 min at 37 C followed by 100 min of starvation for thymine in the presence of required amino acids at 25 C to allow synthesis of initiator proteins in the absence of DNA synthesis. Thymine (2 μg/ml) was then added, and incubation of the aligned cultures was performed at 25 C to slow the rate of chromosome replication. Samples were removed at intervals, and mutations were induced by thymine starvation for 60 min at 37 C or NTG treatment. Unaligned control cultures were prepared from exponential-phase cells; incubation was performed at 25 C and mutations were induced as described above. Figure 6 shows that, by comparing aligned with unaligned cultures, a maximal number of induced revertants was observed at the beginning of the replication cycle for NTG treatment but not for thymine starvation. A similar result was also observed for tryptophan revertants of *E. coli* TAU-bar induced by thymine starvation. Thymineless mutagenesis therefore does not appear to act preferentially at replication growing points.

DISCUSSION

We interpret the experiments described here as suggesting that thymineless death and thymineless mutagenesis may be due to the same primary event. Conditions that permit one to occur also permit the other, and both require continued RNA synthesis. Thymineless death and mutagenesis are initiated at the same suboptimal thymine concentration. However, the induction of mutations appears to be the more sensitive effect. Thymineless mutagenesis occurs during the lag before the onset of thymineless death and during sublethal thymine starvation. Maximal mutagenesis can also be detected at thymine concentrations which cause only limited loss of viability. These observations suggest that the induction of mutations may be a better assay for the changes induced by thymine starvation than loss of viability. Muta-

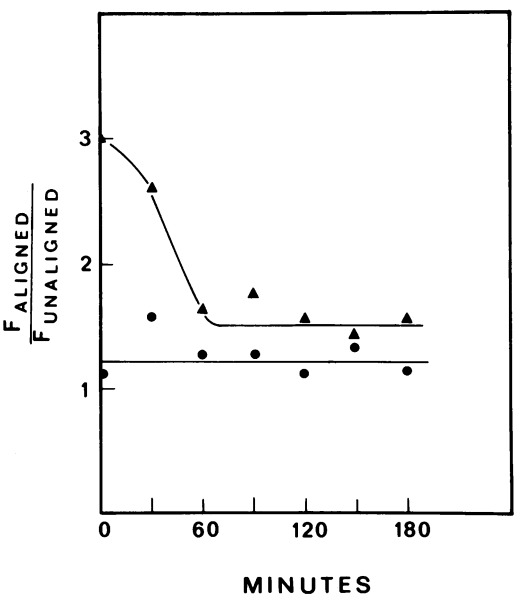

FIG. 6. *Relative frequency of Arg$^+$ revertants between aligned and unaligned cultures after thymine starvation* (●) *or nitrosoguanidine treatment* (▲).

genesis may be less subject to the strain differences and assay effects which have resulted in conflicting data in the past.

It is not clear whether mutations resulting from thymine starvation are fixed in the cell's genome during the period of thymine deprivation or, alternatively, after plating on medium supporting growth. The results obtained with Arg^+ revertants of *E. coli* TAU-bar suggest that postplating growth may not be as important in thymineless mutagenesis as it is in the case of ultraviolet light-induced mutations (25). The data presented do suggest that thymineless mutagenesis does not involve simply errors in base pairing. Mutations do not appear to be induced preferentially at the growing point of DNA replication. Furthermore, thymineless mutagenesis does not require complete thymine deprivation or the complete inhibition of DNA synthesis. Although the experiments described here do not show that DNA synthesis was occurring in the cells that were mutagenized in limiting thymine, it has been shown that thymine limitation reduces the rate of chromosome replication (28). Therefore, reduced incorporation of [^{3}H]uracil in the presence of limiting thymine probably reflects reduced synthesis in all the cells, rather than normal synthesis in some cells and no synthesis in others.

A number of modifications of bacterial DNA have been detected during or after thymine starvation. These include double-strand breaks (27), single-strand breaks (11, 20, 24), degradation of the DNA (2, 21), and alterations in the normal proportions of methylated bases (9). Exactly how these modifications, which are not observed universally, result from thymine starvation is not clear. Thymine starvation has been shown to stimulate excision repair (19), and it has been suggested that thymineless mutagenesis is the result of a more error-prone recombinational repair process (4).

A hypothesis that would incorporate some of these observations and which would be consistent with the data presented here is that single-strand breaks in bacterial DNA occur normally as a result of transcription. In the absence of thymine or during thymine limitation, such breaks would not be completely repaired since all the repair pathways known require some type of DNA synthesis. During prolonged thymine starvation, some of these single-strand breaks might be extended by nucleases to give long single-strand gaps in the DNA (and apparent DNA breakdown). Such partially degraded DNA would be sensitive to additional breakage, and loss of viability (thymineless death) might ultimately result. After plating on medium containing thymine, viable cells containing single-strand breaks might undergo repair by the excision pathway, a constitutive recombinational pathway, or an inducible recombinational pathway (26; M. Radman, Conference on Environmental Toxicity, 1973). Repair of the single-strand breaks by an error-prone recombinational pathway would lead to mutagenesis whereas repair by the more accurate excision pathway would not.

This hypothesis allows the following predictions to be made: (i) thymineless mutagenesis should occur in genes which are being transcribed during thymine starvation, but not in genes which are repressed; (ii) thymineless mutagenesis should be increased if thymine-starved cells are plated under conditions favoring recombinational repair (e.g., on medium containing thymine and caffeine or acriflavine); (iii) thymineless mutagenesis should occur in bacterial strains carrying mutations affecting excision repair (e.g., *uvrA*, *polA*), but not in strains carrying mutations in one of the recombinational pathways (e.g., *recA*, *recBC*, *lex*). Testing these predictions should provide additional insight into the mechanism of thymineless mutagenesis.

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