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REGULATION OF GMP REDUCTASE IN *SALMONELLA TYPHIMURIUM*

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Summary

The levels of guanosine 5′-phosphate reductase (EC 1.6.6.8) in *Salmonella typhimurium* appear to be modulated by changes in the ratio of the adenine and guanine nucleotide pools. Alterations of this ratio may be induced by high levels of guanosine in the culture medium or by genetic lesions in one of several purine interconversion enzymes, such as *pur* A or *pur* B mutants. The induction of the reductase requires transcription and translation processes and, in contrast to earlier observation with *Escherichia coli*, is not dependent on cyclic adenosine 3′,5-phosphate or the cyclic adenosine 3′,5-phosphate receptor protein.

Introduction

Guanosine 5′-monophosphate (GMP) reductase (NADPH : GMP oxidoreductase, EC 1.6.6.8) mediates the irreversible NADPH-dependent catalysis of GMP to inosine 5′-monophosphate (IMP). Its role in the interconversion of purine nucleotides in enteric bacteria was first described by Mager and Magasanik [1]. They showed that slight variations in the level of the reductase occurred with different growth conditions, but these were insufficient to indicate that the enzyme was inducible. However, Magasanik and Karibian [2] observed that guanine had to be added to the growth medium in order to obtain significant activity of GMP reductase in *Escherichia coli* strain HP-1. Nijkamp and DeHann [3] showed that expression of the reductase gene (*guaC*) was noncoordinate with expression of the genes involved in GMP synthesis from IMP (*guaA* and *guaB*). They also showed that reductase activity increased as the guanine in the growth medium was increased. In addition, the reductase gene was not linked to the *guaA-B* operon [4]. Nijkamp [5] implicated adenine nucleotides in the regulation of the *gua* operon and presented an argument for the physiological importance of the *guaA* and *guaB* products in maintaining the

balance in the synthesis of adenine and guanine nucleotides. Momose et al. [6] showed the presence of GMP reductase and its overall importance in the interconversion of the nucleotides in *Bacillus subtilis* but did not indicate environmental control of enzyme activity.

We previously reported [7] the requirement of cyclic adenosine 3′,5′-monophosphate (cyclic AMP) for the induction of GMP reductase in *E. coli* even though the induction process did not appear to be sensitive to catabolite repression. The report presented here provides evidence that GMP reductase in *Salmonella typhimurium* is inducible, that it is controlled independently of the *guaA* and *guaB* genes, it is not dependent on cyclic AMP or its receptor protein, and that the expression of *guaC* is controlled by the intracellular levels of adenine and guanine nucleotides.

Methods

Bacterial strains

All strains used in this study are derivatives of *Salmonella typhimurium*, strain LT-2 obtained from the Demerec collection through the courtesy of the late M. Demerec or K. Sanderson. *Escherichia coli* strain KL 132 carrying the episome F′117 *purA*$^+$ was obtained from K.B. Low [8]. The episome was transferred to a *purA*$^-$ *metE*$^-$ strain of *S. typhimurium* by selection for *purA*$^+$. This served as the donor for transfer to other *purA* strains (e.g. Pur70) of *Salmonella* by selecting for *pur*$^+$ in the absence of methionine. *PurA*$^+$ transductants of strain Pur70 were selected following exposure to P-22 transducing bacteriophage derived from strain LT-2. Production of donor transducing phage lysates and their assay followed standard procedures [9].

Media and culture techniques

The minimal salts medium was medium E described by Vogel and Bonner [10] with 0.2% glucose as the carbon source. Amino acid requirements were fulfilled by the appropriate amino acid at 40 μg/ml or 0.1% casein hydrolysate. For purpose of maintenance, or when an inoculum was grown up, the purine auxotrophs were supplied with 20 μg purine/ml culture medium. All cultures were incubated at 37°C. Tube cultures (5.0 ml) were aerated on a roller drum apparatus and large broth cultures (100—1000 ml) were inoculated from log phase cultures into flasks on a rotary shaker waterbath. Difco nutrient broth served as a medium for preparation of bacteriophage lysates.

Chemicals

All chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.) or Calbiochem (LaJolla, Calif.) Psicofuranine was a gift from G.B. Whitfield, The Upjohn Co., (Kalamazoo, Michigan) and rifampin was obtained through the courtesy of C.L. Hake, Dow Chemical Co. (Zionsville, Indiana). Chloramphenicol was a gift from H.E. Machamer, Parke-Davis (Detroit, Michigan).

Induction and enzyme assays

Induction of GMP reductase was performed either by incubating the organisms overnight or by a 2-h procedure in medium containing guanosine. In

the latter method, an overnight culture was collected by centrifugation, diluted 1/8 with fresh medium, and incubated to mid-log growth, as monitored by a Zeiss spectrophotometer at 540 nm. At mid-log phase, a sample (100—200 ml) was collected as the initial sample and the rest of the cell culture was divided equally among the various test systems. These flasks were incubated an additional 2 h, and then collected by centrifugation. The 2-h induction was useful for inhibition studies or with cyclic AMP mutants, or when there were problems with reversion of mutants. All cell preparations were washed, sonicated and assayed for GMP reductase as described previously [7,11]. IMP dehydrogenase was assayed according to the procedure of Udaka and Moyed [12] and was measured by increased absorbancy at 340 nm. EDTA was 10 mM in the 3 ml reaction system.

Protein concentrations were determined by the Lowry method [13] with bovine serum albumin as the standard.

Results

Induction of GMP reductase

The effect of several purines on GMP reductase in overnight cultures of *S. typhimurium* is illustrated in Table I. Guanosine (200 μg/ml of medium) gave a 4—5-fold induction during the overnight growth or in a 2-h period following shift of mid-log phase cells to the inducing medium. Hypoxanthine and xanthine were relatively weak as inducing agents even though the conversion to their respective ribonucleotides (IMP or XMP) ultimately results in producing, via the interconversion pathway (see Fig. 1), the substrate of the reductase, GMP. The IMP formed from the exogenously supplied hypoxanthine also contributes to AMP synthesis; the possible significance of this conversion in regulation will become evident later in this paper. For some undetermined reason, xanthine was a poor inducer. Guanine or guanosine were equally effective inducers, however we routinely used guanosine because of solubility problems with the high levels of guanine. Several analogues of guanine and guanosine

TABLE I

INDUCTION OF GMP REDUCTASE IN *SALMONELLA TYPHIMURIUM* LT-2

Supplements	(μg/ml)	GMP reductase (nmol/min/mg protein)
None		0.60
Adenine	50	0.90
Guanosine	10	0.89
	200	3.10
Hypoxanthine	200	0.94
Xanthine	200	0.88
8-Azaguanine	100	0.83*
2-Deoxyguanosine	100	2.33*
6-Mercaptoguanosine	100	3.23*
2-Thio-6-oxy-azapurine	100	5.82*
Guanosine	100	3.19*

* All analogues were tested in a 2-h induction system (initial activity was 0.7—0.8 nmol/min/mg protein).

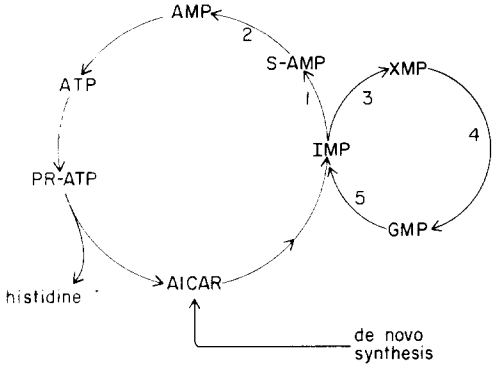

Fig. 1. Purine nucleotide interconversion pathway. 1, *purA* (adenylosuccinate synthase); 2, *purB* (adenylosuccinate lyase); 3, *guaB* (IMP dehydrogenase); 4, *guaA* (XMP aminase); 5, *guaC* (GMP reductase). PR-ATP, 1-phosphoribosyl-ATP; AICAR, amino imidazole carboxamide ribonucleotide; IMP, inosine 5'-monophosphate; S-AMP, succinyl-adenosine 5'-monophosphate; AMP, adenosine 5'-monophate; ATP, adenosine 5'-triphosphate; XMP, xanthosine 5'-monophosphate; GMP, guanosine 5'-monophosphate.

were capable of stimulating synthesis of GMP reductase and 2-thio-6-oxyazapurine appeared to be even more efficient than guanosine.

Cyclic AMP studies

The induction of GMP reductase by guanosine in *E. coli* requires the presence of a functional cyclic AMP generating system [7]. Comparable studies (Table II) were performed with adenyl cyclase-deficient mutants of *Salmonella* (GP-21) and a cyclic AMP protein mutant (GP-61). In all instances, the induction index was identical, thus indicating that induction of GMP reductase apparently does not require the participation of cyclic AMP or cyclic AMP receptor protein in *Salmonella.*

Endogenous induction

The high levels of GMP reductase in the non-induced *Salmonella* implied induction or activation by an endogenously synthesized intracellular compound. We have previously suggested [7] that a guanine derivative appeared the most likely candidate for the inducer. If the endogenous synthesis of this

TABLE II

INFLUENCE OF CYCLIC AMP IN GMP REDUCTASE INDUCTION

Induction index: induced activity/endogenous activity, 2-h induction procedure used; all strains were grown in medium E plus glucose, 0.1% casein hydrolysate and guanosine (200 μg/ml) as indicated. Spec. act. is nmol IMP formed per min per mg protein. Gene designation: cya = adenyl cyclase; crp = cyclic AMP receptor protein.

Strain	Genotype	GMP reductase (spec. act.)		Induction index
		Endogenous	Induced	
LT-2	cya^+ crp^+	1.0	3.6	3.6
GP-21	cya^- crp^+	0.5	1.8	3.6
GP-61	cya^+ crp^-	0.7	2.7	3.6

TABLE III

EFFECT OF PSICOFURANINE ON GMP REDUCTASE IN LT-2 (WILD TYPE)

Enzyme activities expressed as nmol product formed per min per mg protein. Overnight induction system: the level of psicofuranine permits growth at a slightly slower rate but nearly the same A_{540} is reached by all cultures within 18 h.

Media supplements	GMP reductase	IMP dehydrogenase
None	0.83	0.34
Psicofuranine (50 μg/ml)	0.05	1.61
Guanosine (200 μg/ml)	4.42	0.18
Guanosine 200 μg/ml plus psicofuranine (50 μg/ml)	3.20	0.16

compound could be blocked or greatly reduced, there should be a concomitant reduction in the endogenous levels of the reductase. Psicofuranine, an adenosine analogue, inhibits XMP aminase and causes an overall reduction in the synthesis of GMP and its derivatives [14—16]. This inhibition decreases the GMP pool and causes the derepression of the guanine operon resulting in an increase in IMP dehydrogenase activity. This increase can be monitored as an experimental index of the effectiveness of the drug. Table III shows that the basal level of GMP reductase is drastically depressed by psicofuranine inhibition of endogenously synthesized GMP. This condition results in a concomitant derepression of IMP dehydrogenase. Exogenously supplied guanosine replenishes the guanine nucleotide pools and thus stimulates the synthesis of the reductase in the presence or absence of psicofuranine (lines 3 and 4), and represses IMP dehydrogenase.

Synthesis of GMP reductase

Since the level of endogenous induction of GMP reductase seemed to be relatively constant, we tried to define whether induction was due to derepression or to activation of a pre-existing protein by inhibition of translation via chloramphenicol or amino acid starvation. Induction of GMP reductase could be inhibited by chloramphenicol as indicated in experiment 1, Table IV. The

TABLE IV

REQUIREMENT FOR PROTEIN SYNTHESIS IN THE INDUCTION OF GMP REDUCTASE

2—h induction system as described in Methods. Spec. act. = nmol IMP formed per min per mg protein. Concentrations of additions are given in parenthesis as μg/ml medium. CM = chloramphenicol. Experiment 1: Wild type strain LT-2: Initial spec. act. 0.8; Experiment 2: Proline auxotroph, strain Pro 25: Initial spec. act. = 0.64.

Experiment	Strain	Additions	GMP reductase (spec. act.)	Fold increase
1	LT-2	None	0.6	0.8
		CM (200)	0.9	1.1
		Guanosine (200)	1.66	2.8
		CM + guanosine	0.75	1.0
2	Pro 25	None	0.52	0.8
		Proline (40)	0.55	0.9
		Guanosine (200)	0.79	1.2
		Proline + guanosine	2.69	4.2

simultaneous addition of chloramphenicol and guanosine did not result in a significant increase in the enzyme levels, while guanosine alone affected a several fold increase in specific activity. Additional evidence that protein synthesis is required for induction is found in the starvation of *proB-25* (a proline auxotroph) for proline (experiment 2, Table IV). The effect of adding guanosine to the starved culture was slight compared to the increase when both proline and guanosine were simultaneously supplied to the cells. Thus, the induction of GMP reductase requires de novo protein synthesis.

Induction of GMP reductase in purine auxotrophs

Adjunctive to other studies, the GMP reductase levels were routinely measured in purine auxotrophs which were deficient in one of the enzymes of the interconversion pathway. Only a few strains are listed in Table V, however, these data are consistent with those of over 25 mutants examined. An interesting observation was that all those mutants carrying a block in the conversion of IMP to AMP showed constitutive levels of the reductase regardless of the purine concentration in the medium. The addition of guanosine did not affect the levels nor did the presence or absence of a defective regulator gene (*purR*). The high levels of GMP reductase were in both *purA* and *purB* mutants thus making it unlikely that the constitutive levels are due to specific defective products of the mutant genes. Strain Pur70 is a leaky mutant and will grow on guanine or guanosine in media supplemented with either casein hydrolysate or histidine. As can be seen in Table V this strain appeared to be superinducible, gave unusually high enzyme levels, and hence was used for additional studies.

TABLE V

INDUCTION OF GMP REDUCTASE IN PURINE AUXOTROPHS

Overnight induction procedure, all values for a particular strain were measured at the same time: GMP reductase activity were expressed as nmol IMP formed per min per mg protein.

Strain	Genotype	Media Supplements (μg/ml)		GMP reductase
		Ade	GuR	
LT-2	Wild type	—	—	0.6
		200	—	0.68
		—	100	6.40
Pur 155	*purA, purR*	20	—	4.40
		200	—	3.98
		20	100	2.67
Pur 81	*purA*	200	—	3.38
		20	100	3.42
Pur 70	*purA* (leaky)	50	—	6.25
		—	50	31.70
Pur 124	*purA, purB*	50	—	3.13
		50	200	4.06
Pur 73	*purB*	50	—	2.53
		50	200	4.42
Gua 1	*guaA*	—	10	0.39
		—	200	2.72
Gua 5	*guaB*	—	10	0.72
		—	200	1.74

The endogenous levels of the reductase found in *guaA* and *guaB* mutants are also listed in Table V. Although the presence of guanine or guanosine in the culture fluid resulted in a 5—10-fold increase in the reductase, the values were not as high as those observed in wild type or adenine-requiring strains. Differences in the level of the reductase, but not the magnitude of increase, could be caused by the relative proportion of the intracellular concentration of the nucleotide pools. This observation is particularly significant since *gua* mutants are dependent on an outside source for their guanine but they can synthesize adenine de novo. All IMP synthesized via the de novo pathway would be shunted toward AMP synthesis since the *gua* operon would be repressed by the exogenously supplied guanine. Thus *gua* mutants should be starved only for guanine but not adenine nucleotides, and high endogenous levels of GMP would not be expected in this situation.

GMP reductase in Pur70

Since both the endogenous and induced levels were significantly higher in strain Pur70 than any other strain, we reinvestigated the effects of psicofuranine, chloramphenicol, and rifampin with this strain. It was important to compare the psicofuranine and chloramphenicol results with those previously obtained with strain LT-2 to establish that the high levels of the reductase in Pur70 were a direct result of endogenous induction. Rifampin was included in the study to assess the role of transcription in the induction process. The results of these experiments are summarized in Table VI. There was little change in the base level of the reductase in the adenine-grown cells, while a 6-fold increase was observed when guanine was the purine source. Psicofuranine caused a decrease in the level of the reductase of the adenine-grown cells indicating that the high reductase levels in Pur70 is due to an endogenous induction rather than a defective regulatory gene or function. The source of the inducing molecule could be from the exogenously supplied adenine or the de novo synthesis of IMP which, in a *purA* mutant, would be shunted into GMP synthesis. However, at the levels of adenine used in this experiment, the de novo enzymes are known to be repressed, so we must consider an alternate explanation. Besides the possible conversion to IMP, AMP can be converted to ATP which serves as an important source of energy and as a precursor in

TABLE VI

INHIBITION OF GMP REDUCTASE INDUCTION IN PUR 70

Inhibitors	Activity after 2 h*	
	Media supplements	
	Adenine (50 μg/ml)	Guanosine (50 μg/ml) + CH*
None	3.70	20.30
Psicofuranine (50 μg/ml)	0.15	15.70
Chloramphenicol (200 μg/ml)	3.63	2.77
Rifampin (150 μg/ml)	4.58	2.95

* Initial activity: 3.2 nmol IMP formed/min/mg protein; CH, casein hydrolysate 0.1%.

histidine biosynthesis (see Fig. 1). All this would lower the adenine nucleotide pool and increase the guanine pool. Under these conditions, simulating an adenine starvation of the cell, induction of GMP reductase might occur to equalize the adenine:guanine nucleotide balance. Since the Pur70 lesion is "leaky" it can be starved for adenine by growth with guanine or guanosine as the purine source as long as histidine (singly or in casein hydrolysate) is supplied in the culture medium. This condition results in adenine starvation and under these conditions psicofuranine caused only a small decrease in the specific activity of the reductase. These studies signify that the high levels of the reductase in adenine-grown cells result from the endogenous induction by a guanine derivative. The fact that guanosine can increase the levels of reductase in the presence of psicofuranine implies that the control system is not defective. The inhibition obtained with chloramphenicol and rifampin implicates both transcription and translation as requirements for the induction process.

Genetic alteration of Pur70

The *purA* defect in Pur70 was repaired by P-22 mediated transduction or by episomal complementation with F'17. The genetic derivatives were induced by adenine or guanosine and the levels of GMP reductase measured (Table VII). The effect of the genetic alteration is clearly evident; GMP reductase values have dropped to the range observed with LT-2. This effect might imply that the *purA* gene product plays a role in the regulation of GMP reductase and indeed all *purA* strains show high levels of the reductase when grown on adenine (see Table V). However, since the *purB* mutants also show elevated reductase levels, it is therefore more likely that the intracellular levels of adenine nucleotides play an important role in the induction and repression of GMP reductase. It would be expected that maximal induction should occur when adenine pools are low and guanine pools high. Any type of drain on the adenine nucleotide pool would alter the intracellular balance and cause derepression of GMP reduc-

TABLE VII

GMP REDUCTASE IN PUR 70 AND DERIVATIVE STRAINS

Pur 70 was grown in adenine and shifted to fresh induction media containing adenine or guanosine plus casein hydrolysate. The pre-induction levels of GMP reductase (t = 0) were obtained for the maintenance medium of strain.

Strain	Genotype	Media supplements*	GMP reductase**		Fold increase
			t = 0	t = 2 h	
Pur 70	*purA*$^-$	adenine	1.7	2.24	1.3
		guanosine + CH		14.74	8.7
Pur 70 (tr)	*purA*$^+$ (transductant)	none	0.54	0.99	1.8
		adenine		0.44	0.8
		guanosine		2.31	4.3
Pur70/F'117	*purA*$^-$/*purA*$^+$	none	0.79	0.43	0.6
		adenine		0.72	0.9
		guanosine		2.17	2.8

* Purine concentrations were 50 μg purine/ml media; CH, casein hydrolysate was 0.1%.

** GMP reductase was measured after 2 h induction.

TABLE VIII

EFFECT OF HISTIDINE ON ENDOGENOUS INDUCTION IN PUR 70

Spec. act.: nmol IMP formed per min per mg protein. Values are averages of two separate experiments of overnight induction in Medium E-glucose as supplemented below. Concentrations are listed in parenthesis as μg/ml.

Additions	GMP reductase
Adenine (50)	1.53
Adenine + histidine (50)	0.38
Guanosine (50) + histidine	12.21

tase which would function to restore a proper balance between the two nucleotide pools. Since histidine "spares" adenine via feedback inhibition of the histidine pathway, inclusion of histidine in the adenine medium of Pur70 should result in a lowering of the GMP reductase levels. Pur70 was grown overnight on the three types of medium described in Table VIII and the levels of GMP reductase measured. The values are somewhat lower than reported elsewhere in this paper, but the experiment has been repeated with nearly identical results. The high levels of reductase are lowered to a level similar to non-induced wild type when histidine is added to the medium. We conclude that changes in the ratio of the adenine:guanine nucleotide concentrations appear to serve as the corepressor-inducer signals in the control system of GMP reductase.

Discussion

GMP reductase catalyzes a reaction which appears biochemically essential only when a purine auxotroph requires exogenously supplied xanthine or guanine as a source of purines. Therefore, the loss of this enzyme would have no apparent consequence for prototrophic strains, or for those purine auxotrophs (e.g., *gua* mutants) which can synthesize their own adenine via the de novo route, and hence, are not required to utilize exogenous guanine in the synthesis of adenine. The data presented in this report indicate an important physiological role for GMP reductase in the overall maintenance and regulation of purine interconversion enzymes.

Previous studies reported by us [7] indicated that GMP reductase was an inducible enzyme in *E. coli* not subject to catabolite repression, and that cyclic AMP was required for the induction. We have reported here that the reductase is inducible in *Salmonella* by a mechanism that apparently does not involve cyclic AMP or its receptor protein.

The endogenous synthesis of GMP was sufficient to moderately induce GMP reductase synthesis. In *Salmonella* strain LT-2 and a mutant with high constitutive levels of GMP reductase, psicofuranine, an inhibitor of XMP aminase, decreased GMP synthesis and caused a concommitant drop in the specific activity of the reductase. This finding implicates GMP as the most likely inducer. We have also demonstrated that increases in the levels of the reductase result from a specific induction process involving transcription and

translation. Mutants with lesions in either *purA* or *purB* genes or both, were found to have constitutive levels of the reductase. Restoration of these genetic defects restored the induction-repression pattern to normal. There was the possibility that these gene products participated in the regulation of GMP reductase, but an alternate concept was that the genetic defects in purine interconversion lead to alterations in the metabolism of the purine nucleotides. This latter suggestion implies that the ratio of the adenine and guanine nucleotides pools would influence the induction of the reductase.

If an imbalance in the nucleotide pools is responsible for the high derepression of GMP reductase, it should be possible to examine various mutants during induction and demonstrate a shift in the flow of radioactive-labelled purine. ^{32}P-labelling of the nucleotide pools of LT-2 and Pur70 have been examined by this method, but no conclusive data has been obtained (Tai, personal communication).

Pur70 is a leaky mutant and when grown on guanosine+casein hydrolysate (or histidine), adenine starvation occurs. In this circumstance, the nucleotide ratio of adenine to guanine is lowered and the GMP reductase is induced. This induction represents a metabolic effort to restore concentration of the adenine nucleotides to normal. When Pur70 is grown on adenine, the purine moiety is partially lost to histidine and GMP synthesis, allowing some induction by the nucleotide imbalance. It would seem that anything that lowers the adenine pool or raises the guanine pool level would cause the necessary imbalance to allow for induction of GMP reductase. Thus the histidine requirements of the guanine grown cells actually serves to conserve adenine [2] and we demonstrated histidine would lower the reductase levels in Pur70 grown on adenine (see Table VIII). This result supported the concept of nucleotide balance in regulation since the adenine pool would not be drained by the cellular demands for histidine and exogenously supplied adenine would be spared for other functions. During studies on the regulation of guanosine kinase in *Salmonella*, Jochimsen (personal communication) has found with several purine mutants, that either adenine starvation or excess guanosine causes induction of the reductase. The simultaneous addition of adenine, guanosine and histidine resulted in the repression of the reductase.

We conclude that GMP reductase appears to be regulated by changes in the adenine:guanine nucleotide pool. The data with Pur70 can easily be interpreted to fit this definition. We must reason that *gua* mutants are capable of producing sufficient adenine de novo and have no apparent cause for GMP to be wasted in the synthesis of AMP. Nijkamp suggested that adenine nucleotides perform regulatory functions in the control of GMP biosynthesis (i.e. the *gua* operon) by inducing and activating the gene products while the guanine nucleotides repress the *gua* operon. He further suggested the physiological importance of IMP dehydrogenase and XMP aminase to the adenine nucleotides resided in the maintenance of a balance in biosynthesis of adenine and guanine nucleotides. Our results focus on the role of guanine nucleotides as an inducer, and also indicate that the balance between the nucleotides is important in genetic regulation. The interconversion enzymes appear to be considerably more important in the overall function of the metabolite flow of purines than previously recognized. When normal flow is interrupted via mutation, regulatory bal-

ances are impaired. Construction of the proper mutants and experiments to elucidate the mechanism of regulation of the purine operons should be done only with careful consideration of the influence of the interconversion enzymes in their role to maintain the nucleotide balance.

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