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Pyrimidine Nucleotide Metabolism and Pathways of Thymidine Triphosphate Biosynthesis in *Salmonella typhimurium*

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The nucleoside triphosphate pools of two cytidine auxotrophic mutants of *Salmonella typhimurium* LT-2 were studied under different conditions of pyrimidine starvation. Both mutants, DP-45 and DP-55, are defective in cytidine deaminase and cytidine triphosphate (CTP) synthase. In addition, DP-55 has a requirement for uracil (uridine). Cytidine starvation of the mutants results in accumulation of high concentrations of uridine triphosphate (UTP) in the cells, while the pools of CTP and deoxy-CTP drop to undetectable levels within a few minutes. Addition of deoxycytidine to such cells does not restore the dCTP pool, indicating that *S. typhimurium* has no deoxycytidine kinase. From the kinetics of UTP accumulation during cytidine starvation, it is concluded that only cytidine nucleotides participate in the feedback regulation of de novo synthesis of UTP; both uridine and cytidine nucleotides participate in the regulation of UTP synthesis from exogenously supplied uracil or uridine. Uracil starvation of DP-55 in presence of cytidine results in extensive accumulation of CTP, suggesting that CTP does not regulate its own synthesis from exogenous cytidine. Analysis of the thymidine triphosphate (dTTP) pool of DP-55 labeled for several generations with ^{32}P-orthophosphate and ^{3}H-uracil in presence of ^{12}C-cytidine shows that only 20% of the dTTP pool is derived from uracil (via the methylation of deoxyuridine monophosphate); 80% is apparently synthesized from a cytidine nucleotide.

The de novo biosynthesis of pyrimidine nucleotides seems to follow a common scheme in most organisms (for review, *see* 20). The gene loci for the first six enzymes responsible for the de novo synthesis of uridine monophosphate (UMP) have been mapped in *Escherichia coli* (2, 22) and in *Salmonella typhimurium* (25), and it has been shown that five of these enzymes are derepressed when the cells are starved for pyrimidines (2, 27). Two enzymes of the pathway in *E. coli* are subject to allosteric regulation. (i) Aspartate transcarbamylase, the first enzyme specific for the entire pathway, is feedback-inhibited by cytidine triphosphate (CTP; 6, 26). (ii) The activity of CTP synthase, the first enzyme specific for the de novo synthesis of cytidine nucleotides, is influenced by several low-molecular-weight compounds, of which guanosine triphosphate (GTP) and uridine triphosphate (UTP) act as activators and CTP as an inhibitor (4, 12).

Our knowledge of the pathways involved in the metabolism of exogenous uracil and uridine is somewhat more limited. Although it has been shown that *E. coli* contains a UMP pyrophosphorylase (3, 5) and a uridine kinase (1), as well as a uridine phosphorylase (18), very little is known about the function (3) and regulation (1) of these enzymes in vivo.

Enteric bacteria contain a very active cytidine deaminase (24). By using an *E. coli* mutant deficient in this enzyme, Karlström and Larsson were able to show that this strain possessed a separate deaminase for cytosine and was capable of converting exogenous cytidine to CTP (9).

In the present work, we used mutants of *S. typhimurium* with specific requirements for cytidine (17) to study the regulation and pathway of pyrimidine nucleotide synthesis. Since these mutants have lost both cytidine deaminase and CTP synthase, their pathways for UTP and CTP synthesis are completely separated. By measuring the pool sizes of the pyrimidine nucleoside triphosphates in these mutants, it has been possible to study these pathways and their regulation in vivo.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The isolation of the mutants used in the present work has been described previously (17). Two of them, DL-38 and DP-45, are derived from prototrophic *S. typhimurium* LT-2; DP-39 and DP-55 are derived from *S. typhimurium* LT-2 PyrA 81, having a deletion in the structural gene for carbamyl phosphate synthase (25). The phenotypes of the mutants are presented in Table 1.

Bacteria were grown in minimal medium (13) with 0.2% glucose as carbon source. Required nutrients were added to the medium in the following concentrations: ribonucleosides and deoxyribonucleosides, 20 μg/ml; uracil and cytosine, 10 μg/ml; arginine, 50 μg/ml. Cultures were grown at 37 C in Erlenmeyer flasks in a Metabolyte water-bath shaker (New Brunswick Scientific Co., New Brunswick, N.J.). Increase in cell mass was followed at 450 nm in a Zeiss M4 Q III spectrophotometer. (A 1-ml amount of bacterial culture with an absorbancy at 450 nm of 1,000 contains approximately 4×10^8 cells or 0.2 mg, dry weight.)

Determination of acid-soluble nucleoside triphosphate pools. Labeling of cells, preparation of extracts, thin-layer chromatographic separation, and counting of the labeled nucleoside triphosphates from the chromatograms have been described previously (15, 16). A new solvent system was used in the present work for the second dimension of the thin-layer chromatograms. It gives an improved separation of ribonucleoside triphosphates from the corresponding deoxy-compounds. Also in this sytem a stepwise elution is used (14): (i) 2 cm in 0.75 M LiCl, 7.5% boric acid brought to *p*H 7.0 by addition of solid LiOH, followed by (ii) 0.75 M LiCl, 10% boric acid, *p*H 7.0 (with solid LiOH), up to 15 cm above the start line.

Enzyme assays. Toluenized cells were used as enzyme source throughout. They were prepared as follows. Washed cell suspensions containing about 10^{10} cells per ml in 0.05 M tris(hydroxymethyl)aminomethane (Tris)-chloride buffer, *p*H 7.1, were treated with 10 μliters of toluene per ml for 20 min at room temperature and then aerated for 10 min.

Deoxycytidine, cytidine, and cytosine deaminases. The reaction mixtures contained (in 1 ml): 25 μmoles of Tris-chloride (*p*H 7.05), 1 μmole of deoxycytidine, cytidine, or cytosine, and toluenized cells. At different times, 0.1-ml samples were added to 0.9 ml of 0.5 M perchloric acid (0 C) and the mixture was centrifuged. The extinction of the supernatant fluids was measured at 290 nm when deoxycytidine and cytidine were used as substrates, and at 282 nm when the deamination of cytosine was studied. The difference in the molar extinction coefficients between cytidine (deoxycytidine) and uridine (deoxyuridine) is 10.1×10^6 cm² per mole, and 7.3×10^6 cm² per mole between cytosine and uracil under the conditions employed.

Uridine phosphorylase. The difference in absorption in alkali at 290 nm between uracil and uridine is used in this assay to measure the rate of arsenate-stimulated degradation of uridine to uracil. The difference in the molar extinction coefficients between these two compounds, at *p*H 12, is 5.41×10^6 cm² per mole. The reaction mixtures contained (per ml): 20 μmoles of Tris-chloride (*p*H 7.05), 5 μmoles of uridine, 10 μmoles of sodium arsenate, and toluenized cells. At different times, 0.2-ml samples were added to 0.8 ml of 0.5 M perchloric acid (0 C) and the mixture was centrifuged. An 0.5-ml amount of the supernatant fluid was added to 0.5 ml of 1 M NaOH and read at 290 nm. For each assay, a parallel reaction was run in which arsenate was omitted. The values obtained here were subtracted from the former to get the uridine phosphorylase activity.

Chemicals. 5-Fluoro-2′-deoxycytidine (FCdR), 5-fluoro-2′-deoxyuridine (FUdR), 5-fluorocytosine (FC), and 5-fluorouracil (FU) were gifts from L. Hoffmann-La Roche & Co., Basle, Switzerland, through the courtesy of Waerum & Co., Copenhagen, Denmark. 2′-Deoxycytidine (essentially free of

TABLE 1. *Growth characteristics of the S. typhimurium strains used*[a]

Strain	Carbon source[b]		Sensitivity towards analogues[c]				Growth requirement[d]
	Uridine (1 mg/ml)	Adenosine (1 mg/ml)	FCdR (5 μg/ml)	FUdR (5 μg/ml)	FC (10 μg/ml)	FU (10 μg/ml)	
LT-2	+	+	S[e]	S	S	S	none
DL-38	+	+	R	S	R	S	none
DP-45	+	+	R	S	R	S	cyd
PyrA 81	+	+	S	S	S	S	arg, U
DP-39	−	+	R	S	S	S	arg, U
DP-55	−	+	R	S	S	S	arg, U, cyd

[a] All growth tests were performed on minimal agar plates containing minimal medium, agar (15 g/liter), growth requirements, and carbon source (0.1%).

[b] Ability (+) and (−) to use the nucleoside as carbon source.

[c] In experiments with FCdR and FUdR, 10 μg of uracil per ml was added to the plates, together with the analogues. Glucose was used as carbon source throughout.

[d] Cytidine (cyd), arginine (arg), uracil (U).

[e] Sensitivity (S) and resistance (R) to the drug.

cytidine) was from Calbiochem, Los Angeles, Calif. All other pyrimidines, ribonucleosides and deoxyribonucleosides, and amino acids were obtained from Sigma Chemical Co., St. Louis, Mo. ^{32}P-orthophosphate was supplied by the Radiochemical Centre, Amersham, England. 6-^{3}H-uracil, from New England Nuclear Corp., Boston, Mass., was kindly given to us by N. Fiil.

RESULTS

Characterization of mutants. To select for cytidine-requiring mutants, it is first necessary to select for mutants in which some of the enzymes responsible for the catabolism of cytidine are blocked by mutation (17). Three possible candidates were considered: cytidine deaminase [apparently identical with deoxycytidine deaminase (10, 24)], cytosine deaminase, and uridine phosphorylase. Of these, cytidine deaminase is the most active. Although uridine phosphorylase in *E. coli* seems to be specific for uridine (9, 18), we considered it possible that the enzyme in *S. typhimurium* could have some activity towards cytidine as well. Accordingly, mutants defective in some of these enzymes were isolated and used as parents for the selection of the cytidine-requiring mutants. Table 2 gives the specific activities of these enzymes in two cytidine-requiring strains, DP-45 and DP-55, their parents, DL-38 and DP-39, and in the two original strains, LT-2 and PyrA 81.

By comparing Table 2 with Table 1, it can be seen that the phenotypic traits, FCdR^{r}-FUdR^{s} (resistance to FCdR and sensitivity to FUdR), are a consequence of the loss of cytidine (deoxycytidine) deaminase; and the traits FC^{r}-FU^{s} (resistance to FC and sensitivity to FU) are a consequence of the loss of cytosine deaminase. DL-38 and DP-45 still have low levels of cytosine deaminase activity left, but apparently this activity is so low that the cells are FC-resistant. The inability to use uridine as a carbon source is due to a lack of uridine phosphorylase. Table 2 also shows that cytidine, which cannot be deaminated to any significant extent in DP-45, induces uridine phosphorylase in this strain.

TABLE 2. *Enzyme activities[a] in the different strains of S. typhimurium[b]*

Strain	Specific activity[a]			
	Deoxycytidine deaminase	Cytidine deaminase	Cytosine deaminase	Uridine phosphorylase
LT-2	79	29	7.2	6.1
DL-38	<1	<1	1.1	4.0
DP-45	<1	1	1.6	45.8
PyrA 81	73	29	4.0	4.2
DP-39	1	1	6.5	<0.4
DP-55	<1	<1	3.8	<0.4

[a] Expressed as nanomoles per minute per gram (dry weight).

[b] For all assays, toluenized cells were used (*see* Materials and Methods).

Since the cytidine requirement of DP-45 and DP-55 cannot be satisfied by cytosine (17), it can be concluded that neither a cytidine monophosphate (CMP) pyrophosphorylase nor a cytidine phosphorylase acting in reverse is operative in *S. typhimurium*. The only pathway for synthesizing cytidine nucleotides in these mutants seems therefore to be by phosphorylation of cytidine to CMP.

Figure 1 gives a schematic representation of the pathways involved in the metabolism of cytosine and uracil compounds, together with an indication of the enzymes blocked in the various mutants used in this study.

Cytidine starvation of DP-45. Removal of cytidine from an exponentially growing culture of DP-45 results in immediate cessation of growth. At the same time, ultraviolet-absorbing materials start to accumulate in the medium. As shown in Fig. 2, the excretion continues linearly for several hours. The compound(s) excreted has previously been shown to support growth of a uracil auxotrophic mutant of *S. typhimurium* (17). Its absorption spectrum in acid and alkali corresponds to that of uridine; however, spectral data cannot exclude the possibility that it is a uridine nucleotide. Further characterization of the compound(s) has not been achieved.

Figure 3 shows the changes in the ribonucleoside and deoxyribonucleoside triphosphate pools when cytidine is removed from the medium of a growing culture of DP-45. CTP and deoxy-CTP (dCTP) immediately drop to undetectable levels, whereas the pool of UTP increases markedly (15- to 20-fold by 60 min). In contrast, adenosine triphosphate (ATP), GTP, and the three remaining deoxyribonucleoside triphosphates only increase two- to threefold. These results strongly support our previous findings (17) that the cytidine requirement of DP-45 is caused by a mutational loss of CTP synthase. The changes induced by removal of cytidine from the medium are reversible; readdition of cytidine to the medium rapidly restores the triphosphate pools to normal levels (Fig. 3).

The findings that cytidine starvation of DP-45 causes a continued excretion of uridine (Fig. 2), together with an expansion of the UTP pool, strongly suggest that pyrimidine biosynthesis in *S. typhimurium* as in *E. coli* (7) is feedback-controlled by a cytidine nucleotide in vivo.

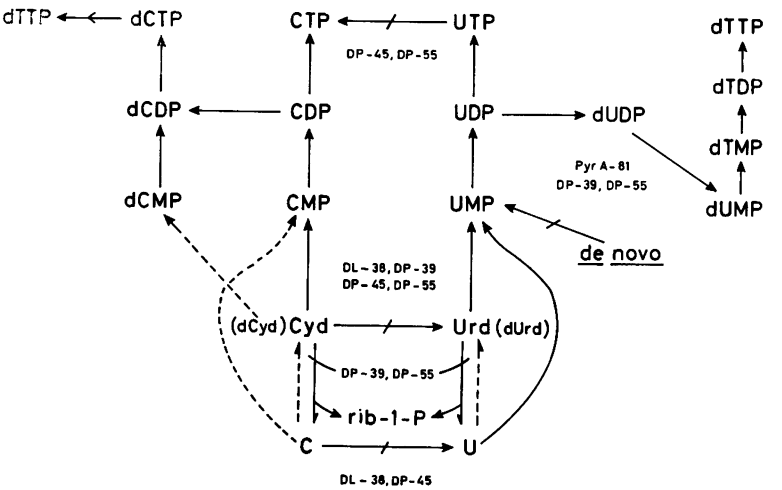

FIG. 1. *Schematic representation of pyrimidine nucleotide metabolism in S. typhimurium. Reactions blocked in the different mutants are shown. Broken lines indicate pathways that do not operate in vivo.*

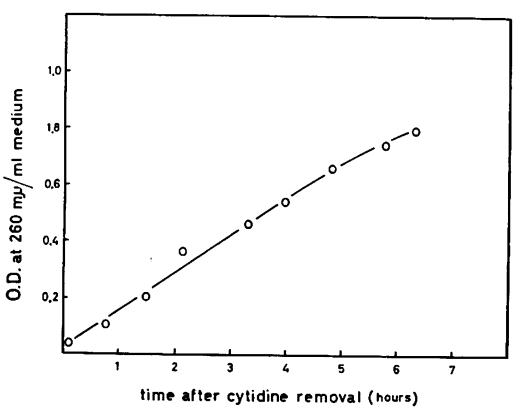

FIG. 2. *Appearance of ultraviolet-absorbing material in the medium during cytidine starvation of S. typhimurium DP-45. At time-zero, an exponentially growing culture of DP-45 was filtered, washed, and resuspended in glucose minimal medium to a density of 4 × 10⁸ cells/ml. At times indicated, 1 ml was filtered on membrane filters, and the optical density of the filtrate at 260 nm was determined.*

Since the cytidine nucleotide pathway in DP-45 is completely separated from the uridine nucleotide pathway (Fig. 1), one might expect that addition of high concentrations of cytidine to the medium would raise the CTP pool of this mutant. If CTP is the main feedback regulator of pyrimidine biosynthesis, such an increase in the CTP pool should result in a decreased UTP pool, eventually accompanied by a lowered growth rate of the mutant. To test this possibility, nucleoside triphosphate pools and growth rates were determined in cultures of DP-45 grown in media containing from 10 to 500 μg of cytidine per ml. No difference in the size of the pools nor in the growth rate could be detected, indicating that the rate-limiting step in the conversion of cytidine to CTP is not influenced by the concentration of exogenous cytidine (19).

The pool of dCTP in DP-45 drops rapidly if cytidine is removed from the medium (Fig. 3B), as would be expected from the fact that deoxyribonucleotides are synthesized from the corresponding ribonucleotides via the reaction catalyzed by ribonucleoside diphosphate reductase (9). Thus, one can test the cells' ability to synthesize dCTP from deoxycytidine by starving DP-45 for cytidine in presence of deoxycytidine. The results (Table 3) show that under such conditions the dCTP pool is depleted, suggesting that *S. typhimurium* is not able to phosphorylate deoxycytidine to the corresponding triphosphate.

Uracil starvation of DP-55. With strain DP-55, which has a double pyrimidine requirement (*see* Table 1 and Fig. 1), one can study both the effect of uridine nucleotide depletion on the CTP pool and the effect of cytidine nucleotide depletion on the UTP pool. Figures 4A and 4B show the changes occurring in the nucleoside triphosphate pools during uracil starvation in presence of cytidine. UTP falls immediately to very low levels, while CTP increases linearly, reaching 10-fold normal values at 60 min. (Since DP-55 is able to grow, with a doubling time of about 4 hr, with cytidine as the only pyrimidine source, there seems to be a residual slow conversion of cytidine to a uracil compound, thus explaining why the UTP pool does not fall to zero.) ATP and GTP showed a threefold increase during the

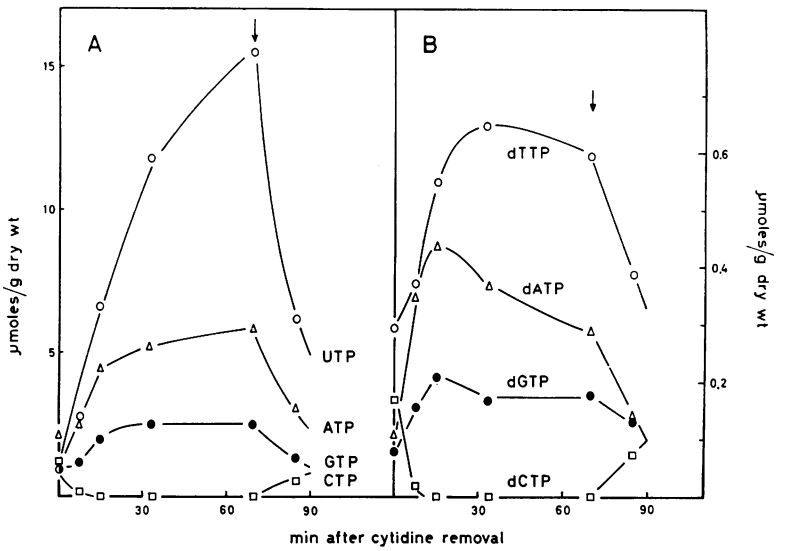

FIG. 3. *Changes in the nucleoside triphosphate pools of S. typhimurium DP-45 during cytidine starvation. At 70 min (indicated by arrows) cytidine was added to the cytidine-starved cells. (A) Ribonucleoside triphosphates; (B) deoxyribonucleoside triphosphates.*

TABLE 3. *dCTP pool of S. typhimurium DP-45 under different conditions of cytidine starvation*[a]

Expt no.	Addition	Concn (μg/ml)	dCTP (μmole per g, dry wt)
1	None		<0.03
2	Cytidine	20	0.33
3	Deoxycytidine	30	0.03
4	dCMP	50	<0.03

[a] Exponentially growing cells were filtered, washed free of cytidine, and resuspended in four flasks containing glucose minimal medium plus the additions indicated. At 60 min, the cells of the four cultures were analyzed for their dCTP content.

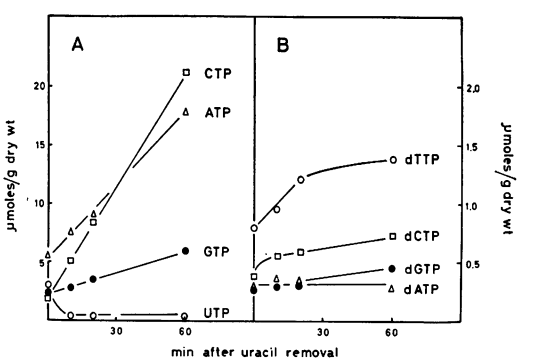

FIG. 4. *Changes in the nucleoside triphosphate pools of S. typhimurium DP-55 during uracil starvation in presence of cytidine. (A) Ribonucleoside triphosphates; (B) deoxyribonucleoside triphosphates.*

same period of time. It should be noted (Fig. 4B) that the pool of deoxythymidine triphosphate (dTTP), which might have been expected to decrease in parallel with UTP (compare the CTP and dCTP pools of Fig. 3), increases somewhat.

Cytidine starvation of DP-55. Since DP-55 requires two pyrimidines and is deficient in the enzyme which catabolizes uridine, uridine phosphorylase (*see* Fig. 1 and Table 2), exogenous uridine and uracil should be converted to UTP by different pathways. If these pathways are regulated differently from each other and from the de novo synthesis of UTP, one would expect to find differences in the pattern of UTP accumulation in DP-55 when cytidine starvation is performed in presence of uracil or uridine. Accordingly, three experiments were performed, the results of which are shown in Fig. 5. The figure shows only the changes in the ribonucleoside triphosphate pools, since the pattern for the deoxyribonucleoside triphosphates is essentially the same as that shown in Fig. 3B. From Fig. 5, it is evident that higher levels of UTP accumulate with uridine as precursor than with uracil, regardless of which one was present in the medium prior to starvation. It should be noted, however, that the zero-time value for UTP in the culture grown on uracil (Fig. 5A) is different

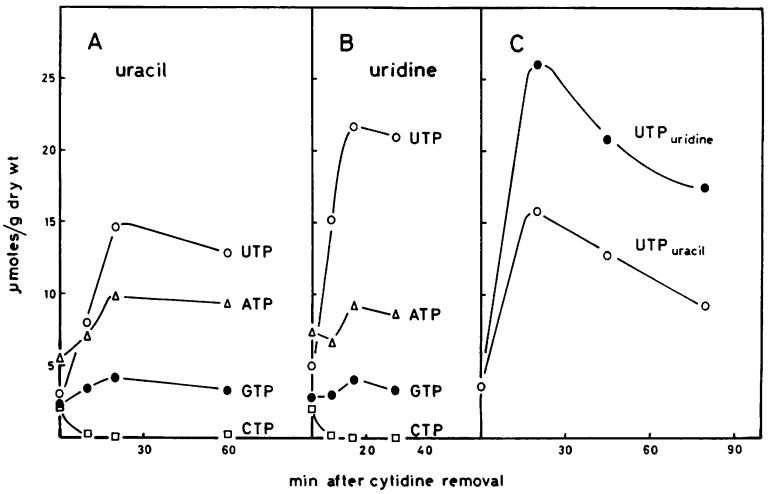

FIG. 5. *Changes in the ribonucleoside triphosphate pools of S. typhimurium DP-55 during cytidine starvation. (A) Pregrown with uracil + cytidine and starved in presence of uracil. (B) Pregrown with uridine + cytidine and starved in presence of uridine. (C) Pregrown in uracil + cytidine and starved in presence of uracil, ○; or uridine, ●.*

from that of the culture grown on uridine (Fig. 5B).

A summary of the effects of cytidine starvation on the UTP pools of the two mutants, DP-45 and DP-55, is presented in Fig. 6. The relative increase in the UTP pool is plotted as a function of duration of starvation. Two things are apparent. (i) The kinetics of UTP accumulation differ for the two mutants. (ii) The maximal UTP pools obtained in DP-55, measured as percentage of control, are the same whether uracil or uridine is used as exogenous precursor, although the absolute values (Fig. 5) differ significantly in the two cases.

Biosynthetic pathways for thymidine nucleotides. In a recent report of Karlström and Larsson (9), strong experimental evidence is presented against the generally accepted belief that dTTP in *E. coli* is synthesized de novo exclusively via methylation of deoxy-UMP (dUMP) catalyzed by thymidylate synthase (23). By growing a uracil auxotrophic *E. coli* mutant lacking deoxycytidine deaminase, with totally labeled ^{14}C-cytidine as the sole pyrimidine source, they showed that only 25% of the deoxythymidine monophosphate (dTMP)-residues in deoxyribonucleic acid are derived from a uridine nucleotide. The remaining 75% of the residues are synthesized directly from the exogenous cytidine without cleavage of the glycosidic bond.

The CTP synthase-deficient strain DP-55, in which the uridine and cytidine pathways are completely separated, should be ideally suited for investigating the pathway of dTTP synthesis in *S. typhimurium*. Table 4 gives the results of an

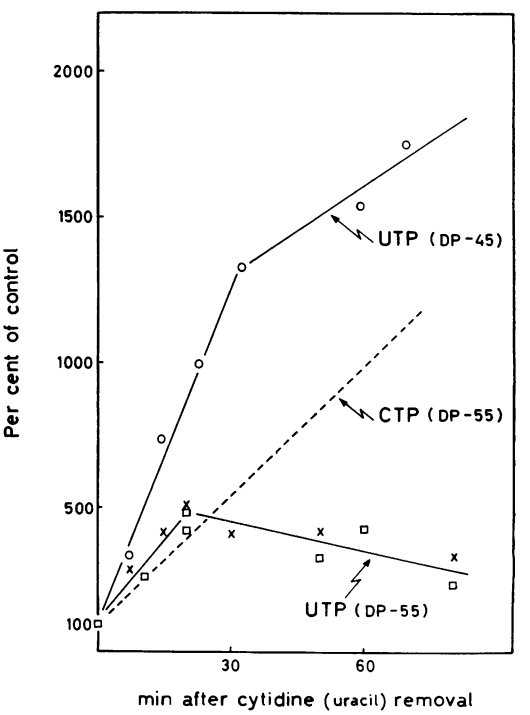

FIG. 6. *Relative changes in the UTP pool of S. typhimurium DP-45 and DP-55 induced by cytidine starvation. Symbols: ○, DP-45; □, DP-55 with uracil as second pyrimidine source before and after starvation; ×, DP-55 with uridine as second pyrimidine source before and after starvation. Broken line indicates the increase in the CTP pool induced by uracil starvation in DP-55, taken from Fig. 3.*

TABLE 4. *$^3H/^{32}P$ ratios of the nucleoside triphosphates in S. typhimurium DP-55 grown with ^3H-uracil and ^{32}P-orthophosphate*[a]

Growth conditions	Compound	Counts per min in spot						Per cent[b] derived from ^3H-uracil
		Chromatogram I			Chromatogram II			
		^{32}P	^3H	$^3H/^{32}P$	^{32}P	^3H	$^3H/^{32}P$	
Exponential growth (uracil + cytidine)	ATP	2,375	5	0.002	2,205	7	0.003	
	CTP	877	0	0.000	896	2	0.002	0.5
	UTP	1,371	304	0.222	1,285	260	0.203	100
	dCTP	169	0	0.000	158	0	0.000	0
	dTTP	330	14	0.042	321	16	0.050	22
Cytidine starvation (70 min)	ATP	4,152	3	0.001	4,558	5	0.001	
	CTP	260	1	0.004	66	0	0.000	0.9
	UTP	5,190	1,090	0.211	6,052	1,379	0.228	100
	dCTP	18	0	0.000	8	0	0.000	0
	dTTP	291	61	0.209	326	68	0.208	98

[a] Cells were grown for several generations in presence of ^3H-uracil, ^{32}P-orthophosphate, and ^{12}C-cytidine. The $^3H/^{32}P$ ratios in the nucleoside triphosphates were determined in extracts of an exponentially growing culture and extracts of the same culture after 70 min of cytidine starvation. Thin-layer chromatograms of the extracts were run in duplicate.

[b] Average of the two chromatograms. Calculated as the $^3H/^{32}P$ ratio for each compound in per cent of the ratio for UTP.

experiment in which a culture of DP-55 was labeled with ^3H-uracil and ^{32}P-orthophosphate in presence of ^{12}C-cytidine. After several generations of exponential growth in presence of the labeled compounds, the acid-soluble nucleoside triphosphate pools were analyzed for their content of ^{32}P and ^3H. Table 4 also gives data for a sample of the same culture taken 70 min after cytidine was removed from the growth medium. The following conclusions can be drawn from Table 4. (i) Since the $^3H/^{32}P$ ratio of CTP is less than 1% of that found in UTP, the mutant seems to be completely blocked in the CTP synthase reaction. (ii) Comparison of the $^3H/^{32}P$ ratio of UTP in the exponential culture (containing ^{12}C-cytidine in the medium) with that of the cytidine-starved culture, in which the UTP pool has increased four- to fivefold, shows that during exponential growth there is practically no conversion of cytidine to uridine nucleotides. (iii) About 80% of the dTTP pool is synthesized from cytidine and only 20% from uracil, in complete accordance with the results of Karlström and Larsson (9). (iv) During cytidine starvation there is an appreciable turnover of the dTTP-pool, and the dTTP synthesized under these conditions is derived entirely from uracil.

DISCUSSION

The results of the present work give some new information about the pathways involved in the pyrimidine metabolism of *S. typhimurium* (*see* Fig. 1). (i) Cytosine is able to satisfy the uracil but not the cytidine requirement of DP-55, indicating that *S. typhimurium* does not contain a CMP pyrophosphorylase. (ii) Like *E. coli* (9), *Salmonella* has two deaminases which act on cytosine compounds (Table 2)—one specific for cytosine and another for cytidine and deoxycytidine. (iii) As can be seen from Table 2, uridine phosphorylase is induced in strain DP-45 by cytidine. (Cytidine cannot be deaminated and serve as a source of uridine in this strain.)

(iv) Neither deoxycytidine nor deoxy-CMP (dCMP) is converted to dCTP in vivo by DP-45 (Table 3). Probably dCMP is not taken up by the cells. Deoxycytidine does enter the cells, since deoxycytidine will serve as a pyrimidine source for auxotrophic mutants requiring uracil. Therefore *S. typhimurium* is devoid of either a deoxycytidine kinase or a dCMP kinase. In view of the similarities between *E. coli* and *Salmonella* and the existence in *E. coli* of a dCMP-kinase (11), we believe that the results of Table 3 reflect that *S. typhimurium* does not contain a deoxycytidine kinase. This might explain why 5-fluorodeoxycytidine is innocuous to cells lacking deoxycytidine deaminase. Such a deficiency, in at least one deoxyribonucleoside kinase, implies that it is impossible to circumvent the ribonucleoside diphosphate reductase in vivo by exogenous addition of deoxyribonucleosides (16, 21).

(v) The labeling data presented in Table 4 show clearly that the main pathway for dTTP synthesis, in strain DP-55 as in *E. coli* (9), is via a cytidine nucleotide and does not involve the

methylation of deoxy-UMP (dUMP; 23). An enzyme system that is able to convert dCTP to dTTP has been isolated from *E. coli* by Förster and Holldorf (Abst. 2nd FEBS Meeting, Vienna, p. 146, 1965). It consists of two enzymes with the following catalytic activities: (i) dCTP + 5,10-methylenetetrahydrofolate → 5-methyl-dCTP + dihydrofolate. (ii) 5-methyl-dCTP → dTTP + NH_3.

Since nothing is known about the existence of such an enzyme system in *Salmonella* nor about the quantitative contribution of this "cytidine pathway" in wild-type cells, we can only say at present that, in *S. typhimurium*, at least two different pathways for the endogenous synthesis of dTTP do exist.

Finally, although 80% of the dTTP in DP-55 is synthesized via the "cytidine pathway" during exponential growth, the results (Table 4) show that during cytidine starvation the "uridine pathway" is capable of taking over the entire synthesis of dTTP, thereby providing the cells with an unaltered pool of this important metabolite.

Control of pyrimidine metabolism. One of the first and most thoroughly studied cases of feedback regulation is that of the de novo synthesis of the pyrimidine nucleotides in *E. coli* (7, 8, 26). The end product of the pathway, i.e., CTP, was shown in vitro to be a potent inhibitor of aspartate transcarbamylase, the first enzyme specific for the biosynthesis of UMP. Data were also obtained (7) that strongly suggested this control mechanism to be operating in vivo.

The results obtained in the present work indicate that a similar mechanism seems to be involved in the control of pyrimidine biosynthesis in *Salmonella* in vivo.

When UTP synthesis from exogenous uracil or uridine is studied during cytidine starvation of DP-55, a somewhat different picture is obtained (Fig. 5). In this case, the UTP pool only increases initially, reaching a plateau after about 20 min, in sharp contrast to what was observed with DP-45 (Fig. 3A). This suggests to us that, when UTP is synthesized from exogenous precursors (uracil or uridine) in a strain lacking CTP synthase, it is able to limit its own synthesis, presumably by feedback inhibition. Since DP-55 is deficient in uridine phosphorylase activity (Table 2), it follows that exogenous uracil and uridine is taken up by different pathways, i.e., via UMP pyrophosphorylase and uridine kinase, respectively. The quantitative differences between UTP accumulation from exogenous uracil and uridine (Fig. 5) and the striking similarities between the same data, when plotted as relative increases (Fig. 6), have led us to propose the following hypothesis for the regulation of uridine nucleotide biosynthesis from exogenous uracil and uridine. UMP pyrophosphorylase and uridine kinase are feedback-inhibited by UTP (or another uridine nucleotide). However, the UTP concentration necessary to limit the two pathways to the same extent differs significantly; it is approximately 50% higher for uridine kinase than for UMP pyrophosphorylase. A direct consequence of this hypothesis is that addition of uridine to cells of DP-55 growing exponentially on uracil (and cytidine) should result in an abrupt increase of about 50% in the UTP pool of the cells. As shown in Fig. 7, this prediction is indeed fulfilled.

Whether CTP also acts as a feedback inhibitor of these two enzymes is less clear from our data. However, since the removal of cytidine from the medium results in a much stronger accumulation of UTP than of any other nucleoside triphosphate (Fig. 5), it might be that CTP, the ultimate end product of the pathway, is also involved in the regulation of the uptake of exogenous uracil and uridine.

In analogy with the hypothesis presented above and the work of Anderson and Brockman (1), one might expect cytidine kinase to be feedback-controlled by CTP. However, the continued accumulation of CTP during uracil starvation in DP-55 (Fig. 4A) seems to exclude this. On the other hand, the small but significant drop in the

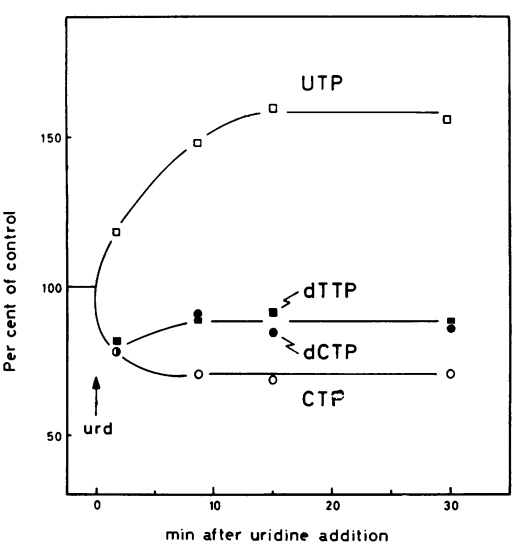

FIG. 7. *Relative changes in the pool sizes of the four pyrimidine nucleoside triphosphates of DP-55 after addition of uridine (20 μg/ml) to a culture growing exponentially in the presence of arginine, uracil, and cytidine.*

CTP pool observed in the experiment shown in Fig. 7 might indicate that in vivo UTP is acting as feedback inhibitor of the cytidine kinase in *Salmonella*.

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