Induction of a Defective Phage and DNA Methylation in Escherichia coli 15T−

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SUMMARY

Growth of Escherichia coli 15T− in a thymine-containing medium with any of several antibacterial agents (5-aminouracil, mitomycin C, nalidixic acid or hydroxyurea) increased the 6-methyladenine content of the extractable DNA and the DNA methylease activity. Similar changes occurred after transient (45 min.) thymine deprivation but not with other antibacterial agents such as penicillin, phenylethyl alcohol, novobiocin. Associated with the increases in 6-methyladenine content and DNA methylase activity was the induction of a defective bacteriophage. Similar effects were noted in the wild-type E. coli 15.

Escherichia coli 15T− is a thymine auxotroph derived from E. coli 15T+. It was selected for its resistance to mitomycin C. Neither phage particles nor changes in DNA methylase or 6-methyladenine content were produced in 15T− under conditions leading to such effects in 15T−.

It is postulated that the rapid killing of 'thymineless death' in Escherichia coli 15T− is secondary to induction of a defective bacteriophage. The increases in 6-methyladenine content and DNA methylase activities that occur with thymine deprivation or treatment with the above antibacterial agents are thought to represent phage-induced changes.

INTRODUCTION

The term 'thymineless death' was introduced by Barner & Cohen (1954) to describe the rapid killing that occurred when logarithmically growing cells of the thymine auxotroph Escherichia coli 15T− were transferred to a thymineless medium. A carbon source and oxygen were required for this rapid killing; later Maaløe & Hanawalt (1961) demonstrated that only cells actively synthesizing DNA were susceptible to thymineless death.

Dunn & Smith (1958) noted an increase in the 6-methyladenine content of the DNA of Escherichia coli 15T− on growth in the presence of the thymine analogues, 5-aminouracil or 2-thiothymine. Theil & Zamenhof (1963) showed that the increase in 6-methyladenine content of DNA occurred only in E. coli 15T− and not in certain other thymine auxotrophs. Gefter, Yudelevich & Gold (1966) recently demonstrated a marked increase in the DNA methylase activity in extracts of E. coli 15T− several hr after exposure to 5-aminouracil. During the past few years, work from several laboratories (Melechen & Skaar, 1962; Korn & Weissbach, 1962) has shown the

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induction, by the thymineless state, of colicin or defective bacteriophage in several thymine auxotrophs other than _E. coli_ I5T-. Mennigmann (1965) demonstrated the defective phage on electron microscopic study of lysates of _E. coli_ I5T- induced by ultraviolet light. Sandoval, Reilly & Tandler (1965) found similar phage particles in the colicin fraction of the u.v. irradiated wild-type _E. coli_ 15.

The foregoing evidence suggests the possibility that the thymineless state or treatment with 5-aminouracil might induce a defective phage in _Escherichia coli_ I5T-, and that the increased DNA methylase activity (and associated increased 6-methyladenine content of DNA) might represent phage-induced changes. This report deals with the effect of a variety of anti-bacterial agents on the induction of defective phage in _E. coli_ I5T- and on the activities, in crude extracts of induced cells, of several enzymes involved in DNA synthesis (polymerase), modification (methylase), and breakdown (deoxyribonuclease). Exposure during growth to mitomycin C or to several other antibacterial agents such as nalidixic acid and hydroxyurea induces a defective phage, and this process is associated with markedly enhanced DNA methylase activity and of 6-methyladenine content of the DNA of treated bacterial cells. These latter alterations appear to represent a phage-induced enzyme and a phage-induced change in DNA. Very recently Yudelevich & Gold (1967) and Medoff & Swartz (1967), using a variety of inducing agents, presented preliminary observations on the induction of a defective phage in _E. coli_ I5T-, accompanied by increases in both DNA methylase activity and DNA methyladenine content.

**METHODS**

_Organisms._ The strain of _Escherichia coli_ I5T- employed was originally obtained from Dr Nathan O. Kaplan at Brandeis University and has been subcultured in our culture collection for several years. This strain is a thymine auxotroph with a low thymine requirement and most closely corresponds to the strain 70 V3-462 described by Breitman & Bradford (1964). _E. coli_ I5-ATCC9723 was obtained from the American Type Culture Collection. _E. coli_ I5T~ is a mitomycin resistant thymine auxotroph derived from the surviving cells of _E. coli_ I5T- after repeated passages of this organism through medium containing increasing concentrations of mitomycin C up to 100 µg./ml. This organism has the same biochemical markers (acid/acid fermentation in Kligler's medium, lack of H2S production, indole + and ornithine decarboxylase +, citrate – etc.) and thymine dependency for growth as _E. coli_ I5T-.

_Media._ Organisms were grown in Salts A medium as described by Dunn & Smith (1958). Thymine was added to the medium to give a final concentration of 2.5 µg./ml.

_Chemicals._ 5-Aminouracil was obtained from Sigma Chemical Company; mitomycin C from Nutritional Biochemical Corporation, and phenylethyl alcohol from Eastman-Kodak. Hydroxyurea was kindly provided by E. R. Squibb and Company; nalidixic acid by Winthrop Laboratories; and chloramphenicol by Parke-Davis and Company. Penicillin G was obtained from E. R. Squibb and Company. [14C-methyl]S-adenosyl-L-methionine with a specific activity of 30 mc/m-mole and [2-14C]-thymine with a specific activity of 59 mc/m-mole were obtained from Tracerlab. Crystalline pancreatic deoxyribonuclease I (electrophoretically purified) and pancreatic ribonuclease were obtained from Worthington Biochemical Company.
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**Growth and harvesting of cells.** Cells were grown in Salts A medium + thymine (2.5 μg./ml.) in 4 l. flasks with vigorous shaking on a Brunswick rotary shaker at 37° until mid-log phase (c. 2 × 10⁸ cells/ml.) as determined by turbidity on the Klett colorimeter. The appropriate antibacterial agents were then added; or the cells were harvested aseptically by centrifugation, washed in Salts A, and transferred to a thymine-less medium. Incubation with vigorous shaking was then continued for an additional one to 15 hr. The cells were harvested by centrifugation and washed twice with one of several solutions. For study of DNA methylating activity, the cells were washed with, and treated ultrasonically in, a 'methylation buffer' containing 0.02 M-tris, pH 8.0; 0.001 M-MgCl₂; 0.002 M-di-Na-EDTA; 0.001 M-2-mercaptoethanol (Fujimoto, Srinivasan & Borek, 1965). Cells to be assayed for DNA polymerase or deoxyribonuclease activity were washed with, and treated ultrasonically in, a solution containing 0.05 M-tris + HCl, pH 7.55; 0.1 M-ammonium sulphate; 0.01 M-2-mercaptoethanol and bovine plasma albumin (1 mg./ml.) (Richardson et al. 1964). Cells to be used for determination of DNA 6-methyladenine content were washed with 0.15 M-NaCl + 0.1 M-EDTA and the DNA was extracted either by the procedure of Marmur (1961) or by the method of Schmidt & Thannhauser (1945).

**Cell extracts.** Sonic extracts were prepared by lysing a chilled suspension of 0.25 to 0.5 g. of cells in 15 to 20 ml. of 'methylation buffer' using a Branson 'Sonifier' at a setting of 8 for three periods of 40 sec. each. Colony counts were made on AC medium, and stock cultures were maintained on the same medium. The addition of the various antibacterial agents, in the concentrations employed, did not alter the pH values of the culture media used. Cells in the presence of penicillin were grown in an osmotically protected medium (Lederberg, 1956).

**Bacterial lysates.** Bacterial lysates examined under the electron microscope for the presence of bacteriophage were prepared by centrifugation of samples for 10 min. at 700 g in an International refrigerated centrifuge to remove bacterial debris. The supernatant fraction was then centrifuged in the 30 rotor of a Spinco Model L centrifuge at 54,000 g for 1 hr, and the pellet was collected, resuspended in Salts A, and centrifuged for 10 min. at 10,000 g in a Serval centrifuge. The supernatant fluid was then centrifuged in the 30 rotor of the Spinco centrifuge at 54,000 g for 1 hr and the pellet was dispersed in 0.5 ml. Salts A. One drop of this suspension was examined by electron microscopy, using phosphotungstate or uranyl acetate staining techniques.

**Assays.** DNA methylation assays were done using the Millipore filter procedure described by Fujimoto et al. (1965). The tests employed sonic extracts of cells harvested at various times after addition of inducing agents which had been clarified by centrifuging at 105,000 g. Each reaction mixture (0.25 ml.) contained sonic extract (20 to 40 μg. of protein); 25 μmole of tris buffer, pH 8.0; 1 μmole MgCl₂; 5 μmole 2-mercaptoethanol; 5 μmole [¹⁴C]-adenosyl-L-methionine (1 × 10⁷ counts/min./μmole); and 100 μmole of either calf thymus or Escherichia coli 15T DNA as methyl-group acceptor. Radioactivity insoluble in 5% cold trichloracetic acid was trapped in the Millipore filter and determined, after drying in an oven at 60°, on a Nuclear-Chicago gas flow end-window counter. For each assay controls were run with incubation mixtures at 37° for 30 min. to which acceptor DNA had not been added. The very low incorporations occurring in these blanks were subtracted from each of the assay results. The reactions were initiated by the addition of the [¹⁴C]-
S-adenosyl-L-methionine to the incubation mixture. The assay was linear; each was repeated twice with two different concentrations of sonic extract. More than 90% of the acid-insoluble counts incorporated in the methylase reaction were eliminated by the addition of 2.5 μg. pancreatic DNAase to the reaction.

DNA polymerase and DNase assays were made on extracts of cells harvested at various times after the addition of the antibacterial agents. Sonic treatment was done as described previously and the enzyme source was the clear supernatant extract after a 20 min. centrifugation at 10,000 g. Samples of extracts containing 20 to 40 μg. of protein were used and each assay exhibited good linearity. The assay of DNA polymerase was performed as described by Lehman et al. (1958). Antibody inhibition studies were made with rabbit antiserum to purified *Escherichia coli* DNA polymerase kindly provided by Dr I. R. Lehman (Aposhian & Kornberg, 1962).

DNase assays in crude sonic extracts of *Escherichia coli* 15T- were done under two sets of conditions. One assay was performed at pH 9.2 utilizing 32P-labelled heated *E. coli* DNA as substrate as described by Lehman (1960) for *E. coli* phosphodiesterase (Exonuclease I). The second assay was made at pH 7.6 utilizing native 32P-labelled DNA as substrate as described for the assay in *E. coli* endonuclease I (Lehman, Roussos & Pratt, 1962).

**DNA base analysis.** DNA obtained from *Escherichia coli* 15T- cells treated with various antibacterial agents was either depurinated in 1 N-HCl at 55° (Theil & Zamenhof, 1963) or hydrolysed in 2 to 5 μmole samples with 0.4 ml. of 85% formic acid in sealed ampoules at 175° in a paraffin bath for 30 min. (Wyatt & Cohen, 1953). Following depurination or hydrolysis the samples were dried in an air stream and dissolved in 50 μl. of 0.1 N-HCl. The base content was then determined by quantitative descending two-dimensional paper chromatography on Whatman no. 1 paper (Wyatt, 1951). The first chromatogram was run for 20 hr in the solvent consisting of isopropanol:conc. HCl: H2O (170:44:36) and for 12 hr in the second solvent of isopropanol: conc. NH3·H2O (85:1.3:15). This system readily separated 6-methyladenine from the other four bases in DNA. The spots (and appropriate blank areas on the chromatograms) were then identified under u.v. light, cut out and eluted overnight in 0.1 N-HCl in acid-cleaned tubes. The amounts of the individual bases were determined in a Zeiss model PMQ II spectrophotometer. The molar extinction coefficients employed were: adenine (263 nm.) 13.1 x 10³, methyladenine (267 nm.) 15.1 x 10³. The amount of 6-methyladenine found was expressed as mole/100 mole of DNA adenine.

To determine the site of incorporation of [14C]methyl group in the *in vitro* enzymic methylation of DNA, the methylation reaction with cell extracts was scaled up 20-fold and the incubation was continued for 1 hr at 37°. The reaction was terminated by the addition of an equal vol. of cold 10% trichloroacetic acid. The precipitate, collected by centrifugation, was washed three times with cold 5% trichloroacetic acid, washed once with 80% ethyl alcohol, and finally washed with cold ethanol + ether (3:1) (Fujimoto et al. 1965). It was then dried over KOH and the dried precipitate hydrolysed with formic acid and processed by the method already described. Markers of 6-methyladenine and 5-methylcytosine were added. As a check for the presence of radioactivity in the 5-methylcytosine spot, one chromatogram was run in another two-dimensional solvent system consisting of isopropanol: conc. HCl: H2O (170:44:36), and water saturated n-butanol ammonia (MacNutt, 1952). As controls, 6-methyladenine and
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5-methylcytosine were treated separately with formic acid and chromatographed in the same way. Formic-acid treatment of 6-methyladenine (but not 5-methylcytosine) produced an additional faint u.v. absorbing spot with a mobility like that of 5-methylcytosine in the first dimension but could be clearly distinguished from it and other bases in the second dimension of the chromatogram. This additional spot represented 10 to 20% of the original 6-methyladenine content.

[2-14C]thymine incorporation in growing cultures (Carr & Rosenkranz, 1966). *Escherichia coli* 15T- cells were grown with aeration at 37° in 10 ml. of Salts A medium + thymine (2-5 µg./ml.) until early log. phase of growth (2 to 3 × 10^8 cells/ml.); and the cells were then harvested by centrifugation, washed with Salts A medium to remove thymine, and finally resuspended in 10 ml. of Salts A medium containing 2-5 µg./ml. of [2-14C]thymine (specific activity = 9.3 × 10^6 counts/min./µmole). The various antibacterial agents were then added at the concentrations indicated (mitomycin C 0.25 µg./ml., 5-aminouracil 10^-8 M, nalidixic acid 40 µg./ml., hydroxyurea 8 mg./ml., penicillin 12 µg./ml., phenylethyl alcohol 3 mg./ml., novobiocin 500 µg./ml.). One ml. samples of culture were taken at intervals and precipitated with an equal volume of cold 10% trichloracetic acid. The precipitate was kept at 4° for 10 min. and then centrifuged at 10,000 rev./min. for 5 min., and washed with 1 ml. of cold 5% trichloracetic acid. The final precipitate was suspended in 1 ml. of 1 N-NaOH and incubated for 16 hr at 37°. The solution was neutralized with 1 N-HCl and then reprecipitated by the addition of an equal vol. of cold 10% trichloracetic acid. The precipitate was collected by filtration through a Millipore filter, dried, and the radioactivity was determined on a gas flow Nuclear-Chicago end-window counter.

RESULTS

6-Methyladenine content of DNA and DNA methylase activity of *Escherichia coli* 15T-

Changes in the 6-methyladenine content and DNA methylase activity of *Escherichia coli* 15T- occurred within 4 or 5 hr of exposure of logarithmically growing cells (in thymine-containing medium) to 5-aminouracil (10^-8 M). By 7 hr there was a tenfold increase in the methyladenine content. The specific activity of DNA methylase increased threefold by 4 to 5 hr. The simultaneous addition of chloramphenicol (10 µg./ml.) with the 5-aminouracil abolished these increases in 6-methyladenine content and in DNA methylase activity.

Exposure of growing cultures of *Escherichia coli* 15T- to mitomycin C (0.25 µg./ml.) similarly effected increases in the 6-methyladenine content of DNA and in the DNA methylase activity of cell extracts (Fig. 1). By 3 hr the 6-methyladenine content increased tenfold and the specific activity of DNA methylase in extracts of the same cell culture increased 20-fold over the activity in comparable untreated cultures. As with 5-aminouracil treatment, the addition of chloramphenicol (10 µg./ml.) and mitomycin abolished the expected increases. In addition to 5-aminouracil and mitomycin treatment, changes in DNA methylase activity were produced by the 'thymineless' state, provided the cells were subsequently re-exposed to thymine (Fig. 2). Further, when thymine was re-added to these cells a tenfold increase in 6-methyladenine content of the DNA occurred. If thymine was not restored to the medium there was no increase in DNA methylase activity or 6-methyladenine content. Neither did the changes in 6-methyladenine and DNA methylase occur when chloramphenicol
(10 μg./ml.) was simultaneously added to the medium with thymine. Mitomycin treatment of cells rendered thymineless did not produce the expected increases in 6-methyladenine content of DNA or of DNA methylase activity. This suggested that some DNA synthesis occurring in the presence of thymine was necessary for the changes in methylation to occur. Mixing experiments, in which sonic extracts of untreated *E. coli* 15T− and mitomycin-treated cells were combined, failed to provide evidence for an inhibitor of DNA methylase in the former.

Fig. 1. Changes in 6-methyladenine content and DNA methylase activity occurring after exposure of *Escherichia coli* 15T− to mitomycin 0:25 μg./ml. (△), and chloramphenicol 10 μg./ml. with mitomycin (▼). ●, Control.

Fig. 2. Changes in DNA methylase activity occurring when *Escherichia coli* 15T− was incubated in a thymineless medium (▲); after 45 min. thymine 2:5 μg./ml. (△) and chloramphenicol 10 μg./ml. with thymine (○) were added to the medium.
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Sites of DNA methylation

Evidence for the in vitro incorporation of [14C]methyl groups from S-adenosyl methionine into DNA (and not into some other trichloracetic-acid-insoluble moiety) was obtained on examining the labelled methylated compounds in the hydrolysate after formic acid hydrolysis of the products of the reaction. Almost 100% of the radioactivity could be accounted for as the bases 6-methyladenine and 5-methylcytosine. In the methylation of calf-thymus DNA by sonic extracts of untreated cells, 61.7% of the radioactivity was found in 6-methyladenine and 35.2% in 5-methylcytosine (Table 1). In contrast, under identical conditions, but with extracts of mitomycin-treated cells as the source of methylase, 98.6% of the radioactivity appeared in 6-methyladenine. The increased DNA methylating activity in extracts of mitomycin-treated cells involved methylation of adenine moieties almost exclusively.

Table 1. Pattern of methylation by the DNA methylase*

<table>
<thead>
<tr>
<th>Base</th>
<th>Untreated cells</th>
<th>Mitomycin-treated cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Counts/min.</td>
<td>Total counts recovered</td>
</tr>
<tr>
<td>Adenine</td>
<td>8</td>
<td>0.5</td>
</tr>
<tr>
<td>6-methyladenine</td>
<td>1008</td>
<td>61.7</td>
</tr>
<tr>
<td>Cytosine</td>
<td>42</td>
<td>2.6</td>
</tr>
<tr>
<td>5-methylcytosine</td>
<td>576</td>
<td>35.2</td>
</tr>
</tbody>
</table>

* Methylation assay system was scaled up 20-fold (as described under Methods) for the determination of the site of incorporation of [14C]methyl group into DNA in vitro. Numbers represent counts/min. in material eluted from the various purine and pyrimidine spots on the two-dimensional chromatogram.

Other enzymes in DNA metabolism

DNA polymerase. No differences in polymerase activity or in DNA primer requirements (heated versus native DNA) were found in extracts of cells grown in the presence of 5-aminouracil or mitomycin under conditions producing increases in the DNA methylating activity. Moreover, both the control and treated extracts are equally inhibited (> 90%) by antibody to Escherichia coli DNA polymerase.

Deoxyribonuclease activity. There was no difference in the DNase activity at pH 7.6 against native 32P-labelled Escherichia coli B DNA between control extracts and extracts of E. coli 15T− cells treated with mitomycin or 5-aminouracil. Deoxyribonuclease activity of the extracts was determined directly and on extracts preincubated 30 min. with 50 μg. pancreatic RNase to remove the endogenous RNA inhibition of endonuclease. Also, deoxyribonuclease activity at pH 9.2 against heated DNA was not significantly different.

Effects of other antibacterial agents on DNA methylation

Several other antibacterial agents were capable of effecting changes in the 6-methyladenine content of DNA and of DNA methylase activity when added to growing cultures of Escherichia coli 15T−. Hydroxyurea (8 mg./ml.) and nalidixic acid (40 μg./ml.) both produced three- to fourfold increases in 6-methyladenine content and approximately twofold increases in methylating activity in cell extracts. Simultaneous
addition of chloramphenicol (10 µg./ml.) prevented these increases. Other antibacterial agents such as penicillin (12 µg./ml.), phenylethyl alcohol (3 mg./ml.) and novobiocin (500 µg./ml.) did not cause discernible alterations in 6-methyladenine content or DNA methylase activity.

Fig. 3. Changes in 6-methyladenine content and DNA methylase activity occurring after exposure of *Escherichia coli* 15 to mitomycin C 0·25 µg./ml. (△), and chloramphenicol 10 µg./ml. with mitomycin (▼). ○, Control.

**DNA methylation in the wild-type *Escherichia coli* 15**

*Escherichia coli* 15T− is a one-step ultraviolet-induced mutant of wild-type *E. coli* 15 (ATCC9723) (Roepke & Mercer, 1947). Therefore *E. coli* 15 should contain the same lysogenic phage present in *E. coli* 15T−. If the observed increases in 6-methyladenine content and in DNA methylating enzyme activity of 15T− were secondary to induction of this phage, then *E. coli* 15 should have exhibited similar changes under the same conditions. Increases in 6-methyladenine content and DNA methylating enzyme activity of *E. coli* 15 were seen after mitomycin C (0·25 µg./ml.) treatment (Fig. 3). Hydroxyurea and nalidixic acid treatment of *E. coli* 15 also produced increases in 6-methyladenine content and DNA methylase activity similar to those produced by these drugs in *E. coli* 15T−. The thymine analogue, 5-aminouracil, on the other hand, did not effect these changes in *E. coli* 15, suggesting that the effect of 5-aminouracil on DNA methylation was dependent on a thymine requirement for growth. In support of this is the observation that the increases in 6-methyladenine content and DNA methylase values of *E. coli* 15T− observed after 5-aminouracil treatment were inhibited by the addition of excess thymine (10 µg./ml.) simultaneously with the 5-aminouracil (10−3 M).

**Induction of defective phage in *Escherichia coli* 15T−**

Those conditions (thymineless medium, mitomycin C, 5-aminouracil) which produced increases in the 6-methyladenine content and of DNA methylating enzyme activity also produced a characteristic filamentous appearance in the bacterial cell (Pl. 1 a). Similar but less marked changes were produced by nalidixic acid and hydroxyurea. Penicillin produced marked elongation of cells as well but did not affect the 6-methyladenine content or methylating activity.
(a) Phase microscopy of *Escherichia coli* 15T-. On the left are untreated *E. coli* 15T- cells 3 hr after logarithmic phase of growth. On the right are cells 3 hr after exposure to mitomycin C 0.25 μg./ml.

(b) Electron micrograph of negatively stained phage particles in the lysate of *Escherichia coli* 15T- after treatment with mitomycin C 0.25 μg./ml.

(c) Electron micrograph of uranyl acetate-stained phage particles in the lysate of *Escherichia coli* 15T- after treatment with hydroxyurea 8 mg./ml.

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Examination by electron microscopy of culture supernatants of Escherichia coli 15T−, grown under conditions producing increases in 6-methyladenine and DNA methylase values, revealed the presence of phage particles. Mitomycin treatment produced the greatest density of such particles as determined by gross number in each photographic field (see Methods) (Pl. 1b). The number of phage particles seen after hydroxyurea or nalidixic acid treatments (Pl. 1c) seemed to be less than with mitomycin C treatment. These phages had heads of size 600 × 600 Å and a tail of 1150 Å, consistent with the dimensions described for the phage ψ (Mennigmann, 1965). None of a variety of other E. coli strains examined served as host for vegetative production of the phage found in lysates of E. coli 15T−.

Fig. 4. Effect of antibacterial agents on DNA synthesis in Escherichia coli 15T− as measured by [2-14C]thymine incorporation into alkali-treated acid-insoluble fractions of cells. Δ, mitomycin C (0.25 μg./ml.); O, 5-aminouracil (10⁻³ M); ■, penicillin (12 μg./ml.); ▼, hydroxyurea (8 mg./ml.); ●, novobiocin (500 μg./ml.); ◇, phenylethyl alcohol (3 mg./ml.); ▽, nalidixic acid (40 μg./ml.); ▲, control.

DNA methylation in Escherichia coli 15T−

No increases in 6-methyladenine content or DNA methylase activity were produced in Escherichia coli 15T− by treatment with mitomycin, 5-aminouracil, nalidixic acid, hydroxyurea or thymine deprivation, conditions which produced such increases in E. coli 15T− or E. coli 15. Thymine deprivation of E. coli 15T− had a bacteriostatic effect as measured by viable colony counts, and when thymine was reintroduced into the medium the number of viable cells increased. Phage particles could not be demon-
strated in the supernatant fluids of $15T_n$ after the various treatments producing such particles in $E. coli$ 15T$^-$ or $E. coli$ 15.

**Effect of antibacterial agents on DNA synthesis and cell division**

Under the conditions employed and concentrations used in the previously described experiments, some antibacterial agents interfered with DNA synthesis while others did not. The concentrations of mitomycin C and 5-aminouracil employed in the earlier experiments had little, if any, effect on DNA synthesis, yet both were potent inducers of DNA methylase activity (Fig. 4). On the other hand, agents like hydroxyurea and nalidixic acid both produced three- to fourfold increases in 6-methyladenine content but interfered grossly with DNA synthesis. The agents novobiocin and phenethyl alcohol, while interfering with DNA synthesis, did not produce increases in methyladenine content. Penicillin treatment of cells in an osmotically protected medium produced little or no interference with DNA synthesis and did not increase the 6-methyladenine content of DNA.

The concentrations of the antibacterial agents used were sufficient to produce their antibacterial effects judged by viable cell counts. For the most part the effects on each cell type were the same. It is notable that the addition of thymine to $Escherichia coli$ 15T$^-$ in a thymineless medium did not produce logarithmic killing or so-called 'thymineless death'.

**DISCUSSION**

The data presented in this report demonstrate that increases in the 6-methyladenine content of DNA and in the DNA methylase activity in $Escherichia coli$ 15T$^-$ can occur under conditions completely divorced from the thymineless state. Mitomycin C, hydroxyurea, and nalidixic acid produce such changes in 15T$^-$ in the presence of normal or excess concentrations of thymine. These latter agents also produce the same changes in wild-type $E. coli$ 15. The increase in DNA methylase activity appears to represent induction of a new enzyme judged: (a) by the different specificity for sites of methylation (adenine alone in contrast to both adenine and cytosine in the untreated cells), and (b) by the inhibition by chloramphenicol.

The concept that 'thymineless death' may be caused by the induction of a bacteriophage has received much support (Mukai, 1960; Sicard & Devoret, 1962). Endo et al. (1965) showed that colicin 15 activity is associated with phage particles seen by electron microscopy. Mennigmann (1965) induced phage particles in $E. coli$ 15T$^-$ with u.v. light. The present studies have established an association between increases in the 6-methyladenine content and DNA methylase activity of $E. coli$ 15T$^-$, and the presence of increased numbers of phage particles in the bacterial lysates. This correlation holds under a variety of conditions such as treatment with various antibacterial agents and thymine deprivation. Those conditions which produce the most marked changes in 6-methyladenine content and DNA methylase activity appear to produce lysates containing the greatest number of phage particles. This correlation is also true for the wild-type $E. coli$ 15. Active phage production occurs in $E. coli$ 15T$^-$ in response to transient thymine deprivation only after the subsequent addition of thymine. This same thymine requirement to complete the lytic cycle of the phage has been reported for phage in a thymine-requiring mutant of $E. coli$ K12 (Korn & Weissbach 1962) and for phage P1b in $E. coli$ B3 (Melechen & Skaar, 1962).
None of the agents described induced the development of phage particles in *Escherichia coli* 15T⁻. DNA methylation studies under the same conditions show no increases. This was true even though the agents could be shown to exert an antibacterial effect on this organism. Thymine deprivation followed by the re-addition of thymine to the medium did not cause logarithmic killing of this organism as in *E. coli* 15T⁻ but rather resulted in bacteriostasis. *E. coli* 15T⁻ presumably is no longer lysogenic for phage Φ. With the loss of the phage, the organism no longer exhibits enhanced DNA methylation and fails to undergo the classic rapid killing of 'thymineless death'.

Recently Donachie & Hobbs (1967) have presented evidence for the thesis that thymineless death in *Escherichia coli* 15T⁻ is not itself caused by phage induction. Survival curves after transient thymine deprivation showed two reductions in viability. Only the second was considered to be the result of killing by released phage. In contrast to this, re-addition of thymine to *E. coli* IG 151 (col₁₄) a thymineless but colicin-free strain derived from *E. coli* 15T⁻ is followed by a complete recovery of viable counts. However, methylation assays were not performed in these studies nor were bacteriophage particles sought in lysates after the reductions in viable cell numbers.

The mechanism of phage induction by the different antibacterial agents employed in the experiments reported here is unknown. Each probably exerts a primary antibacterial effect as well as an inductive effect. 5-Aminouracil is bacteriostatic for *Escherichia coli* 15 but does not appear to act as an inducing agent in this organism. Thus, its properties as an inducer of phage production and DNA methylation seem to be related to the thymine dependence of *E. coli* 15T⁻. This is substantiated by the observation that the inductive effect of 5-aminouracil on *E. coli* 15T⁻ can be overcome with excess thymine. Nalidixic acid and hydroxyurea both exert antibacterial effects on the 'cured' organism 15T⁻ without phage induction. Thymineless conditions in 15T₋ lead to bacteriostasis rather than logarithmic killing. With 5-aminouracil and hydroxyurea, both bacteriostatic agents, there is an increased rate of killing or bacteriocidal effect on *E. coli* 15T⁻ when phage induction commences. We presume, in these instances, that the bacteriostasis represents the antibacterial effect of the agents and that rapid killing is due to phage induction.

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Methylation and phage in E. coli 15T


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