JOURNAL OF BACTERIOLOGY, Oct. 1971, p. 122-131
Vol. 108, No. 1

Printed in U.S.A.

Regulation and Mechanism of Phosphoribosylpyrophosphate Synthetase: Repression by End Products

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Received for publication 5 May 1971

Phosphoribosylpyrophosphate (PRPP) synthetase participates in the biosynthesis in bacteria of purine nucleotides, pyrimidine nucleotides, tryptophan, and histidine. The regulation of the synthesis of PRPP synthetase in *Salmonella typhimurium* was studied. Addition of end products to the growth medium, singly or in combination, resulted in small decreases in the specific activity of PRPP synthetase, but levels of the enzyme were never decreased to less than half of those found when the bacteria were grown on minimal medium. Growth of the bacteria on several different carbon sources or starvation for phosphate had little effect on the specific activity of PRPP synthetase. Over-production of histidine in a histidine regulatory mutant, which would be expected to result in a depletion of intracellular PRPP pools, did not alter PRPP synthetase specific activity. PRPP synthetase levels were examined in auxotrophic strains of *S. typhimurium* that had been starved for the end products of PRPP. In each case derepression of an enzyme in the biosynthetic pathway for the limiting end product was demonstrated. However, only alterations in the levels of pyrimidine bases in the culture medium brought about derepression and repression of PRPP synthetase. Excess pyrimidines do not completely repress the enzyme. Deprivation of exponentially growing cells for pyrimidines by growth of an auxotrophic mutant on media containing orotic acid, which enters the cells slowly, resulted in a 10-fold derepression of PRPP synthetase. Derepression of PRPP synthetase during uracil starvation was prevented by chloramphenicol. The PRPP synthetase activities of extracts from repressed and derepressed cells responded in identical fashion to heat inactivation, cellulose acetate electrophoresis at several *p*H values, and in kinetic experiments.

The biosynthesis of 5-phosphoribosyl-α-1-pyrophosphate (PRPP) from adenosine triphosphate (ATP) and ribose-5-phosphate may be viewed as the first step of a highly divergent biosynthetic pathway leading to pyrimidine nucleotides (15), purine nucleotides (12), histidine (4), tryptophan (27), and pyridine nucleotides (21). Utilization of exogeneous purine and pyrimidine bases via the nucleotide pyrophosphorylase reactions may substitute for de novo synthesis of these compounds, but both metabolic routes require PRPP. Several other branched pathways of biosynthesis have been reported to be subject to elaborate metabolic control (8, 23, 24). The enzyme PRPP synthetase (ATP:D-ribose-5-phosphate pyrophosphotransferase, EC 2.7.6.1) might therefore be expected to be regulated by products of PRPP metabolism.

A previous paper (25) described the isolation of PRPP synthetase from *Salmonella typhimurium* and listed some of the properties of the enzyme. Inhibition of the enzyme by purine and pyrimidine nucleotides and by tryptophan has been reported (R. L. Switzer, Fed. Proc. **26**:560, 1967). This paper is concerned with the regulation of the synthesis of the enzyme by metabolites biosynthetically derived from PRPP. The results indicate that of the metabolites examined —tryptophan, histidine, pyrimidine nucleotides, purine nucleotides, and PRPP itself—only pyrimidine nucleotides appear to function as repressing metabolites for PRPP synthetase. Some possible interpretations of this unusual control mechanism are discussed.

MATERIALS AND METHODS

Bacterial strains. The bacteria used in this study were all derived from *S. typhimurium* LT-2. Table 1 lists the strains used and their origin. Strain E 1-2 was derived from strain *pyr 573* by treatment with *N*-

methyl-*N'*-nitro-*N*-nitrosoguanidine (1) followed by penicillin selection and replica plating to select organisms which grew only on plates supplemented with uracil and adenine, each at 40 μg/ml. Strain E 1-2 requires uracil and either adenine or guanine for growth.

Media and culture methods. The E medium of Vogel and Bonner (26) with 0.5% glucose served as the minimal medium. Supplements to the minimal medium are indicated in context. Effects of metabolites on the specific activity of PRPP synthetase in wild-type bacteria were evaluated by growing the organisms in supplemented medium at 37 C with vigorous aeration after a 2% (v/v) inoculation with a culture grown overnight on minimal medium. The cultures were harvested in the late logarithmic phase of growth, except where otherwise noted, by centrifugation and then frozen until assay.

Derepression experiments were performed by growing the appropriate auxotrophic strain for 14 to 16 hr at 30 C in minimal medium supplemented with an excess of the required end product. The cells were harvested by centrifugation, washed once with 0.85% saline solution, and suspended (about 0.3 mg dry weight of cells per ml) in minimal medium that contained limiting amounts of the end product as described. The bacteria were grown at 30 or 37 C for 4 to 6 hr, harvested, and used for the appropriate enzyme assays. The relative amount of growth was determined from the absorbancy at 660 nm in a Klett-Summerson colorimeter and comparison to a standard curve that related milligrams of dry cells per milliliter to Klett units.

For experiments on the effects of carbon source on PRPP synthetase specific activity, the media contained (per liter): KH_2PO_4, 3.94 g; anhydrous K_2HPO_4, 4.97 g; $NaH(NH_4)PO_4 \cdot 4H_2O$, 3.5 g; and $MgSO_4 \cdot 7H_2O$, 0.2 g (7). Carbon sources were autoclaved separately and added at a final concentration of 0.5% (w/v). The cultures were inoculated with 20% (v/v) of a culture that had been adapted to the appropriate carbon source by overnight growth in medium containing the same carbon source. Cells were harvested in the late logarithmic phase of growth. The *p*H of the media was 6.9 to 7.0 throughout the period of bacterial growth.

To determine the effects of inorganic phosphate on the level of PRPP synthetase, the bacteria were grown in synthetic media modeled after the high- and low-phosphate media of Engelsberg et al. (10). The media were identical except that the high-phosphate medium contained 1% potassium phosphate (*p*H 7.0) and the low-phosphate medium contained only 5 μg of phosphorus/ml (0.16 mM potassium phosphate). Both media contained 0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (*p*H 7.0), 0.1% ammonium sulfate, 0.1% trisodium citrate·$2H_2O$, 0.1% $MgSO_4 \cdot 7H_2O$, and 0.5% glucose. Each culture was grown in duplicate. An inoculum grown in the low-phosphate medium was used to avoid transfer of phosphate into the cultures with the inoculum.

Enzyme assays. Sonic extracts were prepared by suspension of the cells at about 0.1 g (dry weight) per ml in 50 mM potassium phosphate (*p*H 7.5) and exposure to 30-sec bursts at 0 to 5 C with a Branson probe-type sonic oscillator. The sonically treated suspension was centrifuged at 20,000 × *g* for 20 min, and the supernatant liquid was used for assays. Protein was determined by the method of Lowry (16) with crystalline bovine serum albumin as a standard protein.

In most cases, the assay for PRPP synthetase activity was a ^{32}P-transfer assay, which was previously described (25). The assay is based on enzymatic transfer of radioactivity from ATP-γ-^{32}P to PRPP and its acid degradation products. The unreacted ATP is removed from solution by adsorption onto charcoal, and samples of the supernatant fluid are counted by liquid scintillation techniques. The method is suitable for the assay of PRPP synthetase in crude extracts, so long as each assay tube is corrected for a blank from which ribose-5-phosphate is omitted. This correction is necessary because not all of the adenosine triphosphatase activity of crude extracts is inhibited by NaF. The standard assay mixture contained 50 mM triethanolamine-50 mM potassium phosphate buffer (*p*H 8.0), 0.75 mM ethylenediaminetetraacetic acid, 5 mM $MgCl_2$, 3 mM ATP-γ-^{32}P (100,000 to 200,000 counts/min), 5 mM ribose-5-phosphate, and 25 mM NaF. In some experiments, toluene-treated cells were used in the ^{32}P-transfer assay instead of sonic extracts. In these cases, the activity in micromoles per minute per milligram (dry weight) of toluene-treated cells multiplied by 4.4 yields the equivalent activity in micromoles per minute per milligram of protein in sonic extracts of the same cells. The orotate removal assay of Lieberman et al. (15), which is based on conversion of PRPP to orotidylic acid, was used in a few experiments. This assay is less convenient but yields results that are very similar ($\pm$10%) to the ^{32}P-transfer assay.

Histidinol phosphate phosphatase (EC 3.1.3.15) was assayed as described by Ames et al. (2). Before phosphatase assay, the sonic extracts were desalted by passage through small Sephadex G-25 columns and elution with 10 mM triethanolamine-hydrochloride buffer (*p*H 7.6).

The activity of component I of anthranilate synthetase from strain *trpBEDC43* was determined fluorometrically (5). The reaction mixture contained 25 mM Tris-hydrochloride buffer (*p*H 7.8), 5 mM $MgCl_2$, 10 mM L-glutamine, 0.5 mM chorismate, 26 mM 2-mercaptoethanol, and enzyme. The assay was performed at 37 C. The fluorescent emission of the reaction product,

TABLE 1. *Strains of Salmonella typhimurium used in this study*

Strain	Phenotype	Source
LT-2	Wild type	B. N. Ames
LT-2, *pyr573*	Pyr⁻	B. N. Ames
LT-2, *trpA148*	Trp⁻	I. C. Gunsalus[a]
LT-2, *trpBEDC43*	Trp⁻	I. C. Gunsalus[a]
LT-2, *purC452*	Pur⁻	P. Sypherd[b]
LT-2, *hisE11*	His⁻	B. N. Ames
LT-2, *hisA30*	His⁻	B. N. Ames
LT-2, *hisG70*	His⁻	B. N. Ames
LT-2, *hisO1202 hisG1109*	His-constitutive, feedback-resistant	B. N. Ames
LT-2, *pyrA81*	Arg⁻ Pyr⁻	J. L. Ingraham
LT-2, E 1-2	Pyr⁻ Pur⁻	This study

[a] Originally from P. Margolin.
[b] Originally from E. Balbinder.

anthranilate, was measured at 410 nm; the wavelength of the exciting light was 308 nm. Component I of anthranilate synthetase was assayed in the presence of an extract from strain *trpA148* which lacks component I (6). To insure that the extract from strain *trpA148* contained an excess of component II, we grew the organism for 14 hr in minimal medium with a limiting (5 μg/ml) amount of L-tryptophan added. Sonic extracts of strains *trpBEDC43* and *trpA148* were prepared as described above, except that the buffer contained 20 mM Tris-hydrochloride, 10 mM $MgCl_2$, and 13 mM 2-mercaptoethanol in addition to the usual 50 mM potassium phosphate (*p*H 7.5).

The determination of aspartic transcarbamylase (ATCase, EC 2.1.3.2) activity followed the method of Yates and Pardee (28), except that the colorimetric method of Prescott and Jones (22) was used for determination of carbamyl aspartate. In some cases, the ATCase assays were performed with toluene-treated cells rather than sonic extracts. In these cases, toluene (1% by volume) was added to the cell suspension, and the tubes were incubated at 30 C with shaking for 20 min. The cells were collected by centrifugation, washed once with one volume of 50 mM potassium phosphate (*p*H 7.5), and suspended in 0.1 volume of the same buffer. Cells treated in this way were suitable for both ATCase and PRPP synthetase assays.

The procedure of Magasanik et al. (18) was used for assay of inosine 5'-monophosphate dehydrogenase (IMP dehydrogenase, EC 1.2.1.14).

RESULTS

Effect of addition of end products of PRPP metabolism to the growth medium. Table 2 summarizes the results of experiments in which *S. typhimurium* LT-2 (wild type) was grown in the presence of high concentrations of end products derived from PRPP, and the specific activity of the PRPP synthetase was compared with the activity found in cells grown on a minimal glucose-salts medium. The addition of end products—singly or in combination—had only small effects on the specific activity of PRPP synthetase. Small reductions in specific activity were reproducibly found, especially when purine plus pyrimidine compounds were added. The results indicate that either PRPP synthetase is not repressed by end products, or, perhaps more likely, that the enzyme is very nearly fully repressed when grown on minimal medium. This is the case for the enzymes of the histidine biosynthetic pathway in *S. typhimurium*, which are largely repressed during growth on minimal medium. Derepression of the enzymes of histidine biosynthesis can only be demonstrated by starvation for histidine in mutant strains (2).

Effect of growth on various carbon sources. It is possible that the specific activity of PRPP synthetase could depend on the carbon source used by the bacteria, either because of direct effects or because of differences in growth rate or availability of substrates, e.g., ribose-5-phosphate. Table 3 presents a summary of experiments which test this possibility with a wild-type strain of *S. typhimurium*. It is clear that the carbon source generally has very little effect on the specific activity of PRPP synthetase, although we have consistently observed slightly elevated levels of the enzyme after growth of the bacteria on Casamino Acids and appreciably depressed specific activities in succinate-grown cells. There does not appear to be any relation between growth rate and PRPP synthetase activity.

Effects of starvation for inorganic phosphate and PRPP. PRPP synthetase has been shown to require high concentrations of inorganic phosphate for activity (25). Thus, it was possible that the bacteria would respond to phosphate starvation by increased synthesis of PRPP synthetase. The cells were grown in synthetic media modeled after the high- and low-phosphate media of Engelsberg et al. (10). During the logarithmic phase of growth, the doubling time of the low-phosphate cells was 70 min, compared with 62 min for the cells grown in high phosphate. The cells grown in high-phosphate medium reached the stationary phase of growth at an absorbancy at 650 nm of 1.35, whereas the cells grown in low-phosphate medium ceased growing when the absorbancy was 0.66. The *p*H of both media remained above 6.9 throughout the growth period. The cells were harvested in the stationary phase for PRPP synthetase assays. The specific activity of PRPP synthetase was the same in the cells grown in the two media (0.11 μmole per min per mg).

An attempt was made to determine whether derepression of PRPP synthetase occurs in a mutant in which the PRPP supply is under unusually high demand. The organism chosen was a double regulatory mutant which is constitutive for the histidine biosynthetic operon and in which the first enzyme of histidine biosynthesis is resistant to feedback inhibition. This mutant excretes sufficient histidine to feed a histidine-requiring auxotroph (3). Thus, because of an unusual rate of synthesis of histidine and hence excessive demand for PRPP, one might expect derepression of the enzyme if pool sizes of PRPP can regulate synthesis of PRPP synthetase. The results (Table 4) show that no significant increase in PRPP synthetase was found in the regulatory mutant. Elevated levels of histidinol phosphate phosphatase serve to show that the histidine operon was derepressed in the mutant. The experiment confirms the conclusion, reached with other histidine auxotrophs grown under derepressing conditions for histidine, that PRPP synthetase and the histidine biosynthetic enzymes are not coordinately regulated in *Salmonella*.

Effect of starvation for end products of PRPP metabolism. The possibility remains that the synthesis of PRPP synthetase is controlled by an end product (or products) of PRPP metabolism, but that levels of the product(s) are sufficiently high during growth on minimal medium to bring about repression of enzyme synthesis. To test this possibility, mutant strains of the bacteria were grown under conditions that were growth limiting due to lack of end products of PRPP metabolism. In each case, derepression of a biosynthetic enzyme for the end product in question was demonstrated, a result that demonstrates that starvation was indeed brought about by the culture conditions used and that the starved bacteria were capable of protein synthesis.

S. typhimurium cells that had been starved for histidine were obtained by growing three different histidine-requiring mutants (*hisE11*, *hisA30*, *hisG70*) on minimal medium supplemented with 0.05 mM histidinol and 0.4 mM adenine (2, 3). The specific activity of PRPP synthetase in wild-type cells grown on minimal medium was the same or higher (range: 0.06 to 0.13 μmole per min per mg) than in cells which had been starved for histidine (range: 0.03 to 0.07 μmole per min per mg).

A tryptophan-requiring mutant (*trpBEDC43*) which had been grown on limiting amounts of L-tryptophan was derepressed for a tryptophan biosynthetic enzyme, component I of anthranilate synthetase, but starvation for tryptophan did not result in a significant alteration in the specific activity of PRPP synthetase (Table 5).

Purine-requiring auxotrophs of *S. typhimurium* were starved for adenine or guanine by growth in media containing growth-limiting amounts of these compounds (Table 6). At the end of the 5-hr growth period, the bacteria that were grown on media supplemented with low amounts of adenine or guanine (2 to 10 μg/ml) had apparently exhausted the supply of purine bases, with a consequent depletion of their internal purine nucleotide pools. This conclusion follows from the observation that these cells contained elevated concentrations of inosine-5'-phosphate dehydrogenase, an enzyme required for biosynthesis of guanine nucleotides. Inosine-

TABLE 2. *Effect of additions to the culture medium on the level of PRPP synthetase*

Expt	Addition to medium[a]	Doubling time (min)	Relative specific activity[b]
I	None	57	(1.0)
	L-Histidine	57	1.1
	L-Tryptophan	54	0.9
	Adenine	55	0.9
	Uracil	56	0.8
	Histidine + tryptophan	54	0.9
	Adenine + uracil	54	0.7
	Histidine + tryptophan + adenine + uracil	54	0.6
II	None	57	(1.0)
	Adenine + uracil	54	0.9
	Adenosine	54	0.9
	Uridine	56	0.7
	Adenosine + uridine	55	0.6
	Adenosine + uridine + histidine + tryptophan	56	0.5

[a] Each addition was 200 μg/ml, except histidine, which was 250 μg/ml.

[b] Orotate removal assay. Specific activity was 0.13 μmole per min per mg in experiment I and 0.12 μmole per min per mg in experiment II.

TABLE 3. *Effect of carbon source on specific activity of PRPP synthetase*

Carbon source	Doubling time (min)	PRPP synthetase specific activity[a]
Glucose	80	0.19
Casamino Acids	75	0.22
Glycerol	100	0.18
Acetate	200	0.18
Succinate	175	0.10
None[b]	No growth[b]	0.20

[a] Expressed as micromole per minute per milligram.

[b] Glucose-grown cells starved for 4 hr.

TABLE 4. *Relation of PRPP synthetase to expression of the histidine operon*

Strain	Description	Doubling time[a] (min)	PRPP synthetase[b]	Histidinol phosphate phosphatase[c]
LT-2	Wild type	60	0.13	0.56
hisO1202hisG1109	Histidine-constitutive, feedback-resistant	62	0.15	4.35

[a] The bacteria were grown in minimal medium at 37 C and harvested in the late log phase of growth.

[b] Orotate removal assay. Results are expressed as micromole per minute per milligram.

[c] Results are expressed as micromoles per hour per milligram.

5'-phosphate dehydrogenase has been shown to be specifically repressed by some guanine derivative (14). The enzyme can also be derepressed by adenine starvation in mutants with a growth requirement for either guanine or adenine, probably because depletion of adenine nucleotide pools leads to depletion of guanine nucleotide pools (14). Depletion of purine nucleotide pools in this manner did not result in significant alterations in the specific activity of PRPP synthetase in either of two mutant strains that were starved for either adenine or guanine (Table 6).

A similar approach was used to investigate the influence of pyrimidine nucleotide pool size on the synthesis of PRPP synthetase. A pyrimidine-requiring mutant of *S. typhimurium* (*pyr573*) was grown in minimal medium supplemented with various initial concentrations of uracil (Fig. 1). In media containing low concentrations of uracil, the cells ceased growing when the pyrimidine supply was exhausted. The derepression of ATCase in these cells demonstrated depletion of internal pyrimidine nucleotide pools (28). Growth was not limited by pyrimidine supply when the media contained higher concentrations of uracil; in such cultures, ATCase is fully repressed. The growth conditions that led to derepression of pyrimidine biosynthetic enzymes also elicited increased specific activity of PRPP synthetase, an increase that was not found when higher concentrations of uracil were added to the culture medium (Fig. 1). The parallel changes in ATCase and PRPP synthetase specific activities suggests that both activities are regulated by internal pools of pyrimidine nucleotides. Figure 2 summarizes several experiments in which the two enzymes were measured after growth of the bacteria on media containing various levels of uracil. Conditions that lead to the derepression of ATCase result in higher levels of PRPP synthetase, but the synthesis of the two enzymes does not appear to be coordinately regulated. It will be noted that the relative increase in ATCase

TABLE 5. *Relation of PRPP synthetase to tryptophan starvation*

Culture medium[a]	Specific activity[b]	
	Anthranilate synthetase, component I	PRPP synthetase
Minimal + 1 μg of L-tryptophan/ml	2.5	0.085
Minimal + 50 μg of L-tryptophan/ml	<0.1	0.075

[a] *Salmonella typhimurium trpBEDC43* was used in these experiments.

[b] Results are expressed as micromoles per minute per milligram of protein.

TABLE 6. *Relation of PRPP synthetase to purine starvation*

Strain grown	Addition to minimal medium (μg/ml)	Specific activity	
		Inosine-5'-phosphate dehydrogenase[a]	PRPP synthetase[b]
purC452	None	9.6	0.11
	Adenine, 2	24.3	0.082
	Adenine, 5	29.3	0.070
	Adenine, 10	35.4	0.071
	Adenine, 40	<0.2	0.062
	Adenine, 50	<0.2	0.086
E 1-2 (Pur⁻, Pyr⁻)[c]	Guanine, 2	17.4	0.13
	Guanine, 40	3.9	0.15
	Adenine, 2	18.2	0.13
	Adenine, 40	2.3	0.11

[a] Results are expressed as nanomoles of product per minute per milligram of protein.

[b] Results are expressed as micromoles of product per minute per milligram of protein.

[c] Uracil (100 μg/ml) was also added to the minimal medium in experiments with this strain.

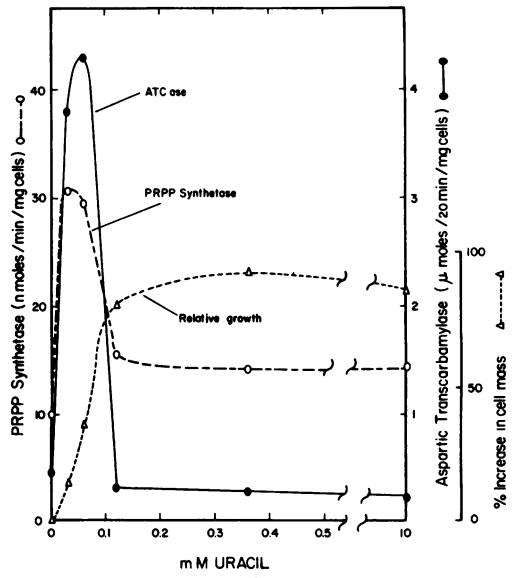

FIG. 1. *Derepression of aspartic transcarbamylase and PRPP synthetase. Salmonella typhimurium pyr573 was grown on minimal medium plus 1 mM uracil, the cells were then washed and grown for 4 hr in minimal media containing the indicated initial concentrations of uracil. The toluenized cell assays for aspartic transcarbamylase (ATCase) and phosphoribosylpyrophosphate (PRPP) synthetase were used in these experiments.*

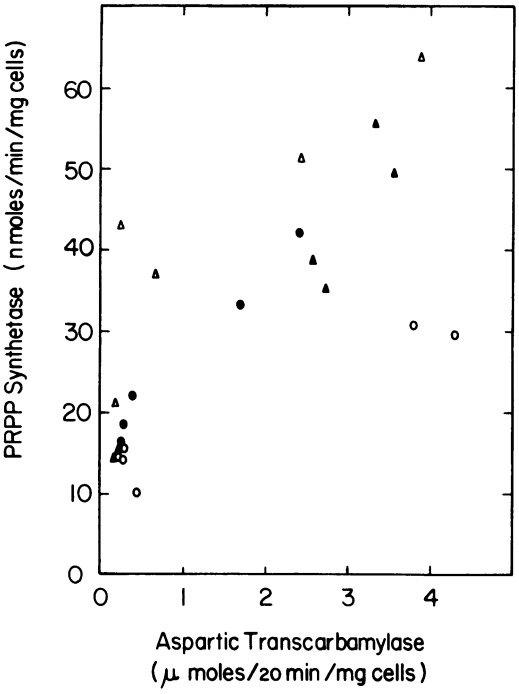

FIG. 2, *Relation between aspartic transcarbamylase and PRPP synthetase during derepression. Each type of symbol* (O, ●, Δ, ▲) *refers to an experiment similar to that described in Fig. 1, where each different symbol corresponds to a different initial concentration of uracil in that experiment. Toluenized cell assays were used.*

(10- to 20-fold) is much greater than for PRPP synthetase (2- to 6-fold). The specific activity of PRPP synthetase in cells grown on media containing excess of uracil is approximately the same as that of wild-type cells grown on minimal medium. Growth of the mutant on minimal medium supplemented with 5 μg of cytosine per ml, instead of uracil, results in similar increases in specific activity of PRPP synthetase and ATCase. Hence the effect appears to be general for pyrimidine starvation, rather than specific for uracil deprivation.

Recently we found a method for starving *S. typhimurium* cells for pyrimidines while the bacteria are growing exponentially rather than ceasing to grow because of depletion of a pyrimidine base in the medium. This was accomplished by culturing a pyrimidine auxotroph in a medium containing orotic acid as the pyrimidine source (9). Orotic acid is taken up from the medium much more slowly than uracil. As a consequence, a pyrimidine auxotroph grows at a rate that is limited by the rate of entry of orotic acid into the cells. The strain used in these studies was strain *pyrA81*, a deletion mutant lacking carbamyl phosphate synthetase (20). A deletion mutant was chosen to avoid reversion of the strain to prototrophy under conditions of pyrimidine starvation. The mutant strain grew only on media containing high concentrations of orotic acid (greater than 100 μg/ml) and at a rate that was dependent upon the concentration of orotic acid in the medium (Table 7). Growth in medium containing 50 μg of uracil per ml was rapid; cells grown in this manner contained repressed concentrations of ATCase and PRPP synthetase. On the other hand, pyrimidine limitation by growth at the expense of orotic acid resulted in a sharp decrease in intracellular pyrimidine nucleotide pools (9). This effect is almost certainly due to the slow rate of entry of orotic acid into the cells, because the enzymes needed for conversion of intracellular orotic acid to uridine monophosphate are derepressed under these culture conditions (9). Pyrimidine-limited cultures exhibited a very large derepression of ATCase and up to 10-fold derepression of PRPP synthetase (Table 7). These experiments strengthen the conclusion that pyrimidine nucleotide pools exert a regulatory control over synthesis of PRPP synthetase. This method of pyrimidine limitation has also provided a convenient means for reproducibly obtaining cells that are maximally derepressed for PRPP synthetase.

The increase in specific activity of PRPP synthetase elicited by pyrimidine starvation is abolished by inclusion of chloramphenicol in the culture medium (Table 8). In this experiment the pyrimidine auxotroph *pyrA81* was grown on excess uracil and then transferred to media containing limiting uracil. Since the cells must grow

TABLE 7. *PRPP synthetase levels in cells limited for pyrimidines by growth on orotic acid*

Addition to minimal medium[a] (μg/ml)	Doubling time (hr)	Specific activity	
		PRPP synthetase[b]	ATCase[c]
Uracil, 50	0.85	0.061	0.18
Orotic acid, 200	12.6	0.52	24.1
Orotic acid, 500	4.4	0.59	5.5

[a] Strain *pyrA81* was used in these experiments. The inoculum was grown on medium supplemented with 50 μg of uracil/ml. The cells were harvested, washed three times, and suspended in the media described above. All media also contained 50 μg of arginine/ml. The cells were harvested while in the exponential phase of growth.

[b] Results are expressed as micromole per minute per milligram.

[c] Results are expressed as micromoles per 20 min per milligram.

TABLE 8. *Effect of chloramphenicol on increase in PRPP synthetase resulting from uracil starvation*

Uracil in growth medium (μg/ml)	Chloramphenicol in growth medium (μg/ml)	Time of adding chloramphenicol (hr after culture inoculated)	Relative increase in cell mass[a]	Specific activity	
				ATCase[b]	PRPP synthetase[c]
5	0	—	3.14	11.3	0.17
50	0	—	5.49	0.37	0.09
5	50	1.5	2.48	0.85	0.11
5	50	1.8	3.37	6.1	0.13
5	50	2.1	3.14	9.7	0.19

[a] Strain *pyrA81* was grown on minimal medium supplemented with 0.5% glucose and 50 μg of arginine and 50 μg of uracil per ml; the cultures were harvested, washed, and suspended in minimal plus 0.5% glucose medium containing the indicated amounts of uracil and arginine (50 μg/ml). Chloramphenicol was added at the times indicated. All cultures were harvested and assayed after 2.5 hr of growth at 37 C.

[b] Results are expressed as micromoles per 20 min per milligram.

[c] Results are expressed as micromole per minute per milligram.

and exhaust the uracil from the medium in order to be derepressed, chloramphenicol was added at various times just before or early in uracil starvation. Uracil limitation had already begun in such cultures, as is indicated by partial derepression of aspartic transcarbamylase. Addition of chloramphenicol at these times largely prevented derepression of PRPP synthetase. On the other hand, addition of chloramphenicol at approximately 30 min after onset of uracil starvation did not block derepression of either PRPP synthetase or ATCase. Control experiments indicated that the concentration of chloramphenicol used (50 μg/ml) did not decrease the viable count of the culture when the antibiotic was diluted out. The results indicate that protein synthesis is required for the increase in specific activity of PRPP synthetase and strongly suggest that uracil starvation brings about derepression of enzyme synthesis.

Are there isozymes of PRPP synthetase? A simple interpretation of the results of the pyrimidine starvation experiments is that there are two isozymes, one which is subject to repressive control by pyrimidines and the other which occurs at constant levels under all growth conditions examined. To test this hypothesis, sonic extracts of strain *pyr573* cells grown under conditions of uracil starvation (specific activity of PRPP synthetase: 0.19 μmole per min per mg) were compared to extracts of the same strain grown on an excess of uracil (specific activity: 0.08 μmole per min per mg). No significant differences in the PRPP synthetase activities of the two extracts could be demonstrated by the following criteria: sensitivity to inhibition by adenosine diphosphate, uridine diphosphate, or Ca^{2+} ions; *p*H activity profile; or rate of inactivation at 60 C. In another series of experiments, extracts of strain *pyrA81* grown on 50 μg of uracil per ml (specific activity: 0.06 μmole per min per mg) were compared with extracts of this strain grown on media supplemented with 200 μg of orotic acid per ml (specific activity: 0.31 μmole per min per mg) by electrophoresis on the Millipore Phoroslide (cellulose acetate) system. Activity of the enzyme was detected with a staining reaction to be described in detail elsewhere (S. Rosenzweig and R. L. Switzer, *unpublished data*). The activity stain is based on formation of ATP from adenosine monophosphate and PRPP in the reverse of the PRPP synthetase reaction. ATP formation is coupled to formation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) via the hexokinase and glucose-6-phosphate dehydrogenase reactions. NADPH is detected by reduction of nitrobluetetrazolium. Electrophoresis of the extracts in buffers with *p*H values from 6.0 to 9.7 revealed a single band of PRPP synthetase activity in all cases. The patterns were identical with repressed and derepressed extracts. We conclude that it is unlikely that multiple PRPP synthetase isozymes are present in *S. typhimurium*, but further attempts to test this possibility are in progress.

DISCUSSION

The experiments in this paper lead to the remarkable conclusion that, of all the end products of PRPP metabolism—histidine, tryptophan, purines, pyrimidines—only pyrimidine compounds exert regulatory influence over PRPP synthetase synthesis. The increases in enzyme activity seen during pyrimidine starvation are specifically associated with pyrimidine deprivation rather than a general effect of growth limitation, because starvation for other metabolites does not bring about changes in the enzyme activity. The correspondence between the response of the synthesis of ATCase and of PRPP synthetase to pyrimidine starvation, either by exhaustion of exogenous pyrimidine bases or by slow

growth on orotic acid, strongly indicates that both enzymes are regulated by intracellular levels of a pyrimidine metabolite, probably a pyrimidine nucleotide. It is clear, however, that AT-Case and PRPP synthetase are not regulated coordinately. The maximal increase in PRPP synthetase specific activity which has been observed (10-fold) is somewhat smaller than the 10- to 25-fold derepression usually seen with unifunctional biosynthetic pathways. However, the comparison is misleading because the unifunctional enzymes are repressed to very low levels, whereas substantial levels of PRPP synthetase are found when the bacteria are cultured with excess pyrimidines. It is likely that the alterations in specific activity of PRPP synthetase result from repression and derepression of enzyme synthesis, since the effect is blocked by chloramphenicol, a specific inhibitor of protein synthesis. Alternative possibilities, such as a protein synthesis-dependent activation of previously present enzyme or decreased rate of PRPP synthetase degradation, seem less likely but have not been excluded.

Repression of PRPP synthetase by its product PRPP would yield a simple and effective control mechanism. Hence, the possibility that the observed alterations in enzyme activity actually result from fluctuations in PRPP pool sizes must be carefully examined. Several lines of evidence militate against the hypothesis that PRPP serves as a co-repressor for PRPP synthetase. Excessive production of histidine by the regulatory mutant (Table 4) would be expected to result in a decreased intracellular concentration of PRPP, and hence derepression of PRPP synthetase. This was not observed. The experiment is not conclusive, however, since the growth rate of the mutant suggests that PRPP pools were not substantially lowered. If PRPP acts as a co-repressor, the results require that the effect of starving auxotrophic strains for pyrimidine bases is to lower PRPP pools. If this were the case, one would expect starvation for purine bases to have the same effects on PRPP pools as pyrimidine bases, yet starvation for purines does not derepress PRPP synthetase. In any event, it is very unlikely that bacteria that cease growth because of exhaustion of pyrimidine bases in the media and contain depleted pools of pyrimidine nucleotides will contain lowered pools of PRPP. The pools of PRPP would be expected to increase under these conditions, because PRPP cannot be used for either reaction with bases or de novo synthesis of pyrimidine nucleotides. Finally, pyrimidine deprivation by growth on orotic acid, which enters the cell very slowly, should not lower PRPP pools, but rather bring about accumulation of PRPP. The hypothesis that PRPP acts as a co-repressor can be accommodated only if pyrimidine deprivation brings about an accumulation of purine nucleotides, which in turn act to lower PRPP pools through feedback inhibition of PRPP synthetase. Because de novo purine biosynthesis is itself under strict feedback control, we consider this possibility to be unlikely. Thus, while it must be admitted that it is very difficult to confidently predict the effects of various culture conditions on the intracellular pool sizes of PRPP, there is no evidence that PRPP acts as the co-repressor for PRPP synthetase. The question cannot be definitively settled until direct measurements of PRPP pools under various growth conditions are made.

It is important to recognize that pyrimidines bring about only a partial repression of PRPP synthetase. In fact, fully repressed levels of the enzyme are similar to those found in wild-type organisms grown on a minimal salts-glucose medium. Thus, growth on an excess of pyrimidine bases does not prevent formation of PRPP needed for synthesis of other end products or for conversion of the pyrimidine bases to the nucleotide form. Indeed, it is difficult to imagine a set of growth conditions where PRPP synthetase activity is not required, since the product is required whether purine and pyrimidine bases are present in the growth medium or synthesized de novo. For this reason, we tend to view the observed regulation of PRPP synthetase as a form of "reserve synthetic capacity" which is called upon in periods of rapid nucleic acid synthesis when nucleotide pools are depleted.

If the total composition of the bacterial cells may be taken as an indication of the relative amount of PRPP used for various end products, it is not surprising that nucleotides serve as the major regulatory metabolites rather than amino acids. If one assumes that the gross composition of *S. typhimurium* cells is the same as *Escherichia coli* cells, the proteins of the organism make up about 50% of the dry weight (17). Of this protein, histidine and tryptophan make up roughly 1% each (17), so that approximately 1% of the cell mass is made up by these products. On the other hand, nucleic acids make up as much as 20 to 24% of the cell mass in rapidly growing cells (17). Thus, approximately 20 times as much PRPP is utilized for nucleotide synthesis as for amino acid synthesis. However, from 50 to 55% of the nucleic acid of the organism is made up of purine nucleotides (19), and free pools of purine nucleotides are probably substantially higher than pyrimidine nucleotide pools (13). Hence it was unexpected that only pyrimidines would serve as repressing metabolites for PRPP synthetase. It might be expected that purines alone, or purines and pyrimidines

together, would be more appropriate repressing metabolites. Such "multivalent repression" (11) by purines and pyrimidines is excluded by the results. The view that the capacity of pyrimidine starvation to derepress PRPP synthetase reflects "reserve capacity" for support of nucleic acid synthesis is tenable only if purine and pyrimidine nucleotide pool sizes fluctuate in parallel during variations in nucleic acid synthesis. It is, of course, possible to vary these pools independently by starving appropriate auxotrophic strains, but in wild-type organisms such independent fluctuations in the nucleotide pools may be rare, if they occur at all. It has been demonstrated that purine and pyrimidine pools do decrease together during the "stringent" response of bacteria to amino acid starvation (13), but further testing of the suggestion is needed. If pyrimidine nucleotide pools are a good general indicator of the supply of precursors for nucleic acid synthesis, certain advantages may accrue from controlling PRPP synthetase levels by pyrimidines alone. Namely, involvement of purine nucleotides in energy metabolism and protein synthesis presents additional causes for fluctuations in these pools to which pyrimidine nucleotide pools may be less sensitive.

An alternative view is that, for PRPP synthetase, repressive control and feedback inhibition act in a complementary rather than cumulative fashion. According to this view, PRPP synthetase is regulated by pyrimidine nucleotides at the level of enzyme synthesis and by purine nucleotide by feedback inhibition. The enzyme is much more sensitive to inhibition by purine nucleotides than pyrimidine nucleotides (R. L. Switzer and D. C. Sogin, *unpublished data*).

The results underscore the highly individualized character of regulatory mechanisms of branched biosynthetic pathways. It would be of great interest to learn whether alternate regulatory patterns for PRPP synthetase are found in other organisms.

ACKNOWLEDGMENTS

The initial phases of this investigation were done when R. L. Switzer was a postdoctoral fellow of the Public Health Service in the laboratory of Earl R. Stadtman at the National Heart Institute, Bethesda, Md. Dr. Stadtman is gratefully acknowledged for his support and valuable suggestions.

We are grateful to B. N. Ames for his useful suggestions and for supplying mutant strains used for this work. Richard Haas is acknowledged for isolating and characterizing strain E 1-2.

This work was supported by Public Health Service grant AM 13488 from the National Institute of Arthritis and Metabolic Diseases. Joyce Olszowy was a predoctoral trainee of the National Institutes of Health (grant 2G 321).

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