Studies on the Biosynthesis of α -Putrescinylothymine in Bacteriophage ϕ W-14-Infected *Pseudomonas acidovorans*

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The α -putrescinylothymine (putThy) in bacteriophage ϕ W-14 DNA is synthesized at the mononucleotide level: it is labeled by uracil or deoxyuridine but not by thymidine, and it appears in the acid-soluble pool of infected cells before the onset of phage DNA synthesis. The methylene group at the C-5 position of the pyrimidine moiety of putThy is derived in vivo from a C₁ unit. Extracts of a phage infected thymidine auxotroph of the host, *Pseudomonas acidovorans*, apparently contain a phage-specific thymidylate synthetase and a phage-specific activity which forms 5-hydroxymethyl dUMP from N₅,N₁₀-methylene-tetrahydrofolate and dUMP.

The DNA of bacteriophage ϕ W-14 contains thymine and α -putrescinylothymine (putThy) (8) so that it appears, at first sight, to be analogous to T2 and T6 DNAs, which contain hydroxymethylcytosine (HMC) and glucosylated HMC (18, 20). However, the glucose molecules are attached to the hydroxymethyl groups of HMC after DNA replication (5), and it is difficult to imagine the methyl groups of thymine reacting with putrescine or a related compound without prior modification. There are several routes by which putThy could appear in ϕ W-14 DNA: (i) some of the thymine methyl groups are modified at the polynucleotide level and then reacted with putrescine molecules; (ii) the DNA initially contains only putThy and some of the putrescinylothymine groups are removed by a reaction which leaves a C-5 methyl group; (iii) a uracil pyrimidine with a suitable C-5 substituent group is synthesized at the mononucleotide level, incorporated into DNA with thymine, and then reacted with putrescine; (iv) putThy is synthesized at the mononucleotide level, and incorporated into DNA with thymine. Routes (iii) and (iv) involve synthesis of DNA from five rather than four bases.

This paper describes the results of experiments to distinguish between these possibilities.

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MATERIALS AND METHODS

Organisms. The bacterial strains used are listed in Table 1. They were maintained as described previously (9), except for strain 3L, for which the medium was supplemented with 1 mg of thymidine and 15 μ g of trimethoprim per ml. Bacteriophage ϕ W-14 stocks (9) were stored in dilute mannitol broth (DMB) at 4 C over chloroform.

Media. The media used were mannitol broth (MB) (9); DMB, which was MB diluted 10-fold; and minimal medium M29 (6). Solid media contained 1.5% agar (Difco).

Growth of bacteria. The methods for growing liquid cultures and determining cell density were described previously (9).

Preparation and titration of phage stocks. Phage stocks were prepared by growing bacterial cultures to a density of 2.5×10^8 to 5.0×10^8 cells/ml, adding phage at a multiplicity of infection (MOI) of about 0.1, and continuing incubation until lysis. Then 1 μ g of DNase was added per ml of culture and incubation was continued for another hour. Bacterial debris was removed by centrifugation at $10,000 \times g$ for 10 min, then the phage was pelleted by centrifugation at $17,000 \times g$ for 2 h and resuspended in DMB. The suspension was again centrifuged at $10,000 \times g$ for 20 min, and the supernatant fluid was removed carefully and stored over chloroform at 4 C. Phage preparations were titrated by the agar overlay method (1).

Isolation and purification of DNA. DNA was isolated and purified from uninfected and infected bacterial cells by the sodium dodecyl sulfate (SDS)-Pronase-phenol method (7). DNA was extracted from phage as follows. A phage pellet was resuspended in Tris-NaCl-EDTA (TNE) buffer (7). An equal volume of water-saturated phenol was added to the suspen-

TABLE 1. Strains of *P. acidovorans*

Strain	Phenotype	Genotype	Source	Reference
29	Wild type		R. Y. Stanier	19
3L	Thymidine auxotroph	<i>thyA</i>	29	6
3L/FU	Resistant to 5-fluorouracil	<i>thyA upp</i>	3L	Kelln and Warren Can. J. Microbiol, accepted for publication
U ₁	Uracil auxotroph		29	

sion and the tube rotated at an angle and at 40 to 60 rpm for 1 h. The aqueous phase was removed, washed with ether, and dialyzed twice against 250 volumes of $0.1 \times \text{SSC}$ at 4 C over a 24-h period. The DNA was precipitated with ethanol and redissolved in $0.1 \times \text{SSC}$.

All DNA samples were hydrolyzed with HCl (8) after precipitation with ethanol and drying with acetone.

Preparation of bacterial extracts. The method used for the preparation of bacterial extracts was described previously (Kelln and Warren, accepted for publication). Protein concentrations were determined by the phenol method (12), using bovine serum albumin as standard.

Enzyme assays. All enzymes were assayed at 28 C. Changes in absorbance were measured with a Unicam SP. 800 B spectrophotometer (Unicam Instruments Ltd., Cambridge, England). Activities were assayed according to the referenced procedure: thymidylate synthetase (i) spectrophotometrically (21), (ii) by tritium release (11, 14), (iii) by the incorporation of labeled formaldehyde (17); dUMP hydroxymethylase (17). It should be pointed out that the formaldehyde incorporation assay can be used to assay both activities because the chromatographic procedure it involves separates dTMP and dHMUMP. All extracts were assayed for fumarase (16); only positive extracts were assayed for the other activities.

Chromatography. Samples of DNA hydrolysates, pool materials or enzyme reactions, and reference standards, were applied to either thin-layer cellulose sheets (Eastman Chromogram Sheet 6064, without fluorescent indicator) or to Whatman 40 SFC grade paper. Chromatograms (descending development for paper) were developed with the following solvents: (i) (2), 2-propanol-concentrated HCl-water (65:17:18, vol/vol); (ii) (8), 2-propanol-concentrated NH_4OH -water (70:10:20, vol/vol); (iii) (3), *t*-butanol-methyl-ethylketone-concentrated HCl-water (40:30:10:20, vol/vol); (iv) (21), 600 g of $(\text{NH}_4)_2\text{SO}_4$ was dissolved in 1 liter 0.1 M phosphate, pH 6.8, the solution was filtered through Whatman no. 1 paper and mixed with 20 ml of *n*-propanol.

Measurement of radioactivity. (i) Chromatograms of DNA hydrolysates and enzyme reaction mixtures: the chromatograms were cut into sections after scanning for UV absorbing areas, and each section was transferred to a scintillation vial. (ii) Intracellular acid-soluble material: at specific time intervals, 3-ml culture samples were filtered through membrane filters (0.45- μm pore size; Millipore Corp.); each membrane was washed twice with 5 ml of $0.01 \times$

MB, then transferred immediately to 5 ml of ice-cold 0.5 N HCl in a 250-ml beaker. After 1 h on ice, the supernatant fluid was removed, passed through a membrane filter (0.45- μm pore size; Millipore Corp.), and the filtrate was evaporated to dryness. The residue was dissolved in 0.2 ml of 6N HCl and hydrolysis, chromatography, and measurement of radioactivity was carried out as described for DNA. (iii) Acid-soluble material: at specific time intervals, 3-ml culture samples were mixed with an equal volume of ice-cold 10% trichloroacetic acid. After 1 h on ice, the precipitates were collected by centrifugation for 20 min at $10,000 \times g$ and 4 C, resuspended in 0.3 N KOH, and incubated at 37 C for 24 h. The solutions were chilled, neutralized with 6 N HCl, then mixed with an equal volume of ice-cold 10% trichloroacetic acid. The precipitate was collected by centrifugation as before, resuspended in 1 N HCl, placed at 90 C for 25 min, and then placed on ice for 2 h. The precipitate remaining was removed on a membrane filter (0.45- μm pore size; Millipore Corp.); the filtrate was evaporated to dryness, dissolved in 0.2 ml of 6 N HCl, and hydrolysis and chromatography were carried out as described for DNA.

After transfer of the samples to scintillation vials, 5 ml of scintillation fluid (Liquifluor, New England Nuclear Corp.) was added and the radioactivity was measured with an Isocap/300 liquid scintillation spectrometer (Nuclear Chicago Corp., Des Plaines, Ill.).

Chemicals. Bases and nucleotides were from Calbiochem (San Diego, Calif.); tetrahydrofolic acid (THFA) was from General Biochemicals (Chagrin Falls, Ohio); other biochemicals were from Sigma Chemical Co. (St. Louis, Mo.). Synthetic putThy was a gift from A.M.B. Kropinski.

Radiochemicals. Radiochemicals were from New England Nuclear Corp. (Boston, Mass.) except for dUMP-5- ^3H , which was from Schwarz/Mann (Orangeburg, N.Y.).

RESULTS

Preliminary studies. The putThy, cytosine, and thymine residues of progeny $\phi\text{W-14}$ DNA were labeled after infection of strain 29 growing in a medium containing radioactive uracil. Adequate separation of these pyrimidines was obtained by using the chromatography systems described in Materials and Methods (see ref. 8). Strain 29 DNA from cultures grown with radioactive uracil yielded only labeled cytosine and thymine.

The conversion of host cells to infected centers was determined under the normal experimental conditions: MOI 10 and a cell density of about 3×10^6 /ml. At 4 min after infection more than 95% of the cells were infective centers, but some 10% of the cells were still colony-forming units. At 10 min after infection >99% of the cells were infective centers and <1% were colony-forming units.

Synthesis of ϕ W-14 DNA in a thymidine auxotroph. Strain 3L, an obligate thymidine auxotroph (6), was used to examine the biosynthetic relationship of thymine and putThy.

Surprisingly, ϕ W-14 produced progeny on strain 3L in the absence of thymidine, an indication that a phage-specific thymidylate synthetase appeared in the infected cell. Uracil-2- 14 C labeled thymine and putThy in the DNA of phage grown on strains 29, and on 3L supplemented with thymidine (Table 2). Furthermore, the ratios of thymine-cytosine and putThy-thymine remained virtually constant, regardless of the host strain, indicating the preferential incorporation of de novo dTMP rather than that formed from added thymidine. This was an unfortunate complication because it meant that the labeling of phage DNA with thymidine would be marginal. However, although the amount of labeled thymidine incorporated into ϕ W-14 DNA was very small, it was sufficient to show that putThy was not derived from thymine (Table 3). Strain 3L/FU, a derivative of 3L selected for resistance to 5-fluoroura-

cil (5-FU), was used in the experiment with thymidine-6- 3 H. This compound was contaminated with uracil. Strains resistant to 5-FU cannot incorporate uracil (15).

Deoxyuridine can be converted to dTMP by the sequential action of thymidine kinase and thymidylate synthetase (15). Deoxyuridine was more effective than thymidine for labeling ϕ W-14 DNA (again using 3L/FU because the deoxyuridine was significantly contaminated with uracil) and putThy was also heavily labeled (Table 4).

Therefore, putThy was synthesized from a deoxynucleotide independently of thymine.

Role of tetrahydrofolate in putThy biosynthesis. The methyl group of thymine and the hydroxymethyl group of hydroxymethyluracil both arise by transfer of a C_1 unit from N_5, N_{10} -methylene-THFA to the C-5 of dUMP. The C-3 of serine is the source of the methylene group of N_5, N_{10} -methylene-THFA. When ϕ W-14 was grown on strain 29 in the presence of serine-3- 14 C, both the thymine and putThy of progeny phage DNA were labeled (Table 5). Therefore, the methylene bridge between the pyrimidine and putrescine moieties of putThy was derived from a C_1 unit.

It was possible, however, that N_{10} -formyl-THFA, not N_5, N_{10} -methylene-THFA, was the C_1 donor for the methylene of putThy. The resultant 5-formyldUMP might react readily with putrescine. However, when ϕ W-14 was grown on strain 29 in the presence of serine-2,3-

TABLE 2. Labeling of bacteriophage ϕ W-14 and host DNAs with uracil-2- 14 C^a

Strain	Base ^b	Radioactivity in uninfected host DNA ^c	Radioactivity in isolated progeny phage DNA ^d	Ratios in phage DNA	
				T/C	PutThy/T
Wild-type	Adenine	40	46	0.42	0.86
	Guanine	27	33		
	Cytosine	5,276	6,789		
	Thymine	2,387	2,877		
	PutThy	26	2,451		
3L	Adenine	44	40	0.42	0.83
	Guanine	34	39		
	Cytosine	8,822	7,844		
	Thymine	38	3,492		
	putThy	32	2,759		

^a Wild-type and strain 3L were grown in M29 (250 μ g of dT/ml was added to the 3L culture) to 3×10^8 cells/ml, then uracil-2- 14 C was added (0.1 μ mol/ml; 0.25 μ Ci/ml). Each culture was divided into two portions and 10 min later one portion of each was infected with bacteriophage ϕ W-14 at an MOI of 10. At 65 min a sample of each uninfected culture was transferred to 10% trichloroacetic acid on ice. Infected cultures were incubated until lysis was complete (approximately 120 min). Analyses of incorporated label were carried out as described in Materials and Methods.

^b Bases were separated by thin layer chromatography with solvent A.

^c Values are for alkali-resistant, acid-insoluble material and are expressed as counts per minute in the section cut from the chromatogram.

^d Values are for isolated DNA and are expressed as for the uninfected host.

TABLE 3. Labeling of bacteriophage ϕ W-14 deoxyribonucleic acid with thymidine-2- 14 C and thymidine-6- 3 H^a

Radioactive compound added	Radioactivity ^b in DNA bases of progeny phage				
	Adenine	Guanine	Cytosine	Thymine	PutThy
Thymidine-2- 14 C ^c	36	28	62	582	31
Thymidine-6- 3 H ^d	41	39	48	648	36

^a Cultures were grown in M29 plus 250 μ g of dT/ml to 3×10^8 cells/ml and then infected with bacteriophage ϕ W-14 at an MOI of 10. The labeled compounds were added at the time of the inoculation of the growth medium. After lysis, the progeny phage were collected and analyses of incorporated label was carried out as described in Materials and Methods.

^b Bases were separated by descending paper chromatography with solvent C. Values are expressed as counts per minute in the section cut from the chromatogram.

^c Thymidine-2- 14 C was added at 2.5 μ Ci/ml to a culture of strain 3L.

^d Thymidine-6- 3 H was added at 25 μ Ci/ml to a culture of strain 3L/FU.

TABLE 4. Labeling of bacteriophage ϕ W-14 DNA with deoxyuridine-2- 14 C^a

Base ^b	Radioactivity in DNA of progeny phage ^c	Ratio of putThy/T
Adenine	63	0.89
Guanine	61	
Cytosine	58	
Thymine	5,625	
PutThy	4,974	

^a Conditions were as described in Table 3, using strain 3L/FU as host. Deoxyuridine-2- 14 C was added at 2.5 μ Ci/ml.

^b Bases were separated by paper chromatography in solvent C.

^c Values are expressed as counts per minute in the section cut from the chromatogram.

3 H, the ratio label in putThy/label in thymine was the same as that observed for serine-3- 14 C (Table 5). This meant that the methyl of thymine and the methylene of putThy both came from N₆, N₁₀-methylene-THFA because the formation of N₁₀-formyl-THFA would result in the loss of a 3 H from the C₁ unit, and a halving of the labeling ratio. (The serine-2, 3- 3 H experiment was conceived and performed by Earl Karrer in this laboratory).

Synthesis of DNA during ϕ W-14 infection. The incorporation of uracil into alkali-resistant, acid-insoluble material was measured during the latent period of phage infection. DNA synthesis continued at the control rate for 12 to 14 min, slowed for approximately 6 min, and resumed at an increased rate after 18 to 20 min (Fig. 1). PutThy was not detected in alkali-resistant, acid-insoluble material before the resumption of DNA synthesis at 18 to 20 min.

Analysis of the nucleotide pools of phage-infected cells. The results so far had indicated that putThy was synthesized at the nucleotide level. Therefore, the acid-soluble pools of in-

fect cells were examined to see if putThy was present before the onset of phage DNA synthesis, phage-specific DNA being defined as that containing putThy.

Strain U₁ was chosen for the experiments because the label of choice was radioactive uracil and with strain U₁ added uracil would not be diluted by the uracil synthesized intracellularly via the de novo pathway.

Infection of strain U₁ in the presence of radioactive uracil, followed by extraction of the pool material, demonstrated the presence of putThy in the pool before it appeared in the DNA (Table 6).

Again the results showed that putThy was synthesized at the mononucleotide level, then incorporated into DNA. (The isolation and characterization of putThy nucleotides will be the subject of a separate communication).

Enzyme activities in infected cell extracts. Infection of *P. acidovorans* with ϕ W-14 apparently resulted in the synthesis of phage-specific enzymes which were involved in deoxynucleotide metabolism; e.g., thymidylate synthetase and an enzyme(s) for putThy deoxynucleotide synthesis.

Thymidylate synthetase was assayed spectrophotometrically in extracts from two infected and the corresponding uninfected strains (Table 7). Excellent activity was observed with the uninfected wild type; the infected wild-type extract gave comparable activity. This was somewhat surprising since the earlier in vivo studies had indicated the synthesis of a phage-specific thymidylate synthetase after infection, and an increase in total activity was anticipated in the infected extract. The uninfected strain 3L extract was inactive, as expected. The strain 3L phage-infected extract also appeared to be inactive, which again was surprising even though it agreed with the observation made with the wild-type infected extract.

The extracts were assayed by using dUMP-5-

TABLE 5. Labeling of bacteriophage ϕ W-14 deoxyribonucleic acid with serine-3- 14 C and serine-2,3- 3 H^a

Radioactive compound added	Radioactivity ^b in DNA bases of progeny phase					Ratio of putThy/T
	Adenine	Guanine	Cytosine	Thymine	PutThy	
Serine-3- 14 C	3,624	10,493	33	2,133	1,796	0.88
Serine-2,3- 3 H ^c	1,095	800	28	639	579	0.90

^a The wild-type was grown to 3×10^8 cells/ml in M29 supplemented with 20 μ g of serine, histidine and methionine/ml and 300 μ g of adenosine/ml. Radioactive serine was added at 0.125 μ Ci/ml, followed by the addition of bacteriophage ϕ W-14 at an MOI of 10. Cultures were incubated until lysis and the analyses of incorporated label were carried out as described in Materials and Methods.

^b Bases were separated by two dimensional thin layer chromatography: first dimension, solvent C; second dimension, solvent B. Values are expressed as counts per minute in the section cut from the chromatogram.

^c Data were obtained from E. Karrer, (personal communication).

FIG. 1 Deoxyribonucleic acid synthesis in uninfected and phage ϕ W-14 infected cells. *P. acidovorans* was grown in M29 to 3×10^8 cells/ml and then uracil-2- 14 C (0.1 μ mol/ml; 0.25 μ Ci/ml) was added. The culture was divided into two portions and one of them was infected with phage ϕ W-14 at an MOI of 10 after 10 min. At intervals, samples of infected and uninfected bacteria were transferred to an equal volume of 10% trichloroacetic acid on ice. RNA was removed and the radioactivity in alkali-resistant, acid-insoluble material was measured as described in Materials and Methods. Symbols: $\circ$, infected; $\blacktriangle$, uninfected.

3 H as substrate (Table 7). The spectrophotometric assay is limited in that any activity with dUMP as substrate can only be measured if the reaction results in oxidation of THFA; any nonoxidative reactions involving dUMP would not be detected. The tritium release assay would measure any reactions involving addition at the 5-position of dUMP with displacement of the tritium which were independent of THFA oxidation. The infected cell extracts were shown to be highly active when assayed in this manner;

TABLE 6. Labeling with uracil-2- 14 C of the pyrimidine bases in the nucleotide pools and of alkali-resistant, acid-insoluble material: uninfected and bacteriophage ϕ W-14 infected cultures of strain U₁^a

Base ^b	Min after infection	Radioactivity ^c in the areas cut from the chromatograms			
		Uninfected culture		Infected culture	
		Pool materials	Alkali-resistant acid-insoluble material	Pool materials	Alkali-resistant acid-insoluble material
Cytosine	12	1,924	12,418	5,896	16,089
	18	2,720	22,944	9,760	27,090
	24	2,262	44,953	11,950	41,437
PutThy	12	130	120	765	46
	18	123	82	1,175	178
	24	62	125	1,754	1,447

^a Strain U₁ was grown in M29 plus 15 μ g of uracil/ml to a cell density of 3×10^8 /ml. The culture was divided into two portions and uracil-2- 14 C was added to each (4 μ Ci/ml), immediately followed by the addition of bacteriophage ϕ W-14 at an MOI of 10 to one culture. Samples were removed at specific times and treated as described in Materials and Methods.

^b Bases were separated by paper chromatography with solvent C.

^c Values are expressed as counts per minute in the section cut from the chromatogram.

dUMP was obviously a substrate and the tritium release was THFA dependent (Table 7). However, the reaction(s) did not result in detectable oxidation of THFA. The biosynthesis of dHMCMP in T-even infections or of 5-hydroxymethyldeoxy UMP (dHUMP) in ϕ e infections is THFA-dependent, but the THFA is not oxidized to DHFA. Substantial tritium release was also observed with the uninfected wild type, whereas no activity was seen with strain 3L extract. The tritium release observed with the

TABLE 7. *Thymidylate synthetase activity in uninfected and bacteriophage ϕ W-14 infected cells^a*

Extract	Sp. act. ^b		
	Spectrophotometric assay	Tritium release assay	
		Complete reaction mixture	Minus THFA ^c
Wild-type, uninfected	0.33	0.28	0
Wild-type, infected	0.30	0.80	0
3L, uninfected	0	0	0
3L, infected	0	1.0	0

^a Extracts were prepared as described in Materials and Methods. For infected extracts, bacteriophage ϕ W-14 was added at an MOI of 10 and the infective centers collected after 20 min of incubation.

^b Expressed as nanomoles of DHFA formed per minute per milligram of protein for the spectrophotometric assay and nanomoles of dUMP converted per minute per milligram of protein for the tritium release assay.

^c Water was substituted for THFA.

wild-type extract was assumed to be the result of thymidylate synthetase activity. The reaction rate assayed by tritium release was 85% of that observed spectrophotometrically; a reduction of similar magnitude was reported for *Lactobacillus casei* (4) and *E. coli* (11) and attributed to an isotope effect.

Infected cell extracts mediated a THFA-dependent release of tritium with dUMP-5-³H as substrate without detectable oxidation of THFA. Since in vivo experiments had indicated that putThy was formed at the mononucleotide level, infected cell extracts were assayed for the conversion of dUMP to other deoxynucleotides.

A nonenzymatic reaction between formaldehyde and THFA generates N₅,N₁₀-methylene-THFA. Since tritium release from dUMP-5-³H was THFA dependent, it seemed reasonable to assume that any nucleotide formed could be effectively labeled by radioactive formaldehyde via the THFA cofactor.

Assays of infected and uninfected 3L extracts under these conditions strongly indicated a phage-specific thymidylate synthetase: the infected extract produced labeled dTMP but the uninfected extract did not (Table 8). Furthermore, a significantly greater amount of label appeared to be associated with dUMP. In solvent system D, dHMUMP has the same relative mobility as dUMP (15). The dUMP areas of the chromatograms were excised and the nucleotide(s) was eluted with very dilute HCl. The solutions were evaporated to dryness, the residues were dissolved in 6 N HCl, and were hydrolyzed

as described for DNA. The hydrolysates were evaporated to dryness, the residues were dissolved in water, and were cochromatogrammed with uracil and 5-hydroxymethyluracil on cellulose sheets by using solvent A in the first and solvent C in the second dimension (see ref. 8). All the radioactivity was associated with the 5-hydroxymethyluracil.

DISCUSSION

The results presented in this investigation show that putThy is synthesized via the fourth of the four possible routes considered in the introduction i.e., it is formed at the mononucleotide level and incorporated into DNA during replication.

DNA is synthesized from five rather than four bases in other phage systems. When ϕ e mutants deficient in phage-specific dTTPase are propagated on thymine auxotrophs of *Bacillus subtilis* growing with thymine, some 20% of the hydroxymethyluracil residues of the progeny phage DNA are replaced with thymine without deleterious effects on the phage (13). Mutants of T4 lacking dCTPase can produce progeny in which some 20% of the hydroxymethylcytosine residues are replaced with cytosine, but the particles promote inefficient late protein synthesis, in spite of generating almost normal levels of phage DNA (10). It is unlikely, however, that ϕ W-14 is simply a defective strain like these mutants of ϕ e and T4 because (i) the ratio of putThy to thymine in the DNA is constant, and (ii) phage infection results in an increase in thymidylate synthetase activity and in the appearance of dUMP hydroxymethylase activity.

TABLE 8. *Incorporation of radioactive formaldehyde into deoxynucleotide monophosphates in bacteriophage ϕ W-14 infected cells^a*

Extract	Radioactivity ^b in area corresponding to	
	dTMP	dUMP ^c
3L, infected	508	2994

^a Extracts were as described in Table 7.

^b Expressed as counts per minute per milligram of protein. Uninfected 3L was used to prepare a control extract. The counts from a reaction mixture containing this extract were subtracted from those given by a mixture containing infected 3L extract.

^c With solvent system D, the position of radioactive dHMUMP is the same as dUMP and the latter (R_f = 0.59 compared with 0.47 for dTMP) was easily detected in ultraviolet light.

The formation of dHMUMP in infected extracts agrees with the *in vivo* data using serine-2,3- 3H as label, which indicates that the biosynthesis of putThy proceeds through a 5-hydroxymethyldeoxyuridine nucleotide intermediate rather than a 5-formyl intermediate. Also, the addition of a hydroxymethyl group to the 5-position of dCMP or dUMP does not result in the oxidation of THFA, which would explain the inability to detect any activity by the spectrophotometric procedure.

The level of thymidylate synthetase activity measured in 3L-infected extracts by the radioactive formaldehyde assay was rather low, and this level would be difficult to detect with the spectrophotometric assay (see Table 7).

The synthesis of ϕ W-14 DNA from five bases makes the distribution of putThy within the DNA very important. It is present in both strands of the DNA, probably to an almost equal extent (8). Experiments are in progress to determine the distribution of putThy within ϕ W-14 DNA.

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