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Repression of Enzyme Synthesis of the Pyrimidine Pathway in *Salmonella typhimurium*

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It has been reported by other workers that a uridine and probably also a cytidine nucleotide are required for maximal repression of aspartate transcarbamylase encoded by the gene *pyrB* in *Salmonella typhimurium*. We have identified the repressing metabolites for three more biosynthetic enzymes, namely, dihydroorotate dehydrogenase (encoded by *pyrD*), orotidine-5'-monophosphate pyrophosphorylase (encoded by *pyrE*), and orotidine-5'-monophosphate decarboxylase (encoded by *pyrF*), as well as examining the repression profiles of aspartate transcarbamylase in more detail. Using a specially constructed strain of *S. typhimurium* (JL1055) which lacks the enzymes for the interconversion of cytidine and uridine compounds, thus allowing the independent manipulation of endogenous cytidine and uridine nucleotides, we found that a cytidine compound is the primary effector of repression in all cases except for aspartate transcarbamylase where little repression is observed in excess cytidine. For aspartate transcarbamylase, we found that the primary repressing metabolite is a uridine compound.

The pathway of de novo biosynthesis of pyrimidine nucleotides has been well established (for review see reference 16). Even though the scheme is common to all organisms, the control of enzyme activity within the pathway varies extensively from genus to genus. At least three sites, namely, carbamyl phosphate synthetase (ATP:carbamate phosphotransferase; EC 2.7.2.5), aspartate transcarbamylase (carbamoylphosphate:L-aspartate carbamoyl transferase; EC 2.1.3.2), and cytidine triphosphate (CTP) synthetase (UTP:ammonia ligase; EC 6.3.4.2), are the targets of control in *Escherichia coli* and *Salmonella typhimurium*.

Carbamyl phosphate synthetase has been studied extensively in *E. coli* by Anderson and co-workers (2, 3) and in *S. typhimurium* by Ingraham and co-workers (1, 13). It is feedback-inhibited by uridine monophosphate (UMP) and activated by ornithine in both genera. Aspartate transcarbamylase is one of the best-studied allosteric enzymes (6, 7, 15) being feedback-inhibited by CTP and activated by adenosine triphosphate in both organisms. CTP synthetase has also been extensively studied in *E. coli* (10). Since its substrate, uridine triphosphate (UTP), and its product, CTP, are both obligately required for ribonucleic acid (RNA) it is not surprising to find that both triphosphates are involved in its control (for a more detailed discussion see reference 9). It therefore appears that the control of enzyme activity is well understood. Such is not the case, however, for the control of the synthesis of the pyrimidine nucleotide enzymes.

Conventionally, when one measures the repression of enzyme synthesis, a potential source of the repressing metabolite(s) is added to the growth medium, and enzyme activities are then measured. It would be convenient if the actual repressing metabolite could be added, as indeed appears to be the case in tryptophan biosynthesis (20). To achieve histidine repression, however, a complex containing histidyl-transfer RNA (tRNA) is probably needed (8, 18). In pyrimidine nucleotide biosynthesis, the involvement of tRNA may be eliminated. However, the addition of uracil as the source of the repressing metabolite expands the endogenous levels of both CTP and UTP, but does not distinguish between them. It was not until Neuhard and Ingraham (14) isolated a cytidine-requiring mutant that such a test could even be attempted. Cytidine-requiring mutants are such that their pyrimidine source can not be supplied by uracil. All those described are defective in the enzyme CTP synthetase, and their genotype is *pyrG* (14). To isolate such

mutants, several known interconversions at the base and nucleoside levels must be blocked by mutation. Accordingly, *pyrG* mutants are obligately *cdd* as well (Fig. 1; reference 4). In such strains, it is therefore possible to separate individually and measure quantitatively the endogenous uridine and cytidine nucleotides (Fig. 1).

Although the effectors (negative and positive) that control enzyme activity of the pathway are well known, the identification of the nucleotides that may cause repression of the enzymes has only recently been attempted (1, 13, 14). In *S. typhimurium* JL1045, which requires cytidine for growth, it has been shown for carbamyl phosphate synthetase that a cytidine and not a uridine compound is the repressing metabolite. The presence of arginine was required as well for maximal repression (1). Endogenous nucleoside triphosphate pool measurements performed under appropriate conditions corroborated the result obtained from enzyme assays (13).

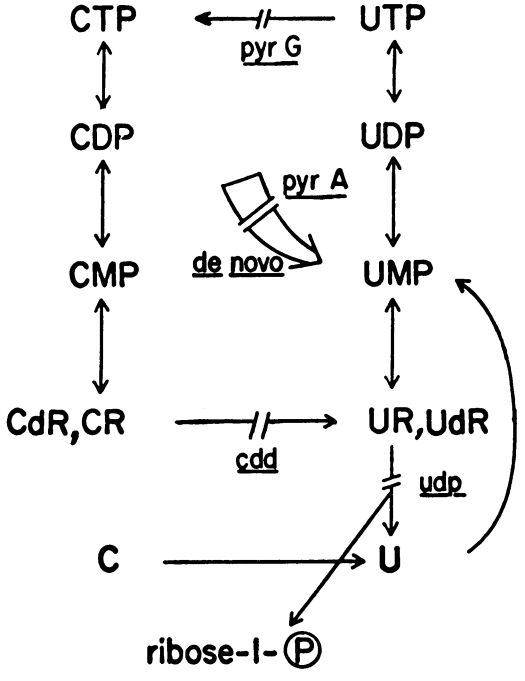

FIG. 1. *Schematic representation of the relevant pyrimidine interconversions in Salmonella typhimurium strain JL1055. Reactions blocked by mutation are indicated by the broken lines. Genotypic symbols are pyrA, specifies carbamyl phosphate synthetase; udp, uridine phosphorylase; cdd, cytidine (deoxycytidine) deaminase and pyrG, CTP synthetase. Abbreviations are C, cytosine; U, uracil; CdR, 2-deoxycytidine; UdR, 2-deoxyuridine; CR, cytidine; UR, uridine.*

Neuhard and Ingraham (14) also examined aspartate transcarbamylase in JL1055, a strain of *S. typhimurium* that requires arginine, uracil, and cytidine for growth. By limiting uracil in the presence of excess cytidine, about sevenfold derepression was observed, but when cytidine was limited in excess uracil about a threefold derepression was noted. Similar results have been obtained by Olszowy and Switzer (17). These results, taken together with the pool data, suggested that a uridine compound and probably a cytidine compound as well were repressing metabolites for aspartate transcarbamylase. They showed clearly that the pyrimidine nucleotide primarily responsible for repression of carbamyl phosphate synthetase was not the same as for aspartate transcarbamylase.

The present series of experiments, performed as a result of a question by John Ingraham, concern the control of synthesis of some of the later enzymes in the pathway: are these enzymes controlled like carbamyl phosphate synthetase or like aspartate transcarbamylase? Using strain JL1055, the same strain of *S. typhimurium* used by Neuhard, we have examined aspartate transcarbamylase again in more detail, as well as dihydroorotate dehydrogenase (L-4,5-dihydro-orotate:oxygen oxidoreductase; EC 1.3.3.1), orotidine-5'-monophosphate (OMP) pyrophosphorylase (orotidine-5'-phosphate:pyrophosphate phosphoribosyl transferase; EC 2.4.2.10), and OMP decarboxylase (orotidine-5'-phosphate carboxylase; EC 4.1.1.23). In the present paper we attempt to identify the repressing metabolites for these four enzymes of the biosynthetic pathway.

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MATERIALS AND METHODS

Bacterial strains. *S. typhimurium* LT2 and JL1055 (*pyrA*, *cdd*, *udp*, *pyrG*; Fig. 1) were used. The wild type is LT2. Strain JL1055 (formerly DP55) requiring arginine, uracil, and cytidine (14) was generously supplied by John Ingraham.

Growth media. Bacteria were grown in TF (12) liquid medium containing the following components (in grams per liter of distilled water): tris(hydroxymethyl)aminomethane (Tris), 12; KCl, 2; NH_4Cl, 2; $MgCl_2 \cdot 6H_2O$, 0.5; $Na_2HPO_4 \cdot 2H_2O$, 0.178; Na_2SO_4, 0.05; and D-glucose, 2.0. After the pH was adjusted to 7.7, 1 ml of $FeCl_3 \cdot 6H_2O$ solution (10 mM) and 5 mg of anhydrous $ZnSO_4$ were added per liter. Supplements, when required, were (in grams per liter): L-amino acids, 0.05; pyrimidines, 0.05.

Growth conditions. With the two strains of *S. typhimurium*, LT2 and JL1055, growth was carried

out at 37 C to measure the repression-derepression of the enzymes in the de novo pyrimidine pathway. Growth experiments were performed in Precision Scientific Co., reciprocating shaking water baths at 37 C. All cultures of LT2 and JL1055 were grown in TF liquid medium (the three supplements added) to 100 Klett units (approximately 10^9 cells per ml) and used to inoculate individual flasks with various supplements as indicated. The change in absorbancy at 660 nm was followed in a Klett-Summerson colorimeter at 30-min intervals. Since JL1055 has an absolute requirement for arginine, uracil, and cytidine, full growth (comparable to LT2) is seen only in the presence of all three required nutrients. Thus, by limiting one or more of these nutrients, growth will stop and derepression of enzyme synthesis will begin. As a control, the wild type was always examined under similar nutritional conditions. Cells from each flask were harvested at exactly 1 h after growth ceased. This allowed sufficient time for derepression to be observed.

Preparation of extracts. Extracts were prepared by sonic disruption for 2 to 3 min (tubes kept in ice) with a Biosonik III microtip (BPiii-40T) sonic oscillator at 80% power. The cells were harvested by centrifugation, washed once in 40 mM potassium buffer (pH 7.0), and resuspended in an equal volume of the same buffer. The disrupted cell material was divided into two fractions. Fraction I was centrifuged at 4 C at 5,000 rpm for 10 min, and the supernatant fluid was used for aspartate transcarbamylase and dihydroorotate dehydrogenase assays (which is membrane bound). Fraction II was centrifuged at 4 C at 20,000 rpm for 1 h, and the soluble supernatant fluid was used for OMP pyrophosphorylase and OMP decarboxylase assays.

Protein determinations on the extracts were carried out by the method of Lowry (11). Recrystallized bovine serum albumin was used as the standard.

Aspartate transcarbamylase. Aspartate transcarbamylase was assayed by the method of Gerhart and Pardee (7). One unit of specific activity for aspartate transcarbamylase is defined as that causing a change of 1.0 optical density unit at 560 nm per mg of protein at 30 C in 30 min in the colorimetric assay (7).

Dihydroorotate dehydrogenase. To assay dihydroorotate dehydrogenase, the method of O'Donovan and Gerhart (15) was used. One unit of specific activity for dihydroorotate dehydrogenase is defined as that causing a change of 1.0 optical density unit at 480 nm per mg of protein at 30 C in 20 min. The amount of reaction was proportional to enzyme concentration up to two units of activity.

OMP pyrophosphorylase. OMP pyrophosphorylase was assayed by the method of Beckwith et al. (5) as modified by J. Wild (Ph.D. thesis, University of California, Riverside, 1971). The assay mixtures contained (per milliliter): 0.1 ml of 1.0 M Tris buffer, pH 8.8; 0.01 ml of 25 mM L-orotate; 0.1 ml of 60 mM $MgCl_2 \cdot 6H_2O$; 0.1 ml of 6 mM 5'-phosphoribosylpyrophosphate (PRPP); 0.05 ml of enzyme extract, and 0.64 ml of deionized water. The mixture was preincubated at 37 C for 5 min, and the reaction was started by the addition of L-orotate. The reaction was followed at 37 C by observing the decrease in optical density at 295 nm compared with that of a blank that contained no PRPP. A change of 3.67 optical density units was equivalent to 1.0 μmol of L-orotate converted to OMP (5).

OMP decarboxylase. OMP decarboxylase was assayed by the method of Beckwith et al. (5) with modifications by Stockert and Wild (R. J. Stockert and J. Wild, unpublished observations). The assay mixture contained (per milliliter): 0.1 ml of 1.0 M Tris buffer, pH 8.6; 0.1 ml of 20 mM $MgCl_2 \cdot 6H_2O$; 0.02 ml of 10 mM OMP; 0.05 ml of enzyme extract; and 0.75 ml of water. The assay mixture containing Tris, $MgCl_2$, enzyme extract, and water was preincubated for 10 min at 37 C, and the reaction was started by addition of OMP. The reaction rate was determined by following the decrease in optical density at 285 nm compared with that of a blank that contained no OMP. A change of 1.38 optical density units was equivalent to 1.0 μmol of OMP converted to UMP (5).

Chemicals. OMP was obtained from Calbiochem, La Jolla, Calif. Arginine, uracil, cytidine, carbamyl phosphate (dilithium salt), L-aspartate, L-orotate, 5'-phosphoribosylpyrophosphate, and L-dihydroorotate were obtained from Sigma Chemical Co., St. Louis, Mo. All other chemicals were reagent grade and obtained commercially.

RESULTS

Starvation of JL1055. Since *S. typhimurium* JL1055 requires arginine and both uracil and cytidine, one can manipulate the uridine and cytidine nucleotide pools independently of each other (13). Such manipulations are required to determine whether a cytidine compound, a uridine compound, or both, function as repressing metabolites for the synthesis of the pyrimidine enzymes. Wild-type growth, comparable to that of strain LT2, is seen only in the presence of arginine, uracil, and cytidine. From the data of Neuhard (13) two key points can be made. (i) Cytidine limitation in the presence of excess uracil drops the CTP pool to undetectable levels (from 2.0 to 0.0 μmol per g dry wt) with a concomitant increase in the UTP pool (from 2.0 to 13.0 μmol per g dry wt). (ii) Starvation for uracil in the presence of excess cytidine rapidly elicits an expansion of the CTP pool (from 2.0 to 22.0 μmol) while the UTP pool is simultaneously decreased (from 2.0 to 0.5 μmol). But unlike the CTP pool during cytidine starvation, a low steady-state level of UTP is maintained (0.5 μmol per g dry wt). All data are recalculated for 60 min after the appropriate starvation (13).

In accordance with this fact, the UTP requirement of strain JL1055 can be satisfied, albeit slowly, with cytidine as the sole pyrimidine source. Indeed, as found by Neuhard (13), there is a residual conversion of cytidine to

uridine, thus explaining why the UTP pool does not fall to zero and why growth does not cease. Cytidine starvation, however, caused an immediate and permanent cessation of growth. Arginine was present in all experiments.

Table 1 gives a summary of the results obtained with strain JL1055 and the wild-type strain LT2. We wish to make the following points about Table 1, by way of summary.

(i) A uracil compound is the predominant species of corepressor in the repressing metabolite population for aspartate transcarbamylase. By growth in uracil with cytidine limiting (thus allowing a large UTP pool), almost maximal repression was observed. On the other hand, in accordance with previous data (13), starvation for uracil in the presence of excess cytidine, gave an enormous CTP and a very low UTP pool and afforded near maximal derepression of aspartate transcarbamylase.

(ii) In direct contrast to aspartate transcarbamylase, a cytosine compound is the primary repressing metabolite for the other three enzymes namely, dihydroorotate dehydrogenase, OMP pyrophosphorylase, and OMP decarboxylase. Starvation for cytidine with excess uracil provides maximal derepression. As can be seen in most cases, a slightly higher level of derepression can be attained if the cytidine starvation is carried out in the presence of uracil. Likewise,

TABLE 1. *Specific activities of four enzymes of the pyrimidine nucleotide biosynthetic pathway of Salmonella typhimurium wild type LT2 and mutant JL1055 grown in minimal medium, and in the presence of uracil and cytidine*

Strain[a] (genotype)	Addition to minimal medium[a]	Concn of supplement (μg/ml)	Enzyme spec act			
			ATCase[b]	DHOdehase[c] × 10^{-2}	OMPpase[d] × 10^{-4}	OMPdecase[e] × 10^{-4}
LT-2 (wild type)	Uracil	0	3.67	1.77	9.60	7.34
	Cytidine	0				
	Uracil	50	1.61	1.50	3.49	6.82
	Cytidine	5				
	Uracil	50	1.65	0.42	1.48	2.05
	Cytidine	50				
JL1055 (*pyrA81, udp, cdd, pyrG*)	Uracil	0	17.02	14.40	15.15	30.20
	Cytidine	0				
	Uracil	50	0.62	12.70	20.00	32.90
	Cytidine	5				
	Uracil	50	0.48	2.00	7.52	0.64
	Cytidine	50				
	Uracil	5	45.10	2.70	6.92	1.98
	Cytidine	50				

[a] *Salmonella typhimurium* LT-2 is the wild-type strain. Genotype designations are: *pyrA*, carbamyl phosphate synthetase; *udp*, uridine phosphorylase; *cdd*, cytidine (deoxycytidine) deaminase; *pyrG*, cytidine triphosphate synthetase. The cells were grown in minimal medium (12) with the indicated amounts of pyrimidines. All cultures had 50 μg of arginine per ml. Bacteria were harvested in the exponential phase of growth, centrifuged, and suspended in the same medium containing the indicated supplements. Single starvations (i.e., starvation for one requirement only) were performed in 5 μg of the limiting nutrient per ml, and the starvation was continued for 1 h after exhaustion of the limiting nutrient. For double starvation (i.e., simultaneous starvation for both cytidine and uracil) cells were harvested in the exponential phase, as for the single starvations, washed once, and suspended in glucose minimal medium (no pyrimidine added) with arginine.

[b] Aspartate transcarbamylase (ATCase) activity was measured as previously described (7), using a sonically treated extract at 30 C for 30 min. One unit of specific activity for aspartate transcarbamylase is defined as that causing a change of 1.0 optical density unit at 560 nm in micromoles of product formed per milligram of protein in 30 min.

[c] Dihydroorotate dehydrogenase (DHOdehase) activity was assayed as previously described (15) using a sonically treated extract at 30 C for 20 min. One unit of specific activity for dihydroorotate dehydrogenase is defined as that causing a change of 1.0 optical density unit at 480 nm per mg of protein at 30 C in 20 min.

[d] Orotidine-5'-monophosphate pyrophosphorylase (OMPpase) activity was measured as previously described (see Materials and Methods). One unit of specific activity is expressed as micromoles of orotate converted to OMP per milligram of protein per minute.

[e] Orotidine-5'-monophosphate decarboxylase (OMPdecase) activity was measured as previously described (see Materials and Methods). One unit of specific activity is expressed as micromoles of OMP converted to UMP per milligram of protein per minute.

maximal repression is observed for all three enzymes when the cytidine concentration in the medium is high (Table 1; reference 13). Although little difference is noted for maximal repression of dihydroorotate dehydrogenase and OMP pyrophosphorylase in excess cytidine with or without excess uracil, we consistently observe a two- to three-fold increase in repression of OMP decarboxylase when both cytidine and uracil are in excess. As can be seen, depletion of cytidine is all that is needed for maximal derepression.

(iii) We performed an identical set of experiments with wild-type LT2 for comparison. Since LT2 contains an active cytidine deaminase and can convert UTP to CTP, one would not anticipate much difference in repression patterns whether cytidine or uracil is supplied. Thus for pyrimidine biosynthesis and quite probably most other biosynthetic systems, that which is observed for the wild type must be treated with care. In general, though, growth in minimal medium gave more derepressed levels than growth in either uracil or cytidine. When both nutrients were added, repression was greater than if either alone was added.

(iv) The term "maximal repression" frequently is used to calculate the extent of derepression for a particular set of conditions. An assay of the enzymes from the wild-type strain grown in a source of the repressing metabolite is general practice to obtain maximal repression (e.g., grown in excess uracil for pyrimidine biosynthesis). Maximal repression can not be extrapolated from such wild-type measurements (Table 1). Only in suitably constructed strains as JL1055 (14) can such a measurement of maximal repression be obtained.

DISCUSSION

Although it has been shown (1) that the synthesis of carbamyl phosphate synthetase requires both arginine and a cytidine but not a uridine compound for maximal repression, and although previous workers have demonstrated that aspartate transcarbamylase requires both a uridine and a cytidine compound for maximal repression, the fundamental question remains, namely, what are the effectors of repression (i.e., corepressors) for the remaining four enzymes in UMP biogenesis. In previous experiments it has been necessary to use two different *S. typhimurium* strains, namely, (i) JL1045 (*cdd*, *pyrG*, *udp*, but *pyrA*$^+$) in which the repression of carbamyl phosphate synthetase was computed accurately and contrasted with the nucleotide triphosphate pools under conditions where uridine and cytidine nucleotides were independently manipulated (13). No doubt exists that CTP (or a cytidine compound) is a potent effector of repression, but arginine is needed for the expression of full repression. For growth, strain JL1045 requires only cytidine. (ii) In the other strain, JL1055, the one used in our study, requiring arginine, uracil, and cytidine (*pyrA*, *cdd*, *pyrG*, *udp*; see Fig. 1), it is not possible to measure carbamyl phosphate synthetase since the gene specifying the enzyme, *pyrA*, carries a deletion. Still it is possible to measure the effects on aspartate transcarbamylase and the subsequent enzymes to UMP under different conditions of growth. The role of arginine was not measured in previous studies nor in our own. Earlier work by Williams (J. C. Williams, M.S. thesis, Texas A&M University, 1971) seemed to indicate that arginine not only played a role in the regulation of enzyme synthesis for carbamyl phosphate synthetase, but also in some of the other de novo enzymes to UMP. The part played by arginine will only be mentioned here and will be the subject of a future communication wherein specially constructed strains carry mutant loci for other amino acids in addition to arginine (e.g., lysine auxotrophs in JL1045 and JL1055). Only the more fundamental question of how the regulation of synthesis of the final four enzymes to UMP has been dealt with here.

In agreement with previous workers (1, 6, 13–15) it seems to us that the putative effectors of repression are indeed a cytidine and a uridine compound, and in the present experiment we maintained arginine constant in all cases to avoid unnecessary complications. The role played by arginine as a possible corepressor must be determined with care. Experiments by Womack in *E. coli* K-12 and *E. coli* B (J. E. Womack and G. A. O'Donovan, manuscript in preparation) seem to indicate that arginine plays only an indirect role. The availability of *argR*$^+$ and *argR*$^-$ in *E. coli* K-12 (from W. K. Mass) will greatly facilitate in these determinations.

As clearly shown by Abd-El-Al and Ingraham (1), a cytidine compound alone is the repressing metabolite of carbamyl phosphate synthetase (in the presence of arginine). Likewise, it has been noted that aspartate transcarbamylase requires both a cytidine and uridine compound to elicit repression (14, 17). However, we found only a minor effect on the repression of aspartate transcarbamylase by a cytidine compound, the major effect being by a uridine compound. Using strain JL1055 we found little, if any effect on dihydroorotate dehydrogenase by a uridine

compound while a cytidine compound is the primary effector of repression. The final two enzymes, OMP pyrophosphorylase and OMP decarboxylase (especially the latter), seem to benefit from the presence of both a uridine compound in addition to their primary effector of repression, a cytidine compound.

Although the experiments reported here have been repeated several times, we are unable to obtain an unequivocal all-or-none answer regarding the effector(s) of repression for each of the six de novo enzymes. Indeed it may well be that each enzyme has different corepressors. Pyrimidine biosynthesis is carried out by enzymes with unlinked genes. Thus, on the reasonable assumption that a common apo-repressor protein is synthesized by a *pyrR* (pyrimidine regulatory) gene (cf. *trpR* for tryptophan biosynthesis), we should not expect the apo-repressor, on binding its corepressors, to bind to the six de novo promoters with the same affinity. It has been shown in this work and elsewhere (1, 16, 17) that there appears to be at least two corepressors for each of the de novo enzymes. Since it seems from our studies that, for some enzymes, both a cytidine and a uracil compound are needed for maximal repression, it might be important to ascertain the preferred order of binding to the putative apo-repressor. Only a repressor with an active conformation should bind to the operator for each gene and several combinations may exist.

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