Inhibition of the Synthesis of Thymine Nucleotides by Azaserine

ALAN C. SARTORELLI AND BARBARA A. BOOTH

Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut 06510

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SUMMARY

The synthesis of purine nucleotides *de novo* in sarcoma 180 ascites cells propagated in mice was essentially completely inhibited for a prolonged period of time by azaserine (*O*-diazoacetyl-L-serine); this metabolic blockade resulted in a 60% decrease in the amount of adenine nucleotides in the cells. Concurrently, in the antibiotic-treated cells, the incorporation of thymidine-^{3}H, formate-^{14}C and deoxycytidine-^{3}H into the thymine of DNA was markedly depressed, while the utilization of orotic acid-^{14}C and leucine-^{14}C for the formation of RNA and proteins, respectively, was unaffected. Azaserine did not decrease the rate of incorporation of adenine-^{14}C into the purine nucleotides of DNA and RNA, suggesting that the polymerization processes per se were not altered by the drug. The inhibition of the incorporation of labeled precursors into the thymine of DNA in azaserine-treated cells was accounted for by a pronounced depression in the levels of thymidine kinase (ATP:thymidine 5′-phosphotransferase, EC 2.7.1.21) and thymidine monophosphate kinase (ATP:thymidine monophosphate phosphotransferase, EC 2.7.4.9). The availability to the cells of a supply of preformed adenine restored both the azaserine-depleted pool of adenine nucleotides and the activities of the thymine nucleotide-synthesizing enzymes; in this capacity, hypoxanthine was only slightly effective, while guanosine and azapurines were ineffective in reversing the azaserine-induced metabolic lesions. The results suggested a possible role for adenine nucleotides in regulating the activities of thymidine kinase and thymidine monophosphate kinase in intact cells.

INTRODUCTION

Azaserine (*O*-diazoacetyl-L-serine), an antibiotic produced by a *Streptomyces*, has been shown to inhibit cellular growth and development in both mammalian and microbial systems. The antibiotic functions biochemically as an antagonist of glutamine; thus, potentially all glutamine-requiring enzymic reactions are sensitive to the inhibitory activity of this agent. Specificity does exist, however, and the biosynthesis of purine nucleotides *de novo* is considered to be the metabolic area most sensitive to the action of the antibiotic (1–10). The enzyme phosphoribosylformylglycineamidine synthetase [5′-phosphoribosyl - formylglycineamide:L - glutamine amido-ligase (ADP); EC 6.3.5.3] is the primary site of inhibition on the purine nucleotide synthetic pathway and is irreversibly titrated by extremely small quantities of azaserine (8, 11–14), whereas other azaserine-sensitive enzymes, e.g., GMP synthetase [xanthosine-5′-phosphate:L-glutamine amido-ligase (AMP); EC 6.3.5.2] (8, 15), NAD synthetase [deamido-NAD:L-glutamine amido-ligase (AMP); EC 6.3.5.1] (16), CTP synthetase (UTP:L-glutamine amido-ligase) (9, 17), and phosphoribosylpyrophosphate amidotransferase [ribosylamine - 5 - phosphate : pyrophosphate phosphoribosyltransferase (glutamate-amidating); EC 2.4.2.14] (8, 18), require considerably larger quantities of the glutamine analog to produce inhibition. The results of studies in several systems implicate the

blockade of purine nucleotide biosynthesis *de novo* in the phenomenon of growth inhibition produced by this agent (e.g., refs. 6 and 7). The consequences of such a metabolic lesion (inhibition of purine nucleotide formation *de novo*), with regard to the maintenance of normal metabolic cellular balance, have not been ascertained. Furthermore, the findings have implicated an additional site or sites in some mammalian systems in the mechanism by which the drug causes a retardation of growth; thus, the presence of a source of preformed purines, a situation that theoretically would be expected to circumvent the blockade of the *de novo* biosynthetic route produced by the antibiotic, did not abolish the inhibition of growth caused by the drug (19, 20).

This report, therefore, deals with the ability of azaserine-stressed sarcoma 180 cells to synthesize DNA, RNA and proteins in the presence of a drug-induced deficiency in the supply of purine nucleotides. Not only was a decrease in the activity of enzymes involved in the synthesis of thymine nucleotides found in azaserine-treated cells, but also a role for adenine nucleotides in the regulation of normal cellular levels of these enzymes has been proposed.

MATERIALS AND METHODS

Experiments were performed on 9- to 11-week-old female CD-1 mice (Charles River Breeding Laboratories, North Wilmington, Massachusetts, U.S.A.). The animals were inoculated intraperitoneally 6 days before use with 2×10^6 sarcoma 180 ascites cells. The chemical agents were dissolved in isotonic saline just prior to use and were injected intraperitoneally. At selected intervals thereafter, either 200 μg of thymidine-methyl-^{3}H (6.6×10^3 cpm/μg), 100 μg of orotic acid-6-^{14}C hydrate (1.6×10^4 cpm/μg), 125 μg of DL-leucine-1-^{14}C (1.5×10^4 cpm/μg), 660 μg of deoxycytidine-^{3}H (1.1×10^3 cpm/μg), 90 μg of formate-^{14}C (4.8×10^3 cpm/μg), 50 μg of adenine-8-^{14}C (2.7×10^4 cpm/μg), or 100 μg of glycine-2-^{14}C (1×10^4 cpm/μg) was administered by intraperitoneal injection to each mouse, and 1 hr was allowed for metabolic utilization. Sodium nucleates were isolated by the method of Tyner *et al.* (21). In experiments involving the incorporation of either thymidine, orotic acid, deoxycytidine, or formate into nucleic acids, the sodium nucleates were hydrolyzed with 70% perchloric acid for 1.5 hr (22) and desalted on charcoal columns, the desired purine and pyrimidine components of the nucleic acids were purified and analyzed as described previously (23–25), except that radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer. After exposure of the cells to either leucine-^{14}C or glycine-^{14}C, residual protein and cold acid-soluble adenine, respectively, were isolated and analyzed as previously reported (26).

The size of the acid-soluble adenine nucleotide pool was estimated by isotope dilution (24); adenine-8-^{14}C HCl hemihydrate (280 mμmoles, 2.7×10^3 cpm/mμmole) was added to cold 4% perchloric acid extracts, purines were liberated by hydrolysis for 1 hr at 90°, and adenine was purified and its specific radioactivity determined as described earlier. Chromatography of unhydrolyzed extracts on columns of Dowex 1-formate has indicated that essentially all the isolated adenine was present in nucleotide form.

Enzyme extracts were prepared, and the activities of thymidine kinase (ATP:thymidine 5′-phosphotransferase, EC 2.7.1.21), thymidine monophosphate kinase (ATP:thymidine monophosphate phosphotransferase, EC 2.7.4.9), and thymidine monophosphate nucleotidase (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5) were assessed by methods described in an earlier report (27).

RESULTS

Sarcoma 180 ascites cells exposed to azaserine exhibit a prompt inhibition of purine nucleotide biosynthesis *de novo* that persists for a relatively long period of time; this finding, measured by the incorporation of glycine-2-^{14}C into acid-soluble adenine nucleotides, is shown in Fig. 1. Essentially complete blockade of the incorporation of glycine occurred at 3 hr after the administration of azaserine, and even 48 hr after the antibiotic the rate of synthesis of adenine-containing compounds

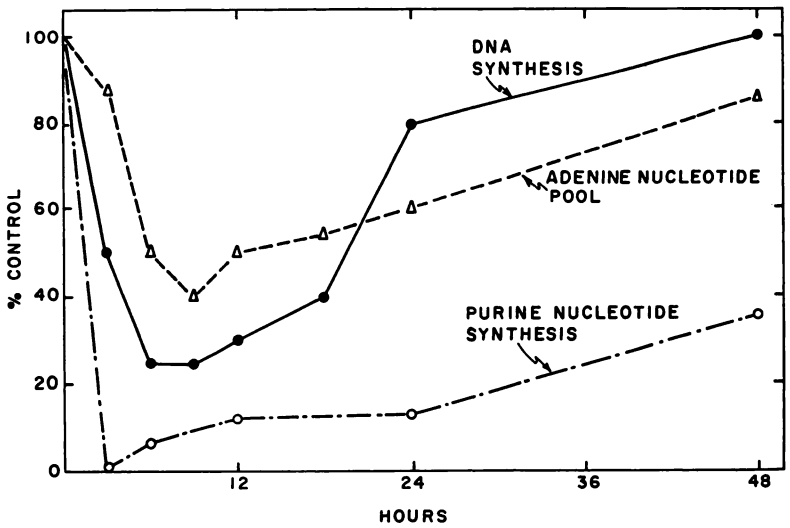

FIG. 1. *The effects of azaserine on the incorporation of glycine-2-^{14}C into acid-soluble adenine nucleotides, the cellular content of acid-soluble adenine nucleotides, and the incorporation of thymidine-^{3}H into the thymine of DNA*

Mice bearing 6-day implants of sarcoma 180 ascites cells received a single intraperitoneal dose of 0.2 mg of azaserine per kilogram of body weight. At selected intervals thereafter, either 100 μg of glycine-2-^{14}C (1×10^4 cpm/μg) or 200 μg of thymidine methyl-^{3}H (6.6×10^3 cpm/μg) was injected into each mouse and 1 hr was allowed for metabolic utilization. In other animals the cellular content of cold acid-soluble adenine nucleotides was measured by isotope dilution. Each value represents the mean obtained from the separate analyses of ascites cells from 3–48 animals.

was only approximately 35% that in control cells. The inability of the cells to synthesize purine nucleotides *de novo* resulted in a gradual depletion of the acid-soluble pool of adenine nucleotides to a maximum of about 40% of the normal level (Fig. 1). Interestingly, the replenishment of the cellular pool of adenine nucleotides occurred under conditions in which the cells appeared to recover only slightly their ability to incorporate glycine into adenine nucleotides. The data in Fig. 1 also show that the exposure of cells to azaserine resulted in a depression of the incorporation of thymidine-^{3}H into the thymine of DNA; a maximum inhibition of 65% occurred by 6 hr after the azaserine; however, this inhibition was relieved completely by 48 hr after the administration of the drug. LePage *et al.* (4) have shown that azaserine induced inhibition of purine nucleotide formation *de novo* almost immediately in ascites cells; nevertheless, in the present study no inhibition of thymidine-^{3}H incorporation into DNA occurred when the isotope was administered 15 min after the azaserine. Thus, although inhibition of the synthesis of purine nucleotides occurred, no inhibition of thymidine utilization for the formation of DNA was observed; the data in Fig. 1 show, however, that at this time little or no depression in the level of the adenine nucleotide pool was produced by the antibiotic.

Since the synthesis *de novo* of purine nucleotides was inhibited essentially completely by azaserine, the blockade of this metabolic pathway could not be increased further by increasing the concentration of the drug. It was reasoned, therefore, that if the decrease in the rate of incorporation of thymidine-^{3}H into the DNA of azaserine-treated cells is related to the inability to synthesize purine nucleotides *de novo*, then no increased effect on the utilization of thymidine should be obtained with an increase in the quantity of azaserine. In agreement with such a concept, it was demonstrated that the exposure of the cells to a 200-fold increase in the level

of azaserine (20 mg/kg), and the subsequent administration of thymidine-^{3}H 12 hr later, did not result in greater inhibition than was produced by 0.2 mg of azaserine per kilogram. Furthermore, the depression in the size of the acid-soluble adenine nucleotide pool induced by the drug was similar for azaserine concentrations ranging from 0.2 to 20 mg/kg.

TABLE 1

Incorporation of orotic acid-6-^{14}C and DL-*leucine-1-^{14}C into RNA and protein, respectively, of sarcoma 180 cells treated with azaserine*

Mice bearing 6-day implants of sarcoma 180 ascites cells received a single intraperitoneal dose of 0.2 mg of azaserine per kilogram of body weight. Twelve hours later, either 100 μg of orotic acid-6-^{14}C hydrate (1.6×10^{4} cpm/μg) or 125 μg of DL-leucine-1-^{14}C (1.5×10^{4} cpm/μg) was injected into each mouse; 1 hr was allowed for metabolic utilization. Each value represents the mean (± the standard error) obtained from the separate analyses of ascites cells from 6–8 animals.

Substrate	Azaserine	cpm/μmole $\times 10^{-2}$ RNA uracil	cpm/mg $\times 10^{-3}$ Residual protein
Orotic acid-6-^{14}C	−	45.0 ± 6.1	—
	+	44.4 ± 6.4	—
Leucine-1-^{14}C	−	—	32.0 ± 3.4
	+	—	27.4 ± 3.7

The effects of azaserine on the rates of synthesis of RNA and protein were assessed with orotic acid-6-^{14}C and leucine-1-^{14}C, respectively (Table 1). No significant inhibition of these metabolic processes was produced under circumstances that markedly lowered the rate of formation of purine and thymine nucleotides. The utilization of the large purine nucleotide pool would appear to account for the apparently normal incorporation of orotic acid-^{14}C into the RNA of cells unable to fabricate purine nucleotides *de novo* because of blockade of this pathway by azaserine.

To determine whether the inhibition by azaserine of thymidine incorporation into DNA was a reflection of a generalized decrease in the rate of synthesis of DNA or was unique to this isotopic tracer, other labeled precursors of DNA were employed. The data presented in Table 2 show that pronounced inhibition of the utilization of both formate-^{14}C and deoxycytidine-^{3}H for the formation of DNA occurred in azaserine-treated cells; however, the utilization of adenine-8-^{14}C by cells exposed to the drug resulted in DNA and RNA purines with a greater specific radioactivity than the polynucleotide purines isolated from untreated cells (Table 3). Correction of these values to account for the lowered acid-soluble adenine nucleotide precursor pool in the drug-treated sarcoma 180 ascites cells indicated that there were no significant differences in the specific radioactivities of the DNA and RNA purines of the two groups. Thus, the activity of DNA nucleo-

TABLE 2

Incorporation of labeled precursors into the nucleic acids of sarcoma 180 cells treated with azaserine

Mice bearing 6-day implants of sarcoma 180 ascites cells received a single intraperitoneal dose of 0.2 mg of azaserine per kilogram of body weight. Twelve hours later, either 90 μg of sodium formate-^{14}C (4.8×10^{3} cpm/μg) or 660 μg of deoxycytidine-^{3}H (1.1×10^{3} cpm/μg) was injected into each mouse; 1 hr was allowed for metabolic utilization. Each value represents the mean (± the standard error) obtained from the separate analyses of ascites cells from 3–14 animals.

Substrate	Azaserine	cpm/μmole $\times 10^{-2}$ DNA thymine	NA[a] cytosine	NA adenine	NA guanine
Formate-^{14}C	−	29.5 ± 5.0	—	23.1 ± 3.7	37.4 ± 5.3
	+	9.0 ± 1.4	—	5.4 ± 0.9	10.4 ± 2.2
Deoxycytidine-^{3}H	−	13.0 ± 1.4	10.4 ± 1.1	—	—
	+	7.5 ± 0.2	6.9 ± 1.0	—	—

[a] NA, nucleic acid (mixed DNA and RNA).

TABLE 3

Incorporation of adenine-8-^{14}C into nucleic acid purines of sarcoma 180 cells treated with azaserine

Mice bearing 6-day implants of sarcoma 180 ascites cells received a single intraperitoneal dose of 0.2 mg of azaserine per kilogram of body weight. Twelve hours later, 50 μg of adenine-8-^{14}C (2.7×10^4 cpm/μg) was injected into each mouse; 1 hr was allowed for metabolic utilization. Each value represents the mean (± the standard error) obtained from the separate analyses of ascites cells from 4 animals.

	cpm/μmole $\times 10^{-2}$			
Treatment	DNA adenine	DNA guanine	RNA adenine	RNA guanine
None	36.1 ± 3.9	1.7 ± 0.5	123 ± 30.5	9.9 ± 1.6
Azaserine	79.1 ± 4.2	3.5 ± 0.6	481 ± 150	18.2 ± 2.5

tidyltransferase appears to be unimpaired by azaserine. These findings also suggested that the synthesis of thymine nucleotides was inhibited in the cells exposed to the antibiotic. This was confirmed by measuring the activities of the enzymes that phosphorylate thymidine and thymidine 5′-phosphate in cell-free extracts. Enzymic extracts prepared from cells 12 hr after exposure to 0.2 mg of azaserine per kilogram showed a pronounced decrease in the activities of thymidine kinase and thymidine monophosphate kinase (Table 4), while thymidine monophosphate nucleotidase activity was unaltered. Dialysis of the enzymic extracts and subsequent measurement of thymidine kinase activity indicated that the degree of inhibition could not be lessened by this procedure; furthermore, the addition of 2×10^{-3} M azaserine to enzyme prepared from untreated cells did not alter the activities of thymidine kinase or thymidine monophosphate kinase. These results indicate that the antibiotic does not directly inhibit these enzymes; the data therefore suggest that either fewer kinase molecules are present or the catalytic properties of the enzyme are altered in cells treated with azaserine.

TABLE 4

Thymidine kinase, thymidylate kinase, and thymidylate nucleotidase activities of sarcoma 180 cells treated with azaserine

Six-day growths of sarcoma 180 ascites cells from untreated mice or from those injected intraperitoneally 12 hr previously with 0.2 mg of azaserine per kilogram of body weight were used as the source of enzyme. Each value represents the mean of duplicate determinations.

		mμmoles/hr/mg		
Treatment	Dialysis[a]	Thymidine kinase	Thymidine monophosphate kinase	Thymidine monophosphate nucleotidase
None	−	18.8	109.8	14.5
Azaserine	−	2.5	47.9	12.1
None	+	8.0	—	—
Azaserine	+	1.8	−	—

[a] The enzymic extract was dialyzed against 100 volumes of 0.05 M tris(hydroxymethyl)aminomethane buffer, pH 7.5, containing 0.05 μmole of thymidine per milliliter.

The availability of a supply of preformed adenine allowed the azaserine-stressed cells to recover almost completely the ability to incorporate thymidine-^{3}H into DNA at a rate comparable to that of controls (Table 5). This suggested that the activities of both thymidine kinase and thymidine monophosphate kinase were restored to nearly normal levels by the availability of preformed adenine. Hypoxanthine was considerably less effective in this regard, and 2-azaadenine, 8-azaadenine, guanosine, and 8-azaguanine were inactive. That the utilization of adenine allowed the cells to circumvent the block on the *de novo* pathway to purine nucleotides is shown in Table 6; in the presence of a source of preformed adenine the nucleotide pool returned to normal levels in azaserine-depleted cells. The involvement of these phenomena in the decreased cellular level of thymidine kinase

TABLE 5

Effect of purines on the azaserine-induced inhibition of thymidine-^3H incorporation into DNA

Mice bearing 6-day implants of sarcoma 180 ascites cells received a single intraperitoneal dose of 0.2 mg of azaserine per kilogram of body weight; a purine was administered 11.5 hr later, and after an additional 0.5 hr 200 μg of thymidine methyl-^3H (6.6×10^3 cpm/μg) was injected into each mouse and 1 hr was allowed for metabolic utilization. Each value represents the mean (± the standard error) obtained from the separate analyses of ascites cells from 4–15 animals.

Treatment	cpm/μmole $\times 10^{-2}$ DNA thymine
None	114.7 ± 11.0
Adenine (30 mg/kg)	88.6 ± 5.2
Azaserine	35.5 ± 4.3
Azaserine + adenine (30 mg/kg)	104.7 ± 14.6
Azaserine + 2-azaadenine (30 mg/kg)	33.3 ± 1.2
Azaserine + 8-azaadenine (30 mg/kg)	41.6 ± 12.3
Azaserine + hypoxanthine (30 mg/kg)	60.5 ± 6.0
Azaserine + guanosine (60 mg/kg)	40.8 ± 5.0
Azaserine + 8-azaguanine (60 mg/kg)	23.5 ± 6.7

activity is shown in Table 7. When the enzymic extracts were prepared from cells which were pretreated with azaserine and then exposed to preformed adenine, a reversal of the azaserine-imposed decrease in the activity of thymidine kinase resulted.

To gain further insight into the mechanism of the drug-induced reduction in the activities of thymidine kinase and thymidine monophosphate kinase, several metabolic inhibitors were given to azaserine-treated cells 0.5 hr prior to their exposure to a source of preformed adenine; 0.5 hr later thymidine-^3H was administered, and its incorporation was used as a measure of the synthesis of thymine nucleotides. The results are presented in Table 8. Actinomycin D, at a concentration (1.0 mg/kg) that caused an 82% inhibition of the incorporation of orotic acid-^{14}C into RNA uracil did not abolish the ability of preformed adenine to restore the synthesis of thymine nucleotides. Furthermore, 2,4-dinitrophenol, sodium cyanide, and 2-deoxyglucose also did not prevent the adenine-induced reversal. *p*-Fluorophenylalanine and puromycin at dose levels which caused 43 and 78% inhibition, respectively, of the fixation of leucine-1-^{14}C into the residual protein of these cells produced ambiguous results. An interpretation of the effects of these agents was complicated by the finding that puromycin alone caused a pro-

TABLE 6

Effect of azaserine on the adenine nucleotide pool of sarcoma 180 cells

Mice bearing 6-day implants of sarcoma 180 ascites cells received a single intraperitoneal dose of 0.2 mg of azaserine per kilogram of body weight; adenine at a level of 30 mg/kg was injected 11.5 hr later and cells were collected after an additional 0.5 hr. The adenine nucleotide pool was measured by isotope dilution. Each value represents the mean (± the standard error) obtained from the separate analyses of ascites cells from 4–16 animals.

Treatment	μmoles/g cells Concentration of adenine nucleotides
None	3.7 ± 0.3
Adenine	3.2 ± 0.1
Azaserine	1.9 ± 0.1
Azaserine + adenine	3.0 ± 0.7

TABLE 7

Effect of adenine on the azaserine-induced inhibition of the activity of thymidine kinase

Six-day growths of sarcoma 180 ascites cells from (a) untreated mice, (b) animals injected intraperitoneally 12 hr previously with 0.2 mg of azaserine per kilogram of body weight, or (c) those receiving 30 mg of adenine per kg 10.5 hr after the azaserine, with cells collected 1.5 hr later, were used as the enzyme source. Each value represents the mean of duplicate determinations.

Treatment	mμmoles/hr/mg Thymidine kinase
None	13.5
Azaserine	3.5
Azaserine + adenine	11.1

TABLE 8

Effect of inhibitors on the adenine-induced reversal of inhibition of thymidine-^{3}H incorporation into DNA

Mice bearing 6-day implants of sarcoma 180 ascites cells received a single intraperitoneal dose of 0.2 mg of azaserine per kilogram of body weight. Eleven hours later a metabolic inhibitor was administered at the indicated level, and 0.5 hr later 30 mg of adenine per kilogram was given intraperitoneally. After an additional 0.5 hr, 200 μg of thymidine methyl-^{3}H (6.6×10^3 cpm/μg) was injected into each mouse; 1 hr was allowed for metabolic utilization. Each value represents the mean (± the standard error) obtained from the separate analyses of ascites cells from 4–46 animals.

Inhibitor	Azaserine	Adenine	cpm/μmole $\times 10^{-2}$ DNA thymine
None	−	−	123.8 ± 4.2
None	−	+	125.4 ± 23.6
None	+	−	38.6 ± 3.1
None	+	+	91.2 ± 5.4
Actinomycin D (0.3 mg/kg)	−	+	130.5 ± 15.9
Actinomycin D (1.0 mg/kg)	−	−	123.6 ± 14.9
Actinomycin D (1.0 mg/kg)	−	+	116.5 ± 18.8
Actinomycin D (0.3 mg/kg)	+	+	87.9 ± 22.6
Actinomycin D (1.0 mg/kg)	+	+	74.0 ± 13.4
2,4-Dinitrophenol (20 mg/kg)	−	−	116.1 ± 13.7
2,4-Dinitrophenol (20 mg/kg)	−	+	100.0 ± 8.0
2,4-Dinitrophenol (20 mg/kg)	+	+	72.9 ± 15.0
Sodium cyanide (2 mg/kg)	−	−	104.4 ± 6.7
Sodium cyanide (2 mg/kg)	−	+	113.2 ± 23.1
Sodium cyanide (2 mg/kg)	+	+	99.7 ± 10.5
2-Deoxyglucose (1000 mg/kg)	−	−	75.9 ± 5.5
2-Deoxyglucose (1000 mg/kg)	−	+	81.5 ± 9.0
2-Deoxyglucose (1000 mg/kg)	+	+	89.3 ± 3.2
p-Fluorophenylalanine (400 mg/kg)	−	−	70.8 ± 6.1
p-Fluorophenylalanine (400 mg/kg)	−	+	95.6 ± 16.1
p-Fluorophenylalanine (400 mg/kg)	+	+	59.2 ± 10.8
Puromycin (50 mg/kg)	−	−	16.1 ± 4.2
Puromycin (50 mg/kg)	−	+	20.7 ± 4.1
Puromycin (50 mg/kg)	+	+	31.0 ± 7.9

nounced depression of the utilization of tritiated thymidine for the synthesis of DNA, while *p*-fluorophenylalanine produced only marginal inhibition of the adenine-induced rescue. Nevertheless, the findings indicate that the synthesis of thymine nucleotides is sensitive to inhibitors of protein synthesis.

DISCUSSION

The azaserine-induced suppression of the biosynthesis of purine nucleotides *de novo* in sarcoma 180 ascites cells was accompanied by a subsequent inhibition of the incorporation of thymidine-^{3}H, formate-^{14}C, and deoxycytidine-^{3}H into DNA. This depression in the rate of incorporation of these isotopic precursors into DNA was not simply the result of the death of the cells, since utilization of radioactive orotic acid and leucine for the formation of RNA and protein, respectively, was not impaired by the azaserine treatment.

Evidence that the retardation of incorporation of thymidine into DNA produced by exposure of the cells to the antibiotic was not attributable to either direct inhibition of DNA nucleotidyltransferase or the impairment of the primer function of DNA in this reaction was provided by the finding that the utilization of adenine-^{14}C for the formation of the purine nucleotides of DNA was not decreased in cells exposed to azaserine. The inhibition induced by azaserine

of the incorporation of labeled precursors into the thymine of DNA was accounted for, however, by a pronounced decrease in the cellular activities of both thymidine kinase and thymidine monophosphate kinase. Azaserine at a concentration of 2×10^{-3} M was not directly inhibitory to these enzymes, nor did the inhibition appear to be due to a metabolic product of azaserine, since the antibiotic did not decrease the rate of incorporation of thymidine into DNA when the isotopic substrate was given 15 min after the drug, a circumstance that results in essentially complete suppression of the incorporation of glycine-^{14}C into the polynucleotide purines (4). Furthermore, dialysis of enzymic extracts did not revive the lowered activity of thymidine kinase in extracts prepared from azaserine-treated cells; this finding also would eliminate the possibility that the inhibition was the result of an antibiotic-induced accumulation of deoxythymidine triphosphate or some other deoxynucleoside triphosphate which acted as a feedback inhibitor of this enzyme in the assay system (28–36).

Although a direct correlation between the absolute size of the adenine nucleotide pool and the degree of inhibition of thymidine-^{3}H incorporation into DNA was not observed (Fig. 1), it was evident that a relationship existed. Thus, provision of preformed adenine to cells unable to fabricate purine nucleotides *de novo* because of blockade of this pathway by azaserine, enabled these cells to restore their pool of adenine nucleotides to normal levels and to recover their ability to synthesize thymine nucleotides; other purines and purine analogs were either inactive or less active in this regard (Tables 5 and 6). These results would appear to account for the ability of azaserine-treated cells to incorporate adenine-8-^{14}C into the purine deoxyribonucleotides of DNA, since the isotopic adenine presumably functions in this system not only as a precursor of polynucleotides, but also to facilitate a restoration of the activities of thymidine kinase and thymidine monophosphate kinase. Additionally, the inhibition of the incorporation of deoxycytidine-^{3}H into the deoxycytidylic acid moiety of DNA induced by the azaserine is considered to be an expression of a limiting supply of thymine nucleotides for the formation of these polymeric molecules.

Although the results obtained with puromycin and *p*-fluorophenylalanine (Table 8) did not clearly indicate that the adenine-induced rescue of the ability to synthesize thymine nucleotides by intact cells involved the resynthesis of new enzymic protein (thymidine kinase and thymidine monophosphate kinase) we would tentatively favor this interpretation. The inability of actinomycin D to influence this process would appear to suggest that azaserine-treated cells possess molecules of messenger RNA with the information necessary for the synthesis of the kinases. If this mechanism is operative, the restoration of the activity of the kinases in azaserine-stressed cells exposed to a source of adenine would indicate a relatively rapid turnover time for these enzymes and suggests the possibility of a role for purine nucleotides in the regulation of these activities. The depression in the rate of incorporation of thymidine-^{3}H into the DNA of cells treated with either puromycin or *p*-fluorophenylalanine is consistent with the observation that the activity of thymidine kinase declines in cells exposed to agents capable of inhibiting protein synthesis (37–41).

Two alternatives appear to be available to explain the lower activities of thymidine kinase and thymidine monophosphate kinase in azaserine-treated cells. The first possibility requires a drug-induced lesion in the protein-synthesizing mechanism which results in a decrease in the rate of formation of both kinase enzymes. This mechanism would appear to be unlikely since azaserine is not a general inhibitor of protein synthesis (Table 1). However, an antibiotic-produced alteration may well be subtle and not detected by measurement of the formation of proteins *in toto*, but may be amplified in the case of proteins undergoing rapid turnover. The second possible mechanism is that ATP contributes to the intracellular stability of these enzymes. This alternative implies that the

rate of formation of these enzymic proteins is not altered in azaserine-stressed cells, but that the cellular concentration of the enzymes decreases because of the unavailability of ATP which is involved in the maintenance of a conformation of both kinase proteins providing stability against either natural decay in the cellular environment or proteolytic enzymes. Evidence is available that both enzymes can be stabilized by substrates *in vivo* and *in vitro*. Thus, thymidine kinase and thymidine monophosphate kinase activities have been protected in intact cells by exposure to thymidine (38, 40, 42, 43). *In vitro*, thymidine kinase can be significantly protected against thermal inactivation by either thymidine, thymidine triphosphate, or ATP (44), and thymidine monophosphate kinase can be protected against inactivation due to dilution by either thymidine or thymidine 5′-phosphate (45). In the latter case, under the conditions employed, ATP was inactive as a protecting agent.

Other studies with mammalian cells indicate that the cellular levels of thymidine, thymidine monophosphate, and thymidine diphosphate kinases are related to the growth rate of cells (e.g., 38, 40–42, 46). Processes that decrease the proliferation rate would thus be expected to lower the activities of these enzymes. The azaserine-induced decrease in the formation of thymine nucleotides in sarcoma 180 ascites cells and the reversal of this lesion afforded by a supply of preformed adenine also would be in accord with this concept and can be interpreted in this manner as a nonspecific effect. This possibility, however, does not provide an adequate explanation for the observed phenomena in precise molecular terms. Thus, the use of azaserine and subsequent reversal of inhibition by adenine to study the factors regulating the activities of these enzymes may provide information concerning the mechanisms of cellular control of the activities of the thymine nucleotide-forming kinases, processes that appear to be involved in the regulation of growth. Investigation of the factors involved in these phenomena are now underway in this laboratory.

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