Thymineless Death in Escherichia coli: Deoxyribonucleic Acid Replication and the Immune State

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Thymineless death (TLD) and nalidixic acid (NA) inactivation were studied in multiple auxotrophic strains of Escherichia coli B and B/r. As expected, it was found that both E. coli B and B/r exhibited an "immune state," i.e., a fraction of the population survived inactivation to both TLD and NA. With glucose as a carbon source in minimal medium, 0.1 to 0.3% of strain B and 0.2 to 0.5% of strain B/r survived inactivation; with acetate as the carbon source, the surviving fractions were increased to 1 to 2% and 5 to 7%, respectively. These immune fractions could be increased in magnitude by preincubation in minimal media containing thymine. Systematic analysis of the particular supplements necessary for the immune state indicated that the absence of the required amino acids was essential for the maximal expression of immunity. However, immunity was not abolished in acetate medium even in the presence of the required supplements. Further studies on the replication of deoxyribonucleic acid (DNA) during preincubation indicated that the degree of immunity did not necessarily correlate with the completion of a round of DNA replication. This finding was supported by examining the immune state in synchronous populations. In both glucose and acetate medium, there was no significant change in the degree of immunity to inactivation within the cell cycles of E. coli B and B/r. We concluded that some other event, possibly inhibition of protein synthesis, was necessary in determining the degree of the immune state. DNA replication was investigated after TLD and NA inactivation, and, as expected, it was found that both events led to premature initiation of replication. The only differences observed in the effects of these two processes on DNA synthesis were the following. (i) NA-induced replication was less sensitive to chloramphenicol than was TLD. (ii) TLD-induced replication was unaffected by pretreatment of the cells with mitomycin C, but this pretreatment prevented the replication of DNA after NA treatment. It was suggested that the mechanism of action of NA could involve a monofunctional attack on the DNA.

The mechanism by which thymine-requiring microorganisms lose viability when deprived of thymine is still unknown (8, 11, 18, 27, 39). Many attempts have been made to elucidate such a mechanism, but, to date, few positive results have been obtained. Hanawalt (18) suggested that the synthesis of some messenger ribonucleic acid (RNA) was involved in the thymineless death (TLD) observed in Escherichia coli (15). However, it has been shown (39) that the rapidly labeled RNA synthesized during TLD in E. coli K-12 has the same base composition as total RNA, indicating that no gross changes occur in the RNA synthesized. In addition, Luzzati (27) found that E. coli 15 exponentially lost its ability to synthesize messenger RNA during thymine starvation, because of the inability of the deoxyribonucleic acid (DNA) to serve as a template for RNA polymerase. Purification of the DNA after thymine starvation led to an increase in the priming efficiency for RNA polymerase. Similarly, Schaiberger et al. (38) observed that crude DNA in extracts of cells undergoing TLD lost the ability to function as a template for DNA polymerase and that this function was restored upon purification of the DNA. These authors concluded that DNA, under thymineless conditions, either became associated with some substance that rendered it inactive as a template and that purification removed this substance, or that an active
fraction of the total DNA was obtained upon purification. Lindahl and Forchhammer (26) found that messenger RNA is more stable when transcription or translation is blocked, enabling messenger RNA to remain attached to the crude DNA. Recently, our laboratory presented evidence (11) that protein synthesis affected the kinetics of TLD in E. coli B and B/r, but it was pointed out that RNA synthesis could not be excluded.

In this paper, we will present information on another aspect of TLD. As shown by Maaløe and Hanawalt (28), cells which required an amino acid and uracil, as well as thymine, for growth did not lose colony-forming ability in the same manner as did cells which required only thymine. When these E. coli 15 cells were deprived of all their growth requirements, death ensued until a survival level of 1 to 3% was reached and then no further death was noted. When in the presence of the amino acid and uracil but in the absence of thymine, these cultures lost viability without exhibiting a surviving fraction. These data were interpreted (28) as indicating that a certain proportion of the bacterial population were “immune” to TLD when their DNA had completed a round of replication, but that RNA or protein synthesis, or both, was required to initiate a new round. Our previous findings (11, 13, 14) were on TLD in E. coli B and B/r. These organisms offer several advantages. First, they are not known to harbor a prophage, as does E. coli 15 (17, 31), which could affect macromolecular synthesis; second, synchronous populations of E. coli B and B/r are readily obtainable (20, 21); and third, these organisms exhibit two types of kinetics of TLD, sensitive and resistant (11, 13, 14). If the “immune state” is a result of a certain number of the cells in the population having completed a round of DNA replication, the immunity should be dependent on the position within the cell cycle. We have found that exponentially growing cultures of multiple auxotrophs of E. coli B and B/r exhibit an immune state and that the degree of immunity is strongly dependent on the growth rate. Although this immune state appeared to be related to the conditions which limited the synthesis of DNA, there was no position within the cell cycle of a synchronously growing population when the cells were more or less immune to thymine deprivation. Throughout this study, we compared the kinetics of TLD to the kinetics of inactivation with nalidixic acid (NA), a process which resembles TLD in some respects (5, 6, 9, 11, 15, 22).

MATERIALS AND METHODS

Bacterial strains. Thymine-requiring strains of E. coli B and B/r were obtained as previously described (13, 14). Additional auxotrophs of these strains were isolated by using a modified treatment with nitrosoguanidine (1, 29) followed by ampicillin enrichment (32). Care was taken to obtain single-step mutations; this was done by limiting the treatment with nitrosoguanidine such that very few mutants arose. Initially, pyrimidine-requiring mutants were selected which required both uracil and arginine (pyr A; 43). Additional amino acid or purine mutants were then obtained by subsequent treatment with nitrosoguanidine and then by ampicillin enrichment. In this manner we produced mutants which all required thymine, arginine, and uracil; additional requirements included histidine, leucine, proline, methionine, or adenine. All these mutants grew well in minimal medium with generation times of less than 60 min. The pyrimidine requirement was satisfied by either 10 μg of uracil or cytosine per ml; and the purine requirement was satisfied by 15 μg of adenine or guanine per ml. Amino acids were added at concentrations of 20 μg/ml for optimal growth. All these organisms exhibited absolute requirements for the supplements stated; after 18 hr of growth in the systematic absence of one requirement, turbidity increased at most two- fold. In the absence of the amino acid requirements, protein synthesis, as measured by 14C-leucine incorporation, was reduced 15-fold. These organisms were “stringent” (4, 40) in that total RNA synthesis was reduced eightfold, as measured by 14C-uracil incorporation in the absence of amino acids. A “relaxed” mutant of E. coli K-12 requiring arginine and methionine (obtained from B. Davis) exhibited at most a twofold reduction in RNA synthesis under the same conditions.

Growth and inactivation. The bacterial strains were aerobically grown in a phosphate salts minimal medium with either 0.5% glucose (generation time, 45 to 60 min) or 0.15% sodium acetate (generation time, 80 to 100 min) as a carbon source and assayed for viability on tryptone broth plates supplemented with thymine. Routinely, the bacteria were first grown in complete medium (14), adapted to minimal medium, and were then inoculated into the minimal medium. Growth was allowed for seven to nine generations until the titer reached 6 × 10^9 to 10 × 10^9 bacteria/ml. Inactivation kinetics were obtained by diluting the bacteria 800- to 3,000-fold into the appropriate minimal medium (minus thymine or in the presence of thymine and NA).

Preincubation of the bacteria in particular medium prior to inactivation was achieved by collecting the cells on a filter membrane (pore size, 0.60 μm; Millipore Corp., Bedford, Mass.) and then by resuspension in that medium. Some care had to be taken here to insure that we were not affecting inactivation by packing the cells on the membrane filter. The following procedure was used when bulk transfer of the bacteria from one medium to another was necessary. First, the membrane filter was wetted by filtration of sterile broth containing 0.8% tryptone (Difco), 0.7% NaCl,
and 30 μg of thymine per ml. Then the bacteria (6 × 10⁷ to 10 × 10⁹/ml in 25 to 35 ml) were collected and washed once with the minimal salts solution. The membrane filter was then placed into the appropriate medium. This entire operation was carried out in an environmental room at 37 C and took no more than 1 min. No other precaution was necessary for those bacteria which required only thymine. However, the strains which had additional requirements were not inactivated immediately after filtration in the same manner as they had been before. For example, E. coli BT−, when deprived of thymine, was inactivated without a lag (11, 13, 14). Cultures of E. coli BT− (AU)−, requiring arginine and uracil, also commenced inactivation immediately when diluted into thymineless medium. However, after this filtration procedure, E. coli B TAU was not inactivated immediately but only after a lag. This behavior was reminiscent of some strains which were inactivated best when in the presence of Casamino Acids (Difco; 13). Hence, when resuspending the multiple auxotrophic strains of B and B/r, a mixture of 10 to 12 amino acids (30 μg/ml each) was added. The only restriction in the types of amino acids in the mixture was that those used were not required by (or were not analogues of) the organism; therefore the mixture varied, depending on the strain. As expected, the inactivation kinetics were the same before and after filtration, and little if any loss in viability or cell number because of the filtration procedure was noted.

Measurement of DNA synthesis. DNA synthesis was measured by using either density labeling with ¹⁵N (obtained as 98% ¹⁵N-ammonium chloride from Isomet Corp.; 2.0 g/liter) or incorporation of ³H-thymine (obtained as thymine-methyl-³H from New England Nuclear Corp.; a concentration of 1.0 μC/ml in the presence of 30 μg of thymine per ml was adequate).

Density labeling. Samples (30 ml) were grown and medium changes were performed as described. At the end of the ¹⁵N incorporation period, 1 ml of 0.2 M NaCN (pH 8) was added and the sample chilled on crushed ice. The bacteria were harvested by centrifugation at 28,000 × g for 10 min in an International B20 Centrifuge. The pellets were washed and resuspended in 4 ml of DNA buffer [0.10 M NaCl, 3 mM ethylenediaminetetraacetate (EDTA), 0.02 M phosphate, pH 6.8]. Sodium dodecyl sulfate (150 mg) was added, and the bacteria were heated at 60 C for 5 to 10 min. The viscous, clear solution was then mixed with an equal volume of buffer-saturated phenol (30), and the DNA was extracted three times. The final DNA solution was then dialyzed against DNA buffer to remove residual phenol. (An alternate method using lysozyme-prepared protoplast for isolating DNA, which yielded results similar to those obtained on DNA extracted with phenol, is given below.) Approximately 2 μg of DNA per ml, as judged by optical density at 260 nm, was added directly to solid CsCl (optical grade; Gallard-Schlesinger Chemical Mfg. Corp.), final density 1.70 g/cm³ at 25 C. The samples were centrifuged in the four-place rotor of the Spinco model E for 18 to 20 hr at 104,000 × g, and pictures were taken by using monochromatic ultraviolet (UV) optics (12). Tracings of these pictures were then made on the Spinco Microdensitometer.

³H-thymine incorporation. As before, 30-ml cultures were used, and medium changes were performed by using 30 μg of thymine (1.0 μC/ml) to label the DNA. The extent of labeling was examined in two independent ways. Initially, we simply followed the kinetics of incorporation into intact cells. At various times, 2-ml portions were taken and placed into 2 ml of 10% trichloroacetic acid containing all the required supplements, and the mixture was chilled on crushed ice. The precipitated cells were collected on a Millipore membrane filter (pore size, 0.45 μm), and were washed five times with 15 ml of boiling water containing 30 μg of thymine per ml and once with cold ethyl alcohol. This procedure was necessary to remove all the unlabeled ³H-thymine. Similar precautions have previously been noted (25). The filters were dried in air, and the radioactivity was measured in a Beckman CPM-100 liquid scintillation counter by using toluene containing 15.1 g of 2, 5-diphenyloxazole per gal and 189 mg of 1, 4-bis-2-(5-phenyloxazoyl)-benzene per gal as counting fluid.

Alternatively, the amount of incorporation into DNA was determined directly. Samples (30 ml) were labeled and harvested as before, washed twice, and suspended in 1.5 ml of cold TCN solution [0.10 M tris (hydroxymethyl) aminomethane (Tris) and 5 mM NaCl (pH 8)]; 0.30 ml of lysozyme (0.80 mg/ml in TCN) was added and mixed at 0 C for 2 min; 0.30 ml of 0.07 M EDTA was then added, and the mixture was incubated at 25 C for 25 min to obtain protoplasts (37); 0.45 ml of 0.5 M K₂HPO₄ was added both to facilitate lysis of the protoplasts and to assist in the isolation of all the DNA (19). Solid CsCl (1.25 g per ml of solution to give a final density of 1.70 g/ml at 5 C) was added, and the solution was placed in the cold overnight, mixed again, and then centrifuged in an SW 39 rotor of a Spinco model L for 44 hr at 30,000 rev/min at 5 C. The tubes were punctured, and fractions were collected; 0.1-ml portions were precipitated with cold 5% trichloroacetic acid directly onto 1-inch (2.54-cm) squares of Whatman no. 3 filter paper, were washed twice with cold 5% trichloroacetic acid, twice with cold ethyl alcohol, once with cold ether (3), and were dried and counted as above.

Chemicals. NA (Sterling-Winthrop Research Institute, Rensselaer, N.Y.) was dissolved in 0.01 N NaOH (1 mg/ml) and then diluted into the appropriate media.

Chloramphenicol (CAP; Parke, Davis & Co., Detroit, Mich.) was used at concentrations varying from 10 to 50 μg/ml.

Mitomycin (MC; Kyowa Hakko Kogyo Co.) was dissolved in acetone at a concentration of 2 mg/ml, and then diluted into the appropriate media to a final concentration of 2 μg/ml for E. coli B and 15 μg/ml for strain B/r. The cells were incubated at 37 C for 25 min in the presence of thymine. This procedure resulted in a loss of viability to 0.005 to 0.05% survival (14).
RESULTS

Immune state. TLD in strains of *E. coli* B and B/r requiring thymine, arginine, and uracil was examined. Initially, glucose was used as a carbon source, and, in this medium, the generation times at 37°C for strains B and B/r were 41 to 44 min and 44 to 47 min, respectively. As expected (11, 13, 14), in the absence of all the required supplements, cultures of *E. coli* B commenced death immediately and *E. coli* B/r lost colony-forming ability after a lag of about 50 min. Inactivation proceeded (Fig. 1) to a survival level of 0.1% for strain B and 0.3% for strain B/r (Fig. 2).

Both these survival levels were less than that obtained with *E. coli* 15, but we will see later that this was probably because of differences in growth rate. In the absence of thymine but in the presence of arginine and uracil (not shown), inactivation proceeded beyond these levels in the same manner as the parental Thy− strains. Preincubation of these organisms for progressively longer times in the presence of thymine but in the absence of arginine and uracil led to increased survival levels (Fig. 1 and 2). After such preincubation for a time equivalent to two generations, *E. coli* B/r TAU, similar to *E. coli* 15, exhibited an immune state of about 60%. However, the inactivation of *E. coli* B TAU never exceeded 10% survival even after three generations of preincubation. This level of survival could be increased by altering the growth rate of the bacteria. When *E. coli* B TAU was grown in acetate medium (Fig. 3), the immune state was increased to about 2% without preincubation and to a maximum of about 35% after preincubation. It would appear that interference with protein and RNA synthesis in the presence of thymine gives rise to a population of cells immune to TLD. However, we examined the kinetics of inactivation of the parental Thy− strains in acetate medium (Fig. 4 and 5) and found that these cells, in spite of having no additional requirements for growth, also exhibited an "immune state"; 1 to 2% for strain B and 6 to 8% for strain B/r. Moreover, addition of arginine and uracil during TLD had no effect on the immune state noted for the auxotrophic strains. We therefore concluded that these *E. coli* strains

![Figure 1](http://jb.asm.org/)

**Fig. 1.** Control of immune state in *E. coli* B in glucose medium. *E. coli* B (TAU)− was grown to about $8 \times 10^9$/ml in glucose medium containing all the supplements, and was then transferred to a similar minimal medium which lacked arginine and uracil but contained 30 μg of thymine per ml. At the times indicated (0, 5, 40, and 90 min), 800- to 3,000-fold dilutions were made into medium lacking thymine, arginine, and uracil, and samples were taken at the indicated times for assay of viable bacteria.
were susceptible to immunity from TLD under conditions which limited initiation of DNA synthesis, but that the absence or presence of protein/RNA synthesis may not be a controlling factor. In addition, it should be noted that E. coli B differed from B/r in two ways: no lag in TLD was observed (11, 13, 14), and a smaller percentage of this bacterial population was susceptible to immunity. If a completion of a round of DNA replication is the only factor which influences immunity, it is difficult to understand why B should differ from B/r to the degree of immunity established after prolonged preincuba-
tion.

Conditions for the immune state. As indicated, preincubation in the presence of thymine led to a progressive increase in the number of cells immune to TLD. To explore whether the preincuba-
tion conditions could alter the ultimate immunity, additional mutants were necessary. The (TAU)− strains were again treated with nitrosoguanidine and ampicillin, and strains requiring another amino acid or adenine were isolated. These strains were then subjected to a variety of pre-
incubation conditions, and inactivation resulting from either thymine deprivation or NA treatment was then examined in glucose minimal medium. Both E. coli B and B/r exhibited an immunity to NA (11, 15) as well as to TLD (Table 1). The greatest degree of immunity was achieved whenever the required amino acids were absent from both the preincubation and the inactivation media. However, even in the persistent presence of the required amino acids, considerable immunity (2 to 3% for TLD or NA in strain B, 25% for TLD, and 7% for NA in strain B/r) was achieved. This immunity was always lower than that observed in the persistent presence of uracil, in sharp contrast with the results of Hanawalt (18), who suggested that the absence of uracil (i.e., RNA synthesis) protected E. coli 15 against

![Graph showing control of immune state in E. coli B TAU in acetate medium.](image)

**Fig. 3.** Control of immune state in E. coli B TAU in acetate medium. E. coli B (TAU)− was grown in acetate medium and then transferred to acetate medium lacking arginine and uracil and containing 30 μg of thymine. The degree of the immune state was then examined as before.

![Graph showing immune state in E. coli B in acetate medium.](image)

**Fig. 4.** Immune state in E. coli B in acetate medium. E. coli B T− and (TAU)− were grown in acetate medium and then diluted 800- to 3,000-fold into acetate medium lacking thymine but with the indicated supplements.
TABLE 1. Effect of amino acids* or uracil on immune state*

<table>
<thead>
<tr>
<th>Additions during preincubation to <em>Escherichia coli</em> strain</th>
<th>Per cent survival after preincubationβ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TLD</td>
</tr>
<tr>
<td></td>
<td>− T</td>
</tr>
<tr>
<td>B</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0.1</td>
</tr>
<tr>
<td>+ T</td>
<td>12</td>
</tr>
<tr>
<td>+ T + AA</td>
<td>3</td>
</tr>
<tr>
<td>+ T + U</td>
<td>18</td>
</tr>
<tr>
<td>B/r</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0.2</td>
</tr>
<tr>
<td>+ T</td>
<td>71</td>
</tr>
<tr>
<td>+ T + AA</td>
<td>25</td>
</tr>
<tr>
<td>+ T + U</td>
<td>66</td>
</tr>
</tbody>
</table>

* Strains used required uracil (U), thymine (T), and arginine plus one other amino acid (AA), i.e., histidine, leucine, or proline. The data reported here represent an average of at least two separate experiments on each of three different mutants, and approximately 10 to 15% variation from experiment to experiment was noted.

β Per cent survival after 4 hr of inactivation for strain B (generation time, 45 to 50 min), and 5 hr for strain B/r (generation time, 50 to 55 min). Preincubation was 110 min for strain B and 120 min for B/r. TLD, thymineless death; NA, nalidixic acid.

* Concentration was 30 μg/ml for strain B and 45 μg/ml for strain B/r.

TLD. When shifting from amino acid preincubation to the inactivating media containing uracil, or conversely, the degree of immunity was greatly reduced in both types of inactivation. Whether this was because of some rapid inactivating event or insufficient removal of either the amino acids or uracil when shifting to the inactivating medium is not clear. Filtration or a 3,000-fold dilution gave the same results as did an 800-fold dilution, indicating that the exogenous supplements were sufficiently depleted, but this could not eliminate the possibility that the intracellular concentrations were not reduced rapidly enough. It should also be pointed out that even after two generations of growth, no differences were observed in the cell number during all the preincubation conditions. These results supported the hypothesis (11) that inhibition of protein synthesis reduced inactivation because of either NA treatment or thymine deprivation.

Further examination of the preincubation conditions was performed with the strains which also required adenine (or guanine) for growth. Table 2 lists the degree of the immunity found under various conditions of preincubation. Here too, little change in cell number was detected under any of the preincubation conditions, even after 120 min. The greatest immunity to TLD was found after preincubation in the presence of thymine and adenine, but NA inactivation was most inhibited after preincubation with only thymine. Curiously, this maximal NA immune state was abolished when the inactivation medium contained adenine. Although the B/r strain exhibited greater immunity than did strain B, the general pattern of immunity established under the various preincubations was the same. It is not clear what the gross differences in immunity mean. For example, preincubation in the presence of thymine, adenine, and uracil but in the absence of arginine, a condition which should insure the completion of a round of DNA replication, yielded the least immunity to either TLD or NA. We will see shortly that the extent of DNA replication does not necessarily correlate with the degree of immunity established. Other factors may be involved. Neuhard (33) indicated that when cultures of *E. coli* were deprived of thymidine, the cells responded by accumulating deoxyadenosine triphosphate, and Biswas et al. (2) showed that thymine deprivation resulted in an 11-fold increase in ribonucleotide reductase activity which could greatly affect the pool sizes of the precursors for DNA synthesis (23). Perhaps the regulation of nucleotide pools is a prime factor in determining the degree of immunity to either TLD or NA treatment. The effect on the immunity to NA of adenine may be explained in this manner. It is obvious that immunity is not an entity in itself, but is subject to the conditions for both preincubation and inactivation.
Effect of synchrony on the immune state. As previously indicated, a critical test for ascertaining whether the state of DNA replication affects the degree of immunity to TLD or NA is to study the inactivation during the states of the cellgrowth cycle. Synchronous populations of E. coli B and B/r, requiring thymine, arginine, uracil, and an additional amino acid, were obtained in both glucose and acetate medium by using a well described method (20, 21). In glucose medium, samples were taken every 7 min during the growth cycle of 45 min, and the degree of immunity to TLD and NA was measured. After 4 hr for strain B, 0.1 to 0.3% immunity was observed throughout and 0.2 to 0.5% after 5 hr for strain B/r. These data were the same as those obtained in the exponential population (Table 1). An objection to these negative findings could be that the generation time (45 min) was too close to the time required for one round of DNA replication (41 min; reference 10). To circumvent this, we repeated the synchrony by using acetate as a carbon source. Here the generation times were about 80 min for strain B and about 100 min for strain B/r, and the time required to replicate DNA was not significantly altered (10). Samples were taken every 15 min during the cell cycle; 1 to 3% survival was obtained for strain B, and 4 to 11% for strain B/r at all times within the growth cycle. We conclude from these results that completion of a round of DNA replication is not a necessary factor for establishing immunity to either TLD or NA. Similar results on TLD have been found by Pierucci (personal communication), who used an independently isolated auxotroph of E. coli B/r with glucose and succinate as carbon sources.

DNA replication during preincubation. Inherent throughout these studies was the assumption that we were comparing immunity under different conditions of DNA replication. The degree of replication was measured in two independent ways. 3H-thymine incorporation into E. coli B TAUAde was determined, and the kinetics of incorporation are depicted in Fig. 6. As expected, continuous incorporation was found in cultures containing all the requirements. The culture containing thymine, adenine, and uracil but lacking arginine incorporated 3H-thymine for a time and then ceased incorporation at 40 to 50% of the control culture after one generation of growth. This is in good agreement with those data obtained previously (25, 28). Little incorporation was observed in cultures lacking adenine in the presence or absence of uracil. We could not detect great differences in labeling in the cultures both containing adenine, but one with and one without uracil. We considered the possibility that this was
from the difficulty of exhaustively removing all the unlabeled \(^3\)H-thymine. Consequently, the bacteria were isolated, and their DNA was extracted and banded in CsCl. The activity of the DNA made under various preincubation conditions is listed in Table 3, which includes the degree of immunity recorded after each preincubation. Again, the absence of adenine had more of an effect on the extent of labeling than did the absence of uracil. The differences observed in the labeling in the absence of either purines or pyrimidines may be related to the influence on nucleotide pools, or it may indicate that incorporation is influenced by turnover of ribosomal RNA. Doudney et al. (16) showed that there is a great deal of ribosomal degradation during TLD in the presence and in the absence of required amino acids. Nevertheless, the results clearly show the extent of labeling bears little relation to the degree of immunity.

**DNA replication during inactivation.** An attempt was made to study DNA replication in *E. coli* B and B/r TAU during regimes of amino acid or uracil starvation by using \(^1\)N incorporation to measure the degree of replication. As reported for *E. coli* 15 (5, 36), bacteria undergoing TLD or NA initiated new growing points, which is evidenced by the premature appearance of doubly labeled DNA. Other than to again demonstrate (25) that deprivation of the amino acid during either TLD or NA inactivation greatly limited the ultimate rate of DNA synthesis, it was not possible to examine critically the effects of amino acid or uracil on the labeling of DNA. For example, in the complete absence of uracil (and consequently cytosine), DNA was still replicated, as measured by both \(^1\)N- and \(^3\)H-thymine incorporation, after TLD, but was not labeled after NA inactivation (unpublished data). This may suggest that there is less breakdown of ribosomes during NA treatment than TLD (16).

Further analysis of DNA replication during inactivation was done with the parental Thy\(^-\) strains. It was found (Fig. 7) that TLD or NA prematurely led to the doubly labeled DNA. *E. coli* B, which was inactivated much earlier than strain B/r, was labeled about 20 min sooner than was B/r. There was no synergistic effect of NA and TLD; the presence or absence of thymine in the presence of NA yielded the same pattern.

<table>
<thead>
<tr>
<th>Preincubation (110 min)</th>
<th>Per cent (^3)H in DNA</th>
<th>Per cent survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TLD</td>
</tr>
<tr>
<td>+ T + Ade + U + A</td>
<td>100</td>
<td>0.1</td>
</tr>
<tr>
<td>+ T + Ade + U</td>
<td>24</td>
<td>0.5</td>
</tr>
<tr>
<td>+ T + Ade</td>
<td>15</td>
<td>30</td>
</tr>
<tr>
<td>+ T + Ade + A</td>
<td>6</td>
<td>22</td>
</tr>
<tr>
<td>+ T + U</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>+ T + U + A</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>+ T</td>
<td>2</td>
<td>18</td>
</tr>
</tbody>
</table>

* Abbreviations: DNA, deoxyribonucleic acid; TLD, thymineless death; NA, nalidixic acid; T, thymine; Ade, adenine; U, uracil; and A, arginine.

Protoplasts were prepared as described, and the DNA was centrifuged to equilibrium in CsCl. Fractions were collected and assayed for \(^3\)H. Of all the activity, 80 to 90% was found in the DNA banded at its density. About 5 to 20% of the activity was found at the meniscus, indicating that some DNA was trapped with a light-density component. The total counts obtained under each preincubation condition were then compared by using the normal growth condition as the standard (which yielded about 44,000 counts/min).

* Survival was measured in the absence of all the requirements, i.e., only thymine-inactivating medium was used (see Tables 1 and 2). The results reported here were obtained by using the B strain; similar patterns of survival were observed with the B/r auxotroph.
DNA replication patterns for TLD or NA. E. coli B and B/r were grown in glucose minimal medium and transferred to medium lacking thymine or containing thymine and NA at 30 μg/ml for the indicated times. The cultures were then transferred into the appropriate media containing thymine and 15N ammonium chloride. Except for the lower left set, where the effectiveness of NA in preventing DNA synthesis is presented, the cells were then transferred to media containing thymine and 14N for 40 min. The conditions for mitomycin C (MC) treatment are as indicated. L designates light DNA containing all 14N; H indicates DNA containing all 15N. Both methods for extracting the DNA (phenol or lysozyme) yielded similar patterns; the conditions for centrifugation are presented in the text.

Fig. 7. DNA replication patterns for TLD or NA. E. coli B and B/r were grown in glucose minimal medium and transferred to medium lacking thymine or containing thymine and NA at 30 μg/ml for the indicated times. The cultures were then transferred into the appropriate media containing thymine and 15N ammonium chloride. Except for the lower left set, where the effectiveness of NA in preventing DNA synthesis is presented, the cells were then transferred to media containing thymine and 14N for 40 min. The conditions for mitomycin C (MC) treatment are as indicated. L designates light DNA containing all 14N; H indicates DNA containing all 15N. Both methods for extracting the DNA (phenol or lysozyme) yielded similar patterns; the conditions for centrifugation are presented in the text.

of DNA replication. These patterns illustrate the fact that cytosine was also supplied by the uracil requirement in the TAU derivative strains. The density difference between the light and hybrid DNA in the parental Thy− strains was 6.1 mg/cm³. However, in the TAU strains, this density difference was 3.8 mg/cm³ and at times was as low as 2.6 mg/cm³. In comparing the effects of NA versus TLD on DNA replication, a difference was noted after mitomycin (MC) inactivation. E. coli B was inactivated by MC to about 0.01% and was then subjected to either NA or TLD for one generation, followed by incorporation of 15N for one generation. Essentially the same pattern of light, hybrid, and heavy DNA was obtained as without the prior MC treatment. However, after MC treatment, NA did not induce new growing points; in fact, no DNA was repli-
cated. *E. coli* B/r did not replicate DNA after MC plus NA or TLD treatment. This may reflect a difference in the inactivation mechanism of MC. For both B and B/r, we chose to achieve the same amount of inactivation by MC. This required about 2 \( \mu \)g of MC per ml for strain B, and 15 \( \mu \)g/ml for strain B/r. These were the conditions described (41) for differentiating monofunctional versus bifunctional (42) attack of MC on DNA; the mechanism of the action of NA is not known (6). These results on *E. coli* B after MC inactivation may imply that NA exerts a monofunctional attack on DNA and that NA, coupled with a prior monofunctional attack (limited MC treatment), inhibits subsequent DNA synthesis. Preliminary experiments indicated that NA does not specifically alter the density of double-stranded DNA with respect to single-stranded DNA, as does actinomycin D (7), but further work is necessary.

**Effect of CAP on DNA replication.** The replication patterns of \(^{15}\)N DNA in *E. coli* B under conditions which limit protein synthesis are illustrated in Fig. 8; the patterns obtained with B/r were essentially the same. When CAP was present at any time prior to \(^{15}\)N incorporation, the rate of incorporation was greatly decreased. After inactivation by either TLD or NA, the presence of CAP had little or no effect on the appearance of the prematurely doubly labeled DNA. The only difference noted between TLD and NA was that CAP had less of an effect on DNA synthesis when NA was used to inactivate (Fig. 8, series 3). This may again reflect some basic difference between the mechanism(s) involved in TLD or NA inactivation (11). Similar results on the effect of CAP in *E. coli* 15 undergoing TLD have also been reported (24). It is noteworthy that CAP had little effect on the replication of DNA after inactivation. We indicated (11) that the addition of thymine and CAP after inactivation led to a recovery of *E. coli* B. However, we should point out that thymine alone had less of an effect on recovery, and *E. coli* B/r

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**Fig. 8.** Effect of CAP on DNA replication patterns in *E. coli* B. *E. coli* B was grown and transferred as described in the legend to Fig. 7. Whenever \(^{15}\)N was present, thymine was also present. The concentration of chloramphenicol used varied from 10 to 50 \( \mu \)g/ml, with no apparent difference in the incorporation of \(^{15}\)N. In Fig. 7 and 8, we examined the kinetics of appearance of the heavy, and hybrid DNA; the relative concentrations of each peak are to be noted.
did not recover under any conditions. These results indicate that regardless of whatever is involved in recovery from either TLD or NA, some event other than DNA replication must also be necessary.

**DISCUSSION**

The results presented here were directed at examining the relationship between immunity to inactivation by either thymine starvation or NA and DNA replication. Some of the conditions which led to immunity were also those which allowed completion of a round of DNA replication. However, a major difficulty was the interpretation of conditions, since it was not possible to affect a single macromolecular event without affecting others (2, 16, 26, 33). Deprivation of uracil led to apparent scavenging, presumably because of ribosomal breakdown (16). Withdrawal of amino acids, or addition of CAP, could have had an effect on RNA (26, 34). From a variety of experiments, we concluded that there was no causal relationship between immunity and DNA replication. It may well be that some other macromolecular process is directly involved in these inactivations resulting from thymine starvation or NA. The presence of amino acids during both preincubation and inactivation decreased, but did not eliminate, immunity to either TLD or NA. Amino acids also prevented recovery from TLD (11), therefore protein synthesis may be directly involved in inactivation, immunity, and recovery. RNA synthesis cannot be directly eliminated, but we were not able to detect any difference between the inactivation or immunity of a "relaxed" strain of *E. coli* K-12 (4, 40; unpublished data).

The apparent effect of protein synthesis on inactivation may not necessarily be relevant. In terms of immunity, slow growth in acetate medium greatly affected the degree of immunity in both *E. coli* B and B/r. However, the presence or absence of arginine and uracil did not noticeably alter the degree of immunity. Even the parental Thy- strains exhibited considerable "immunity" in acetate medium. This could indicate that thymine deprivation affects the regulation of a critical process other than the direct synthesis of DNA. We might add that, in the course of our studies, thymineless death was also examined in an *E. coli* K-12 strain which was deficient in the synthesis of L-rhamnose-thymidine diphosphate, an intermediate in cell wall synthesis (35). This organism required only thymine and grew well in both glucose and acetate medium. Yet, we found that 15 to 20% of these bacteria were "immune" to TLD in glucose medium and 50 to 60% in acetate medium. These results serve to support the conclusion that TLD may involve processes which do not directly involve DNA replication.

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**LITERATURE CITED**


