Purine Analogs as Feedback Inhibitors.*† (25045)

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A feedback mechanism of biosynthetic control which shuts down formation of purine precursors has been described for auxotrophic mutants of *Escherichia coli* that accumulate the substrate of their genetically blocked reaction(2,3). One such mutant, strain B-96, is lacking in transformylase activity and hence accumulates the ribotide of 5-amino-4-imidazolecarboxamide (AICAR) which is dephosphorylated in the process of its excretion and appears in the culture fluid as riboside form. Accumulation of AICAR comes to an immediate halt when minute amounts of any purine which can support growth of mutants are added to the medium. Thus, a direct correlation exists between those purines which can be used for nucleic acid synthesis and those which are able to trigger the shut-down mechanism of biosynthetic control. For a clearer insight into the action of purines as feedback inhibitors, it became desirable to find substances which could prevent this inhibitory action. The "antipurines," inhibitory structural analogs of naturally-occurring purines, appeared to be likely candidates for such a role. A number of antipurines were tested, but instead of nullifying feed-back inhibition, those which were active simulated natural purines as potent inhibitors of AICAR formation. This suggested that the structural similarities which made them competitive antimetabolites were sufficient to mimic the natural purines in activating feedback mechanisms of control. This report deals with analysis and description of effects of purine analogs on formation of the purine precursor, AICAR.

Materials and methods. Escherichia coli, strain B-96, is a purine-requiring mutant isolated in this laboratory from the wild-type, strain B, by penicillin-selection technic. It is non-discriminating in its choice of purines for growth. The accumulation of AICA (as the riboside) was observed in non-proliferating suspensions of bacteria by methods previously described(4). Non-proliferating cell suspensions (0.5 mg dry weight/ml) were incubated in phosphate buffer (0.04 M at pH 7.4) containing $MgSO_4$ (0.01%), glucose (0.02 M) as source of carbon, and ammonium chloride (0.1%) as source of nitrogen. Casein hydrolysate (0.1%) was also added to increase the yield of AICA. At appropriate time intervals, aliquots were removed, cells were precipitated with trichloroacetic acid and AICA content of supernates was measured as diazotizable amine by the method of Bratton and Marshall(5).

Results. The amount required for 50% inhibition of AICA formation was determined for 15 analogs and related compounds (Table I). The most active inhibitors were the 3 antimetabolites which have gained considerable popularity as growth inhibitors in many other systems, *viz.*, 6-thioguanine, 6-mercaptopurine and 2,6-diaminopurine. As little as 1 μg of these compounds/ml of test system reduced the yield of AICA by at least 50%. The 8-azapurines were comparatively less active as inhibitors.

Dose response curves are shown in Fig. 1. It is evident from these curves that a proportionality exists between degree of inhibition and logarithm of concentration of the antimetabolites. With increased concentrations, a plateau level of maximal inhibition was reached at 70 to 85%; complete inhibition was never achieved. The kinetics of inhibition are shown in Fig. 2 with 6-mercaptopurine as an example. With 6-mercaptopurine present from the beginning, rate of formation of AICA comes to nearly a complete halt after 30 minutes. When addition of the antimetabolite is delayed, the decelerative action becomes more pronounced. After production of AICA has been under way for one hour, addition of 6-mercaptopurine causes immediate and complete cessation of further formation. This

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TABLE I. Effect of Purine Analogs on Formation of AICAR by *Escherichia coli*, Strain B-96.

Compound*	Purine substituents 2	6	8	Other	Amt for 50% inhibition (μg/ml)
6-thioguanine	NH_2	SH			.2
6-mercaptopurine		SH			.5
2,6-diaminopurine	NH_2	NH_2			1.0
8-aza-adenine		NH_2	(—N=)†		20.0
8-azaguanine	NH_2	OH	(")		100
8-azahypoxanthine		OH	(")		"
Pyrazoloadenine		NH_2	(")	7: (—CH=)	"
2-thioxanthine	SH	OH			160
8-azadiaminopurine	NH_2	NH_2	(")		200
Kinetin		NHC_4H_3O			500
8-azaxanthine	OH	OH	(")		700
2-thio-8-azaxanthine	SH	OH	(")		>1000
2-thioadenine	SH	NH_2			"
Benzimidazole				1,3: (—CH=)	"
Puromycin riboside		$N(CH_3)_2$		9: 3′-NH_2-ribose	"

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† Symbols in parentheses refer to substitutions within the purine ring, *e.g.*, 8: (—N=) means that the —CH= at position 8 has been replaced with —N=; 7: (—CH=) means that the —N= at position 7 has been replaced with —CH=.

Yield of AICA was determined after 3 hr of incubation at 37°C.

experiment not only shows direct and immediate inhibitory action of 6-mercaptopurine but also serves to rule out the possibility that inhibition may have been at the level of enzyme formation. The effects observed are strikingly similar to those obtained with adenine as a feedback inhibitor(3).

When the analogs are classified as to their

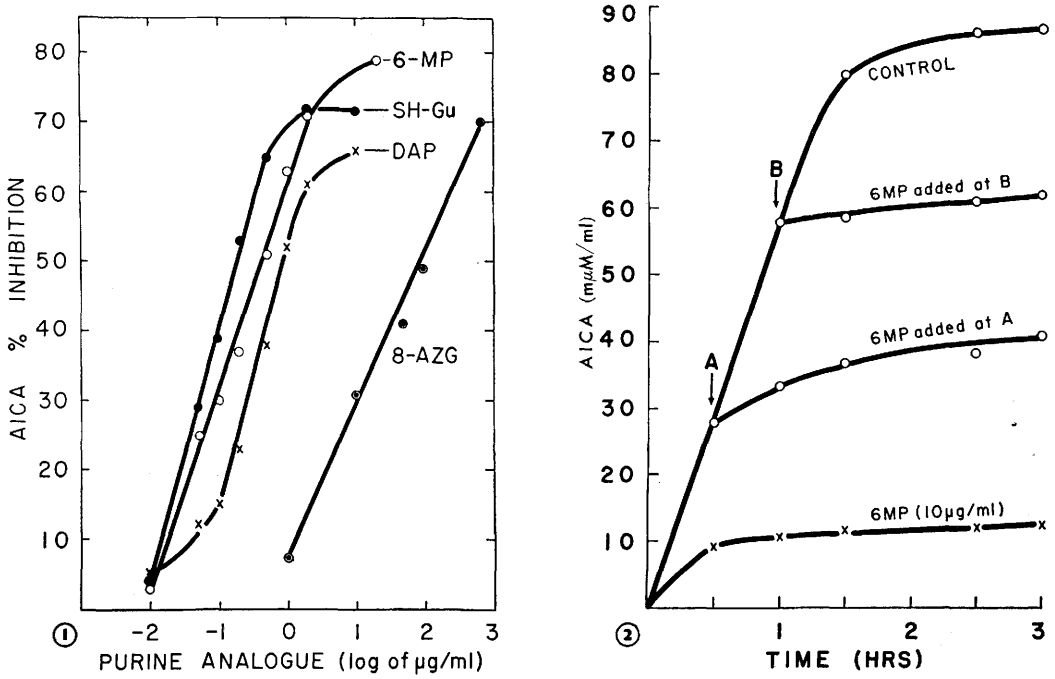

FIG. 1. Dose response curves of various purine analogs as inhibitors of AICA accumulation by *E. coli*, strain B-96. 6-mercaptopurine (6-MP); 6-thioguanine (SH-Gu); 2,6-diaminopurine (DAP); 8-azaguanine (8-AZG).

FIG. 2. Inhibition of AICA accumulation by 6-mercaptopurine (6-MP). Additions of 6-MP to give final concentration of 10 μg/ml were made as indicated.

effectiveness as inhibitors of bacterial growth, this is seen to correlate with their effectiveness as inhibitors of AICAR formation. The best inhibitors of growth are those which are potent inhibitors of AICAR formation; those which are inactive as inhibitors of biosynthesis are also inactive as inhibitors of growth. No simple quantitative correlation was found. For example, 2,6-diaminopurine, which gives a half-maximal inhibition of AICA formation at 1 μg/ml, requires 10 times this concentration to give half-maximal inhibition of growth. The quantitative discrepancies may reside partially in the fact that complete inhibition of AICAR formation is never obtained and partially in the possibility that metabolic diversions of analogs may occur during growth.

Discussion. In addition to the traditional action of successful antipurines as competitive inhibitors at the level of interconversions and utilization of nucleic acid purines, apparently at the nucleotide level, it is becoming increasingly evident that they may also inhibit at the level of *de novo* biosynthesis of nucleotides. Incorporation of isotopically labeled glycine into nucleic acid purines of tumor cells is inhibited by 2,6-diaminopurine and, to a lesser extent, by 6-mercaptopurine(6). A multi-site inhibitory action of 6-thioguanine has also been revealed in metabolism of purines by tumor cells, with inhibition of *de novo* biosynthesis occurring before formation of the precyclic precursor, formylglycinamide ribotide (7). Inhibition at this level would prevent formation of the cyclic aminoimidazole precursor studied here. It is suggested that inhibitory antipurines act as sequential inhibitors by preventing both biosynthesis and eventual metabolic utilization of purine nucleotides. Indeed, those which are effective chemotherapeutic agents may owe their success to this sequential blockade.

The results reported here suggest that the very property of structural similarity which endows the analogs with competitive action may also allow them to trigger the feedback mechanism of biosynthetic control. This is particularly evident for those analogs which prevent formation of AICA at levels of 0.01 micromole or less. For those requiring more than 1 micromole for effective inhibition, as with most 8-azapurines, a pseudo-type of feedback inhibition may be operating. This could occur by limiting availability of 5-phosphoribosyl-1-pyrophosphate (PRPP) for *de novo* biosynthesis. The utilization of PRPP in formation of analog-ribonucleotides has been reported for a variety of purine analogs(8,9). Final evaluation of the mechanism of action of antipurines, particularly as inhibitors of *de novo* synthesis, must also take into account possible competition with purine-containing cofactors which participate in the biosynthetic reactions.

Summary. Accumulation of ribotide of 5-amino-4-imidazole carboxamide (AICAR), excreted in the riboside form by a purine-requiring mutant of *Escherichia coli,* was inhibited by a number of structural analogs of purines. The most potent inhibitors were 6-thioguanine, 6-mercaptopurine, and 2,6-diaminopurine. The type of inhibition obtained suggests that the antimetabolites sufficiently resemble natural purines in structure to act as feedback inhibitors in biosynthetic control.

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