Influence of Thymine Starvation on the Integrity of Deoxynucleobnucleic Acid in *Escherichia coli*

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The influence of thymine starvation on the single-strand molecular weight of deoxynucleobnucleic acid (DNA) from *Escherichia coli* was determined by sedimentation through gradients of alkaline sucrose. Growth of cells for as long as 150 min in thymineless medium did not significantly reduce the molecular weight below the control value of 2.4 ± 0.3 x 10^6 daltons. Incubation of cells in thymineless medium after exposure to 500 ergs/mm^2 of ultraviolet light or 20 krads of ^14^C gamma rays did not appear to block the rejoining of single-strand breaks associated with irradiation. Thus, DNA repair enzymes, presumably including DNA ligase, are not significantly inhibited by thymine starvation.

Freifelder (5) reported that thymine starvation of nonlysogenic *Escherichia coli* K-12 produced single-strand breaks in the exogenous sex factors F and F'lac. Since the breakage exhibited first-order kinetics at a rate proportional to molecular weight, a rate constant of 0.98 break per genome per min at 37 C was predicted for the *E. coli* chromosome. Interference with deoxynucleobnucleic acid (DNA) ligase as a consequence of thymine starvation was proposed as the cause of the strand breakage.

We tested the predicted rate constant in *E. coli* C thy-321 by determining the effect of thymine starvation on the sedimentation characteristics of DNA in alkaline sucrose gradients by the procedure developed by McGrath and Williams (12). In addition, we investigated the influence of thymine starvation on the sedimentation characteristics of cellular DNA in ultraviolet light (UV)- or gamma ray-exposed cells to determine the relationships between thymine starvation, excision repair of UV-irradiated DNA, repair of radiation-induced single-strand breaks, and DNA ligase.

**MATERIALS AND METHODS**

**Bacterial growth.** A thymine-requiring mutant of *E. coli* strain C was used in these studies. The derivation and properties of this strain, designated thy-321, were described previously (9, 21).

*E. coli* C thy-321 was grown in M-9 buffer (2) supplemented with glucose at a concentration of 0.04 or 1%. For normal growth, thymine was added at a final concentration of 5 μg/ml. All incubations were at 37 C in a rotary shaker water bath. The cell generation time, as measured by a doubling of the optical density at 420 nm, was 54 min in this medium.

Overnight cultures were grown in limited glucose (0.04%) medium to approximately 10^9 cells/ml. These cultures were then diluted 1/20 into complete glucose (1%) medium and grown to mid-log phase, about 3 x 10^9 cells/ml, for experimentation. If labeled cells were desired, thymine-methyl-^3^H (14.9 Ci/m mole, Schwarz BioResearch) was incorporated into the complete growth medium at a concentration of 20 μCi/ml. After growth to log phase, the cells were harvested by centrifugation, washed in unsupplemented M-9 buffer, recentrifuged, and then suspended in the desired medium.

In the thymineless studies, cultures were grown and harvested as described above, suspended at the same concentration in medium containing no thymine, and returned to incubation.

**Molecular-weight analysis.** The method of McGrath and Williams (12) was used for sedimentation of DNA through alkaline sucrose gradients. After the desired treatment (i.e., thymineless incubation or irradiation, or both) cells were converted to spheroplasts (19), layered onto 0.1 ml of 0.5 NaOH on top of a 4.5-ml gradient of 5 to 20% (w/v) sucrose adjusted to pH 12.5 with NaOH. Approximately 6 x 10^9 cells were placed on each gradient. The tubes were centrifuged in the SW 50.1 rotor of a Spinco model L2 ultracentrifuge for 90 min at 30,000 rev/min at 20 C.

After centrifugation, the tubes were punctured, and 10-drop fractions were collected directly into counting vials. Scintillation fluid was then added, and the samples were counted in a liquid scintillation counter. The counting solution was Triton X-100-toluene (1:2) with Packard Permafluor as the scintillator.

Bacteriophage T2 was lightly labeled with thymine-methyl-^14^C (30 mCi/m mole, Schwarz BioResearch) by using the precautions suggested by Thomas (22). The phage particles were placed in 0.1 ml of 0.5 NaOH...
for release and denaturation of the DNA. Centrifugation and radioactivity determinations were as previously described.

In the molecular-weight calculations from the sedimentation data, the value of $60 \times 10^6$ daltons for single-stranded T2 DNA was used (1, 13). The T2 value was also confirmed for this system by using λ-bacteriophage labeled with $^{14}C$-thymine.

For calculation of the molecular weights, the centroid of each profile was determined by the equation

$$X = \frac{1}{N} \sum_{i=1}^{N} x_i Y_i$$

where $X_i$ is the percentage of radioactivity found in gradient fraction number $Y_i$.

In the interest of reproducibility, the counts in fractions corresponding to fragments with molecular weights of $10^6$ daltons, or less, were not included in the calculations.

Relating the centroid determined from equation 1 to the molecular weight value of $60 \times 10^6$ daltons for single-stranded T2 DNA, the molecular weight for a particular sedimentation profile was determined from the equation

$$D_1/D_2 = \left(\frac{M_1}{M_2}\right)^{0.38}$$

A more complete description of this method of calculation has been presented by Rupp and Howard-Flanders (19).

Irradiation. For ultraviolet irradiation, labeled cultures were harvested, washed, and suspended in unsupplemented M-9 buffer at a concentration of $2 \times 10^7$ to $3 \times 10^8$ cells/ml. The cell suspension was then exposed, while being stirred in a sterile petri dish, to two Sylvania germicidal lamps at a distance of 95 cm. The average incident dose rate was 10 ergs per mm$^2$ per sec as measured by chemical actinometry with ferrioxalate (17).

Cells were exposed, in unsupplemented M-9 buffer, to gamma radiation from a $^{60}Co$ source. The dose rate was 6,700 rad/min as determined by lithium fluoride thermoluminescent dosimetry.

For postirradiation incubation, the M-9 buffer was replaced with the desired medium. For zero time samples, cells were taken from the irradiation device directly to an ice bath for spheroplasting.

RESULTS

Although the properties of this bacterial strain were previously described (21), certain properties are critical to this work (Fig. 1a and b). For the data in Fig. 1a, the cells were incubated either in complete medium or in medium lacking thymine, and cell viability was measured periodically. These cells undergo thymineless death in the classical manner (4, 11, 21), with a half-time of about 20 min after a 60-min lag period.

In their recent paper, Medoff and Overholt (14) emphasized that in any study of thymineless

![Fig. 1. Effects of thymine starvation on viability and growth of E. coli C thy-321. (a) Kinetics of thymineless death for this strain. Exponentially growing cells were transferred to complete medium without thymine and incubated for various periods of time before plating on nutrient agar plates. The zero time value was taken as 100% survival, and all other values were related to it. Symbols: $\times$, increase in cell number associated with normal growth; $\bullet$, decrease in cell viability in thymine-starved cells. (b) Optical density at 420 nm plotted against time. The data indicate no difference in cells in medium with or without thymine. Further, addition of thymine to cells starved for thymine for 120 min produced no change from control for the next 240 min.](http://jb.asm.org/Downloaded from)
death, the strain should be tested for induction of phage. Fig. 1b shows the results of such a test. Cells were incubated in complete medium or in medium lacking thymine, and the changes in their optical density at 420 nm were periodically determined. At 2 hr, thymine was added to one of the thymineless cultures, and incubation was continued for 4 hr. If the cells were lysogenic, lysis should have taken place under these conditions (10). Electron microscopy after thymineless incubation also failed to show any phagelike particles after attempted induction of this strain (8).

Figure 2 shows typical sedimentation patterns for 14C-thymine-labeled DNA of T2 (Fig. 2a) and 3H-thymine-labeled DNA of untreated control cells (Fig. 2b). By using the equations given previously with sedimentation profiles such as these, the molecular weight of E. coli C thy-321 DNA appears to be about 2.4 ± 0.3 × 10^6 daltons. The mean and standard deviation were determined from 20 analyses performed over 12 weeks. The values are in good agreement with those given by McGrath and Williams (12, 13). Thus, this system appears to analyze fragments representing about one-sixth of a single strand of the E. coli chromosome (3, 12).

Table 1 shows the results of a series of studies in which the cells were incubated in the absence of thymine. Labeled, exponentially growing cells were transferred into medium lacking thymine and then incubated for various periods. Although the values are somewhat less than the 2.4 × 10^6 dalton figure for control, only the 60-min value is outside the one standard deviation range of control. Thus, a progressive accumulation or production of single-strand breaks did not accompany 150 min of thymineless incubation.

The postulated mechanism for the repair of radiation damage to DNA involves a sequence of steps (7). First the damage must be recognized. The damaged portion is then excised, and a new portion is synthesized. The rejoicing of the newly synthesized region to the original parental strand represents the final step in the repair process. The enzyme implicated in this final step is DNA ligase (16). If this enzyme is inhibited by thymine starvation, as suggested by Freifelder (5), incubation for various periods. Although the values are somewhat less than the 2.4 × 10^6 dalton figure for control, only the 60-min value is outside the one standard deviation range of control. Thus, a progressive accumulation or production of single-strand breaks did not accompany 150 min of thymineless incubation.

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**Table 1. Summary of molecular weights after thymine starvation**

<table>
<thead>
<tr>
<th>Time of thymine starvation (min)</th>
<th>Molecular weight (daltons)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.4 ± 0.3 × 10^6</td>
</tr>
<tr>
<td>30</td>
<td>2.1 × 10^6</td>
</tr>
<tr>
<td>60</td>
<td>2.0 × 10^6</td>
</tr>
<tr>
<td>90</td>
<td>2.2 × 10^6</td>
</tr>
<tr>
<td>120</td>
<td>2.2 × 10^6</td>
</tr>
<tr>
<td>150</td>
<td>2.3 × 10^6</td>
</tr>
</tbody>
</table>

*Mean and standard deviations based on 20 sedimentation profiles.

![Fig. 2. Alkaline sucrose gradient sedimentation profiles for DNA from T2 bacteriophage and E. coli C thy-321.](http://jb.asm.org/DownloadedFrom)
tion of irradiated cells in thymineless medium might then result in an increase in the number of single-strand breaks in the DNA, assuming that the preceding steps of the repair sequence occur normally in spite of inhibition of the final step. Shuster and Boyce (20) have shown that at least one E. coli strain is capable of excising dimers during thymine deprivation.

Figures 3 and 4 show the results of a series of experiments to test this hypothesis of ligase inhibition. Labeled, exponentially growing cells were exposed to either 500 ergs/mm² of UV radiation, or 20 krad of gamma radiation. The samples were then incubated for various time periods. Figures 3a and 4a show the sedimentation profiles for cells analyzed immediately after irradiation. In each case, the position of the peak indicates a reduction of molecular weight due to the introduction of single-strand breaks. Figures 3b and 4b show the results of growth in complete medium for 60 min after exposure. For the profiles in Fig. 3c and 4c, the cells were irradiated and incubated after irradiation as before, except that the cells were in thymineless medium.

Table 2 summarizes the molecular-weight values for Fig. 3 and 4. The zero time values for both figures show a reduction of molecular weight due to the induction of single-strand breaks. After a 60-min incubation in complete medium, there is an increase in molecular weight to a value approaching that of control (Table 1). Also, the shapes of the sedimentation profiles change from the almost symmetrical distributions seen immediately after irradiation to the asymmetrical shape characteristic of normal E. coli DNA (Fig. 2; 13). Intermediate time studies showed that this increase is rapid in the first few minutes after irradiation, and then gradually slows as the control value is approached. Cells incubated in thymineless medium also undergo the increase in molecular weight with postirradiation incubation. Cells exposed to gamma radiation showed the same molecular weights, whether or not thymine was present. There is a difference in the weights for the UV-irradiated cells, but both values do represent an increase from the zero time values.

**DISCUSSION**

Freifelder (5) reported that thymine starvation caused changes in the sedimentation properties of exogenotic covalent circular sex factors which were consistent with a conclusion that single-strand breaks are caused in DNA by thymine starvation. This conclusion supported several earlier suggestions by others implicating single-strand break production in thymineless death. To account for their observation of decreased viscosity and increased sensitivity to shearing forces in the DNA obtained from thymine-starved cells,

![Fig. 3. Alkaline sucrose gradient sedimentation of DNA from ultraviolet light-irradiated cells. Radioactivity on the ordinate is plotted against fraction number on the abscissa with fraction 1 being the bottom of the tube. The cells in part a were irradiated and spheroplasted immediately. In part b, the cells were incubated for 60 min in complete medium before spheroplasting. In part c, the cells were incubated for 60 min in medium complete except for thymine. Molecular weights for the profiles are shown in Table 2.](image-url)
Menningmann and Szybalski (15) suggested that thymineless death might involve alteration of DNA structure. Hanawalt (6) suggested that DNA breaks might be associated with continuing ribonucleic acid transcription on which thymineless death was found to be dependent. The subsequent observation of repair replication in thymine-starved cells by Pauling and Hanawalt (18) further supported the expectation that single-strand breaks are produced in the DNA of thymine-starved cells.

Freifelder (5) determined that 0.029 breaks per 7.4 × 10⁶ daltons per min at 37°C occurred in F'lac during thymine starvation. Extending this value to a whole E. coli chromosome, 20 to 25 breaks per 2.4 × 10⁷ daltons should have been observed after 150 min of growth in thymineless media. The data presented in Table 1 failed to indicate any significant reduction in molecular weight as measured by sedimentation through gradients of alkaline sucrose. Thus, thymineless death in E. coli C thy-321 is not accompanied by single-strand breakage at the frequency predicted by Freifelder (5).

Freifelder's (5) suggestion that DNA ligase might be inhibited during thymine starvation was very appealing in the context of repair of radiation damage, since this enzyme is thought to be required to complete the repair of both UV and X-ray damage. Our data (Fig. 3 and 4, Table 2) are the results of some experiments to examine the effect of thymine starvation on the rejoining of single-strand breaks associated with irradiation. After exposure to UV, the single-strand molecular weight is reduced while excision repair takes place, with 15 to 18 strand breaks per chromosome (Table 2) apparent at any time before completion. This is in good agreement with the value of 15 strand breaks given by Setlow, Carrier, and Williams (Biophys. Soc. Abstr. 11th Annu. Meeting, p. 83, 1967). We assume that the molecular-weight reduction at time zero represents the very rapid onset of excision, even at low

**Table 2 Summary of molecular weights after irradiation**

<table>
<thead>
<tr>
<th>Sample (min)</th>
<th>Ultraviolet, 500 erg/mm² (daltons)¹</th>
<th>Gamma, 20 krad (daltons)²</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.6 × 10⁶</td>
<td>1.2 × 10⁶</td>
</tr>
<tr>
<td>60</td>
<td>2.1 × 10⁶</td>
<td>1.9 × 10⁶</td>
</tr>
<tr>
<td>60 (Thy⁻)</td>
<td>1.8 × 10⁶</td>
<td>1.9 × 10⁶</td>
</tr>
</tbody>
</table>

¹ Molecular-weight values for the profiles in Fig. 3.
² Molecular-weight values for the profiles in Fig. 4.

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Fig. 4. Alkaline sucrose gradient sedimentation of DNA from gamma-irradiated cells. Radioactivity on the ordinate is plotted against fraction number on the abscissa with fraction 1 being the bottom of the tube. The cells in part a were irradiated and spheroplasted immediately. In part b the cells were incubated for 60 min in complete medium before spheroplasting. In part c, the cells were incubated 60 min in medium complete except for thymine. Molecular weights for profiles are shown in Table 2.
temperature. By 60 min, the molecular weights of cells grown in complete or thymineless media seem to be returning to the control value. Thymineless incubation may extend the time for completion of dark repair, but it does not cause the accumulation of broken regions that would be expected if the incision-excision steps continue while the rejoining step was blocked. After gamma-ray exposure, the rejoining process took place at identical rates in complete and thymineless media. Since the role of DNA ligase in DNA repair is not known, these results do not permit a conclusion that thymine starvation does not inhibit the effective participation of DNA ligase in repair of radiation damage. However, the results show that the changes in single-strand molecular weight after irradiation are not markedly influenced by thymine starvation. Therefore, the enzymes involved in repair are probably not subject to inhibition by thymine deficiency.

Our investigation does not suggest why exogenous sex factors are broken in thyme starved E. coli K-12, but chromosomal DNA of E. coli C thy-321 is not broken. Possible strain differences could be investigated by examining the fate of exogenetic sex factors in E. coli C thy-321.

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LITERATURE CITED