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Pyrimidine Biosynthetic Enzymes of *Salmonella typhimurium*, Repressed Specifically by Growth in the Presence of Cytidine

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The repressive effects of exogenous cytidine on growing cells was examined in a specially constructed strain in which the pool sizes of endogenous uridine 5'-diphosphate and uridine 5'-triphosphate cannot be varied by the addition of uracil and/or uridine to the medium. Five enzymes of the pyrimidine biosynthetic pathway and one enzyme of the arginine biosynthetic pathway were assayed from cells grown under a variety of conditions. Cytidine repressed the synthesis of dihydroorotase (encoded by *pyrC*), dihydroorotate dehydrogenase (encoded by *pyrD*), and ornithine transcarbamylase (encoded by *argI*). Moreover, aspartate transcarbamylase (encoded by *pyrB*) became further derepressed upon cytidine addition, whereas no change occurred in the levels of the last two enzymes (encoded by *pyrE* and *pyrF*) of the pyrimidine pathway. Quantitative nucleotide pool determinations have provided evidence that any individual ribo- or deoxyribonucleoside mono-, di-, or triphosphate of cytosine or uracil is not a repressing metabolite for the pyrimidine biosynthetic enzymes. Other nucleotide derivatives or ratios must be considered.

Regulation of the pyrimidine biosynthetic pathway at the levels of gene expression has received limited investigation. However, the regulation of the activity of the first and second enzymes of the pathway has been the topic of several reports (1, 3, 12, 13). The studies presented here focus on the regulation of the synthesis of the pyrimidine biosynthetic enzymes.

The biosynthesis of the pyrimidine nucleotide, uridine-5'-monophosphate (UMP), in *Escherichia coli* and *Salmonella typhimurium* is accomplished by the sequential action of six enzymes (Fig. 1): carbamyl phosphate synthetase (CPSase; EC 2.7.2.5), aspartate transcarbamylase (ATCase; EC 2.1.3.2), dihydroorotase (DHOase; EC 3.5.2.3), dihydroorotate dehydrogenase (DHOdehase; EC 1.3.3.1), orotate phosphoribosyl transferase (OMPppase; EC 2.4.2.10), and orotidylate decarboxylase (OMPdecase; EC 4.1.1.23). These six enzymes in the above two organisms are encoded by six unlinked genes (26, 29). The route of synthesis of UMP is not unique to these two bacteria but rather it appears to be of a universal nature (23).

In 1953, Back and Woods (4), using a *pyrA* mutant of *E. coli* and following the growth of this mutant on uracil or carbamyl aspartate, provided the first evidence that uracil may repress the formation of an enzyme (or enzymes) participating in pyrimidine biosynthesis. Yates and Pardee (33) subsequently showed that uracil starvation resulted in an increased synthesis of all six enzymes of the pathway. Beckwith et al. (8) conducted further studies on the regulation of pyrimidine enzyme synthesis and concluded that the rate of synthesis of CPSase and ATCase was independently regulated but the synthesis of the last four enzymes was coordinately controlled. It was later observed through experiments creating a partial starvation for pyrimidines that the synthesis of DHOdehase and OMPppase was not coordinate (10). Schwartz and Neuhard (27) have presented further evidence for the coordinate synthesis of DHOase and DHOdehase.

In most of the previous studies (8, 10, 31, 33) on the regulation of pyrimidine enzyme synthesis, the approach used was one of limiting the cell for the pyrimidine nucleotide, UMP. However, starvation for UMP results in starvation for all pyrimidine nucleotides (Fig. 2) and these earlier studies offered no insight into the nature of the repressing metabolites of the pyrimidine biosynthetic enzymes. In addition, starvation for pyrimidines may lead to nonspecific derepression (30).

It was not until Neuhard and Ingraham (21) succeeded in the isolation of a cytidine-requiring mutant due to mutations in the structural

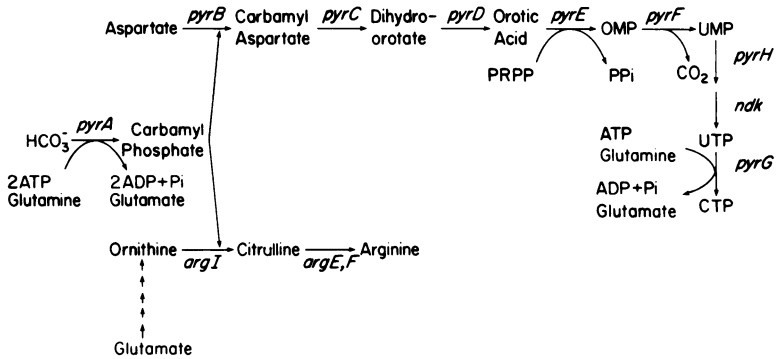

FIG. 1. *Pyrimidine and arginine biosynthetic pathway of Salmonella typhimurium. Genetic symbols for the enzymes are shown in italics. The gene designations are as follows: pyrA, CPSase (EC 2.7.2.5); pyrB, ATCase (EC 2.1.3.2); pyrC, DHOase (EC 3.5.2.3); pyrD, DHOdehase (EC 1.3.3.1); pyrE, OMPppase (EC 2.4.2.10); pyrF, OMPdecase (EC 4.1.1.23); pyrH, UMP kinase (EC 2.7.4.4); ndk, nucleoside-diphosphokinase (EC 2.7.4.6); pyrG, CTP synthetase (EC 6.3.4.2); argI, OTCase (EC 2.1.3.3); argE, argininosuccinate synthetase (EC 6.3.4.5); and argF, argininosuccinase (EC 4.3.2.1).*

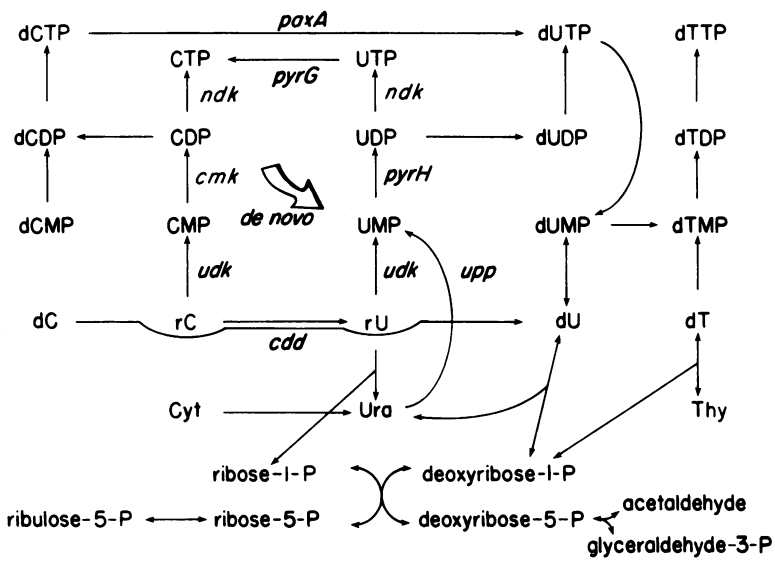

FIG. 2. *Pathways for the utilization of pyrimidine bases and nucleosides and the subsequent interconversions occurring at the nucleotide level. Genetic symbols for the enzymes are shown in italics and the gene designation are as follows: upp, UMP pyrophosphorylase (EC 2.4.2.9); udk, uridine kinase (EC 2.7.1.48); cdd, cytidine (deoxycytidine) deaminase (EC 3.5.4.5); paxA, deoxycytidine triphosphate deaminase (EC 3.5.4. unassigned); pyrG, CTP synthetase (EC 6.3.4.2); ndk, nucleoside-diphosphokinase (EC 2.7.4.6); and cmk, CMP kinase (EC 2.7.4. unassigned).*

genes for cytidine (deoxycytidine) deaminase (*cdd*) and cytidine 5'-triphosphate (CTP) synthetase (*pyrG*) that independent alteration of the cytosine nucleotide pools without direct and simultaneous alteration of the uracil nucleotide pools became possible.

In *E. coli* and *S. typhimurium*, carbamyl phosphate synthesis is catalyzed by a single enzyme (24, 32) encoded by the gene *pyrA*. Carbamyl phosphate is required for the synthesis of arginine as well as UMP (Fig. 1). Using cytidine-requiring mutants it has been shown that the greatest degree of repression of CPSase occurs when both cytidine and arginine are added to the growth medium (1). It was concluded that both a cytosine compound and arginine are involved in regulating the expression of the *pyrA* gene.

To date no true regulatory mutants of the pyrimidine biosynthetic pathway have been isolated in either *E. coli* or *S. typhimurium*. Mutants have been isolated that mimic a constitutive phenotype for all six pyrimidine enzymes (22, 27). These mutants are not deficient in any regulatory function but rather they contain leaky mutations in UMP kinase (encoded by *pyrH*; see Fig. 2) and the cell is limited in its ability to synthesize uridine 5'-diphosphate (UDP) and consequently all other pyrimidine nucleotides. We chose to study the patterns of pyrimidine enzyme repression in exponentially growing cultures of a specially constructed *pyrH* mutant (UMP kinase leaky; it is neither an auxotroph nor a conditional lethal) and its "isogenic" derivative, thereby circumventing the problem of nonspecific derepression that may occur during pyrimidine starvation experiments. In this report we present the results of these experiments and our attempts at the identification of the repressing metabolites for the pyrimidine biosynthetic enzymes of *S. typhimurium*. Also included in this presentation are experiments on the regulatory interrelationships of the arginine and pyrimidine pathways.

Very recently a similar study using *pyrH* mutants of *S. typhimurium* was carried out by Schwartz and Neuhard (27) and a discussion of their results and interpretations in comparison to ours is presented.

(This paper is partial fulfillment of the requirements for a doctorate degree for J. J. Kinahan.)

MATERIALS AND METHODS

Organisms. All bacterial strains used in this study are derivatives of *S. typhimurium* LT2. Table 1 lists the strains used and their genotypes. The method of isolation of strain HD1100 (formerly HD11) has been reported (22). Strain KD1104 was isolated as a 5-fluorodeoxycytidine-resistant, 5-fluorodeoxyuridine-sensitive derivative of HD1100; strain KD1104 will henceforth be referred to as UMP kinase leaky. The loss of cytidine (deoxycytidine) deaminase activity was confirmed by the enzyme assay. The *pyrH* gene (encoding UMP kinase) has been located on the chromosome of *S. typhimurium* and represented on the *Salmonella* map at approximately 8 min (15). The gene is 30% co-transducible with *pan* using phage P1*kc* but no co-transduction between these loci was observed with phage P22 (15). It was necessary, as a control, to obtain an isogenic strain differing only with respect to the *pyrH* allele. Rather than carrying out the involved procedure of isolating a mutant strain and making this mutant susceptible to P1*kc* infection (Gal$^-$), it was considered more feasible to introduce the *pyrH*$^+$ allele by conjugation. The genes for proline biosynthesis are represented on the map near 10 min, *proA* and *proB* being located closer to *pyrH* than *proC*. Thus, as a recipient strain for transfer of the *pyrH*$^+$ allele, a *proA* (or *proB*) auxotroph, strain KD1107, was isolated from an *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NG; 2) mutagenized culture of KD1104 followed by penicillin counter-selection (28). Strain KD1107 could be cross-fed by a known *proC* mutant, indicating the mutation as *proA* or *proB* (16). Strain KD1107 was mated with strain JL84 (refer to Table 1) and proline prototrophic recombinants were selected. One of these recombinants, KD1109, was screened for the introduction of the *pyrH*$^+$ allele by enzyme assay (Table 2) and for increased sensitivity to 5-fluorouracil (15). Strains KD1104 and KD1109 are considered "isogenic" for the purposes of these experiments with the exception of the *pyrH* allele.

Media. The synthetic medium was Medium A (9) with added $FeCl_3 \cdot 6H_2O$ (0.8 μg/ml) and $ZnSO_4 \cdot 6H_2O$ (5 μg/ml) and 0.2% glucose as carbon source; this glucose minimal medium will be referred to as AG medium. Amino acid and pyrimidine supplements, when used, were added at a final concentration of 50 μg/ml. For the quantitation of the nucleotide pools, medium TF (20) was used. Solid media were prepared by the addition of agar to 1.5%.

Growth conditions. The incubation temperature for the growth of cells was 37 C. Liquid cultures were grown in an Environ-Shaker model 3597 (Lab-Line Instruments, Inc., Melrose Park, Ill.) at a shaker speed of 200 rpm. Growth was followed by measuring the increase in absorbancy at 450 nm.

Preparation of cell extracts. Cultures were grown to a cell density of approximately 5×10^8/ml, harvested by centrifugation at 10,000 × *g* for 5 min. The cell pellet was resuspended to one-half of the original volume in ice-cold 40 mM potassium phosphate buffer, pH 7.0, and recentrifuged. The cell pellet was then resuspended in sufficient ice-cold 40 mM potassium phosphate-1 mM dithiothreitol buffer, pH 7.0, to give about 10^{10} cells/ml. The cells

TABLE 1. *Bacterial strains*

Strain	Sex	Genotype	Source[a]
LT2	F$^-$	Wild type	P. E. Hartman
JL84	HfrA	*metB406, hisD23, gal-50*	J. L. Ingraham
HD1100	F$^-$	*pyrH11*	Ref. 6
KD1104	F$^-$	*pyrH11, cdd-4*	NG, FdC^r
KD1107	F$^-$	*pyrH11, cdd-4, pro-13*	NG
KD1109	F$^-$	*cdd-4*	Conjugation

[a] Abbreviations: NG, mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; FdC^r, selected for resistance to 5-fluorodeoxycytidine.

TABLE 2. *Specific activity of the enzyme UMP kinase in strains KD1104 and KD1109 grown in minimal medium*

Strain (genotype)	Sp act
KD1104 (*pyrH11, cdd-4*)	3.1
KD1109 (*cdd-4*)	15

were disrupted by treatment with a Heat Systems model SW185 sonic oscillator (Plainview, N.Y.) at a setting of 40 for a total of 50 s (5 × 10-s bursts with a 30-s cooling period between each burst). The extract was centrifuged at 10,000 × *g* for 5 min at 4 C and 0.5 ml of the supernatant was withdrawn for the assay of dihydroorotate dehydrogenase. The remaining extract was centrifuged at 27,000 × *g* for 30 min at 4 C, and the supernatant was removed and, in general, used for enzyme assays without further treatment.

Enzyme assays. All assays were carried out at 30 C. Changes in absorbancy were measured with a Beckman model 25 recording spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). Specific activities of the enzymes assayed were determined under conditions in which product formation (or substrate utilization) was proportional to extract and time and are expressed as nanomoles per minute per milligram of protein. Protein was determined by the method of Lowry et al. (18) using bovine serum albumin as standard. (i) The enzyme reaction mixture with aspartate transcarbamylase was as described previously (13). (ii) The dihydroorotase assay mixtures contained the following in 1 ml: 100 μmol of tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, pH 8.6; 1 μmol of L-dihydroorotate; 1 μmol of ethylenediaminetetraacetate; extract and water. (iii) The ornithine transcarbamylase reaction mixture contained the following in 1 ml: 100 μmol of Tris-hydrochloride buffer, pH 8.6; 2.5 μmol of dilithium carbamyl phosphate; 10 μmol of L-ornithine; water and extract. In the above three enzyme assays, the formation of ureido groups was measured by the method of Prescott and Jones (25). (iv) Dihydroorotate dehydrogenase, the spectrophotometric assay described by Beckwith et al. (8), was used but with a slight modification. The reaction mixture contained the following in 1 ml: 100 μmol of Tris-hydrochloride buffer, pH 8.6; 1 μmol of L-dihydroorotate; extract and water. The increase in absorbancy at 290 nm was followed using a molar extinction of 6.0×10^3. (v) Orotidine-5'-monophosphate (OMP) pyrophosphorylase and OMP decarboxylase were assayed as previously described (31) but with minor modification. The $MgCl_2$ was omitted from the OMP decarboxylase assay and a difference in molar extinction of 1.65×10^3 was used, whereas 3.95×10^3 was used for the OMP pyrophosphorylase assay, both values as used by Fyfe et al. (11). (vi) Histidinol dehydrogenase (L-histidinol:NAD^+ oxidoreductase; EC 1.1.1.23) was assayed as described by Loper and Adams (17). This enzyme served as the internal control enzyme to ensure that the effects observed under pyrimidine-supplemented conditions were specific for pyrimidine or arginine biosynthetic enzymes. (vii) The uridine-5'-monophosphate (UMP) kinase assay was carried out as previously described (10) without modification.

Quantitation of endogenous nucleotide pools. For nucleoside triphosphate measurements the acid-soluble pools were extracted as described by Bagnara and Finch (5). For the determination of other nucleotides, formic acid was used for extraction rather than acetic acid (19). The separation, identification, and quantitation of the various nucleotides was carried out according to previously published procedures (19).

Chemicals. Bases, nucleosides, nucleotides, and sugar nucleoside diphosphates were purchased from Sigma Chemical Co. Various other chemicals were reagent grade.

Radiochemicals. All radiochemicals were obtained from New England Nuclear Corp.

RESULTS

Growth conditions. The generation times in minimal media of strains KD1104 (the *pyrH* leaky strain) and KD1109 (the *pyrH*$^+$ strain containing the wild-type allele) were 59 and 60 min, respectively. However, a 2-h lag in reaching logarithmic growth was consistently observed when the *pyrH* leaky mutant was grown in the presence of cytidine (50 μg/ml). This did not occur with the *pyrH*$^+$ strain, nor with either when grown in minimal or minimal plus uracil.

Enzyme levels of the pyrimidine and arginine biosynthetic pathways of strains KD1104 and KD1109 grown under various conditions. Table 3 summarizes the specific activities obtained for five enzymes of the pyrimidine pathway and one enzyme of the arginine pathway from the above two strains grown under four different conditions. The following are key points we wish to make regarding Table 3. (i) Strain KD1104 grown on minimal medium showed derepression of all five pyrimidine enzymes. (ii) When uracil was added to the growth medium of strain KD1104 no change in the enzyme levels resulted. (iii) Strain KD1109 showed repression of the pyrimidine enzymes upon the addition of uracil alone, a finding consistent with previously reported results. (iv) Strain KD1109 when grown in uracil had only two-sevenths of the level of ATCase as when grown in minimal medium; the other pyrimidine enzymes did not show quite as large an effect. (v) Strain KD1104 (grown with and without uracil) showed a dramatic increase in the level of only one enzyme, that being a 70-fold derepression of ATCase relative to the ATCase level of strain KD1109 grown with uracil. (vi) The level of ornithine transcarbamylase (OTCase) observed with these strains was greater than that seen for wild-type LT2 when grown in the absence of arginine. Yet the OTCase level observed when the two strains were grown in the presence of arginine was comparable to that seen for wild-type LT2 (Table 4).

Again referring to Table 3, upon the addition of cytidine to strain KD1104, a change in the level of four of the enzymes assayed was observed. The level of ATCase increased 2.8-fold

TABLE 3. *Specific activities of five enzymes of the pyrimidine biosynthetic pathway and one enzyme of the arginine biosynthetic pathway of S. typhimurium strains KD1104 and KD1109 grown in minimal medium, and in the presence of uracil (50 μg/ml) and/or cytidine (50 μg/ml)*

Strain (genotype)	Additions to AG medium	Enzyme sp act[a]					
		ATCase	DHOase	DHO-dehase	OMP-ppase	OMP-decase	OTCase
KD1104 (*pyrH11, cdd-4*)	None	225	328	24.6	125	38.6	209
	Uracil	228	370	27.5	132	42.2	200
	Cytidine	649	51.7	9.6	120	45.0	165
	Uracil and cytidine	321	64.3	6.8	139	41.4	173
KD1109 (*cdd-4*)	None	11.2	180	12.0	34.3	20.9	185
	Uracil	3.2	100	7.6	13.6	13.0	187
	Cytidine	9.8	49.3	6.9	27.7	19.5	154
	Uracil and cytidine	3.0	55.6	5.6	15.5	13.6	142

[a] The level of the control enzyme, histidinol dehydrogenase, remained constant for all growth conditions.

TABLE 4. *Levels of ornithine transcarbamylase in strains LT2, KD1104, and KD1109 grown in the presence and absence of arginine*[a]

Strain (genotype)	Sp act	
	−Arg	+Arg
LT2 (wild type)	62.5	4.8
KD1104 (*pyrH11, cdd-4*)	209	5.1
KD1109 (*cdd-4*)	185	4.9

[a] Arginine was added at a final concentration of 50 μg/ml.

above the plus and minus uracil levels. The level of DHOase was one-sixth the minimal medium level and that of DHOdehase was one-third; as can be noted, a significant change in the level of OTCase occurred also. The levels of OMPppase and OMPdecase, the last two enzymes, however, were unchanged. Strain KD1109 showed a general repression of all the enzymes but the amount of repression was not as pronounced for three of them (ATCase, OMPppase, OMPdecase) as that seen under the plus uracil condition. The repression of DHOase and DHOdehase was greater than that observed when uracil was added to the medium. Upon the addition of cytidine, OTCase was partially repressed in both strains, an occurrence that was not observed with added uracil.

When both uracil and cytidine were added to a culture of strain KD1104, the 2.8-fold increase in the level of ATCase observed with cytidine addition was markedly reduced, but this level was still greater than that observed in the conditions with and without uracil. DHOase and DHOdehase were repressed, whereas no change in the levels of OMPppase and OMPdecase occurred. Repression of OTCase was still observed. With the KD1109 strain repression of all six enzymes occurred.

Nucleotide pool quantitation studies. Dramatic changes in enzyme levels were observed when comparing the "isogenic" strains KD1104 and KD1109 under a variety of growth conditions. These changes should be reflected in the endogenous pool(s) of the repressing metabolite(s). Repression and/or derepression occurred as a result of the addition of pyrimidines to the growth medium and, thus, experiments were conducted to quantitate the endogenous pool sizes of the various pyrimidine nucleotides. In addition, we measured the pool sizes of various purine nucleotides and certain sugar nucleoside diphosphates.

Table 5 summarizes the results of the nucleotide pool determinations. When the triphosphate pools of strain KD1104 are compared to strain KD1109 under the different growth conditions, we observed that in the nonsupplemented medium the size of the uridine 5′-triphosphate (UTP) pool in strain KD1104 (harboring the *pyrH* leaky mutation) was smaller than that of strain KD1109 but the cytidine 5′-triphosphate (CTP) pool was larger. This is consistent with a previous observation made with *pyrH* cold-sensitive mutants (15). In this previous study it was also observed that the guanosine 5′-triphosphate (GTP) pool size was greater than the adenosine 5′-triphosphate (ATP) pool size, whereas the opposite is true for the wild type. As can be seen from our results, the ATP pool was greater than the GTP pool (analogous to wild type) with our *pyrH* leaky mutant.

When uracil was added, the UTP and CTP

pool of strain KD1104 showed little or no change, whereas with strain KD1109 an expansion of both pools was observed. Upon cytidine addition a 2.5-fold increase in the CTP pool size of strain KD1104 was found, concomitant with a decrease to 50% in the UTP pool size.

With both cytidine and uracil present in the medium, an increase in the size of the CTP pool was again observed with strain KD1104. The UTP pool in this case increased in size as compared to that observed upon cytidine addition alone, but did not increase to that observed under plus and minus uracil conditions. With strain KD1109 the addition of both pyrimidines resulted in an increase in the pool size of both UTP and CTP when compared to the pool sizes of the AG-grown culture (minimal medium).

In general, pyrimidine deoxyribonucleoside triphosphate pools did not show any significant pool size changes, although a reduction in the purine deoxyribonucleoside triphosphate pools was observed in the cytidine-supplemented conditions.

Mono- and disphosphate pools. An examination of the diphosphate pool sizes (minimal grown cultures only) presented no dramatic differences in any pool when comparing the two strains (Table 6). The greatest difference observed was in the uridine 5'-diphosphate (UDP) pool; strain KD1104 had a UDP pool approximately one-half that of strain KD1109. We quantitated the monophosphate pool sizes of cells grown with and without uracil (Table 7). In the absence of uracil strain KD1104 showed lower pool sizes of deoxyribonucleoside monophosphates and a greater pool of UMP when compared to strain KD1109. In the presence of uracil, the UMP pool of strain KD1104 can be expanded over that of minimal-grown cells but this expansion was not reflected in the UTP pool. Strain KD1109 showed an expansion of the UMP pool in the presence of uracil and this was reflected in the UTP pool.

Sugar nucleoside diphosphate pools. Measurements of the pools of uridine-5'-diphosphoglucose, uridine-5'-diphosphogalactose, and cytidine-5'-diphosphoglucose, also showed no significant differences between the two strains (Table 8).

DISCUSSION

These studies were carried out for three reasons. Firstly, previous work on the regulation of pyrimidine enzyme synthesis had centered on starvation experiments, with the exception of one recent study (27). The possibility existed

TABLE 5. *Nucleoside triphosphate pools[a] of strains KD1104 and KD1109 under different growth conditions[b]*

Strain (genotype)	Additions to AG medium	Nucleoside triphosphate							
		GTP	ATP	CTP	UTP	dGTP	dATP	dCTP	dTTP
KD1104 (*pyrH11, cdd-4*)	None	6.00	11.2	2.48	2.52	0.44	0.64	0.52	0.9
	Uracil	6.08	11.2	2.7	2.75	0.46	0.67	0.62	1.0
	Cytidine	3.27	7.1	6.2	1.28	0.31	0.35	0.55	1.3
	Uracil and cytidine	4.29	9.5	7.43	1.8	0.36	0.38	0.72	1.6
KD1109 (*cdd-4*)	None	3.84	10.3	2.21	3.42	0.36	0.48	0.54	1.0
	Uracil	3.0	8.75	2.68	4.48	0.35	0.41	0.54	1.0
	Cytidine	3.5	9.2	4.1	3.54	0.39	0.40	0.58	1.2
	Uracil and cytidine	3.1	8.31	3.34	4.25	0.37	0.39	0.53	1.1

[a] Nucleotide pools are presented as micromoles of nucleotide per gram of bacterial dry weight.
[b] Uracil and cytidine were each added at 50 μg/ml.

TABLE 6. *Nucleoside diphosphate pools[a] of strains KD1104 and KD1109 under different growth conditions*

Strain	Additions to AG medium	Nucleoside diphosphate						
		GDP	ADP	CDP	UDP	dGDP	dCDP	dTDP and dUDP
KD1104	None	0.75	2.0	0.45	0.3	0.08	0.13	0.36
KD1109	None	0.59	2.7	0.63	0.66	0.08	0.14	0.45

[a] Nucleotide pools are measured as micromoles of nucleotide per gram of bacterial dry weight.

TABLE 7. *Nucleoside monophosphate pools[a] of strains KD1104 and KD1109 under different growth conditions*

Strain	Additions to AG medium	Nucleoside monophosphate[b, c]						
		AMP	CMP	UMP	dAMP	dCMP	dUMP	dTMP
KD1104	None	0.89	0.55	5.2	0.21	0.05	0.29	0.27
	Uracil	0.63	0.52	8.37	0.22	0.05	0.27	0.25
KD1109	None	0.94	0.59	4.1	0.21	0.16	0.43	0.31
	Uracil	0.97	0.97	4.8	0.12	0.06	0.53	0.29

[a] Nucleotide pools are presented as micromoles of nucleotide per gram of bacterial dry weight.
[b] The nucleotides GMP and dGMP were not adequately separated for determination.
[c] The deoxyribonucleotide, dADP, could not be adequately separated for measurement.

TABLE 8. *Sugar nucleoside diphosphate[a] pool sizes of strains KD1104 and KD1109 grown on minimal medium*

Strain (genotype)	Sugar nucleoside diphosphate[b]		
	UDP-glu	UDP-gal	CDP-glu
KD1104 (*pyrH11, cdd-4*)	7.1	2.38	0.29
KD1109 (*cdd-4*)	8.0	3.34	0.30

[a] Abbreviations used: UDP-glu, uridine-5'-diphosphoglucose; UDP-gal, uridine-5'-diphosphogalactose; CDP-glu, cytidine-5'-diphosphoglucose.
[b] Values are expressed as micromoles of sugar nucleotide diphosphate per gram of bacterial dry weight.

that such methodology might have led to general derepression (30) rather than a specific effect on the pyrimidine pathway and hence an accurate reflection of in vivo regulation. Therefore, we chose to investigate the patterns of repression-depression in actively growing cells. A similar approach was adopted by Schwartz and Neuhard in their studies (27). Secondly, we wanted to determine if any correlation between nucleotide pool sizes and pyrimidine enzyme levels existed. Thus, it was necessary to quantitate the nucleotide pools in cells that were repressed and in cells that were derepressed for pyrimidine enzyme synthesis. Any reproducible correlation between pool size and enzyme level might resolve the nature of the repressing metabolites of pyrimidine enzyme synthesis. Our third objective was to assess the possible effects on the arginine pathway as a result of pyrimidine derepression or additions of pyrimidines to the growth medium.

Previous workers using starvation conditions have intimated that the repressing metabolites for the various pyrimidine enzymes were a uracil nucleotide, a cytosine nucleotide, or an additive of both. These studies can be summarized as follows: *pyrA*, repressed by both a cytosine compound and arginine (1); *pyrB*, repressed by a uracil nucleotide (15, 31), though starvation for cytidine showed some derepression (21); *pyrC*, *pyrD*, *pyrE*, and *pyrF*, primarily repressed by a cytosine compound (31). Schwartz and Neuhard, using *pyrH* mutants, conclude that *pyrB*, *pyrE*, and *pyrF* are controlled by a uridine nucleotide and *pyrC* and *pyrD* by a cytidine nucleotide, but continue to emphasize the di- or triphosphates as the repressing metabolites.

We did not carry out any studies on the regulation of *pyrA* and therefore cannot make any comparisons to previous studies. For the other enzymes of the pyrimidine pathway, comparisons can be made and we are in accord in part with statements of Schwartz and Neuhard. We concur with the starvation experiments regarding cytosine control of DHOase and DHOdehase. However, we found no repression whatever of OMPppase or OMPdecase upon the addition of cytidine and we suggest that the synthesis of these enzymes is under the control of a uracil compound. As we pointed out, with strain KD1104 (the UMP kinase leaky mutant) the size of uracil nucleotide pools cannot be varied (except for UMP) by the addition of uracil and/or uridine, and we cannot eliminate the possibility that both uracil and cytosine compounds are involved. However, with strain KD1109 (the *pyrH*$^+$ wild type) the amount of repression observed with added cytidine appears insignificant relative to that observed when uracil is added. If both uracil and cytosine compounds were possibly involved in causing repression, we feel that the addition of both to the medium would result in a greater amount of repression than would the addition of either alone. Yet when both were added, the amount of repression was comparable to that observed with uracil alone.

In the starvation experiments by previous workers (21), cytidine starvation resulted in a

17-fold increase in the level of ATCase. In our study, the addition of cytidine resulted in a 2.8-fold increase in the level of ATCase and this was coupled with a decrease in the OTCase level. It is well established that CTP is a feedback inhibitor of ATCase in vitro (13) and most likely in vivo as well. The above observations tend to place far more responsibility on feedback inhibition than previously ascribed. As evidence, we offer the decrease in the OTCase levels as an indirect effect of feedback inhibition of ATCase resulting in the increased availability of carbamyl phosphate, even though in the presence of cytidine carbamyl phosphate synthetase would be partially repressed (1). When cytidine is added, DHOase and DHOdehase are also repressed and thus predictably more carbamyl phosphate should be available as a result of the repression of these two subsequent enzymes. Further, possibly an initial temporary rise in the CTP pool with concomitant inhibition of ATCase (and subsequent increase in ATCase synthesis) may account for the initial lag in growth of the leaky strain grown with cytidine, or cytidine inhibition of ATCase itself occurs, but these have not been investigated.

Alternatively, cytidine addition may cause repression of the synthesis of OTCase directly. Indeed, greater repression of OTCase occurs when both cytidine and arginine are added than when arginine is added alone; in agreement with this result is that derepression of OTCase was found to be greatest when cells were starved for both arginine and cytidine (J. C. Williams, Ph.D. thesis, Texas A & M Univ., College Station, Tex., 1973).

As mentioned earlier, the OTCase level of strains KD1104 and KD1109 was elevated on minimal medium when compared to wild-type LT2. This could have increased the sensitivity of the system with regard to the repressive effect of added cytidine. Moreover, an observation such as this could not be demonstrated had "isogenic" strains not been used. Had we used a wild-type LT2 for comparison, one might be tempted to conclude that the elevated OTCase level was a reflection of the derepressed ATCase level and competition for carbamyl phosphate. It can be borne in mind that our parent strain, HD1100 (see Table 1), had been subcultured numerous times before these studies and such subculturing may have resulted in a second mutation, giving rise to the increased OTCase level.

In the presence of cytidine, only the level of ATCase was found to be derepressed in strain KD1104, whereas the OMPppase and OMPdecase levels were unchanged. If all three enzymes are under the regulation of the same repressing metabolite, then it would appear that the *pyrE* and *pyrF* genes were already fully derepressed.

In strain KD1109 some repression was observed on all the enzymes when cytidine was added, even though the strain was deficient in cytidine (deoxycytidine) deaminase activity. However, it has been shown conclusively that in vivo the *cdd* mutation does not result in the complete inability to deaminate cytidine (6). Pyrimidine auxotrophs containing a *cdd* mutation are capable of using cytidine as the sole pyrimidine source unless a second mutation is introduced in the *upp* (UMP pyrophosphorylase) gene (6). Thus, with strain KD1109 growing in the presence of cytidine, one is observing the combined repressive effects of (i) added cytidine and (ii) a partial cytidine breakdown to uracil.

Nucleotide pool quantitation experiments. In comparing the various enzyme levels of strains KD1104 and KD1109, dramatic differences were apparent and we believed these differences would be reflected in a marked change in the pool size of the repressing metabolites. We therefore measured the nucleotide pool sizes of both deoxy- and ribonucleotides for the express purpose of detecting a significant difference in certain endogenous pools. Only then could a correlation with the dramatic differences in enzyme levels be ascribed.

We measured the triphosphate pools under all four growth conditions (see Table 5), but since large differences in enzyme levels were apparent on minimal medium we decided to quantitate the diphosphate pools of minimal-grown cells only. For the monophosphate pools, we measured the pools of cells grown, with and without uracil. The primary purpose was to ascertain whether or not the addition of uracil to a culture of strain KD1104 affected the size of the UMP pool without causing any significant change in the UTP pool.

Analyzing the triphosphate pool data, we concluded that no differences in these pool sizes could account for the differences found in the enzyme levels of the two strains. In fact, the CTP pool of strain KD1104 was larger than that of KD1109 when the cells were grown on minimal medium, and yet DHOase and DHOdehase were derepressed in strain KD1104. If these enzymes were under CTP repressive control they should have been repressed, not derepressed. The *cmk* mutants (7, 27) have been instrumental in eliminating cytidine 5'-monophosphate (CMP) as a repressing metabolite,

and our observation here apparently eliminates CTP. We do know that added cytidine causes repression and that the cytidine 5'-diphosphate (CDP) pool was smaller in strain KD1104 than in strain KD1109 (see Table 6), but the difference did not appear very great. Moreover, when the two strains were grown in the presence of uracil, a situation where the CTP pools were virtually equivalent, the DHOase and DHOdehase of strain KD1109 were repressed, whereas they were still derepressed in strain KD1104. The synthesis of CTP and other cytosine nucleotides is dependent on the enzyme CTP synthetase under these conditions (refer to Fig. 1). In strain KD1109, the CTP so synthesized was being converted to the repressing metabolite(s) as evidenced by the repressed enzyme levels, but in strain KD1104 this conversion would appear to be inhibited. We feel that these data suggest that the regulation may be associated with uracil nucleotide pool sizes. It appears unlikely that CTP, CDP, or CMP alone are responsible for repression. Also, to achieve repression all available data suggest that added cytidine must be converted to the diphosphate level at least.

The same arguments developed in the above paragraph would seem applicable to the uracil ribonucleotide pools. Previous work with cold-sensitive *ndk* (nucleoside diphosphokinase) mutants (14) adds the greatest support to the statement. Under conditions where the activity of this enzyme is blocked, the pools of UTP and UDP decrease, yet all of the enzymes assayed were repressed (14).

We have quantitated the deoxyribonucleotide pools and believe that none appear to be candidates for repressing metabolites. We did not, however, separate the deoxy-UDP and deoxy-TDP pools from each other and thus these compounds cannot be eliminated.

Because of the lack of significant differences in the deoxyribonucleotide and ribonucleotide pools between the *pyrH*$^+$ and *pyrH* leaky strains, we measured the pool sizes of certain sugar nucleoside diphosphates, UDP-glucose, UDP-galactose, and CDP-glucose. Thus study too showed no significant differences between the two strains (Table 8). More appropriate mutants (altered in sugar nucleotide synthesis) have now been constructed and are presently being investigated and will be the topic of a separate report.

A major problem in studying the regulation of synthesis of the enzymes of the pyrimidine de novo pathway is assessing what compound(s) is the true end product of the total pathway. One can assign UMP as the end product of the de novo pathway per se, but pyrimidine synthesis obviously does not stop with this compound. Repression studies with other biosynthetic pathways are simplified because in certain cases (e.g., the tryptophan pathway) the end product can be added directly to the medium. In the pyrimidine pathway we do not know what product(s) is the end product(s), and even if we did evidence suggests that the compound(s) is a nucleotide and actively growing cells are probably not permeable to such compounds.

In the first report on *pyrH* leaky mutants, UDP or UTP were intimated as the repressing metabolites for the pyrimidine biosynthetic enzymes under uracil compound(s) control (22). In the Schwartz and Neuhard paper (27) these compounds were again emphasized as the repressing metabolites; however, the possibility of sugar nucleotides was suggested. The only endogenous nucleotide pools measured in their report were those of UTP, CTP, GTP, and ATP. The pool size data were expressed as a percentage of the total ribonucleoside triphosphate pool rather than as the individual concentration of each nucleotide, as has been the manner of expression of such data in previous reports and as we have presented here. By expressing their data in this percentage manner they were able to derive a correlation between the relative pool size of UTP and the amount of derepression of uracil-controlled pyrimidine biosynthetic enzymes. However, this same correlation is not evident for the cytosine-controlled enzymes (see Table 3 in their report). Further, expressing the data as relative pool sizes means that specific changes occurring solely in purine nucleoside triphosphate pool sizes directly alter the relative pyrimidine nucleoside triphosphate pools. Thus, if the relative pool sizes of nucleoside triphosphates represent the controlling elements for synthesis of pyrimidine biosynthetic enzymes, then under conditions of purine nucleoside triphosphate pool expansion (by the addition of purine precursors to the culture medium) and where the pyrimidine nucleoside triphosphate pools remain constant, that is, do not increase proportionately, then the relative pool sizes of pyrimidine nucleoside triphosphates would decrease, resulting in the derepression of pyrimidine biosynthetic enzymes. Under purine-limiting conditions the opposite situation would hold. No evidence was provided regarding either of these cases. Also, since only ribonucleoside triphosphates were measured, this assumes that any changes in repressing metabolites are directly reflected in these triphosphate pools, or, alternatively, that these triphosphates are the repressing metabo-

lites. We wish to emphasize our belief that neither pyrimidine ribo- nor deoxyribonucleoside di- or triphosphates, by themselves, are repressing metabolite candidates, as evidenced by the lack of significant differences in the pool sizes of these compounds when comparing *pyrH*$^+$ and *pyrH* leaky strains. In addition, the usefulness of expressing the endogenous pool measurements as relative concentrations of the total nucleotide pool and relating these interpretations to the regulatory aspects of pyrimidine enzyme biosynthesis is questionable.

In summary, we now believe that the synthesis of DHOase and DHOdehase are solely under the control of a cytosine compound, and that ATCase, OMPppase, and OMPdecase are regulated by a uracil compound (or possibly, but we believe unlikely, by both a uracil and cytosine compound). In addition our evidence suggests that cytosine and uracil ribonucleotides are not repressing metabolites and the same statement appears probable with regard to deoxyribonucleotides. We have also provided, during the course of these studies, evidence for a coupled repression of arginine and pyrimidine biosynthetic enzyme synthesis that may be related to the availability of carbamyl phosphate.

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