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Inhibition of De Novo Purine Biosynthesis and Interconversion by 6-Methylpurine in *Escherichia coli*

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The inhibition of *Escherichia coli* strain B and strain W-11 by 6-methylpurine depended on the formation of 6-methylpurine ribonucleotide by the action of adenine phosphoribosyltransferase (AMP: pyrophosphate phosphoribosyltransferase, EC 2.4.2.7). 6-Methylpurine ribonucleotide inhibited the de novo synthesis of purines, presumably via pseudofeedback inhibition of phosphoribosylpyrophosphate amidotransferase (EC 2.4.2.14). The same mechanism accounted for its inhibition of adenylosuccinate synthetase [IMP: L-aspartate ligase (GDP), EC 6.3.4.4]. Adenine and 6-methylaminopurine prevented inhibition by competing for the action of adenine phosphoribosyltransferase. In addition, adenine reversed this inhibition by replenishing the AMP to bypass both sites of inhibition. Nonproliferating suspensions of strain B-94, which lacked adenylosuccinate lyase (EC 4.3.2.2), converted exogenous hypoxanthine and aspartate to succinoadenine derivatives which accumulated in the medium. Compounds which inhibited adenylosuccinate synthetase inhibited accumulation of the succinoadenine derivatives. A method was described for the isolation of mutants which potentially possessed an altered adenylosuccinate synthetase.

The initial steps in the synthesis of the pyrimidine moiety of thiamine (B_1-pyrimidine) and purines are implemented by the same enzymes, with 5-aminoimidazole ribonucleotide being the last common intermediate (25). Adenine or adenosine inhibition of *Salmonella typhimurium* (24) and *Escherichia coli* (62) is presumably because of feedback inhibition of this branched route, resulting in the requirement for B_1-pyrimidine or thiamine to relieve the inhibition.

Similarly, 6-methylaminopurine inhibits the growth of *E. coli* B unless the medium is supplemented with hypoxanthine and thiamine (or B_1-pyrimidine) to supply both products of the dual purpose pathway. The limited conversion of 6-methylaminopurine to inosine monophosphate (IMP) by *E. coli* B leads to the additional requirement for hypoxanthine in this system. The inhibition exerted by adenine and 6-methylaminopurine is not expressed in organisms which lack adenine phosphoribosyltransferase; therefore, the active derivatives occur on the ribonucleotide level (*unpublished results*).

6-Methylaminopurine can satisfy the purine requirement of *E. coli* strain W-11 in which the de novo pathway is blocked after the synthesis of 5-aminoimidazole ribonucleotide (19). Since this organism was not susceptible to inhibition by adenine, adenosine, or 6-methylaminopurine, despite the ability of these purines to exert marked feedback inhibition of purine biosynthesis, we examined 6-methylpurine (MeP), which was known to inhibit *E. coli* (7), for its ability to inhibit more effectively the de novo pathway. MeP inhibited the growth of *E. coli* strain B and strain W-11. Unlike 6-methylaminopurine, MeP inhibition was not relieved by the combination of hypoxanthine and thiamine but was relieved by adenine. Adenine was known to prevent MeP inhibition of other organisms (5, 6, 23), but the mechanism required clarification. The present study was initiated to determine the inhibitory form of MeP, the inhibited site(s), and the mechanism by which adenine relieved MeP inhibition.

MATERIALS AND METHODS

Microorganisms and culture conditions. *E. coli* B was maintained on a minimal medium of ammonium salts and glucose (19). *E. coli* strain W-11, a purine auxotroph blocked after 5-aminoimidazole ribo-

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nucleotide synthesis (18), was maintained in the minimal medium supplemented with 0.15 mM hypoxanthine. Strains B/DAP and W-11/DAP, which lack adenine phosphoribosyltransferase (18), were cultured in the same media as listed for the parent strains. Strain B-94 (10) requires adenine (0.15 mM) because of its lack of adenylosuccinate lyase; it has an unrelated requirement for arginine (40 μg/ml). Better growth resulted when the medium was further supplemented with 0.1% casein hydrolysate. Unless otherwise indicated, the inocula were derived from overnight cultures which were diluted 10^{-2} into the test media (10 ml) in Erlenmeyer flasks (50 ml) fitted with Morton closures. Cultures were shaken at 37 C in a water bath for the designated time (usually 8 hr). Turbidity was measured with a Klett-Summerson colorimeter at 540 nm.

Feedback inhibition of de novo purine synthesis. The extracellular accumulation of purine precursors by purine-requiring auxotrophs served as an index of the de novo pathway (11). *E. coli* strains W-11 and W-11/DAP were grown for 12 hr at 37 C, with aeration, in minimal medium containing 0.1 mM hypoxanthine. The cells were harvested, washed, and suspended (0.5 mg/ml) in minimal medium containing 50 μg/ml of glycine and different concentrations of MeP. The suspensions (10 ml) were incubated for 3 hr at 37 C with aeration prior to determining the 5-aminoimidazole ribonucleoside (AIR) by the method of Bratton and Marshall (2).

Strain B-94 was grown for 24 hr in minimal medium supplemented with adenine (0.15 mM), arginine (50 μg/ml), and casein hydrolysate (0.1%). The cells were harvested, washed twice with cold water, and resuspended (0.5 mg/ml) in minimal medium with various concentrations of MeP. The suspensions (10 ml) were incubated at 37 C for 3 hr with aeration prior to determining the ribonucleoside and ribonucleotide derivatives of 5-amino-4-imidazole-*N*-succinylocarboxamide (S-AICA; reference 10).

Chemicals. All routine chemicals were obtained from commercial sources. MeP was purchased from Mann Research Laboratories, Inc., New York, N.Y. Hadacidin was a gift from H. T. Shigeura, Merck Institute for Therapeutic Research, Rahway, N.J. MeP-8-^{14}C was prepared and supplied by I. W. F. Davidson. Other radiochemicals were purchased from New England Nuclear Corp., Boston, Mass.

Preparation of MeP ribonucleotide. In preliminary experiments, an autolysate (17) of bakers' yeast was shown to contain adenine phosphoribosyltransferase by its conversion of adenine to adenosine monophosphate (AMP) in the following system: tris-(hydroxymethyl)aminomethane(Tris) - hydrochloride, 0.012 M (p*H* 7.4); $MgSO_4$, 0.02 mM; adenine-8-^{14}C, 0.0924 mM; 5-phosphoribosyl-1-pyrophosphate, 0.1 mM; KF, 0.01 mM; autolysate, 0.1 ml; and water added to a final volume of 1.0 ml. The reaction mixture was incubated for 30 min at 37 C and terminated by heating for 3 min at 100 C. MeP was substituted for adenine in a 100-ml system which was incubated for 1 hr. The analogue ribonucleotide (MePRP) was isolated by ionexchange chromatography on a diethylaminoethyl (DEAE)-Sephadex A-25 (formate) column (2.5 by 19 cm) by gradient elution with 0.025 M formic acid (500 ml) in the mixer flask and 0.5 M formic acid (500 ml) in the reservoir. The flow rate was approximately 10 ml per 18 min. The ultraviolet (UV)-absorbing (260 nm) fractions were pooled, and the formic acid was removed by repeated lyophilization. The identity and purity of the ribonucleotide was verified by paper chromatography.

Extraction of ribonucleotides. After an exposure to radioactive hypoxanthine or MeP, the bacteria were harvested and the culture fluid was analyzed by paper chromatography. The intracellular ribonucleotide pool and ribonucleic acid (RNA) were obtained by methods described by Zimmerman and Magasanik (32). When appropriate, the ribonucleotides were hydrolyzed in 1 N HCl at 100 C for 30 min.

Separation of purine derivatives. The normal bases were identified by descending chromatography on Whatman no. 1 paper with either 86% butanol (20) or butanol-glacial acetic acid-water (58.1:15.7:26.2; reference 27) as solvent for 18 to 20 hr. MeP derivatives were separated by descending chromatography with ethyleneglycol-monomethyl ether-butanol-glacial acetic acid-water (7:7:2:4) for 24 hr (I. W. F. Davidson, *personal communication*). The R_F values for the base, ribonucleoside, mono-, di-, and triphosphoribonucleosides were 0.70, 0.54, 0.45, 0.27, and 0.17, respectively.

Ribonucleotides were separated by electrophoresis in 0.05 M formate buffer (*p*H 3.5) for 2.5 hr at 1.5 mamps per strip of Whatman no. 1 paper (1.5 by 8.0 cm; 21).

Detection and quantitation of radioactive compounds. The purine standards were located as UV-absorbing areas, whereas the radioactive areas were detected with a radiochromatogram scanner. Radioactivity was measured by cutting the paper into strips and counting the sections in a Beckman liquid scintillation system.

Biosynthesis, isolation, and characterization of succinoadenine derivatives. Cultures of strain B-94 (12 hr) were harvested by centrifugation and suspended to a density of 200 Klett units (540 nm) in ammonium salts-glucose medium containing hypoxanthine (1 mM), aspartate (40 μg/ml), and histidine (20 μg/ml), but lacking arginine. The suspension was incubated for 4 hr at 37 C, with aeration. The supernatant fluid was adjusted to *p*H 7.4 to 8.0, applied to a Dowex-1-formate column (1.2 by 6.0 cm), and eluted with formic acid (1). The peak fractions (267 nm) were pooled and lyophilized repeatedly to remove the formic acid.

UV spectra were obtained on each pooled fraction before and after a 30-min acid hydrolysis. The spectral constants for succinoadenylate (S-AMP) and succinoadenine (S-Ad) were described (3, 4). Succinoadenosine (S-AdR) resembled S-AMP, spectrally, and the same constants were employed. Pentose was measured by the orcinol method (22) using adenosine or AMP as the standard, depending on the absence or presence of organic phosphate (30) in the test sample.

Preparation of extracts for the in vitro synthesis of S-AMP. Strain B-94 was grown for 12 hr in a

highly buffered minimal medium (27) supplemented with glucose (0.4%), arginine (40 μg/ml), and adenine (0.15 mM); the cells were collected by centrifugation, washed twice with cold saline, and suspended in 0.005 M potassium phosphate buffer, *p*H 7.2 (16), to a density of 240 Klett units (540 nm) prior to disruption by sonic oscillation with the Biosonik oscillator (Bronwill Scientific, Rochester, N.Y.). The cellular debris was removed by centrifugation at 12,100 × *g* for 30 min at 4 C, and the nucleic acids were precipated with 1.3 ml of 5% streptomycin sulfate per 5 ml of extract (16). The protein content of the extracts was estimated spectrophotometrically (15). The unfractionated extracts served as the source of adenylosuccinate synthetase and were substituted for the fractionated extracts as described in Fromm's modification (8) of assay II of Lieberman (16). Streptomycin sulfate was omitted from the reactants. A linear relationship between the quantity of S-AMP synthesized [increase in absorbance at 280 nm (4)], and the extract added was obtained, provided the protein content was less than 0.12 mg/ml.

Isolation of mutants with altered adenylosuccinate synthetase. *E. coli* B (0.1 ml) was spread on ammonium salts-glucose agar plates containing MeP (3.0 mM), hypoxanthine (0.6 mM), and thiamine (50 ng/ml). The plates were incubated at 37 C for 30 hr to permit colonies (30 to 50 per plate) to develop. Colonies were transferred to minimal medium plates containing thiamine and either MeP (3 mM) or MeP plus hypoxanthine (0.6 mM). After overnight incubation at 37 C, the colonies were scored for growth in the presence and absence of hypoxanthine. Colonies which were sensitive to MeP in the absence of hypoxanthine but resistant to MeP in the presence of hypoxanthine were selected. These cultures remained prototropic with respect to their growth requirements. The rationale for this selection method was described in the text.

RESULTS

Inhibition of E. coli by MeP. MeP inhibited the growth of *E. coli* B and, to a lesser degree, strain W-11, but it did not inhibit strains B/DAP and W-11/DAP (Fig. 1). Therefore, the inhibitory properties of the analogue depended on its conversion to MePRP by adenine phosphoribosyltransferase.

Mutants resistant to 2,6-diaminopurine are devoid of adenine phosphoribosyltransferase or contain the enzyme with an altered specificity (13). Mutants of *E. coli* B selected for their resistance to MeP (3 mM) also were resistant to 2,6-diaminopurine. Extracts of five different mutants were examined for their adenine phosphoribosyltransferase contents; three mutants lacked adenine phorphoribosyltransferase and two exhibited markedly reduced activity. This represents the simplest mechanism for acquiring resistance to these analogues.

Analysis of the ethyl alcohol extracts of exponential phase cells (*E. coli* strains B, B-94, and W-11) incubated in the presence of MeP-*8*-^{14}C for 30 to 60 min revealed the presence of the mono-, di-, and triphosphoribosyl derivatives; under similar conditions, strain B contained the largest quantities of these derivatives. Strains B/DAP and W-11/DAP did not produce ribonucleotides of the analogue.

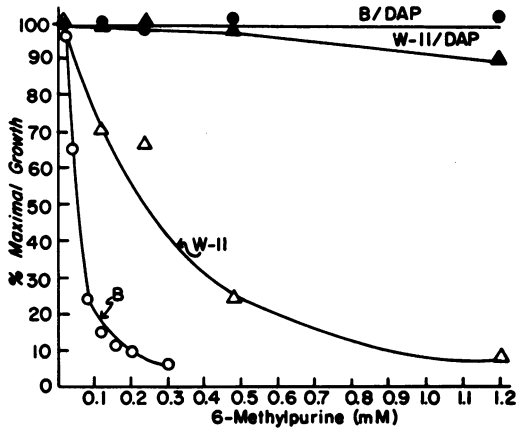


FIG. 1. *6-Methylpurine inhibition of Escherichia coli strains. The organisms were grown for 8 hr in minimal medium with different concentrations of 6-methylpurine. The cultures of strain W-11 and W-11/DAP were supplemented with hypoxanthine (0.15 mM) to satisfy their purine requirement.*

Inhibition of de novo purine synthesis. MeP, at 0.56 mM, inhibited AIR synthesis in strain W-11 by 50%, whereas levels as high as 1.2 mM exerted only a moderate effect in strain W-11/DAP (Fig. 2). Comparable experiments with strains B-94 revealed that MeP inhibited the de novo route of this organism to the same extent. The de novo route of purine synthesis in *E. coli* B was inhibited by MeP as indicated by the inhibition of glycine-*1*-^{14}C incorporation into adenine and guanine (*not illustrated*). Presumably, phosphoribosylpyrophosphate amidotransferase of these organisms was susceptible to pseudofeedback inhibition by MePRP. However, the ability of MeP to inhibit strain W-11, which lacked the de novo purine pathway and consequently was routinely cultured with hypoxanthine as its purine source, demonstrated that MePRP must block a site in addition to the amidotransferase.

Prevention of inhibition. The inclusion of 0.2 mM adenine in the culture media allowed the normal growth rate and maximal yield of strains B and W-11 despite the presence of 0.2 mM MeP. By contrast, higher concentrations of hypoxanthine or guanine were unable to prevent the inhibitory properties of the analogue even at 0.05 mM MeP.

If the mechanism by which adenine specifically prevented MeP inhibition involved the competition of adenine and MeP for the active site of adenine phosphoribosyltransferase, 6-methylaminopurine, another active substrate for the transferase (19) also should prevent inhibition. 6-Methylaminopurine, as intracellular 6-methyladenylic acid, possesses the potential to inhibit both de novo purine and thiamine biosynthesis in *E. coli* B (*unpublished results*); consequently, both hypoxanthine and thiamine were added as supplements to prevent inhibition by 6-methylaminopurine. However, these supplements did not prevent or alter MeP inhibition.

Increasingly higher concentrations of 6-methylaminopurine reduced, but did not completely prevent, the inhibition by MeP in strains B and W-11 (Fig. 3). This suggested a competitive interaction between the two analogues. By contrast, the ability of adenine to prevent inhibition was not of a simple competitive nature (Table 1). In fact, the inhibited cells appeared to be adenine-deficient since the extent to which adenine prevented inhibition was proportional to the response of a purine auxotroph to purines. This data suggests that, in addition to competing with MeP for the active site of adenine phosphoribosyltransferase and thus decreasing the intracellular level of the inhibitory nucleotide, adenine may noncompetively reverse the inhibitory action of MePRP on either adenylosuccinate synthetase or adenylosuccinate lyase by supplying AMP, the end product of the inhibited step.

Reversal of MeP inhibition by adenine. Previous data established that adenine specifically prevented the expression of the potential inhibitory properties of MeP in *E. coli*. Adenine was also effective in relieving the growth inhibition after the maximal degree of inhibition had been imposed, even though MeP remained in the medium (Fig. 4). A lag period of approximately 2 hr was required before a significant rate of growth was resumed. Furthermore, if the inhibited cells were washed free of extracellular MeP and resuspended in minimal medium, the normal growth rate was reestablished even in the absence of adenine. Maintenance of the inhibitory state apparently required a continuous synthesis of the inhibitory nucleotide.

Alteration of nucleotide pools by MeP. That MeP specifically inhibited AMP synthesis was verified by the demonstration that the analogue inhibited the incorporation of exogenous hypoxanthine-*8*-^{14}C into the intracellular AMP pool, although it did not impair its incorporation into the guanosine monophosphate (GMP) pool (Table 2). The altered adenine:guanine (A:G) ratio of the nucleotide pool was only modestly

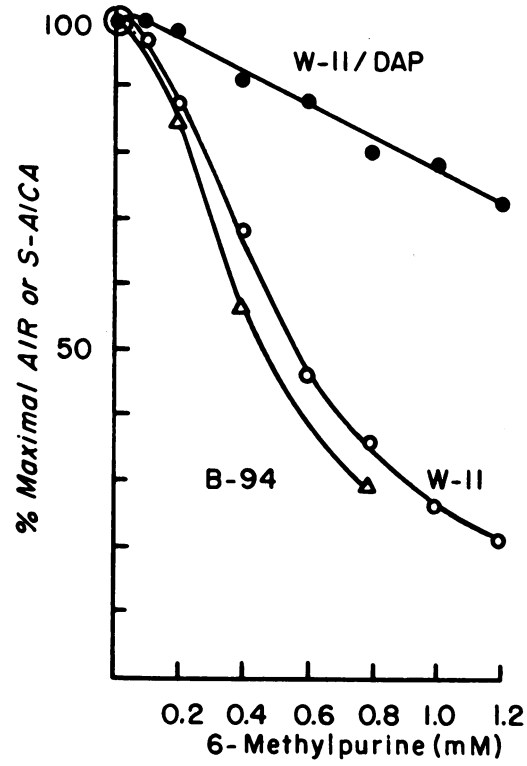


FIG. 2. *Inhibition of de novo purine synthesis by 6-methylpurine. Conditions for the synthesis of 5-aminoimidazole ribonucleoside (AIR) by strain W-11 (○, ●) and succino-5-amino-4-imidazolecarboxamide derivatives (S-AICA) by strain B-94 (△) were described in the text.*

reflected in the base ratio of the RNA, despite a marked inhibition of RNA synthesis. This observation implied that RNA synthesis ceased before the major change in the nucleotide pool occurred and that MePRP was not extensively incorporated into RNA.

Inhibition by MeP of the interconversion of IMP to AMP conceivably could involve either adenylosuccinate synthetase or adenylosuccinate lyase. However, the end product regulation of the adenylosuccinate synthetase by AMP (31) made this enzyme a likely candidate for inhibition by MePRP, an AMP analogue. Therefore, inhibition by the analogue and its reversal by adenine was compared to the inhibition exerted by hadacidin, an aspartate analogue which specifically inhibits adenylosuccinate synthetase (29). The susceptibility of an overnight culture of *E. coli* B to inhibition by adenine, or adenosine, necessitated the addition of thiamine to prevent bacteriostasis (24). The later use of exponential phase cells alleviated adenine inhibition and therefore the

requirement for thiamine. In the presence of thiamine, adenine and adenosine prevented the inhibition exerted by both MeP and hadacidin (Table 3). The extent to which adenosine was converted to adenine rather than to inosine would account for the partial prevention of MeP inhibition. Adenosine more effectively prevented hadacidin inhibition since hadacidin was less toxic than MeP. Supplying the precursor of AMP synthesis via the salvage pathway specifically prevents the inhibition by both hadacidin and MeP.

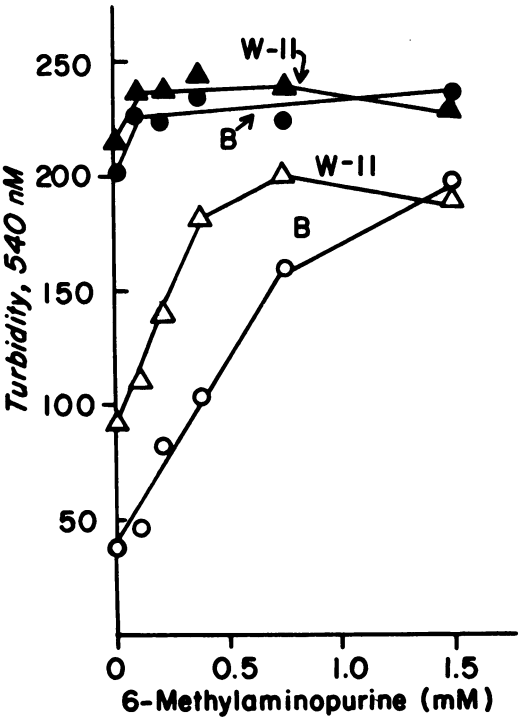


FIG. 3. *Influence of 6-methylaminopurine on 6-methylpurine inhibition. The minimal medium (10 ml) was supplemented with hypoxanthine (0.15 mM) and thiamine (50 ng/ml). Test systems (open symbols) contained 6-methylpurine (0.2 mM). Various concentrations of 6-methylaminopurine were added to all systems.*

TABLE 1. *Relationship between adenine concentration and 6-methylpurine inhibition*[a]

6-Methylpurine	Turbidity[b] of adenine				
	0	0.02	0.1	0.2	0.5
0	248	238	228	234	230
0.2	0	37	165	220	220
0.5	0	16	122	220	222
1.0	0	16	125	220	222
2.0	0	16	108	220	206

[a] Values expressed in millimolar concentration.

[b] *E. coli* B was diluted 10^{-2} in minimal medium (10 ml) supplemented as indicated above. All cultures were incubated 8 hr at 37 C with aeration, and turbidity was measured with a Klett colorimeter at 540 nm.

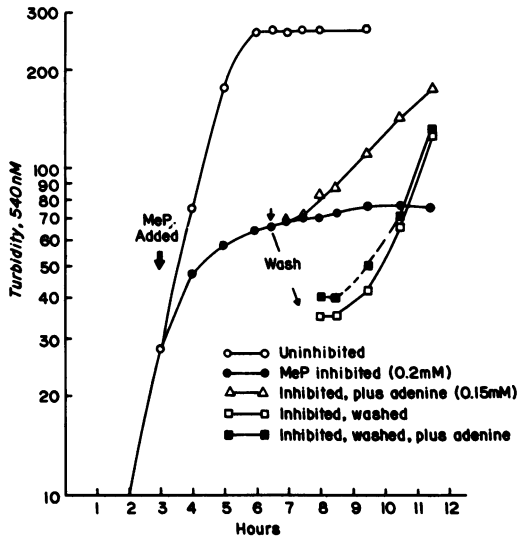


FIG. 4. *Reversal of 6-methylpurine inhibition. E. coli B was grown to exponential phase in minimal medium (50 ml), 6-methylpurine (0.2 mM) was added to 40 ml to obtain inhibited cells, and the remainder served as the uninhibited control. Equal portions of the inhibited cells were treated as illustrated at 6.5 hr.*

TABLE 2. *Hypoxanthine-8-^{14}C incorporation patterns*[a]

Incubation system	Nucleotide fraction[b]			RNA fraction[b]		
	Adenine	Guanine	Ratio of adenine to guanine	Adenine	Guanine	Ratio of adenine to guanine
Control....	20,800	3,650	5.7	244,000	316,000	0.77
Inhibited...	14,450	7,340	1.9	77,500	116,500	0.66

[a] An exponential phase culture (50 ml, 36 Klett units) of *E. coli* B was divided between two flasks. 6-Methylpurine (0.5 mM) was added to one flask, and both cultures were incubated until the inhibition of growth was complete (2 hr). The control was diluted to the same turbidity as the inhibited system (50 Klett units), and hypoxanthine-8-^{14}C was added to both systems. The cells were incubated for another hour prior to determining the distribution of radioactivity.

[b] Values expressed as counts per minute per milligram of cells.

Inhibition of adenylosuccinate synthetase. Suc-

cinoadenine was detected in the medium when hypoxanthine was added to nonproliferating suspensions of *E. coli* strains which lacked adenylosuccinate lyase (10). Nonproliferating strain B-94 was examined for its ability to convert hypoxanthine and aspartate to extracellular S-Ad derivatives which would serve as an index of adenylosuccinate synthetase. The incubation fluids were fractioned by ion exchange on Dowex-1-formate (Fig. 5). In this experiment, two major UV-absorbing fractions were eluted after the residual hypoxanthine. Acid hydrolysis (1 N HCl, 100 C, 15 min) of either product resulted in a shift of the λ maximum from 267 nm to 277 nm; this agreed with Carter and Cohen's (4) data on the acid hydrolysis of S-AMP to its aglycone. The first peak contained S-Ad and pentose in approximately equimolar amounts and was identified as S-AdR. The second peak contained equimolar amounts of the base, pentose, and phosphate, and was identified as S-AMP. If incubation of the nonproliferating suspension was extended to 6 hr, a small amount of S-Ad was also detected and was eluted prior to S-AdR. Therefore, whole cells of strain B-94 actively converted hypoxanthine and aspartate to S-AMP; the aglycone, the ribonucleoside, and the ribonucleotide accumulated in the medium.

The relative amounts of S-AdR and S-AMP synthesized by nonproliferating strain B-94 when arginine is omitted from medium and the effects of hadacidin and adenine on this system are shown in Table 4. In the control, approximately twice as much ribonucleotide as ribonucleoside accumulated. Hadacidin and adenine inhibited their accumulation as anticipated.

This system provided a useful method for determining the intracellular effect of MeP on adenylosuccinate synthetase (Fig. 6). MeP-treated cells decreased their uptake of hypoxanthine and synthesis of S-Ad derivatives after 2 to 4 hr, whereas the control system remained linear in both catagories; the rapidity and severity of these reactions were proportional to the concentration of the analogue. The comparatively high concentration of the analogue and the extended time period required to impose an adequate state of inhibition on strain B-94, as compared to strains B and W-11, reflect this organism's limited ability to synthesize intracellular MePRP.

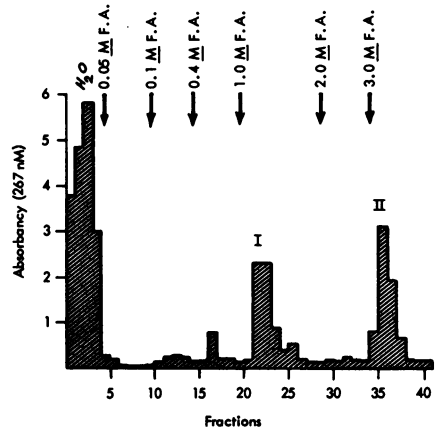
A comparative study of the ability of AMP, hadacidin, and MePRP to inhibit adenylosuccinate synthetase in vitro was performed (Table

TABLE 3. *Prevention of 6-methylpurine or hadacidin inhibition*

Reversing agent[a]	Growth in incubation systems[b]			
	Thiamine	MeP (Hdc) + thiamine	Minimal medium	MeP (Hdc)
None	244 (265)	0 (59)	242 (254)	0 (55)
Adenine	238 (246)	214 (218)	0 (24)	4 (45)
Adenosine	250 (260)	100 (208)	0 (9)	0 (29)

[a] Adenine, 0.15 mM; adenosine, 0.15 mM.

[b] All systems (10 ml) were incubated for 8 hr at 37 C prior to determining their turbidities (540 nm). Thiamine, 5 μg/ml; 6-methylpurine (MeP), 0.15 mM. Minimal medium contained the above combinations of supplements and was inoculated with cells (10^{-2}) derived from an overnight culture of *E. coli* B. Numbers in parentheses represent data obtained with hadacidin (Hdc, 4 mM) as the inhibitor.



	Before Hydrolysis λ max	After Hydrolysis λ max	S-Ad/pentose/phosphate	Identity
Peak I	267 nM	276 nM	1/1.3/0	S-AdR
Peak II	268 nM	277 nM	1/0.9/1	S-AMP

FIG. 5. *Isolation and characterization of succinoadenine derivatives.*

TABLE 4. *Synthesis of succinoadenine derivatives by Escherichia coli strain B-94*[a]

Inhibitor	S-AdR	S-AMP	Total S-Ad derivatives
	μmoles	*μmoles*	*μmoles*
None	1.79	3.69	5.48
Hadacidin (2.0 mM)	0.72	0.91	1.63
Adenine			
0.1 mM			1.84[b]
0.2 mM			2.51[b]

[a] Succinoadenosine (S-AdR) and succinoadenylate (S-AMP), synthesized in 25-ml incubation systems, were separated via ionexchange.

[b] Determined by the amount of UV-absorbing (267 nm) material adsorbed to the ion-exchange column.

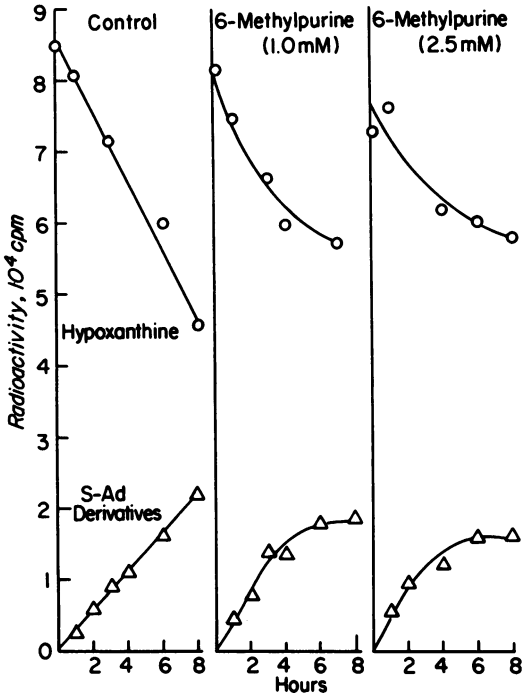


FIG. 6. *Inhibition of intracellular adenylosuccinate synthetase. E. coli strain B-94 was obtained from a mid-log phase culture, washed, and resuspended (0.25 mg/ml) in minimal medium containing hypoxanthine 8-^{14}C (0.48 mM) and aspartate (0.5 mM). Equal quantities of the suspension were transferred to three flasks which contained different amounts of 6-methylpurine. The cultures were incubated at 37 C with aeration, and samples were withdrawn at various times, filtered, and 100 μliters was subjected to electrophoresis prior to determination of the radioactivity associated with the succinoadenine (S-Ad) derivatives. Migration distances towards the anode were: hypoxanthine, 2.8 cm; S-AdR, 6.8 cm; S-AMP, 10.8 cm.*

5). MeP ribonucleotide was an effective inhibitor in this cell-free assay, the 50% inhibition end point being 0.03 mM MePRP (*not shown*).

As a point of interest, we determined the growth conditions of strain B-94 which provided maximal adenylosuccinate synthetase activity. Strain B-94 exhibited maximal growth on 0.15 mM adenine. When the cells were grown for 12 hr on a wide range of adenine, from suboptimal to fourfold excess, the variation in adenylosuccinate synthetase differed by no more than twofold. Maximal activity [50 units per mg of protein, as previously defined (8)] was found with cells which were supplied with 0.45 mM adenine. The small variation in adenylosuccinate synthetase content, and the fact that growth on excess adenine did not reduce the activity, indicated that the adenylosuccinate synthetase in strain B-94 was not regulated appreciably by repression.

TABLE 5. *Inhibition of adenylosuccinate synthetase*

Incubation system[a]	Concn	S-AMP formed
	mM	μM
Basic mixture		
IMP	0.25	82.6
Aspartate	0.82	
GTP	0.17	
$MgCl_2$	6.6	
Basic mixture plus		
AMP	0.2	0.85
Hadacidin	1.65	0
MePRP	0.12	1.5

[a] Abbreviations: IMP, inosine monophosphate; GTP, guanosine triphosphate; AMP, adenosine monophosphate; MePRP, 6-methylpurine ribonucleotide.

Selection of mutants conditionally resistant to MeP. The previous experiments indicated that MePRP inhibited two sites in the biosynthesis of purines, namely, adenylosuccinate synthetase and phosphoribosylpyrophosphate amidotransferase. Although resistance to MeP usually is mediated by the loss or alteration of adenine phosphoribosyltransferase, conditional resistance could result from an alteration in the allosteric site of adenylosuccinate synthetase, provided hypoxanthine and thiamine were added to bypass the inhibited de novo route. In such mutants, the conversion of IMP to AMP would proceed regardless of the intracellular concentration of the analogue nucleotide. If the adenylosuccinate synthetase of *E. coli* B lacked control by repression and derepression, as demonstrated for strain B-94, the observed resistance pattern would not be due to an increased synthesis of adenylosuccinate synthetase. A potential selection method for the isolation of mutants containing altered adenylosuccinate synthetase would involve the isolation of organisms sensitive to MeP in the absence of hypoxanthine but resistant to MeP in the presence of hypoxanthine. Mutants which satisfied these criteria have been isolated (Table 6, group 3).

DISCUSSION

The present study indicated that MeP, like adenine, 6-methylaminopurine, and 2,6-diaminopurine (19, 28), required the action of adenine phosphoribosyltransferase for ribonucleotide synthesis. Analogues of purine ribonucleotides often function as psueofeedback inhibitors of purine de novo synthesis (12). This represents one of the inhibitory sites for MePRP and is important for organisms such as *E. coli* B which synthesize purines de novo. The present data indicates that

MePRP also serves as a pseudofeedback inhibitor of adenylosuccinate synthetase by virtue of its functional resemblance to AMP, the normal regulator of the synthetase (31). This site acquires primary importance in purine auxotrophs such as strain W-11 when hypoxanthine or guanine serves as the purine source. The sensitivity of the two sites of inhibition is apparently of the same magnitude, since hypoxanthine failed to reverse inhibition even at the lowest concentrations (0.02 to 0.05 mM) of the analogue. It may not be necessary for MePRP to exert marked inhibition of either of the two potential inhibitory sites, since RNA synthesis (9) is completely arrested when the purine ribonucleotide pools are reduced by as little as 30 to 40%. Observations relating to the inhibition of RNA and coenzyme (I. W. F. Davidson and J. Fellig, Fed. Proc., **21**:160, 1962) synthesis in other organisms (14, 23) may represent the consequences of the more fundamental inhibition of purine synthesis and interconversion.

Inhibition by MeP required MePRP formation; therefore, organisms which lack or contain altered adenine phosphoribosyltransferase are resistant to MeP in a manner analogous to 2,6-diaminopurine resistance (13). Furthermore, adenine and 6-methylaminopurine effectively compete for the transferase to prevent MePRP formation and subsequent inhibition by MeP.

The adenine phosphoribosyltransferase of *E. coli* B possessed a limited specificity toward MeP as evidenced by the low quantities of the nucleotides detected in extracts of cells inhibited by MeP and by the ease with which adenine prevented the inhibition of MeP. The transferase of strain B-94 appeared to be even more specific for adenine. Although yeast autolysates possessed a very active transferase, its specificity toward MeP was sufficiently low that only limited quantities of the nucleotide were obtained.

The inhibitory effects of MeP may be overcome by the simple expediency of removing exogenous MeP. The requirement for continuous MePRP formation to maintain the inhibited state indicates that the inhibited cells maintain a relatively small pool of the analogue nucleotide and that the pool has a relatively short half-life. This view is supported by the very modest quantity of the analogue nucleotide detected in the cell and by the small quantity that can be synthesized in vitro.

In regard to the mechanism by which a normal purine "relieves" the inhibition of an analogue, our studies indicate the importance of determining whether the normal metabolite prevents establishment of the inhibitory state by competing with the inhibitor for the enzyme responsible for its conversion to the biologically active form, or whether the normal metabolite reverses previously imposed inhibition by supplying the product of the inhibited site(s). In the instance of MeP inhibition in *E. coli*, we present evidence that both mechanisms play a role in the ability of adenine to restore the cell to its normal physiological condition. The "reversibility" of MeP inhibition by adenine in other organisms (5, 6, 14, 23) does not establish that adenine is serving as the end product of the inhibited step. It will be necessary to establish that the analogue nucleotide is serving as a feedback inhibitor of adenylosuccinate synthetase in these organisms before drawing definite conclusions.

TABLE 6. *Isolation of mutants conditionally resistant to 6-methylpurine (MeP)*

Microbial group	Purine supplements		Interpretation
	MeP	MeP and hypoxanthine	
1	Susceptible	Susceptible	Wild type
2	Resistant	Resistant	Altered adenine phosphoribosyltransferase
3	Susceptible	Resistant	Altered adenylosuccinate synthetase; active adenine phosphoribosyltransferase

Although MeP is an effective inhibitor of purine biosynthesis, the dual sites of inhibition by MeP precludes elucidation of its specific effect on thiamine biosynthesis since adenine, which is required to bypass the adenylosuccinate synthetase site, is itself an inhibitor of thiamine biosynthesis.

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