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Deoxycytidine Triphosphate Deaminase: Characterization of an *Escherichia coli* Mutant Deficient in the Enzyme

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A mutant of *Escherichia coli*, previously shown to contain abnormal nucleoside triphosphate pools, was found to be defective in its ability to synthesize thymidine nucleotides. The defect is not in the enzyme thymidylate synthetase but in deoxycytidine triphosphate deaminase, an enzyme that supplies deoxyuridine monophosphate, the substrate for thymidylate synthetase.

We recently reported the isolation and partial characterization of a mutant of *Escherichia coli*, HD1038, with abnormal pyrimidine deoxyribonucleoside triphosphate pools (21). The mutant, originally isolated as a pyrimidine excreter, differs from its parent, JC411, by having a 5- to 10-fold elevated endogenous pool of deoxycytidine triphosphate (dCTP) and a 2-fold reduced endogenous pool of thymidine triphosphate (dTTP) (21). Since pool studies suggested a defective synthesis of thymidine nucleotides, it was of interest to identify the specific block in HD1038.

This paper will identify the mutation as a deficiency in dCTP deaminase, the enzyme described in the accompanying paper (20). The genetic symbol *paxA* is suggested as the genotype for the mutant.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* JC411, a K-12 F^- strain requiring histidine, arginine, leucine, and methionine, was received from A. J. Clark. HD1038 was isolated as a pyrimidine excreting mutant of JC411 after *N*-methyl-*N*-nitroso-*N'*nitroguanidine (NTG) treatment (1). Details of the selection procedure will be described elsewhere (O'Donovan and Gerhart, *in preparation*).

HD1038 cdd. HD1038 *cdd* is a derivative of HD1038 which lacks cytidine (deoxycytidine) deaminase (*see* Fig. 3). It was isolated from a mutagenized (NTG) culture of HD1038, as a mutant resistant to 5-fluorodeoxycytidine (10 μg/ml) in the presence of uracil, but sensitive to 5-fluorodeoxyuridine (5 μg/ml) in the presence of uracil (18).

JC411 cdd. JC411 *cdd* was obtained by transferring the *cdd* mutation from HD1038 *cdd* into JC411 by transduction with phage P1, and selecting for resistance to 5-fluorodeoxycytidine in the presence of uracil as described above. The *cdd* mutation was confirmed by enzyme assays on crude extracts as described previously (17).

HD1038 tpp. HD1038 *tpp* is a thymidine phosphorylase-deficient derivative of HD1038 (*see* Fig. 3). After mutagenesis (NTG), phenotypic expression in minimal medium with glycerol as a carbon source and penicillin counterselection in minimal medium with 0.1% thymidine as a carbon source, HD1038 *tpp* was isolated as a mutant that was unable to utilize thymidine, but could use deoxyadenosine as the sole source of carbon (4). The mutation was confirmed by enzyme assays (14).

The glucose minimal medium used throughout was previously described (16). Required amino acids were added to the medium at 20 μg/ml. The concentrations of bases and nucleosides used in different experiments are indicated in the appropriate tables and figures. Cultures were grown with shaking at 37 C. Increase in cell mass was observed at 436 nm in an Eppendorf photometer (model 1101 M). One milliliter of bacterial culture with an absorbancy at 436 nm of 1.000 contains approximately 4×10^8 cells or 0.2 mg of dry weight.

Determination of acid-soluble nucleotide pools. Nucleoside triphosphates and nucleoside monophosphates were determined by previously published procedures (15, 17).

Incorporation of ^{14}C-thymine into deoxyribonucleic acid (DNA). The ability of the different mutants to incorporate exogenous ^{14}C-thymine into acid-insoluble material was determined by the method of Munch-Petersen (14).

All countings were done in a Packard liquid scintillation spectrometer (type 3003) by using a toluene solution containing 4 g of PPO (2,5-diphenyloxazole) and 50 mg of POPOP [1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene] per liter.

Determination of enzyme activities. Washed cells were suspended in 2 volumes of 0.05 M tris(hydroxymethyl)aminomethane (Tris)-chloride (*p*H

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7.1), 1 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM mercaptoethanol and disrupted by treatment with a Branson sonic oscillator (model S125) for 60 sec. The suspension was centrifuged at 0 C for 30 min at 20,000 × *g*. The supernatant fluid was treated with one-third volume of 10% streptomycin sulfate and centrifuged. The supernatant fluid is designated fraction I. For the dCTP deaminase assays, fraction I was made 50% saturated with respect to ammonium sulfate, allowed to stand for 30 min at 0 C, and then centrifuged. The supernatant fluid was discarded, and the precipitate was dissolved in one-fifth the volume of 0.05 M Tris-chloride (*p*H 7.1), 2 mM $MgCl_2$, and 1 mM mercaptoethanol (fraction II).

dCTP deaminase. dCTP deaminase was assayed as described in the accompanying paper (20). Fraction II was used as enzyme source, after 2 hr of dialysis against 0.05 M Tris-chloride (*p*H 7.1), 2 mM $MgCl_2$, and 1 mM mercaptoethanol.

Thymidylate synthetase. The procedure described by Lomax and Greenberg (11) was used with slight modifications. Fraction I (*see above*) was dialyzed against 0.05 M Tris-chloride (*p*H 7.4) containing 0.01 M mercaptoethanol and 1 mM EDTA for 2 hr. Samples of 10, 30 and 50 μliters were incubated for 5, 10, and 15 min at 30 C. The assay mixture contained in a final volume of 0.2 ml: 42 mM Tris-chloride (*p*H 7.4), 26 mM $MgCl_2$, 15.8 mM formaldehyde, 106 mM mercaptoethanol, 1.06 mM EDTA, 0.30 mM DL-tetrahydrofolate, 0.1 mM deoxyuridine monophosphate (dUMP)-*5-*^3H (2,850 counts per min per nmole), and extract. The reaction was stopped by the addition of 0.5 ml of a slurry of Norite A [100 mg/ml in 1 mM sodium phosphate, 1 mM sodium pyrophosphate, *p*H 7.4 (6)]. The Norite, with adsorbed dUMP-*5-*^3H, was removed by centrifugation. A 0.25-ml amount of the supernatant was mixed with 3 ml of methyl Cellosolve and 6 ml of scintillation fluor (0.4% PPO, 0.05% POPOP in toluene) and counted in a scintillation spectrometer.

Protein was determined by the method of Lowry et al. (12).

Chemicals. Streptomycin sulfate was obtained from Sigma Chemical Co. (St. Louis, Mo.), and NTG was purchased from K & K Laboratories Inc. (Plainview, N.Y.). Thymine-*2-*^{14}C, uracil-*6-*^3H, and dUMP-*5-*^3H were obtained from The Radiochemical Centre (Amersham, England) and carrier-free ^{32}P-orthophosphate was from Atomenergikommisionens Forsøgsanlaeg (Risø, Denmark). The 5-fluoro analogues were generous gifts from W. E. Scott, Hoffmann-La Roche Inc., Nutley, N.J.

RESULTS

As previously reported, a marked difference was noted in the endogenous nucleoside triphosphate pools, when *E. coli* HD1038 was compared with its parent, *E. coli* JC411, after growth in minimal medium (21). Table 1 compares the nucleoside triphosphate pools in exponentially growing cultures of JC411 and HD1038. The most noticeable differences are the fivefold increase in the level of dCTP and the twofold decrease in the dTTP pool in HD1038. Since a similar pattern of pyrimidine nucleoside triphosphate pools is observed when thymine-requiring mutants of *E. coli* and *Salmonella typhimurium* are limited in their supply of dTTP (15, 19, 20), it was tentatively assumed that HD1038 is altered in its ability to synthesize thymidine nucleotides, the high dCTP pool being a result of the low dTTP pool. In spite of the abnormal nucleotide pools, HD1038 has the same generation time as JC411 in glucose minimal medium (i.e., doubling every 55 min at 37 C).

TABLE 1. *Nucleoside triphosphate pools in Escherichia coli JC411 and HD1038*[a]

Pools[b]	JC411[c]	HD1038[c]
GTP	1.18	0.94
ATP	2.94	2.51
CTP	0.57	0.29
UTP	0.92	1.01
dGTP	0.10	0.06
dATP	0.17	0.16
dCTP	0.17	0.97
dTTP	0.12	0.05

[a] Pools were determined in cells growing exponentially in glucose minimal medium containing the necessary nutrients and ^{32}P-orthophosphate (3 μCi/μmole).

[b] GTP, guanosine triphosphate; ATP, adenosine triphosphate; CTP, cytidine triphosphate; UTP, uridine triphosphate; dGTP, deoxyguanosine triphosphate; dATP, deoxyadenosine triphosphate; dCTP, deoxycytidine triphosphate; dTTP, deoxythymidine triphosphate.

[c] Values are expressed as micromoles per gram (dry weight).

To determine whether the levels of the nucleoside triphosphates in HD1038 could be altered, various purines and pyrimidines and their corresponding nucleosides were added to the medium. It was found that if HD1038 is grown in a medium containing the four deoxyribonucleosides (deoxyadenosine, deoxyguanosine, deoxycytidine, and thymidine), the nucleoside triphosphate pools were restored to normal parental levels, whereas the addition of ribonucleosides or bases had little, if any, effect.

Figures 1a and 1b show the effect of individually added deoxyribonucleosides on the dTTP and dCTP pools of HD1038, respectively. Deoxyguanosine (*not shown*) and deoxyadenosine have no effect, whereas the addition of pyrimidine deoxyribonucleosides bring the dTTP and dCTP pool of HD1038 to parental levels.

Since enteric bacteria can only metabolize deoxycytidine via deamination catalyzed by cytidine deaminase (*cdd*; 8, 17), it seems likely that the ability of deoxycytidine to restore the pools of HD1038 to normal is a result of its deamination to deoxyuridine. Figures 1c and 1d show that this is indeed the case. The abnormal pools of a cyti-

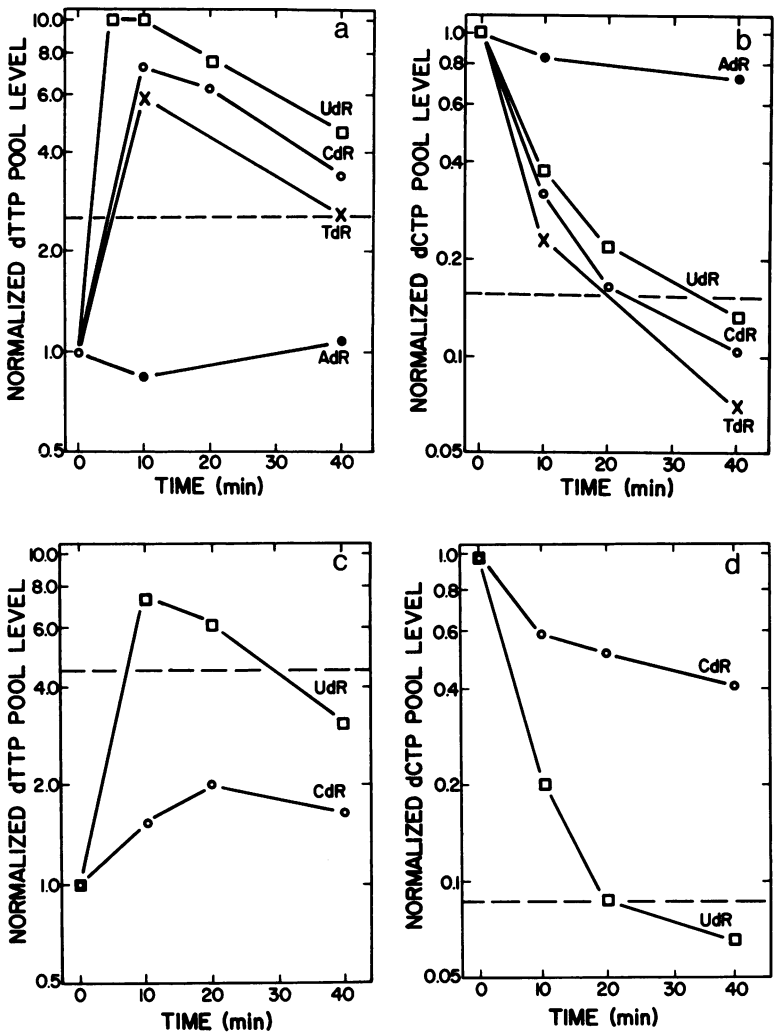

FIG. 1. *Changes in the endogenous dTTP pools (a and c) and dCTP pools (b and d) in Escherichia coli HD1038 (a and b) and E. coli HD1038 cdd (c and d) after addition of the indicated deoxyribonucleosides to the growth medium (at 0 min). The data are normalized by dividing all values by the value obtained before any addition of deoxyribonucleosides. The broken lines indicate the pool size found in the parent strains, JC411 (a and b) and JC411 cdd (c and d), grown in the absence of deoxyribonucleosides. Deoxyribonucleosides were added to give a final concentration of 50 μg/ml.*

dine deaminase-negative derivative of HD1038, HD1038 *cdd*, are not restored to normal levels by addition of deoxycytidine, whereas the addition of deoxyuridine to the medium has the same effect as in the parent strain HD1038.

Based on these findings two alternative explanations for the HD1038 mutation were considered. (i) The mutant contained a "leaky" thymidylate synthetase (*see* Fig. 3), i.e., an enzyme which requires higher endogeneous levels of dUMP to be able to supply the cells with normal amounts of thymidine nucleotides. (ii) HD1038 is mutated somewhere on the biosynthetic pathway for dUMP. In both cases the addition of deoxyuridine or thymidine would be expected to restore the dTTP pool to parental levels, which subsequently would result in a decrease in the dCTP pool (15, 19, 20).

Thymidylate synthetase. Studies of thymine-requiring mutants (*thy*) defective in thymidylate synthetase have established that a low endogenous dTTP pool results in extensive accumulation of dCTP (10) and dUMP (15, 20). These changes are accompanied by an increased catabolism of pyrimidine deoxyribonucleotides to the corresponding deoxyribonucleosides and eventually

further to deoxyribose-1-phosphate, which enables the cells to convert exogenous thymine to thymidine and thus to deoxythymidine monophosphate (dTMP; 15, 20). This in turn explains why cells that are impaired in their ability to convert dUMP to dTMP, in contrast to wild-type cells, are capable of incorporating exogenous thymine into DNA.

Table 2 shows the results of an experiment in which the nucleoside monophosphate pools of HD1038 and JC411 are compared. Included also are the pool sizes in HD1038 grown under conditions where the nucleoside triphosphate pools are brought to normal by addition of deoxyuridine. Although the dTMP pool of HD1038 is only about 30% of that of JC411, this decrease is not accompanied by any significant increase in the dUMP pool. Addition of deoxyuridine to the growth medium of HD1038 results in a normal dTMP pool and a discrete swelling of the dUMP pool (Table 2).

As mentioned above, an alternative diagnostic test for impaired thymidylate synthetase activity in vivo is the ability of the cells to incorporate exogenous thymine into DNA. Figure 2 shows that HD1038, like the parent JC411, is unable to utilize exogenous thymine for DNA synthesis in vivo. However, if a supply of deoxyribosyl groups is simultaneously made available by the addition of deoxyadenosine (7, 13), thymine is readily incorporated into DNA. As a control, thymine incorporation by HD1038 *tpp* is also shown in Fig. 2. This strain lacks thymidine phosphorylase and is therefore unable to convert thymine into thymidine.

Thus, although HD1038 is somewhat limited in its ability to synthesize thymidine nucleotides endogenously, the low dUMP pool and the lack of deoxyribosyl groups necessary for the conversion of exogenous thymine to thymidine seems to indicate that the HD1038 mutation is not affecting thymidylate synthetase activity. Accordingly, Table 3 shows that the specific activity of thymidylate synthetase, in crude extracts of HD1038, is equal to that found in JC411.

A further proof that thymidylate synthetase is not involved was obtained genetically. The gene for thymidylate synthetase, *thyA*, has been shown to map between *lysA* and *argA* on the linkage map of *E. coli* (23). Thus, by growing P1 phage on HD1038 and transducing into a *lysA* and *argA* recipient, and selecting for double transductants (*lysA*$^+$, *argA*$^+$), one is assured of contransducing the *thyA* locus. Five such cotransductants were checked for altered deoxyribonucleoside triphosphate pools. All five had wild-type levels of dCTP and dTTP, indicating that the mutation does not map between *lysA* and *argA* and is

TABLE 2. *Nucleoside monophosphate pools of Escherichia coli JC411 and HD1038*[a]

Pools[b]	JC411	HD1038[c]	HD1038 + UdR[c]
AMP	0.16	0.15	0.13
CMP	0.10	0.08	0.07
UMP	0.38	0.47	1.18
dCMP	0.03	0.03	0.03
dUMP	0.03	0.03	0.09
dTMP	0.37	0.14	0.48

[a] Pools were determined in cells growing exponentially in glucose minimal medium containing the necessary nutrients and ^{32}P-orthophosphate (3 μCi/μmole).

[b] Abbreviations as in Table 1.

[c] Values are expressed as micromoles per gram (dry weight). Deoxyuridine (UdR, 50 μg/ml) was present throughout the entire growth period with HD1038.

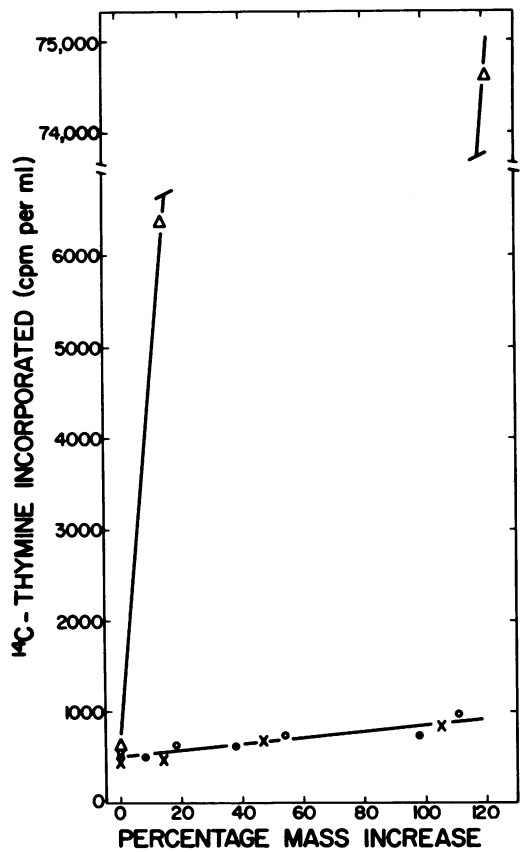

FIG. 2. *Incorporation of thymine-2-^{14}C into acid-insoluble material by exponentially growing cells of the following strains of Escherichia coli: HD1038, ●; HD1038 tpp, ×; JC411, ○; JC411 + deoxyadenosine, △. The concentrations of ^{14}C-thymine and deoxyadenosine used were 5 μg/ml and 50 μg/ml, respectively.*

TABLE 3. *Thymidylate synthetase activity in extracts of Escherichia coli JC411 and HD1038*[a]

Strain	Thymidylate synthetase specific activity[b]
JC411	0.315
HD1038	0.311

[a] Preparation of extracts and enzyme assays were performed as described in the text. A saturating concentration of the substrate, deoxyuridine monophosphate (dUMP), was used.

[b] Expressed as nanomoles dUMP converted per minute per milligram of protein.

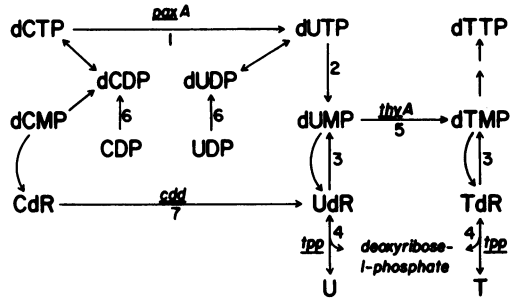

FIG. 3. *Pyrimidine deoxyribonucleotide metabolism in Escherichia coli. 1, dCTP deaminase; 2, dUTP pyrophosphatase; 3, thymidine kinase; 4, thymidine phosphorylase; 5, thymidylate synthetase; 6, ribonucleoside diphosphate reductase; 7, cytidine deaminase.*

therefore not in the thymidylate synthetase gene (*thyA*).

Biosynthesis of dUMP. The results presented above indicate that the HD1038 mutation must affect the biosynthesis of dUMP. In the accompanying paper (20), it is shown that dUMP may be synthesized by two different pathways in *S. typhimurium*. One of these, the uridine diphosphate (UDP) pathway, involves the direct reduction of UDP to deoxyuridine diphosphate (dUDP; reaction 6, Fig. 3), its subsequent conversion to deoxyuridine triphosphate (dUTP) which is then converted to dUMP (reaction 2, Fig. 3 and reference 2). The other, the dCTP pathway, proceeds via deamination of dCTP, catalyzed by dCTP deaminase (reaction 1, Fig. 3) followed by hydrolysis to dUMP, probably catalyzed by dUTP pyrophosphatase (reaction 2, Fig. 3 and references 2 and 5). Furthermore, it was shown that the contribution from each of these pathways to the synthesis of dUMP and thereby dTMP, could readily be determined in strains lacking cytidine deaminase (*cdd*). Thus, if cells are grown for several generations in the presence of uracil-6-^{3}H, ^{12}C-cytidine, and ^{32}P-orthophosphate, the specific activities of the thymidine nucleotide pools ($^{3}H/^{32}P$), compared with the uridine and cytidine nucleotide pools, can be used to calculate the contribution from each of the pathways to thymidine nucleotide synthesis (20).

Table 4 gives the results of an experiment in which JC411 *cdd* and HD1038 *cdd* were subjected to such an analysis. There is an equal contribution from each of these pathways to dTMP in JC411 *cdd*, whereas the contribution from the dCTP pathway in HD1038 is essentially zero (Table 4). This shows that the mutation responsible for the HD1038 phenotype is in the dCTP pathway.

Assays of dCTP deaminase activity in extracts

TABLE 4. *Specific activities of the nucleoside monophosphate pools in Escherichia coli JC411 cdd and HD1038 cdd grown in the presence of ^{32}P-orthophosphate and ^{3}H-uracil*[a]

Strain	Pools[b]	Counts/min[c]			Per cent derived from	
		^{32}P	^{3}H	$^{3}H/^{32}P$	CMP	UMP
JC411 *cdd*	AMP	314	0	0		
	CMP	171	144	0.84	100	0
	UMP	440	1,252	2.85	0	100
	dTMP	416	748	1.79	52[d]	48
HD1038 *cdd*	AMP	417	0	0		
	CMP	223	23	0.10	100	0
	UMP	443	1,306	2.95	0	100
	dTMP	69	213	3.09	0	100

[a] Cells were grown exponentially for several generations in the presence of ^{32}P-orthophosphate and uracil-6-^{3}H. After extraction and thin-layer chromatographic separation (17), the specific activity ($^{3}H/^{32}P$) of the individual acid-soluble nucleoside monophosphates was determined.

[b] Abbreviations: AMP, adenosine monophosphate; CMP, cytidine monophosphate; UMP, uridine monophosphate; dTMP, deoxythymidine monophosphate.

[c] Corrected for background and, in the case of tritium, for ^{32}P counts in tritium channel.

[d] The per cent of dTMP derived from CMP was calculated as follows: dTMP from CMP = [(specific activity of UMP minus specific activity of dTMP)/(specific activity of UMP minus specific activity of CMP)] × 100.

of JC411 *cdd* and HD1038 *cdd* (Table 5) confirm the conclusion drawn from the in vivo experiments (Table 4) and, furthermore, identify the mutation in HD1038 as one affecting the synthesis of dCTP deaminase.

TABLE 5. *dCTP deaminase activity in extracts of Escherichia coli JC411 cdd and HD1038 cdd*[a]

Strain	dCTP deaminase specific activity[b]
JC411 *cdd*	2.03
HD1038 *cdd*	0.15

[a] Preparation of extracts and spectrophotometric assays are described in the text.

[b] Expressed as nanomoles of dCTP deaminated per minute per milligram of protein.

DISCUSSION

The present paper describes the identification of dCTP deaminase in *E. coli* and the phenotype of a mutant which lacks the enzyme (*paxA*). In the accompanying paper (20), the enzyme dCTP deaminase is reported for the first time in *S. typhimurium*. Recently, a phage-specific dCTP deaminase has been reported to be induced in *Bacillus subtilis* after infection with phage PBS1 (24).

Labeling experiments by Neuhard (17) and Karlström and Larsson (8) have indicated that two pathways for the biosynthesis of dTTP may exist. By using specially constructed strains (*pyrA*, *pyrG*, *cdd*) of *S. typhimurium* [for more detailed discussion, see accompanying paper (20) and reference 22], Neuhard (17) was able to show that 80% of the label ultimately appearing in dTTP was derived from a cytosine compound by a pathway not requiring the intermediate formation of uridine nucleotides. Only 20% of the label in dTTP was derived directly from a uridine nucleotide. An identical result was obtained by Karlström and Larsson (8) by using *cdd* mutants of *E. coli*.

As a possible explanation for this apparent dilemma, a pathway described by Förster and Holldorf was drawn upon. In their short report (Förster and Holldorf, Abst. 2nd Meeting Fed. of Eur. Biochem. Soc., Vienna, p. 146, 1965), they described an enzyme system in *E. coli* capable of directly converting dCTP to dTTP through the intermediate, 5-methyl dCTP. Thus, a triphosphate level methylation of dCTP coupled with the sequential action of a deaminase yielded dTTP. If such were the case, it would require a double mutational event to achieve thymine auxotrophy, i.e., a mutation in thymidylate synthetase (*thyA*) as well as a mutation in one of the two enzymes in the above postulated pathway. The identification of dCTP deaminase in *S. typhimurium* (20) led to the elucidation of a previously unreported pathway for dUMP and subsequently dTMP biosynthesis, involving the sequential action of dCTP deaminase and probably dUTP pyrophosphatase (2, 5; Fig. 3). This provided a facile explanation for the labeling data and, at the same time, indicated that a double mutational event would not be required for thymine auxotrophy.

If the contention that dCTP deaminase along with dUTP pyrophosphatase, acting in sequence, is an important supplier of dUMP in wild-type enterobacteria, what then would be the consequences of a mutation eliminating this enzyme? At least four easily distinguishable characteristics would be expected. (i) The endogenous pool of dTTP would be low. (ii) The endogenous pool of dUMP would likewise be low. (iii) dCTP would be expected to be high, as a consequence of the low dTTP pool (15, 19, 20). (iv) All dTTP would be derived from a uridine nucleotide, none coming from a cytosine compound. The mutant HD1038, described herein, has precisely these characteristics. In accordance, it was shown to lack dCTP deaminase activity in vitro. Thus, in the absence of one of the suppliers of dUMP (dCTP deaminase), the other supplier (ribonucleoside diphosphate reductase) is able to take over and supply enough dUMP, and thus thymidine nucleotides, to ensure normal growth. This, however, may only be possible provided the cells (HD1038) have a low dTTP pool, which has been shown to result in derepressed synthesis of ribonucleoside diphosphate reductase (3). It should be kept in mind that the affinity of *E. coli* ribonucleoside diphosphate reductase for UDP, as a substrate, is significantly lower than it is for cytidine diphosphate, adenosine diphosphate, and guanosine diphosphate (9, 10).

Besides providing evidence for the existence of two pathways for dUMP synthesis, this and the accompanying paper (20) suggest a central role for dUTP pyrophosphatase in thymidine nucleotide metabolism. A common reason given for the existence of dUTP pyrophosphatase is the dephosphorylation of dUTP to prevent its incorporation into DNA (2, 5). We believe that the primary function of dUTP pyrophosphatase is to supply all endogenous dUMP for thymidylate synthetase, whether via dCTP deamination or via UDP reduction (Fig. 3).

Any cell which contains a low endogenous dTTP pool due to a decreased capacity to catalyze the conversion of dUMP to dTMP will maintain a high dCTP pool and a high dUMP pool (15, 19). In addition, such cells are able to incorporate exogenous thymine into their DNA,

due to an increase in the availability of endogenous deoxyribose-1-phosphate (15). The finding that HD1038, although low in dTTP and high in dCTP, is unable to utilize exogenous thymine (Fig. 2) indicates that the extra supply of deoxyribose-1-phosphate, generated in *thyA* mutants, is derived via the dCTP deaminase pathway (*see* Fig. 3 and reference 20).

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