Studies On The Effect OF Azaserine On DNA

Charles Zucker
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STUDIES ON THE EFFECT OF AZASERINE ON DNA

Submitted in Partial Fulfillment of the Requirements for Graduation with Honors to the Department of Biology at Carroll College, Helena, Montana

Charles Zucker
March 24, 1981

Research performed at Argonne National Laboratory under the supervision of Dr. Herbert E. Kubitschek.
This thesis for honors recognition has been approved for the Department of Biology.

Dr. John Christensen, Director

Mr. Guido Bugni

Father Joseph Harrington

March 16, 1981

Date
ACKNOWLEDGEMENTS

Many thanks to Herbert E. Kubitschek for his advice on all phases of the research and to Donna Williams-Hill for her kind encouragement.
ABSTRACT

Azaserine (O-diazoacetyl-L-serine) is known to be a non-competitive inhibitor of two enzymes necessary for de novo purine biosynthesis. Work done in this laboratory and others, however, indicates that much of azaserine's effect in cytotoxicity, mutagenesis and carcinogenesis can be attributed to its action on DNA. DNA repair-deficient strains of *Escherichia coli* are found to be more sensitive to azaserine cytotoxicity and, in cases, to azaserine mutagenesis than the wild type *E. coli*. Attempts at showing an in vitro effect of azaserine on DNA have not been conclusive as of yet. An interesting aspect of azaserine cytotoxicity is that it can be partially reversed by growth in low levels of the alkylating agent N-methyl-N'-nitro-N-nitroso-guanidine. This adaptive response suggests that part of the in vivo action of azaserine involves an alkylation of the DNA.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>i</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF ILLUSTRATIONS</td>
<td>v</td>
</tr>
<tr>
<td>INTRODUCTION AND LITERATURE REVIEW</td>
<td>1</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>6</td>
</tr>
<tr>
<td>Media, Reagents, and Strains</td>
<td>6</td>
</tr>
<tr>
<td>WP100 Survival Curves</td>
<td>6</td>
</tr>
<tr>
<td>Adaptation</td>
<td>7</td>
</tr>
<tr>
<td>Thin Layer Chromatography</td>
<td>8</td>
</tr>
<tr>
<td>Transformation</td>
<td>8</td>
</tr>
<tr>
<td>UV Absorption Spectra of DNA</td>
<td>9</td>
</tr>
<tr>
<td>T4 DNA Melting Curves</td>
<td>9</td>
</tr>
<tr>
<td>RESULTS</td>
<td>10</td>
</tr>
<tr>
<td>WP100 Survival Curves</td>
<td>10</td>
</tr>
<tr>
<td>Adaptation</td>
<td>10</td>
</tr>
<tr>
<td>Thin Layer Chromatography</td>
<td>10</td>
</tr>
<tr>
<td>Transformation</td>
<td>11</td>
</tr>
<tr>
<td>UV Absorption Spectra of DNA</td>
<td>11</td>
</tr>
<tr>
<td>T4 DNA Melting Curves</td>
<td>11</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>20</td>
</tr>
<tr>
<td>LITERATURE CITED</td>
<td>25</td>
</tr>
<tr>
<td>APPENDIX</td>
<td>A-1</td>
</tr>
</tbody>
</table>

iii
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>The effect of azaserine treatment of DNA on transformation in B. subtilis</td>
<td>16</td>
</tr>
<tr>
<td>2</td>
<td>Absorption spectra of calf thymus DNA</td>
<td>17</td>
</tr>
<tr>
<td>Figure</td>
<td>Illustration Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>------------------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>1</td>
<td>Azaserine, a glutamine analog</td>
<td>12</td>
</tr>
<tr>
<td>2</td>
<td><em>Escherichia coli</em> WP100 (uvrA, recA) survival curves for 0.1 ug/ml azaserine challenge</td>
<td>13</td>
</tr>
<tr>
<td>3</td>
<td>Adaptation to either MNNG or azaserine challenge by growth in low levels of MNNG</td>
<td>14</td>
</tr>
<tr>
<td>4</td>
<td>Adaptive effect of azaserine on <em>E. coli</em> WP2</td>
<td>15</td>
</tr>
<tr>
<td>5</td>
<td>Melting curves for T4 DNA</td>
<td>18</td>
</tr>
<tr>
<td>6</td>
<td>Nucleophilic attack on azaserine</td>
<td>19</td>
</tr>
</tbody>
</table>
INTRODUCTION AND LITERATURE REVIEW

The goal of this investigation was to determine the mechanisms of azaserine mutagenesis and azaserine cell toxicity. Azaserine (O-diazoacetyl-L-serine) was first discovered in a crude extract of a *Streptomyces* filtrate that had the ability to inhibit Sarcoma 180 (26). The compound was isolated from this crude extract and its structure was determined to be an alpha-diazoester analog of the amino acid L-serine (26). Early investigations with azaserine quickly established that the chemical inhibited *de novo* purine biosynthesis (1). Azaserine resembles the structure of glutamine (Fig. 1) which serves as the nitrogen donor in the biosynthesis of the purine ring. Glutamine and azaserine compete for the active site of two enzymes responsible for catalyzing the addition of nitrogen to the purine ring. Once at the active site azaserine acts as a non-competitive inhibitor by forming a thiol-ether linkage with a cysteine residue near the active site of the enzyme (17).

In addition to its inhibition of purine biosynthesis, azaserine has been shown to be a pancreatic carcinogen in rats (19) and able to cause single-strand DNA breaks in rat liver cells (18). Previous work done in this laboratory has shown that DNA repair-deficient strains of *Escherichia coli* (Migula) Castellani and Chalmers are more sensitive to azaserine cytotoxicity than the wild type *E. coli*. DNA repair-
deficient strains of *E. coli* are generally more susceptible to azaserine induced mutagenesis than wild type cells, although there is a dependence on error-prone repair for such mutagenesis (personal communication, H. E. Kubitschek).

Certain diazo-compounds act as alkylating agents of DNA precursors (11, 12). The possibility that azaserine acts as an alkylating agent is currently being investigated at Dartmouth Medical School using radioactive azaserine. Results to date indicate there is an alkylation adduct, although the structure and biological significance of this adduct has not yet been determined (personal communication, T. J. Curphey).

Experiments were designed to show both the *in vivo* and *in vitro* effects of azaserine on DNA. Survival to azaserine challenge was determined for *E. coli* WP100, a DNA repair-deficient strain of *E. coli*, to confirm earlier results. Stationary phase cells were used for determining survival so that azaserine inhibition of purine biosynthesis would not be a toxic factor. Strain WP100 is deficient in UV excision repair and recombination repair (13). Because it has been suggested that cell transport systems play a large role in azaserine toxicity (3, 28, 29), survival curves were also done using permeabilized WP100. A permeabilized cell will allow small molecular weight compounds like azaserine to pass through the cell membrane. Permeabilization served to lessen any effect membrane transport systems or other cellular intermediates may have had in azaserine toxicity.
Perhaps the most interesting studies were those done on the adaptive response. The adaptive response was first discovered by Cairns and Sampson in 1977 (4, 23). It is the ability of *E. coli* cultures to become more refractory to the cytotoxic and mutagenic effects of alkylating agents when previously grown in a media containing low concentrations of the alkylating agent. Generally, a low concentration of the chemical to be tested is added to an exponentially growing culture. This is the adaptive dose. After 90 minutes (1 - 2 cell doublings), a high concentration of the same chemical is introduced. This is the challenge dose. If the chemical induces the adaptive response the culture will be more resistant to any cytotoxic or mutagenic effects of the chemical. The mechanism of the adaptive response is not yet known and attempts to link it with previously discovered DNA repair pathways have failed (7, 8, 9, 24). It has been shown, however, that adapted cells are more efficient at removing O\(^6\)-methylguanine than nonadapted cells (25). If N-methyl-N'-nitro-N-nitroso-guanidine (MNNG) induced cells were more resistant to azaserine cytotoxicity it could be inferred that at least some of the *in vivo* effect of azaserine is alkylation of DNA.

Various *in vitro* studies were performed to investigate the action of azaserine on DNA. Thin layer chromatography was done on a reaction mixture of azaserine and mononucleoside. Any new component appearing in the reaction mixture would indicate that azaserine was reacting with the mono-
nucleoside to form a modified mononucleoside. Since it is quite possible that azaserine reacts differently with the mononucleosides than with the polynucleotide the interaction of azaserine and DNA was also measured. Three methods were used to measure any changes that occurred in the DNA. First, the effect of azaserine on the transforming ability of DNA was measured. Transformation is the ability of a bacterial cell to incorporate DNA from the external environment and use parts of that DNA, changing its genetic structure and the proteins it produces. We used DNA from Bacillus subtilis (Ehrenberg) Cohn that contained the genetic information necessary to synthesize methionine and tryptophan. If azaserine reacted with the DNA \textit{in vitro} it would be expected to inactivate some of this DNA and decrease the transformation efficiency. The \textit{B. subtilis} strain used for transformation experiments also was deficient in UV excision repair. This guarded against the possibility that the cell would simply repair DNA lesions and use it anyway.

Secondly, UV absorption spectra between azaserine-treated and untreated DNA were compared. Any significant change in the structure of DNA can be detected by comparing ratios of optical density (OD) measurements taken at different wavelengths (27).

Observation of the melting temperature of DNA was the third parameter used to measure the effect of azaserine on DNA. When double stranded DNA is heated, the hydrogen bonds holding the base pairs are weakened and the DNA unwinds.
This phenomenon is commonly called "DNA melting." As DNA melts it absorbs more light at 260 nm. This hyperchromic shift is caused by the changing electronic interactions and resonance patterns when double-stranded DNA changes to single stranded form (17). If azaserine forms an adduct on the DNA one might notice two effects. The $T_m$ (midpoint melting temperature) would be lower or higher, depending on the nature of the adduct. An alkylation adduct which disturbs base pairing would lower the $T_m$ whereas an adduct that cross-linked the DNA helix would raise the $T_m$. The amount of hyperchromic shift might also decrease if an alkylation adduct had disturbed the base pairing. This effect would occur because the DNA strands would have partially separated before melting had occurred. Therefore, the amount of hyperchromic shift observed during melting would be less than the amount seen in unmodified DNA.
MATERIALS AND METHODS

Media, Reagents and Bacterial Strains

Azaserine, all mononucleosides, and all amino acids were purchased from Calbiochem Corp. Calf thymus DNA was obtained from Worthington Biochemical Corporation, N-methyl-N'-nitro-N-nitroso-guanidine (MNNG) from Mann Research Laboratories, and nucleic acids from Nutritional Biochemical Corporation. Inorganic chemicals were from Fisher. Nutrient Agar and Bacto-Agar were from Difco. Thin layer chromatography paper was obtained from EM Reagents, and cellulose dialysis tubing from Spectrum Medical Industries.

*Escherichia coli* strains WP2 (trp<sup>-</sup>) and WP100 (uvrA, recA) were kindly provided by H. E. Kubitschek. *Bacillus subtilis* GSU1027 (uvrA) and *B. subtilis* BC92 DNA were kindly provided by J. G. Peak.

WP100 Survival Curves

Stationary phase cultures were used in azaserine toxicity experiments. Cells were grown on a minimal medium (see Appendix, p.A-2) inoculated with a single colony isolate of WP100. Cultures were incubated at 37°C with mild agitation for 18-21 hrs. A 6-ml aliquot of the culture was removed and centrifuged. The pellet was resuspended in an equal volume
of M9 medium (see Appendix, p. A-2). The resuspended culture, approximately 1-2 x 10^9 cells/ml, was starved for 1 hr at 37°C to insure that cells were in stationary phase. Cultures were challenged with azaserine at 23°C for various periods of time.

To determine survival of permeabilized cells, the starved culture was centrifuged and the pellet resuspended in an equal volume of 40mM Tris (2). This step was repeated. Finally the culture was centrifuged and the pellet resuspended in M9 medium. The culture was then challenged with azaserine at 23°C. To determine cell survival, appropriate dilutions of the azaserine-challenged culture were made and 0.1 ml of the diluted culture was plated onto nutrient agar. Azaserine was diluted by at least 10^4 by this procedure. Colonies were counted after 24 hrs at 37°C.

**Adaptation**

Adaptation trials for cell survival were carried out with wild type strain WP2. Cultures made by serial dilutions into minimal medium from a nutrient broth culture were grown overnight at 37°C. The culture with approximately 1 x 10^9 cells/ml was then diluted 100-fold into fresh minimal medium. The diluted culture was incubated at 37°C and growth was followed by measuring OD_{600} on a Zeiss spectrophotometer. At OD_{600} ca. 0.04 (approximately 5 x 10^7 cells/ml) an adaptive concentration of either MNNG or azaserine was added. After 90 min of growth the culture was challenged with azaserine or MNNG. For cultures adapted with azaserine exogenous
purines were present in the growth medium at concentrations of 40 ug/ml adenine and 25 ug/ml guanine. Percentage survival was computed by the formula:

\[
\text{Number of survivors in challenged cultures} \times \frac{100}{\text{Number of survivors in unchallenged cultures}}
\]

Thin Layer Chromatography

Five mM concentrations of mononucleosides were reacted with 50 mM azaserine for 18 hrs at 37°C. Thin layer chromatography was done on the reaction mixtures to determine whether or not any products had been formed. The reaction mixtures were spotted on a silica gel 60 F-254 (0.2 mm thick) plate. The solvent n-BuOH:EtOH:H₂O, in a 4:1:5 ratio (upper phase) was used for separating the reaction mixture.

Transformation

Details on transformation procedures were kindly communicated by M. J. and J. G. Peak (see Appendix, p.A-2). DNA from \textit{B. subtilis} BC92 (wild type) was treated with azaserine \textit{in vitro}. The DNA was then dialyzed against SSC (see Appendix, p. A-2) to remove the azaserine. After a thorough dialysis (48 hrs) the DNA was added to \textit{B. subtilis} GSU1027 (uvrA) competent cell cultures. Transformation efficiency was measured using the methionine and tryptophan markers by plating onto Spizizen plates lacking either methionine or tryptophan. Plates were incubated for 48 hrs at 37°C and colonies counted.
UV Absorption Spectra of DNA

Calf thymus DNA in 0.1x SSC was treated with 500 ug/ml azaserine for 30 min and dialyzed for 48 hrs. Control DNA was not treated with azaserine but went through the same dialysis procedure. The UV absorption spectrum of the azaserine DNA was compared to the UV absorption spectrum of the control DNA.

T4 DNA Melting Curves

*Escherichia coli* bacteriophage T4 DNA with $^{14}$C labeling was extracted and purified by D. M. Williams-Hill. T4 DNA ($^{14}$C) was treated with azaserine and thoroughly dialyzed. Control DNA was not treated with azaserine but went through the same dialysis procedure. The OD$_{260}$ was measured with a variable temperature cuvette assembly provided by H. E. Kubitschek. The relative concentrations of DNA in various samples were determined from measurements of radioactivity using a Packard Model 3330 Tri-Carb Scintillation Counter. The $T_m$ and the percentage of hyperchromic shift were determined for the control DNA and the azaserine treated DNA.
RESULTS

WP100 Survival Curves

The survival of WP100 (uvrA, recA) exposed to 0.1 ug/ml of azaserine is shown in Fig. 2. The cytotoxicity of azaserine in the DNA repair deficient strain (WP100) was approximately 1500 times greater at the 10% survival level than the cytotoxicity of azaserine in the wild type (WP2). There was no significant difference in survival between the Tris washed cultures and the cultures kept in M9 medium.

Adaptation

MNNG-induced adaptation to challenge doses of either MNNG or azaserine is shown in Fig. 3. The challenge dose was normally 1 mg/ml * 7 min for azaserine or 100 ug/ml * 5 min for MNNG. Not shown on the graph are results of cultures adapted with azaserine and challenged with azaserine. These cultures showed a small adaptive effect equal to approximately one-third of the adaptive effect induced with MNNG (Fig. 4).

Thin Layer Chromatography

Thin layer chromatography of the reaction mixtures revealed no reaction products for any of the mononucleosides.
Transformation

The data obtained from the transformation experiment is summarized in Table 1. No significant decrease in the number of met\(^+\) or trp\(^+\) transformations due to azaserine treatment was observed.

UV Absorption Spectra of DNA

A comparison of absorption ratios is shown in Table 2. Note the differences in the OD\(_{240}/\text{OD}_{260}\).

T4 DNA Melting Curves

No difference in either the \(T_m\) (midpoint melting temperature) or the amount of hyperchromic shift was detected in comparing the azaserine-treated and untreated DNA (Fig. 5).
Figure 1. Azaserine, a glutamine analog.
Figure 2. Escherichia coli WP100 (uvrA, recA) survival curves for 0.1 ug/ml azaserine challenge. Stationary phase cells were washed three times before challenge (see Materials and Methods). Survival was determined by colony formation on nutrient agar plates.
Figure 3. Adaptation to either MNNG or azaserine challenge by growth of E. coli in low levels of MNNG. Challenge was with either 100 μg/ml MNNG for 5 min or 1 mg/ml azaserine for 7 min.
Figure 4. Adaptive effect of azaserine on E. coli WP2. Minimal medium was supplemented with 40 \( \mu g/ml \) adenine and 25 \( \mu g/ml \) guanine. Challenge was with 1 mg/ml azaserine for 5 min. Cultures show a small adaptive effect when previously grown in media containing a low concentration of azaserine.
<table>
<thead>
<tr>
<th>Azaserine ug/ml</th>
<th>Transformants met⁺/ml</th>
<th>Transformants trp⁺/ml</th>
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<tbody>
<tr>
<td>0</td>
<td>53</td>
<td>24</td>
</tr>
<tr>
<td>10</td>
<td>57</td>
<td>29</td>
</tr>
<tr>
<td>100</td>
<td>45</td>
<td>31</td>
</tr>
<tr>
<td>1,000</td>
<td>36</td>
<td>71</td>
</tr>
<tr>
<td>10,000</td>
<td>46</td>
<td>66</td>
</tr>
</tbody>
</table>

Table 1. The effect of azaserine treatment of DNA on transformation in *B. subtilis*. *Bacillus subtilis* BC92 DNA was treated in *vitro* with varying concentrations of azaserine. The BC92 DNA was then used to transform *B. subtilis* GSU1027 (met⁻, trp⁻, uvr⁻) into met⁺ or trp⁺.
Table 2. Absorption spectra of calf thymus DNA. Experimental DNA was treated with 500 μg/ml azaserine for 30 min. Both the control DNA and the experimental DNA were thoroughly dialyzed against 0.1x SSC.

<table>
<thead>
<tr>
<th></th>
<th>Control DNA</th>
<th>Experimental DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak</td>
<td>259</td>
<td>257-259</td>
</tr>
<tr>
<td>Inflection Point</td>
<td>229-231</td>
<td>230-233</td>
</tr>
<tr>
<td>OD\textsubscript{270}/OD\textsubscript{260}</td>
<td>0.83</td>
<td>0.83</td>
</tr>
<tr>
<td>OD\textsubscript{240}/OD\textsubscript{260}</td>
<td>0.59</td>
<td>0.65</td>
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</tbody>
</table>
Figure 5. Melting curves for T4 DNA. T4 DNA was exposed to 500 ug/ml azaserine for 30 min at 37°C. Both the control DNA and the azaserine-exposed DNA were thoroughly dialyzed against SSC. The average $\Delta T_m$ in °C for three experiments (control DNA - azaserine-treated DNA) was $-0.0167 \pm 0.1094$. The average percentage hyperchromic shift was $29.4 \pm 3.3$ for the control DNA and $33.0 \pm 0.7$ for the azaserine-treated DNA.
Figure 6. Nucleophilic attack on azaserine. Dotted line indicates site of esterase action. Azaserine might also spontaneously cleave at this point. The probable adduct is shown above.
DISCUSSION

The survival curves of DNA repair-deficient strains of E. coli provide evidence that azaserine acts on the DNA of the organism. Strain WP100 lacks both the uvrA gene and the recA gene and is about 1500 times more sensitive to the toxic effects of azaserine than wild type E. coli cells. This greatly increased sensitivity of E. coli WP100 to azaserine correlates well with the sensitivities of other DNA repair-deficient strains of E. coli (personal communication, H. E. Kubitschek).

Escherichia coli cells that are washed in 40 mM Tris buffer become more permeable to low molecular weight compounds. Thus, a Tris-washed WP100 cell should become highly permeable to azaserine. With WP100 there is no significant difference between azaserine toxicity in permeabilized cells and non-permeabilized cells (Fig. 2). This would indicate that transport across the cellular membrane does not play a large role in azaserine toxicity in E. coli. It also indicates that the loss of sulfhydryls from the permeabilized cells (2) does not increase azaserine toxicity even though sulfhydryls are known to react with azaserine (6). Although the bacterial cell membrane apparently does not play a large role in azaserine toxicity, a study of mammalian cell lines shows a
correspondence of \( \gamma \)-glutamyl transpeptidase activity and azaserine toxicity (20). Therefore, any conclusions made about azaserine cytotoxicity in bacterial systems must be reconsidered when applied to mammalian systems.

The MNNG-induced adaptation to azaserine challenge provides evidence that azaserine acts as an alkylating agent of DNA \textit{in vivo}. The adaptive response is known to occur with several different alkylating agents (4, 8) and has been shown to be different from previously documented DNA repair pathways (8, 9, 23, 24). As shown by Schendel and Robins (25), adapted cells repair \( O^{6} \)-methyl guanine lesions at a much faster rate than unadapted cells. This indicates that at least part of azaserine's cytotoxicity may be due to an \( O^{6} \)-methylation of guanine or a lesion closely resembling a methylation. Diazo compounds like azaserine are known to be biological alkylating agents (11, 12, 21). It has been suggested that the DNA adduct azaserine produces could be an acetyl group (personal communication, Dr. M. MacCoss). The ester linkage of the azaserine could spontaneously cleave or be cleaved by an esterase \textit{in vivo}. Nucleophilic attack of the molecule would then yield the proposed adduct (Fig. 6). It would be significant if azaserine induced a strong adaptive response but with growing cultures any interference with purine biosynthesis decreases the growth rate and therefore decreases any adaptive response that is seen. Because azaserine does not induce a strong adaptive response, we cannot be certain that the main effect of azaserine is
alkylation of the DNA. All we can say is that the lesion azaserine produces is repaired by the same enzymes induced by the adaptive response.

A previous study done in this laboratory on azaserine mutagenesis conflicts with the theory that azaserine alkylates DNA in vivo. According to that study, azaserine mutagenesis seems to be dependent on error-prone repair (personal communication, Dr. H. E. Kubitschek). When the enzymes necessary for error-prone repair (13, 30) are not present in the E. coli cell, azaserine causes no mutagenesis. This is unlike the behavior of alkylating agents which induce base mispairing in the absence of error-prone repair (16). Therefore, azaserine does not act like an alkylating agent in some mutation studies but does act like an alkylating agent in adaptive survival.

Our in vivo results indicate that much of azaserine's cytotoxicity is due to its effect on DNA. In contrast, most of the in vitro experiments failed to show that azaserine reacted with DNA. One difficulty with most in vitro experiments is the lack of sensitivity. Whereas one hit on a DNA molecule can cause cell death or mutation, it takes hundreds of such hits to produce a noticeable in vitro effect. The fact that azaserine treatment failed to significantly change the melting temperature of phage T4 DNA could be due to the insensitivity of the melting curve method (Fig. 5). If the experiment was repeated with a more intense azaserine treatment, a greater change in the $T_m$ of the DNA might be observed.
The thin layer chromatography performed on the mixtures of azaserine and mononucleosides may also have been an insensitive method. It is possible that any reaction products formed were in such small concentrations that they were unobservable. Repeating the experiment with a 100- or 500-fold molar excess of azaserine might provide enough modified nucleosides for detection. In any event, the chemical behavior of the mononucleosides would not necessarily mimic the behavior of the polynucleotide.

In the transformation experiments, azaserine treatment of the DNA was in vitro but a biological detection method was used. Presumably this method should detect low levels of damage to DNA. Bacillus subtilis GSU1027 (uvrA) cells were used because they lack the enzyme necessary to repair pyrimidine dimers. Thus, GSU1027 cells are less able to repair any damage to the transforming DNA than are wild type B. subtilis cells. Results showed that the number of transformants/ml formed was independent of the azaserine concentration. It therefore appears that if any lesions were formed by this in vitro treatment of DNA with azaserine they did not decrease the transformation efficiency (Table 1).

UV absorption spectra of calf thymus DNA treated with azaserine did differ from the UV spectra of untreated calf thymus DNA (Fig. 6). It is not known at this point whether such a change is due to residual azaserine or an actual reaction with the DNA.
In conclusion, azaserine appears to act as an alkylating agent in adaptive survival tests. However, experiments on azaserine-induced mutation in *E. coli* WP indicate that such mutation is dependent on error-prone repair of DNA. This conflicts with a direct alkylation model for azaserine mutation and toxicity in *E. coli*. Furthermore, *in vitro* experiments do not confirm the existence of an alkylation adduct. Perhaps tests using radioactive azaserine will shed more light on the *in vivo* and *in vitro* action of azaserine on DNA.
LITERATURE CITED


M9

Solution A:  \( \text{NH}_4\text{Cl} \quad 200 \text{ g} \)
\( \text{MgSO}_4\cdot7\text{H}_2\text{O} \quad 40 \text{ g} \)
\( \text{H}_2\text{O} \quad 2000 \text{ ml} \)

Solution B:  \( \text{Na}_2\text{HPO}_4 \quad 300 \text{ g} \)
\( \text{KH}_2\text{PO}_4 \quad 150 \text{ g} \)
\( \text{NaCl} \quad 25 \text{ g} \)
\( \text{H}_2\text{O} \quad 2000 \text{ ml} \)

Add 10 ml Solution A and 40 ml Solution B to 950 ml \( \text{H}_2\text{O} \) and autoclave.

Minimal Media for WP E. coli

Add 0.1% glucose, 25 ug/ml tryptophan, 25 ug/ml thymidine to M9.

SSC

\[
\begin{align*}
\text{NaCl} & : 26.33 \text{ g} \\
\text{Na}_2\text{Citrate} & : 13.23 \text{ g} \\
\text{H}_2\text{O} & : 3000 \text{ ml}
\end{align*}
\]

Adjust to pH 7-8 with either HCl or NaOH.

Transformation in B. subtilis (Courtesy M. J. and J. G. Peak)

Spizizen (SP) Salts

\[
\begin{align*}
\text{NaCitrate} & : 1.0 \text{ g} \\
\text{(NH}_4\text{)}\text{SO}_4 & : 2.0 \text{ g} \\
\text{KH}_2\text{PO}_4 & : 6.0 \text{ g} \\
\text{K}_2\text{HPO}_4 & : 14.0 \text{ g (18 g if hydrated)}
\end{align*}
\]

Stocks

\[
\begin{align*}
\text{Amino acids} & : 10 \text{ mg/ml} \\
\text{MgSO}_4 & : 10\% \\
\text{Glucose} & : 50\% \text{ w/v} \\
\text{CaCl}_2 & : 0.05 \text{ M} \\
\text{MgCl}_2 & : 0.10 \text{ M}
\end{align*}
\]

SP 1 (per 100 ml SP salts)

\[
\begin{align*}
\text{MgSO}_4 & : 0.10 \text{ ml} \\
\text{Glucose} & : 1.20 \text{ ml}
\end{align*}
\]

SP 2 (per 100 ml SP salts)

\[
\begin{align*}
\text{CaCl}_2 & : 1.0 \text{ ml} \\
\text{MgCl}_2 & : 2.0 \text{ ml}
\end{align*}
\]
Minimal Plates
Flask #1: SP salts for 1 liter
  5 ml appropriate amino acids
  300 ml H₂O
Flask #2: 20 g agar
  5 g glucose
  1 ml MgSO₄
  700 ml H₂O

Autoclave, combine, pour.

Transformation Protocol

1. To a 0.4 ml tube of competent cells, add 3.6 ml SP 2 (no amino acids).

2. Add 1 ml of this to 0.10 ml of transforming DNA, shake 30 min.

3. Plate out dilutions upon minimal plates containing appropriate amino acids.