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The Inhibition of DNA(Cytosine-5)Methylases by 5-Azacytidine

The Effect of Azacytosine-Containing DNA

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SUMMARY

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DNA extracted from *Escherichia coli* grown in the presence of 5-azacytidine (azaC-DNA) inhibits the DNA(cytosine-5)methyltransferase extracted from *E. coli* K12 cells without affecting the DNA(adenine-N^6)methyltransferase present in these same cells. The inhibition is time-dependent and the rate of inhibition can be decreased by addition of substrate DNA. The inhibitory capacity of the DNA is destroyed by incubation with pancreatic deoxyribonuclease or micrococcal nuclease; however, the inhibited enzyme cannot be reactivated by treatment with these enzymes. The cytosine methylases methylate only double-stranded DNA. Similarly, the inhibitory DNA loses activity if it is heat-denatured, and regains activity upon reannealing. The DNA will also inhibit the *Eco*RII and *Hpa*II modification methylases which also synthesize 5-methylcytosine in DNA. Digestion of the inhibitory DNA with the respective restriction endonuclease destroys, in part, the inhibitory activity of the DNA for the respective methylase. Base analysis indicated that 5-azacytidine replaced 8.4% of the cytosine in the azaC-DNA. These results suggest that DNA containing 5-azacytidine irreversibly inhibits DNA(cytosine-5)methylases.

INTRODUCTION

5-Azacytidine is a base analogue that inhibits growth of both bacterial and mammalian cells (1, 2). It can act as a mutagen (3) and has been reported to cause differentiation of mouse embryo cells in culture (4). The drug is incorporated into DNA and RNA in both bacterial and mammalian cells (5, 6). The earliest effect of the drug on macromolecular synthesis is the inhibition of protein synthesis (5, 7). This is believed to result from its incorporation into RNA. The ribosomal RNA synthesized in its presence does not mature normally and is undermethylated (8, 9), and tRNA synthesized after its administration to mice lacks 5-methylcytosine (10).

We have previously reported that the drug inhibits the DNA(cytosine-5)methylase in *Escherichia coli* K12 cells without inhibiting the DNA(adenine-N^6)methylase (11) when cells are grown in the presence of the drug. This inhibition could not be reversed by dialyzing the enzyme extracts, nor could it be reproduced *in vitro* by incubating 5-azacytidine or supernatants from extracts of cells grown in 5-azacytidine with DNA methylase preparations. In this paper we report that DNA extracted from cells grown in the presence of 5-azacytidine inhibits the DNA(cytosine-5)methylase irreversibly *in vitro*, and demonstrate some properties of the inhibition. Recently Jones and Taylor (12) have reported that 5-azacytidine inhibits methylation of cytosine in mammalian DNA.

MATERIALS AND METHODS

Bacterial strains. E. coli K12W6 met$^-$ was obtained from Dr. B. Sheid, Downstate Medical Center, Brooklyn, N. Y. *E. coli* RY23 containing the *Eco*RII restriction-modification system and *Haemophilus parainfluenzae* containing the *Hpa*II restriction-modification system were obtained from Dr. R. Roberts, Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.

*Enzymes. Hpa*II endonuclease was obtained from Bethesda Research Laboratories, Bethesda, Md.; *Eco*RII endonuclease was obtained from Miles Laboratories, Elkhart, Ind. The *E. coli* K12 and *Eco*RII methylases were prepared according to the method of Hattman (13). Partially purified *E. coli* K12 preparations were purified through DEAE-cellulose and contained both cytosine and adenine DNA methylases. Crude enzyme used in some experiments was the 100,000 × *g* supernatant fraction. The *Hpa*II methylase was prepared according to the procedure of Mann and Smith (14). Micrococcal nuclease and pancreatic deoxyribonuclease were obtained from Sigma Chemical Company, St. Louis, Mo.

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DNA preparation. *E. coli* B and calf thymus DNA were obtained from Sigma Chemical Company. The azaC-DNA[1] was prepared from *E. coli* K12, unless otherwise indicated, grown in minimal A medium (15) containing DL-methionine, 40 μg/ml. The cells were grown to an A_{550} of 0.5–0.6; 5-azacytidine, 10 μg/ml (unless otherwise indicated), was added and the cells were incubated for 30 min with aeration. The cells were collected by centrifugation and the DNA was extracted according to the procedure of Marmur (16), except that after the final isopropanol precipitation the DNA was again treated with pancreatic ribonuclease, 50 μg/ml (heated at 80° for 10 min), extracted with $CHCl_3$/isoamyl alcohol, and precipitated with ethanol. The azac-DNA, stored frozen in SSC, was found to be stable for up to 6 months.

Enzyme assays. The methylation reaction contained 33 mM Tris-HCl (pH8), 0.7 mM EDTA, 13 mM β-mercaptoethanol, 15 μg of *E. coli* B DNA or 30 μg of calf thymus DNA, 2.5 μM *S*-[*methyl*-^{14}C]adenosylmethionine (53 mCi/mmole, and the indicated amount of enzyme protein, usually 20–30 μg, in a final volume of 0.3 ml. The mixture was incubated 30 min at 37° and the reaction was stopped with the addition of 0.4 ml of 7% $HClO_4$. After addition of 1 mg of yeast DNA, the precipitate was collected by centrifugation, washed with 3% $HClO_4$, dissolved in 0.5 ml of 0.5 M NaOH, and incubated for 10 min at 60°. The DNA was precipitated with addition of 15 μl of concentrated HCl and 0.5 ml of 7% $HClO_4$. The precipitates were collected by centrifugation and dissolved in 0.3 ml of 0.4 M NH_3; 0.2-ml aliquots were counted in Aquasol scintillation fluid (New England Nuclear Corporation, Boston, Mass.) in a liquid scintillation counter at 80% efficiency. In some experiments *S*-[*methyl*-^{3}H]adenosylmethionine, 267 Ci/mole, was used and counted at 30% efficiency. The assays were linear for 30 min.

Enzyme inhibition studies were performed by preincubating enzyme and DNA for the indicated times at 37° in 33 mM Tris-HCl (pH 8), 13 mM β-mercaptoethanol, and 0.7 mM EDTA. Aliquots were removed at the appropriate times and assayed as indicated above. In kinetic studies the amount of azaC-DNA present in the assay reaction caused less than 10% inhibition of enzyme activity.

*Eco*RII digestion was performed in 50 μl of 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, and 5 mM $MgCl_2$; digestions with *Hpa*II were performed in 50 μl of 10 mM Tris-HCl, (pH 7.4), 10 mM $MgCl_2$, 7 mM KCl, and 2 mM β-mercaptoethanol for the indicated times at 37°. The amount of endonuclease used was the amount that destroyed greater than 90% of 3 μg of *E. coli* B DNA when assayed with the respective methylase under standard conditions. For *Eco*RII this was 6 units incubated for 16 hr and for *Hpa*II it was 9 units incubated for 2 hr.

Protein determinations were performed by the method of Lowry *et al.* (17).

DNA base analysis. Base analysis was performed by paper chromatography according to the method of Bendich (18) after hydrolysis of the DNA with 88% formic acid for 30 min at 180° in a sealed tube. The amount of 5-azacytosine in azaC-DNA was calculated from the expression $\alpha = (C_c - C_a)/C_c(1 - C_a)$, where α is the proportion of cytosine replaced by azacytosine and C_c and C_a are the proportions of cytosine recovered from control and azaC-DNA, respectively.

[1] The abbreviations used are: azaC-DNA, DNA extracted from *Escherichia coli* K12 cells grown in 5-azacytidine, 10 μg/ml, for 30 min; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, SSC, 0.15 M NaCl/0.015 M sodium citrate, pH 7.0.

RESULTS

AzaC-DNA is a poor substrate for DNA(cytosine-5)methylase. *E. coli* B is a very poor substrate for the adenine methylase; less than 10% of the methyl groups incorporated into *E. coli* B DNA with crude extracts of *E. coli* K12 as source of enzyme are in N^6-methyladenine (11, 19). However, this DNA is a good substrate for the cytosine methylase, since *E. coli* B DNA does not contain 5-methylcytosine. *E. coli* K12 DNA is a poor substrate for methylases isolated from *E. coli* K12, presumably because the methylation sites have been methylated *in vivo*.

We had previously demonstrated that *E. coli* K12 grown in the presence of 5-azacytidine had decreased DNA(cytosine-5)methylase activity. If inhibition of cytosine methylation had occurred *in vivo*, then DNA isolated from such cells should be a better substrate for DNA methylases than DNA extracted from control cells. In actuality, just the opposite was observed. As shown in Table 1, DNA extracted from cells grown in 5-azacytidine, 10 μg/ml, was a much poorer substrate than DNA extracted from untreated cells.

5-Azacytidine content of azaC-DNA. When *E. coli* K12 cells are grown in 5-azacytidine (10 μg/ml) for 30 min there is little inhibition of DNA synthesis, and the DNA content of the culture increases by 40%. Since 5-azacytosine is incorporated into DNA in place of cytosine (5), we estimated the 5-azacytosine content of our DNA preparations by determining the base content under conditions that destroy 5-azacytosine and compared that value with the control (Table 2). When the cytosine content is adjusted to the control value, 5-azacytosine is found to constitute 8.4% of those bases incorporated as cytosine, or 2% of the total base content of the DNA. Unless otherwise noted, this DNA was used in all experiments.

AzaC-DNA is an inhibitor of DNA(cytosine-5)methylase activity in vitro. When azaC-DNA was tested as an

TABLE 1

Rate of methylation of DNA with Escherichia coli K12 DNA methylase

The source of 5-azacytosine-containing DNA was cells grown in the presence of the indicated concentration of drug for 30 min. *E. coli* K12 DNA (45 μg) or *E. coli* B DNA (30 μg) was assayed with 1 mg of crude *E. coli* K12 methylase enzyme protein as described under Materials and Methods for 10 min.

Source of DNA	Activity
	pmoles ^{14}C-methyl/mg protein/min
E. coli B	0.66
E. coli K12	0.17
E. coli K12 (5-azacytidine, 10 μg/ml)	0.02
E. coli K12 (5-azacytidine, 1 μg/ml)	0.17

TABLE 2

Base content of DNA

DNA from control *Escherichia coli* K12 and from cells grown in the presence of 5-azacytidine (10 μg/ml) for 30 min were prepared and analyzed as described under Materials and Methods.

Source of DNA	Base content[a] (%)			
	T	A	C	G
Control	23.5 ± 0.8	25.7 ± 0.5	24.8 ± 0.4[b]	26.2 ± 1.2
AzaC-DNA	23.9 ± 0.4	26.0 ± 0.4	23.2 ± 0.4[b]	26.7 ± 0.5

[a] Base content of DNA. Values are means ± standard deviation of four determinations for control and three determinations for AzaC-DNA.

[b] $p < 0.005$.

inhibitor of methylation of *E. coli* B DNA by enzyme prepared from *E. coli* K12, it was found that the DNA inhibited methylation. DNA extracted from cells grown in 5-azacytidine, 10 μg/ml, was a better inhibitor than DNA extracted from cells grown in 1 μg/ml of the drug (Table 3). The DNA preparations were much more potent inhibitors if they were preincubated with the enzyme than if they were added after the substrate DNA.

AzaC-DNA does not inhibit E. coli K12 DNA-(adenine-N^6)methylase. If the azaC-DNA were to have the same specificity *in vitro* as azacytidine has *in vivo*, then the azaC-DNA should inhibit the *E. coli* K12 cytosine methylase but not the adenine methylase present in the same cell extracts. *E. coli* B DNA is a very poor substrate for the adenine methylase (19); calf thymus DNA is a much better substrate for this enzyme: 50% of the methyl groups incorporated are present as N^6-methyladenine. When this DNA is used as substrate, azaC-DNA is a much poorer inhibitor of DNA methylation than when *E. coli* B DNA is used as substrate (Table 4). To verify that only the cytosine methylase was being inhibited, the methylated bases synthesized with calf thymus DNA as substrate in the presence and absence of azaC-DNA were determined. As is shown in Table 4, there was no effect of the azaC-DNA on the amount of N^6-methyladenine synthesized, but there was a decrease in the amount of 5-methylcytosine synthesized.

Characteristics of the inhibitor. In order to verify that the inhibition was due to DNA and not a contaminant, we measured the inhibitory activity of the DNA before and after treatment with pancreatic deoxyribonuclease and micrococcal nuclease. In order to assay enzymatic activity, the nucleases were inhibited with EDTA and EGTA, respectively, following digestion. Both enzymes destroy inhibitory activity, but only if the azaC-DNA is

TABLE 3

Inhibition of DNA methylation by DNA extracted from cells grown in 5-azacytidine

The source of 5-azacytidine-containing DNA was cells grown in the presence of the indicated concentration of the drug for 30 min. The first incubation contained 40 mM Tris-HCl, pH 8.0; 16 mM β-mercaptoethanol; and 1.2 mg of crude enzyme protein in a final volume of 0.25 ml. In the second incubation, *S*-[*methyl*-^{14}C]adenosylmethionine was added to a final concentration of 3 μM. The final volume was 0.28 ml. *Escherichia coli* B DNA (25 μg) or *E. coli* K12 DNA (35 μg) was added where indicated.

Source of DNA		Activity
First incubation	Second incubation	
		pmoles/mg protein/min
E. coli B		0.83
E. coli K12	*E. coli* B	0.81
E. coli K12 (5-azacytidine 10 μg/ml)	*E. coli* B	0.03
E. coli K12 (5-azacytidine, 1 μg/ml)	*E. coli* B	0.24
E. coli B	*E. coli* K12	0.69
E. coli B	*E. coli* K12 (5-azacytidine, 10 μg/ml)	0.15
E. coli B	*E. coli* K12 (5-azacytidine, 1 μg/ml)	0.63

TABLE 4

Inhibition of calf thymus or Escherichia coli B DNA methylation by 5-azacytidine-containing DNA

Partially purified enzyme from *E. coli* K12 cells, 40 μg, was incubated in the presence of 1.5 μg azaC-DNA for 10 min prior to assay. The indicated DNA, 30 μg, and *S*-adenosylmethionine was then added as described in Table 3. For base analysis the reaction was scaled up 2-fold. The DNA was extracted as described by Kalousek and Morris (23), washed with ethanol, and dried. It was hydrolyzed in 0.5 ml 88% formic acid at 180° for 30 min, and chromatographed on Whatman No. 1 paper in butanol-water-ammonia, 86:14:5. Recovery of radioactivity was 73% with no additions and 66% with addition of 5-azacytidine DNA. The experiments were performed in duplicate.

Addition to preincubation medium	DNA used in assay	Activity		
		pmoles/mg protein/min	*pmoles ^{14}C-methyl*	
None	Calf thymus	3.1	10.7	10.8
azaC-DNA	Calf thymus	2.0	2.2	12.2
None	*E. coli* B	3.2	—	—
azaC-DNA	*E. coli* B	0.5	—	—

TABLE 5

Inactivation of 5-azacytidine-containing DNA by digestion with nucleases

Experiment 1: 1.2 μg of azaC-DNA was incubated with 1 μg of pancreatic deoxyribonuclease, 10 μmoles of Tris-HCl (pH 8), 4 μmoles of β-mercaptoethanol, and 0.25 μmoles of $MgCl_2$ in a volume of 28 μl for 15 min; 50 μl of 0.1 M EDTA and 0.1 ml of crude enzyme protein, 4.3 mg/ml, were added and the mixture was incubated for 10 min. Last, 30 μg of *Escherichia coli* B DNA and *S*-[*methyl*-^{14}C]adenosylmethionine (0.74 nmole) was added to a final volume of 0.2 ml. Experiment 2: The incubation contained 1.2 μg of azaC-DNA, 10 μmoles of Tris-HCl (pH 8), 4 μmoles of β-mercaptoethanol, 2 μg of micrococcal nuclease, 0.13 μmoles of $CaCl_2$, and 25 μg of purified DNA methylase protein. The nuclease was inhibited with 0.5 μmoles of EGTA and the methylase was assayed as above. The incubation period for nuclease was 15 min and for methylase 5 min at 37°.

Experiment	Addition		Activity
	First incubation	Second incubation	
1	AzaCyd-DNA + DNAse	Methylase	0.89
	AzaCyd-DNA	Methylase	ND[a]
	DNAse	Methylase	1.05
2	AzaCyd-DNA	Methylase	0.29
	AzaCyd-DNA + nuclease	Methylase	5.60
	AzaCyd-DNA + methylase	Nuclease	0.07
	Methylase	Nuclease	5.33

[a] ND, None detected.

incubated with the nuclease prior to addition of the methylase (Table 5). If the methylase is incubated with the azaC-DNA prior to addition of the micrococcal nuclease, the enzyme remains inhibited. The enzyme cannot be reactivated by digestion with nuclease nor by dialysis of the nuclease-treated inhibited enzyme (data not shown).

Since azaC-DNA hydrolysis products do not inhibit the enzyme, the enzyme may be inhibited only if the 5-azacytosine is in intact DNA which can act as a substrate for the methylase. The *E. coli* K12 methylase required double-stranded DNA as substrate; it will not methylate single-stranded DNA (20). We therefore investigated the effect of heat-denaturing the azaC-DNA on its activity as an inhibitor. Since 5-azacytidine is readily hydrolyzed in water to form *N*-(formylamidino)-*N'*-β-D-ribofuranosylurea, (21) it was necessary to demonstrate that loss of inhibitory activity is a function of DNA denaturation and not temperature. AzaC-DNA was therefore denatured in 0.1 × SSC and SSC. As is shown in Fig. 1, when azaC-DNA was denatured in 0.1 × SSC the T_m of the DNA was 75° and the temperature at which 50% of the inhibitory activity was lost was 76°. In SSC the respective values were 96° and 95°.

To prove that the loss of inhibitory activity was not due to destruction of 5-azacytosine after denaturation, DNA was reannealed at 37° in 4 × SSC containing 40% formamide. Inhibitory activity returned when the DNA reannealed, as is shown in Fig. 2.

Inhibition is time-dependent and inhibited by substrate DNA. The inhibition of the methylase was dependent on the concentration of azaC-DNA present in the preincubation medium as well as on the length of time the enzyme was incubated in the presence of azaC-DNA prior to assay (Fig. 3). The experimental data did not give first-order plots at the concentrations of azaC-DNA used. Higher concentrations gave rates of inhibition that were too fast to measure and also caused significant time-dependent inhibition during the assay of enzyme activity. The rate of enzyme inactivation could be decreased by decreasing the temperature of the reaction, but the plots were still not first-order.

The rate of inactivation of enzyme was decreased by the presence of substrate DNA; however, *S*-adenosyl-

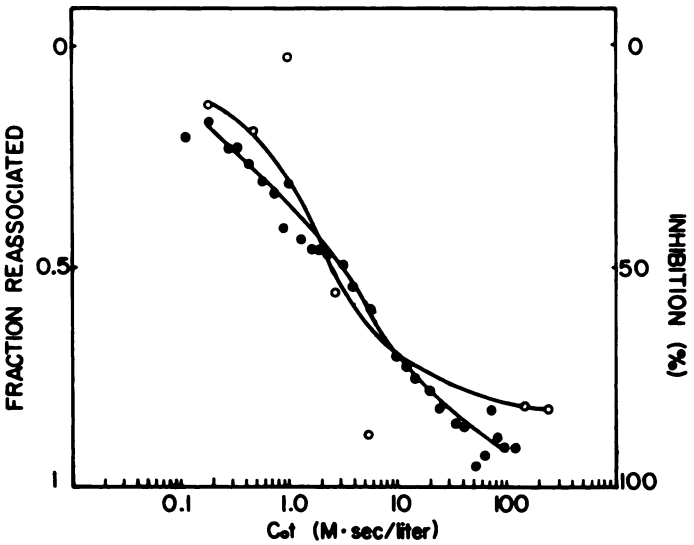

FIG. 2. *Effect of reannealing azaC-DNA on inhibition of DNA methylase*

azaC-DNA (0.55 mg/ml) in 4 × SSC and 40% formamide was overlaid with paraffin oil. Aliquots (1 μl) were removed for assay immediately before and after heating to 100° for 5 min. The solution was then incubated at 37° and aliquots were removed at the appropriate times for assay, which was performed as described under Materials and Methods. Aliquots were rapidly cooled in ice and frozen until asssayed. The fraction of DNA reassociated was calculated from A_{260} measurements made under identical conditions with an azaC-DNA concentration of 36 μg/ml. The Cot values are uncorrected for incubation conditions and were calculated according to the method of Britten *et al.* (24) ●——●, Fraction reassociated; ○——○, percentage inhibition.

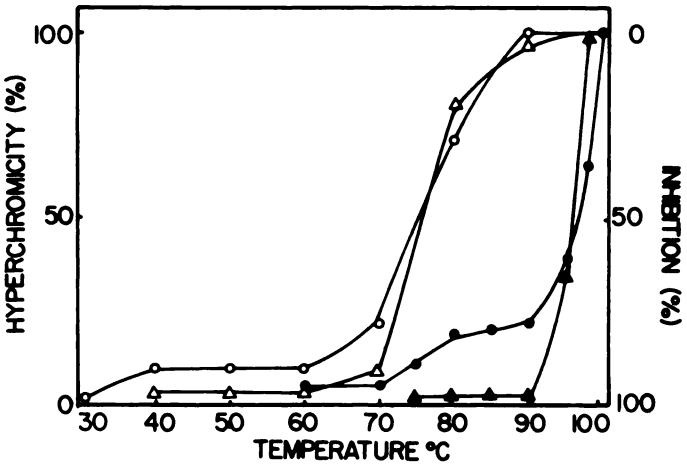

FIG. 1. *Effect of heat denaturation on activity of azaC-DNA*

azaC-DNA (34 μg) was heated in 1.3 ml of either 0.1 × SSC or SSC and the A_{260} was measured. At the indicated temperatures, 40-μl aliquots were removed and assayed for inhibition of DNA methylase. DNA and 75 μg of purified enzyme protein were incubated for 10 min before assay as indicated under Materials and Methods; 0.1 × SSC, ○——○, hyperchromicity; △——△, percentage inhibition. SSC, ●——●, hyperchromicity; ▲——▲, percentage inhibition.

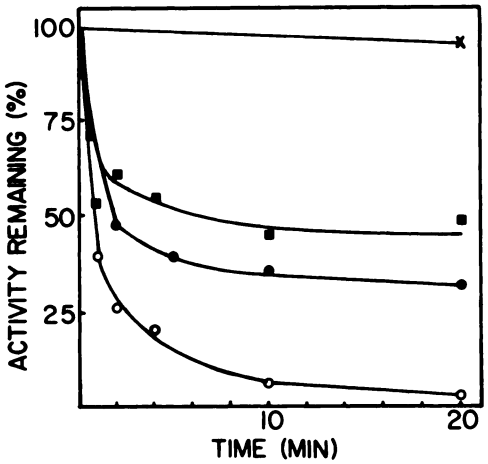

FIG. 3. *Time-dependent inactivation of Escherichia coli K12 DNA methylase by azaC-DNA*

Purified enzyme (25 μg) was incubated with azaC-DNA as described under Materials and Methods in 1.0 ml. Aliquots were removed and assayed at the indicated times; ×——×, control; ■——■, 1.1 μg of azaC-DNA; ●——●, 1.6 μg of azaC-DNA; ○——○, 3.2 μg of (azaC-DNA).

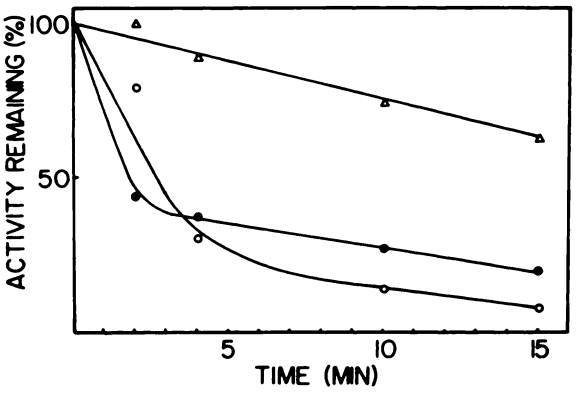

FIG. 4. *Effect of Escherichia coli B DNA and S-adenosylmethionine on the rate of inactivation of E. coli K12 DNA methylase by azaC-DNA*

Purified enzyme (25 μg) was incubated with 3.4 μg of azaC-DNA in the presence of 78 μg of *E. coli* B DNA or 25 μM *S*-adenosylmethionine in 1 ml. Aliquots were removed at the indicated times and assayed as described under Materials and Methods. ○——○, Control; ●——●, 25 μM *S*-adenosylmethionine; △——△, *E. coli* B DNA.

methionine at a concentration of 25 μM [K_m 12 μM (13)] had no effect on the rate of inactivation of the enzyme (Fig. 4). The apparent enhancement of inactivation shown in Fig. 4 was not reproducible.

Inhibition of restriction-modification methylases is decreased by digestion of azaC-DNA by their respective restriction nucleases. The effect of azaC-DNA on other DNA(cytosine-5)methylases was tested. Both the DNA modification enzyme *Eco*RII, which methylates the same cytosine as does the K12 cytosine methylase in the sequence CC$\overset{*}{T}$GG (13)—where the asterisk indicates the base methylated by the methylase, and the *Hpa*II cytosine methylase, which methylates C in the sequence CCGG, were inhibited by azaC-DNA (Fig. 5). 5-Azacytidine did not inhibit the *Hpa*II methylase when added to

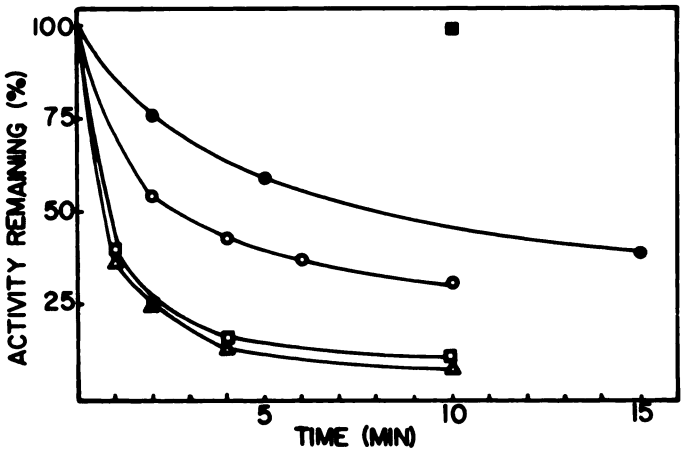

FIG. 5. *Time-dependent inhibition of EcoRII and HpaII methylase by azaC-DNA*

EcoRII methylase (30 μg) or HpaII methylase (6 μg) was incubated with azaC-DNA and aliquots were removed at the indicated times and assayed as described under Materials and Methods. *Eco*RII enzyme, ●——●, control; ○——○, azaC-DNA (2.3 μg/ml); △——△, azaC-DNA (4.3 μg). *Hpa*II enzyme, ■——■, control; □——□, azaC-DNA (3.4 μg/ml).

growing *H. parainfluenzae*, nor did it inhibit growth of this organism.

The characteristics of the inhibition of these enzymes by azaC-DNA were the same as those for the K12 methylase. The inhibition was time-dependent and gave nonlinear first-order plots.

Since 5-azacytidine, pancreatic deoxyribonuclease digests of azaC-DNA, and single-stranded azaC-DNA are not inhibitory to the cytosine DNA methylase, and since *E. coli* B DNA decreased the rate of inhibition, to act as an inhibitor, 5-azacytosine has to be in the same molecular form as the substrate. We therefore attempted to determine whether 5-azacytosine anywhere in DNA could act as an inhibitor, or whether the 5-azacytosine had to be in the sequence that would normally be methylated in order for it to inhibit the DNA methylase. If the latter is the case, then restriction enzymes that hydrolyze DNA at these sequences should decrease the inhibitory activity of the azaC-DNA.

*Eco*RII nuclease does partially destroy the inhibitory activity of azaC-DNA isolated from *E. coli* B, but not azaC-DNA isolated from *E. coli* K12 (Table 6). With the *Hpa*II restriction enzyme we could destroy most, but not all, of the inhibitory activity of azaC-DNA for *Hpa*II methylase under conditions that did not affect the ability of the DNA to inhibit the *Eco*RII methylase. Under these conditions, greater than 95% of *E. coli* B DNA was hydrolyzed within 2 hr, as judged by the destruction of its ability to act as substrate for the *Hpa*II methylase. We could destroy greater than 95% of the activity of the azaC-DNA by incubation for 14 hr with the *Hpa*II endonuclease, but these conditions also caused some decrease in the ability of the DNA to inhibit the *Eco*RII enzyme (data not shown).

DISCUSSION

5-Azacytidine has been shown to be incorporated into DNA in *E. coli*. Paces et al. (5) estimated that 26% of cytosine was replaced by 5-azacytosine in newly synthesized DNA when *E. coli* B was grown in the presence of 5-azacytidine, 5 μg/ml, for 25 min. With a 40% increase in DNA content of the cells this would be equivalent to an over-all 5-azacytosine content of 5% of cytosine.

We have estimated the amount of 5-azacytosine in the DNA by measuring the decrease in cytosine content after growing cells for 30 min in 5-azacytidine, 10 μg/ml. We found that such DNA contains 8.4% of its cytosine as 5-azacytosine.

We find that azaC-DNA is not a substrate for the K12 cytosine methylase but is a potent inhibitor of DNA(cytosine-5)methylases extracted from *E. coli* K12, *E. coli* RY23 containing the *Eco*RII methylase, and from *H. parainfluenzae* containing the *Hpa*II methylase. For azaC-DNA to act as an inhibitor it must have the same characteristics as the substrate used by these enzymes. It must be an intact double-stranded molecule. Digestion of the DNA or melting the DNA destroys inhibitory activity, whereas such activity reappears if the melted DNA is reannealed.

The inhibition is time-dependent and inhibited by substrate DNA. Since digestion of the inhibited enzyme

TABLE 6

Digestion of azaC-DNA with restriction nucleases

DNA, isolated from either *Escherichia coli* B or K12 grown in 5-azacytidine for 30 min, was incubated with *Eco*RII nuclease as described under Materials and Methods for 16 hr; 10 μmoles of Tris (pH 8), 4 μmoles of β-mercaptoethanol, and 5 μmoles of EDTA were added and the volume was made 0.2 ml. *E. coli* K12 cytosine methylase (30 μg) was added and the mixture was incubated for 4 min; 30 μg of *E. coli* B DNA and 1.9 nmoles (0.5 μCi) of *S*-[*methyl*-^{3}H]adenosylmethionine were added and the enzyme was assayed for 30 min. Digestion with the *Hpa*II endonuclease was performed as described under Materials and Methods for 4 hr. Reaction with 6 μg of *Hpa*II methylase or 30 μg of *Eco*RII methylase and assay of the inhibited enzyme were performed as described above. The activity of the control incubations were experiment 1, 5.3 pmoles; experiment 2, 4.6 pmoles; experiment 3, *Hpa*II enzyme, 5.7 pmoles; and *Eco*RII enzyme, 4.7 pmoles.

Experiment	Source of DNA	Endonuclease	Methylase	Activity (% of control)
1	AzaC-*E. coli* B, 2.7 μg	—	*E. coli* K12	9
	AzaC-*E. coli* B, 2.7 μg	*Eco*RII	*E. coli* K12	35
	AzaC-*E. coli* K12, 1.1 μg	—	*E. coli* K12	9
	AzaC-*E. coli* K12, 1.1 μg	*Eco*RII	*E. coli* K12	3
2	AzaC-*E. coli* B, 1.3 μg	—	*E. coli* K12	15
	AzaC-*E. coli* B, 1.3 μg	*Eco*RII	*E. coli* K12	37
	AzaC-*E. coli* K12, 0.4 μg	—	*E. coli* K12	29
	AzaC-*E. coli* K12, 0.4 μg	*Eco*RII	*E. coli* K12	25
3	AzaC-*E. coli* K12, 1.0 μg	—	*Hpa*II	13
	AzaC-*E. coli* K12, 1.0 μg	*Hpa*II	*Hpa*II	67
	AzaC-*E. coli* K12, 1.0 μg	—	*Eco*RII	10
	AzaC-*E. coli* K12, 1.0 μg	*Hpa*II	*Eco*RII	14

with micrococcal nuclease does not restore enzyme activity, it appears that the inhibition is irreversible. These characteristics of the inhibitor suggest that it combines with the active site of the enzyme. At present we cannot differentiate between a covalent or noncovalent complex of the enzyme with the inhibitor.

These findings suggest that the enzyme combines with DNA containing 5-azacytosine in the specific sequence that these methylases recognize; 5-azacytosine elsewhere in the DNA should not inhibit these enzymes. This possibility was tested by digesting the azaC-DNA with the respective restriction endonuclease. With the *Eco*RII nuclease we were able to decrease the inhibitory activity of azaC-DNA isolated from *E. coli* B cells but not the activity of the azaC-DNA isolated from *E. coli* K12 cells, which would have one strand methylated and therefore should be resistant to this enzyme. In the case of the *Hpa*II methylase we could destroy most of the inhibitory activity of the azaC-DNA by digestion with the *Hpa*II endonuclease under conditions that did not affect the ability of the DNA to inhibit the *Eco*RII methylase. However, we could get complete loss of inhibitory activity for the *Hpa*II methylase only under conditions that caused some loss of inhibitory activity of the DNA for the *Eco*RII methylase, presumably because the enzyme was contaminated with other nucleases. Since we achieved 95% digestion of substrate DNA within 2 hr whereas we had to incubate for 14 hr to achieve similar digestion of the azaC-DNA it appears that azaC-DNA is relatively resistant to these nucleases. From these experiments we can attribute most of the inhibitory activity of the DNA to the specific sites recognized by these methylases, but we cannot at present rule out the possibility that 5-azacytosine at other sites can also inhibit these enzymes.

Since the inhibitor appears to be azacytosine in the sequence recognized by the methylase, we can calculate the concentration of inhibitor used in our experiments. DNA from *E. coli* K12 contains 0.25% 5-methylcytosine (22). A chromosome of 2.7×10^9 daltons would therefore have 2×10^4 5-methylcytosines per chromosome, 8% of which are replaced by 5-azacytosine. The concentration of inhibitor used in the experiment shown in Fig. 3, 1–3 μg/ml azaC-DNA, would therefore correspond to 0.6–1.8 $\times 10^{-9}$ M, making azaC-DNA a very potent inhibitor of the enzyme.

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REFERENCES

1. Cihak, A., and F. Sorm. Biochemical effects and metabolic transformation of 5-azacytidine in *Escherichia coli*. *Collect. Czech. Chem. Commun.* **30**:2091–2102 (1965).
2. Hanka, L. J., J. S. Evans, D. J. Mason, and A. Deitz. Microbial production of 5-azacytidine. I. Production and biological activity. *Antimicrob. Agents Chemother.* 619–624 (1967).
3. Fucik, V., S. Zadrazil, Z. Sormova, and F. Sorm. Mutagenic effects of 5-azacytidine in bacteria. *Collect. Czech. Chem. Commun.* **30**:2883–2886 (1965).
4. Constantinides, P. G., P. A. Jones, and W. Gevers. Functional striated muscle cells from non-myoblast precursors following 5-azacytidine treatment. *Nature (Lond.)* **267**:364–366 (1977).
5. Paces, V., J. Dosokocil, and F. Sorm. Incorporation of 5-azacytidine into nucleic acids of *Escherichia coli*. *Biochim. Biophy. Acta* **161**:352–360 (1968).
6. Jurovik, M., K. Raska, Jr., F. Sorm, and Z. Sormova. Anabolic transformations of the new antimetabolite 5-azacytidine and its incorporation into RNA. *Collect. Czech. Chem. Commun.* **30**:3370–3376 (1965).
7. Doskocil, J., V. Paces, and F. Sorm. Inhibition of protein synthesis by 5-azacytidine in *Escherichia coli*. *Biochim. Biophys. Acta* **145**:771–779 (1967).
8. Weiss, J. W., and H. C. Pitot. Inhibition of ribosomal RNA maturation by 5-azacytidine and 8-azaguanine in Novikoff hepatoma cells. *Arch. Biochem. Biophys.* **160**:119–129 (1974).
9. Weiss, J. W., and H. C. Pitot. Alterations of ribosomal precursor RNA in Novikoff hepatoma cells by 5-azacytidine: studies on methylation of 45S and 32S RNA. *Arch. Biochem. Biophys.* **165**:588–596 (1974).

10. Lu, L. W., G. H. Chang, D. Medina, and K. Randerath. Drug effects on nucleic acid modification. 1. A specific effect of 5-azacytidine on mammalian transfer RNA methylation *in vivo*. *Biochem. Biophys. Res. Commun.* **68**: 1094–1101 (1976).
11. Friedman, S. The effect of 5-azacytidine on *E. coli* DNA methylase. *Biochem. Biophys. Res. Commun.* **89**:1324–1333 (1979).
12. Jones, P. A., and S. M. Taylor. Cellular differentiation, cytidine analogs and DNA methylation. Cell **20**:85–93 (1980).
13. Hattman, S. Partial purification of the *Escherichia coli* K-12 *mec*$^+$ deoxyribonucleic acid-cytosine methylase. *J. Bacteriol.* **129**:1330–1334 (1977).
14. Mann, M. B., and H. O. Smith. Specificity of *Hpa*II and *Hae*III DNA methylases. *Nucleic Acid Res.* **4**:4211–4221 (1977).
15. Miller, J. H. *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y., 432 (1972).
16. Marmur, J. A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J. Mol. Biol.* **3**:208–218 (1961).
17. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
18. Bendich, A. Methods for characterization of nucleic acids by base composition. *Methods Enzymol.* **3**:715–723 (1957).
19. Fujimoto, D., P. R. Srinivasan, and E. Borek. On the nature of the deoxyribonucleic acid methylases. *Biochemistry* **4**:2849–2855 (1965).
20. Gold, M., and J. Hurwitz. The enzymatic methylation of ribonucleic acid and deoxyribonucleic acid. *J. Biol. Chem.* **239**:3866–3874 (1964).
21. Pithova, P., A. Piskala, J. Pitha, and F. Sorm. Hydrolysis of 5-azacytidine and its connection with biological activity. *Collect. Czech. Chem. Commun.* **30**:2801–2811 (1965).
22. Vanyashin, B. F., A. N. Belozersky, N. A. Kakurina, and D. X. Kadirova. 5-methylcytosine and 6-methylaminopurine in bacterial DNA. *Nature* (*Lond.*) **218**:1066–1067 (1968).
23. Kalousek, F., and N. R. Morris. The purification and properties of deoxyribonucleic acid methylase from rat spleen. *J. Biol. Chem.* **244**:1157–1163 (1969).
24. Britten, R. J., D. E. Graham, and R. R. Neufeld. Analysis of repeating DNA sequences by reassociation. *Methods Enzymol.* **29**:363–405 (1974).

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Errata

Volume 19, No. 2 (1981), in the article, "The Inhibition of DNA(Cytosine-5) Methylases by 5-Azacytidine: The Effect of Azacytosine-Containing DNA," by Stanley Friedman, pp. 314–320: In Table 4 the fourth and fifth column headings, omitted in the original table, should read 5-MeCyt and 6-MeAde, respectively.

Volume 19, No. 3 (1981), in the article, "Transient Induction of Phenotypic Resistance in Human Lymphoblastoid Cells following Sequential Use of Two Antifolates," by Michael R. Hamrell, John Laszlo, and W. David Sedwick, pp. 491–495: the figure drawings for Figs. 1 and 2 are reversed; the legends and descriptions are correct.