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EFFECT OF 5-AZACYTIDINE ON DEOXYRIBONUCLEIC ACID METHYLATION IN *ESCHERICHIA COLI* K12

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Abstract—5-Azacytidine inhibits *Escherichia coli* DNA(cytosine-5)methylase when added to growing cells. The time-course of recovery of methylase activity and the appearance of 5-methylcytosine in DNA following removal of the drug was studied. When *E. coli* K12 was treated with 5-azacytidine for 30 min, DNA(cytosine-5)methylase levels decreased to less than 10% of control levels and slowly recovered to control levels after seven generations of growth. 5-Methylcytosine synthesis in DNA remained at less than 10% of control levels for three generations after treatment and returned to control levels after six generations of growth. In contrast, β-galactosidase levels in induced cells, which declined to 66% of control one generation after treatment, returned to control by the third generation of growth. The rate of induction of β-galactosidase had returned to the control rate two generations after growth resumed. Since azacytidine-containing DNA inhibits DNA-cytosine methylases *in vitro*, the prolonged inhibition of cytosine methylation in *E. coli* K12 following treatment with the drug could be due to the persistence of the drug in DNA and thus inhibition of newly synthesized enzymes.

5-Azacytidine is incorporated into both DNA and RNA and, as a result, inhibits protein synthesis [1]. The drug also specifically inhibits the methylation of cytosine to 5-methylcytosine in mammalian RNA [2] and DNA [3]. We have demonstrated that 5-azacytidine inhibits *S*-adenosyl-L-methionine : DNA-(cytosine-5)-methyltransferase (EC 2.1.1.37) in *E. coli* K12 without any effect on the activity of DNA-(adenine-N^6)methyltransferase present in the same cell [4]. DNA containing 5-azacytosine will inhibit several bacterial DNA(cytosine-5)methylases *in vitro*, and this inhibition is irreversible [5]. The present experiments were performed to determine if the drug inhibits DNA methylation in growing *E. coli* K12 and to study the time-course of recovery of DNA methylation and enzyme activity after the removal of the drug from the growth medium in order to determine if the inhibition in cells is irreversible, as it is *in vitro*.

MATERIALS AND METHODS

Growth conditions and drug treatment. E. coli K12 ATCC was grown in minimal A medium [6] supplemented with 40 μg/ml DL-methionine in 250-ml side-arm flasks in a reciprocating shaker at 37°. Growth was recorded by turbidity readings in a Klett–Summerson colorimeter with a No. 54 filter. When the cells reached 60 Klett units, 5-azacytidine was added and the cells were incubated for 30 min. Cells were centrifuged for 6 min at 6000 *g* and washed twice with medium. The cells were resuspended in the original volume of medium, and aliquots were transferred to separate flasks for further incubations. Generation time was determined by measuring the rate of growth between 30 and 60 Klett units.

DNA extraction and analysis. DNA was extracted as described by Lark [7]. The DNA was treated with 0.5 ml of 0.1 N sodium hydroxide at 30° for 16 hr. Following neutralization with trichloroacetic acid, the DNA was precipitated with 2 vol. of ethanol. The DNA was dissolved in 0.1 N sodium hydroxide, reprecipitated in 5% trichloroacetic acid, and washed with 5% trichloroacetic acid, alcohol, ether, and dried. The DNA was hydrolyzed in 0.1 ml of 70% perchloric acid at 100° for 1 hr and diluted to 0.5 ml with water. The solution was neutralized with 1 M potassium hydroxide, the $KClO_4$ precipitate was removed by centrifugation, and the bases were analyzed. Base analysis was performed either by two-dimensional paper chromatography or high-pressure liquid chromatography. Paper chromatography was performed in ethyl acetate–propanol–water (4:1:2) followed by *n*-butanol–ammonia–water (96:5:14) [8]. The bases were located under ultraviolet light and the areas containing them cut out. Radioactivity was estimated by counting the paper in a scintillation spectrometer.

When DNA was labeled with [2-^{14}C]uracil, base analysis was performed by high-pressure liquid chromatography on a BioRad Aminex A-7 column (0.3 × 50 cm) in 0.02 M ammonium bicarbonate, pH 10.6, at 40° at a flow rate of 0.2 ml/min [9]. The eluate was monitored at 254 nm with an Altex ultraviolet monitor. Fractions were collected and radioactivity was determined by counting the fractions in a liquid scintillation counter.

Enzyme determinations. β-Galactoside activity was determined by measuring the rate of hydrolysis of *o*-nitrophenyl-β-galactoside as described by Miller [6].

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DNA(cytosine-5)methylase activity measured the incorporation of radioactivity from *S*-adenosyl-[*methyl*-^{14}C]methionine into alkali-stable, acid-precipitable material resistant to deproteinization. *E. coli B* DNA was used as substrate [10].

Measurement of [^{3}H]*leucine incorporation.* Samples of bacteria which had incorporated [4,5-^{3}H]-leucine were treated with 5% trichloroacetic acid in an ice bath. The samples were filtered on membrane filters, washed three times with cold 5% trichloroacetic acid, dried, and counted in organic scintillation fluid.

Protein determinations were by the method of Lowry *et al.* [11].

RESULTS

Effect of 5-azacytidine on growth rate and protein synthesis of E. coli K12. *E. coli* K12 was treated with 5-azacytidine (20 mg/ml) in log phase of growth for 30 min. The cells were washed and reincubated. When growth resumed, the generation time had increased from 44 min before treatment to 74 min after treatment. The growth rate returned to 80% of the pretreatment growth rate six generations after removal of the drug (Table 1). As another correlate of growth rate, measurements of the rate of leucine incorporation into protein were made, since 5-azacytidine inhibits protein synthesis more markedly than it inhibits RNA or DNA synthesis [1]. The rate of leucine incorporation declined to 55% of control one generation after removal of the drug, and recovered to 90% of the control rate by the sixth generation (Table 1).

Effect of 5-azacytidine on DNA methylation. DNA methylation was measured in two ways. Cells were treated with [*methyl*-^{14}C]methionine either during 5-azacytidine treatment or at different times after removal of the drug. The 5-MeCyt/6-MeAde ratio was determined. This is a valid measure of 5-MeCyt formation only if the drug does not inhibit adenine methylation. The 6-MeAde/Ade ratio was measured after labeling the cells with [^{3}H]adenine and was found not to differ from control (Table 2). Alternatively, cells were treated with [2-^{14}C]uracil either during treatment with 5-azacytidine or after removal of drug, when growth had resumed, and the 5-MeCyt/Cyt ratio was determined.

Treatment of cells with 5-azacytidine depressed methylation of cytosine in DNA as measured by the ratio of 5-MeCyt/6-MeAde to 2% of the control (Table 3). Methylation of DNA remained at less than 10% of control levels for as long as three generations of growth after removal of the drug, and reached control levels only after six generations of growth.

Measurement of 5-MeCyt/Cyt ratios gave similar results. One and one-half generations after removal of 5-azacytidine from the growth medium, the 5-MeCyt-Cyt ratio was still less than 13% of control. It did not matter whether the DNA was labeled with [2-^{14}C]uracil during azacytidine treatment or after azacytidine was removed from the medium and the cells had resumed growth. After five generations of growth, the 5-MeCyt/Cyt ratio had reached 79% of the control level, and, again, the ratios were the same whether the DNA was labeled during or after azacytidine treatment (Table 2).

Effect of 5-azacytidine on methylase levels. Treatment of cells with 5-azacytidine caused a marked decrease in cytosine methylase activity. Methylase activity recovered very slowly after removal of the drug. Only after seven generations of growth did methylase activity recover to control levels. When cytidine was added to the growth medium after removal of 5-azacytidine, there was less inhibition of methylase activity, but the time-course for return of activity was similar (Fig. 1). Under these conditions the generation time was increased to 80 min over five generations of growth. The return of activity

Table 1. Growth rate and [^{3}H]leucine incorporation in *E. coli* K12 after treatment with 5-azacytidine*

Generation	Generation time (min)	[^{3}H]Leucine incorporation (nmoles/min/mg protein)
Control	44 ± 8†	7.6 ± 1.6†
1	74 ± 17	4.2 ± 0.7
2	84 ± 14	4.6 ± 0.3
3	87 ± 9	4.9 ± 0.6
4	76 ± 6	6.3 ± 1.7
5	70 ± 9	6.7‡
6	55 ± 5	6.9‡

* *E. coli* K12 was grown to 60 Klett units in 10 ml medium in a 250-ml side-arm flask. Cells were treated with 20 μg/ml 5-azacytidine for 30 min. The cells were washed twice with medium; aliquots were appropriately diluted with fresh medium and reincubated. The cells were grown to 60 Klett units and were treated with 0.14 mM [^{3}H]leucine, 4.4 Ci/mole. Aliquots of 1 ml were removed at 2-min intervals into 1 ml of cold 10% trichloroacetic acid. The precipitate was collected and radioactivity determined as described in Materials and Methods.

† Average of three determinations ± S.D.

‡ Average of two determinations.

Table 2. 5-MeCyt/Cyt and 6-MeAde/Ade ratios in DNA synthesized after treatment of cells with 5-azacytidine*

Treatment	Time of measurement	5-MeCyt/100Cyt	6-MeAde/100Ade
None		0.71 ± 0.02† (4)‡	1.94
5-azacytidine (20 μg/ml)	40 min§	0.08	1.88‖
	1 generation§	0.09	
	3.5 generations§	0.05	
	5 generations§	0.56	
	3.5 generations¶	0.09	
	5 generations¶	0.55	

* Cells were grown to 50 Klett units in 30 ml of minimal A medium supplemented with 40 μg/ml DL-methionine in a 250-ml side-arm flask. Aliquots (5 ml) were treated with 25 μg/ml 5-azacytidine for 40 min. Incubation with radioactive precursors was also performed for 40 min. The cells were washed twice with medium, diluted with fresh medium, and harvested at the indicated times. DNA was extracted and the bases were analyzed by HPLC as described in Materials and Methods. Experiments were performed in duplicate. The 6-MeAde/Ade ratio was determined by paper chromatography. In a typical control experiment, 827 cpm were recovered in 5-MeCyt and 3869 cpm in 6-MeAde.

† ± S.D.

‡ Number of determinations. All other data are the average of two determinations.

§ [2-^{14}C]Uracil (10 μCi), 56 mCi/mmole, added with 5-azacytidine.

¶ [2-^{14}C]Uracil (10 μCi) added after washing cells and resumption of growth.

‖ [2-^{3}H]Adenine (50 μCi), 16.6 Ci/mmole, added with 5-azacytidine.

followed a similar course even if the generation time was prolonged to 140 min by growing the cells with acetate as the sole carbon source (Fig. 1).

Effect of 5-azacytidine on β-galactosidase synthesis. 5-Azacytidine inhibits protein synthesis as a result of incorporation into RNA [1]. The drug completely inhibits induced enzyme synthesis in bacteria if administered before the inducer [12]. To ascertain that the quantity of drug or its metabolites remaining in the cells after removal of the drug from the medium would not inhibit new enzyme synthesis, we measured the rate of recovery of β-galactosidase synthesis. When cells were grown in the presence of inducer, isopropylthiolgalactoside, 5-azacytidine caused a decrease in β-galactosidase levels one generation after the drug was removed. Unlike the effect of the drug on the methylase, there was no immediate decrease in enzyme activity; the decrease in activity was noted only after growth had resumed. Furthermore, β-galactosidase activity recovered to 90% of control

Table 3. DNA methylation with [^{14}C-*methyl*]methionine after treatment with 5-azacytidine*

Treatment	Time of measurement (generations)	5-MeCyt / 6-MeAde	
5-Azacytidine	0	0.01	(2)†
	2	0.01	(1)
	3	0.08 ± 0.01‡	(3)
	5	0.47 ± 0.01	(3)
	6	0.55 ± 0.11	(4)
None		0.56 ± 0.06	(5)

* Cells were grown in 10 ml minimal medium supplemented with 40 μg/ml DL-methionine. When the cells reached 60 Klett units, 5-ml aliquots were treated with 5-azacytidine for 30 min, washed twice with 10 ml medium, and diluted with fresh medium. The cells were treated for 30 min at the time of addition of 5-azacytidine, or at the indicated time after growth resumed, with [^{14}C-*methyl*]methionine (20 μCi, sp. act. 50 Ci/mole). DNA was extracted as described by Lark [7] and hydrolyzed in 70% perchloric acid at 100° for 30 min. Bases were separated by two-dimensional paper chromatography. In a typical experiment, DNA extracted from control culture contained 2387 cpm in 6-MeAde.

† Number of determinations.

‡ S.D.

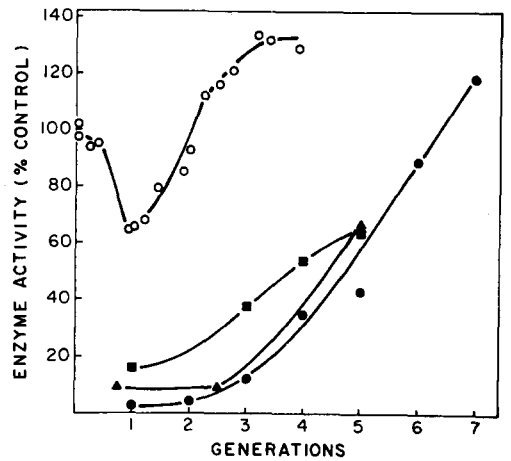

Fig. 1. Recovery of methylase and β-galactosidase activity after treatment of cells with 5-azacytidine. Cells were grown in 100 ml minimal A medium containing 40 μg/ml DL-methionine to 0.6 optical density units and treated with 20 μg/ml 5-azacytidine for one generation. The cells were washed twice by centrifugation with medium, diluted into 100 ml fresh medium, reincubated, and harvested by centrifugation at the indicated time. Cells were washed and methylase levels were determined as described [10]. β-Galactoside levels were determined in cells growing in 20 ml medium supplemented with 10^{-3} M isopropyl β-D-thiogalactoside. Aliquots (1 ml) were removed at the indicated times and assayed according to Miller [6]. Cells were grown in medium containing 2% glucose as carbon source (●, ○); 2% sodium acetate as carbon source (▲); 2% glucose as carbon source, 20 μg/ml cytidine added after treatment with 5-azacytidine (■). β-Galactosidase activity (○); DNA(cytosine-5)methylase activity (●, ▲, ■). Control β-galactosidase activity was 4.8 μmoles per min per mg protein; control methylase levels were 1.5 pmoles per min per mg protein.

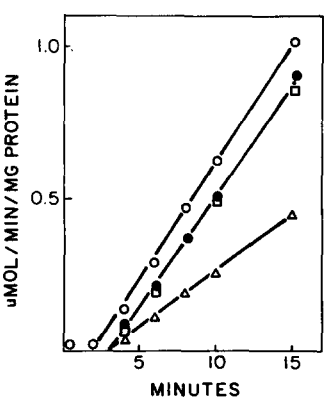

Fig. 2. Effect of treatment with 5-azacytidine on induction of β-galactosidase after removal of the drug. *E. coli* K12 was grown and treated with 5-azacytidine as described in Table 1. The cells were washed and reincubated in 10 ml medium. After one, two or three generations, the cells were treated with 10^{-3} M isopropyl β-D-thiogalactoside, and 1-ml samples were taken for determination of β-galactosidase levels according to Miller [6]. Control (○); one generation (△); two generations (●); three generations (□); after removal of azacytidine from the incubation medium.

levels within two generations of growth after drug treatment (Fig. 1).

The rate of induction of β-galactosidase was also measured at varying times after treatment of the cells with azacytidine. One generation after treatment the rate of induction of enzyme activity was 50% of the control rate and attained the control rate by two generations (Fig. 2), a result consistent with greater than 90% of recovery of activity occurring within two generations after removal of drug (Fig. 1). In contrast, the rate of protein synthesis as measured by the rate of leucine incorporation, as noted above, slowly returned from 55 to 90% of control level over the course of six generations of growth (Table 1). The return of β-galactosidase synthesis to control levels before recovery of general protein synthesis, as measured by leucine incorporation, is confirmed by the increased content of the enzyme in cells at three and four generations as compared to the control (Fig. 1).

DISCUSSION

The rapid inhibition of the methylase enzyme and the slow recovery of activity to control levels over seven generations of growth indicate that 5-azacytidine inhibited the enzyme irreversibly in growing cells. *In vitro* we have shown that azacytosine-containing DNA inhibits the methylase irreversibly. The prolonged time for recovery of methylase activity, as measured by either return of enzyme activity or synthesis of 5-methylcytosine in DNA, indicates that the inhibitor remains in the cell after its removal from the incubation medium, in DNA. The return of activity would appear to be related to cell division rather than to time of incubation following treatment, since it takes the same number of generations for methylase activity to return, independent of the growth rate of the cells.

Paces *et al.* [12] demonstrated that 5-azacytidine is incorporated into DNA and RNA in *E. coli*. Doskocil and Sorm [13] showed that the drug inhibits protein synthesis, presumably because of the incorporation of the drug and its deaminated metabolite, 5-azauridine, into RNA [13]. The effect of the drug on induced enzyme synthesis has also been studied by these authors. They demonstrated that 5-azacytidine would inhibit induction of β-galactosidase if added before, but not if added after, induction with methylthio-β-galactoside [12]. Since 5-azacytidine was less effective as an inhibitor of protein synthesis in mutants lacking cytidine deaminase than in wild-type cells, Doskocil and Sorm [13] proposed that the major inhibitor of protein synthesis was 5-azauridine; however, even in these mutants 5-azacytidine is an effective inhibitor of β-galactosidase synthesis [13].

The effects of 5-azacytidine on β-galactosidase synthesis described in this paper are consistent with the suggestion that 5-azacytidine or its metabolite, 5-azauridine, by being incorporated into RNA, inhibits protein synthesis. The drug or its metabolites do not inhibit the enzyme directly, because the decrease in β-galactosidase activity appears only after cell growth resumes following removal of the drug. Induced enzyme synthesis was still inhibited one generation

after removal of drug but returned to control levels by two generations (Fig. 2). By three generations, β-galactosidase levels were greater than those present in control cells (Fig. 1). This is because the rate of β-galactosidase synthesis had returned to control levels by the second generation of growth but the rate of protein synthesis had not reached control levels by that time (Table 1).

The rapid recovery of β-galactosidase activity after removal of 5-azacytidine indicates that the internal pool of drug declines rapidly, such that functional mRNA can be synthesized at the same rate as in control cells by the second generation after removal of the drug. The fact that the β-galactosidase levels were higher than control levels by the third generation may be due to the presence of a CCAGG sequence in the β-galactosidase promoter [14]. This sequence would normally be methylated in *E. coli* K12 by the *dcm* methylase [15], but should be unmethylated in cells treated with 5-azacytidine. In the one *E. coli* system studied containing a CCAGG sequence in a control region, a regulatory protein binds less efficiently to the methylated *lex*A operator than to the operator if it is unmethylated [16]. The enhanced synthesis of β-galactosidase would be expected to occur if a similar enhancement of binding occurred at promoter-RNA polymerase binding sites when the promoter was unmethylated.

The loss of methylase activity and the prolonged slow recovery of activity, therefore, cannot be due to the effect of the drug on RNA synthesis or to the continued presence of the drug in the ribonucleotide pool. This suggests that the inhibitor in growing cells is also azacytosine-containing DNA. However, DNA synthesized at the time of addition of 5-azacytidine did eventually become methylated five generations after removal of the drug from the medium (Table 2). This suggests that 5-azacytosine or its breakdown products are eventually removed from DNA. The rate of DNA turnover in these cells, as measured by the loss of [^{3}H]thymidine incorporated into DNA at the time of treatment with 5-azacytidine, is very low. Four percent of the incorporated thymidine was lost by the sixth generation. Control cells lose less than 3% of the incorporated thymidine in the same period (unpublished observation). However, there may be an enzymatic system capable of removing 5-azacytosine from DNA which has minimal effects on DNA turnover.

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