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Biochemical Evidence for the Existence of Thymidylate Synthase in the Obligate Intracellular Parasite *Chlamydia trachomatis*

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Since eucaryotic cell-derived thymidine or thymidine nucleotides are not incorporated into *Chlamydia trachomatis* DNA, we hypothesized that *C. trachomatis* must obtain dTTP for DNA synthesis by converting dUMP to dTMP. In most cells, this reaction is catalyzed by thymidylate synthase (TS) and requires 5,10-methylenetetrahydrofolate as a cofactor. We used *C. trachomatis* serovar L_2 and a mutant CHO K_1 cell line with a genetic deficiency in folate metabolism as a host for chlamydial growth. This cell line lacks a functional dihydrofolate reductase (DHFR) gene and, as a result, is unable to carry out de novo synthesis of dTTP. *C. trachomatis* inclusions form normally when $DHFR^-$ cells are starved for thymidine 24 h prior to and during the course of infection. When [6-^{3}H]uridine is used as a precursor to label *C. trachomatis*-infected CHO $DHFR^-$ cells, radiolabel is readily incorporated into chlamydia-specific DNA. When DNA from [6-^{3}H]uridine-labelled infected cultures is acid hydrolyzed and subjected to high-performance liquid chromatography analysis, radiolabel is detected in thymine and cytosine nucleobases. By using the $DHFR^-$ cell line as a host and [5-^{3}H]uridine as a precursor, we could monitor intracellular *C. trachomatis* TS activity simply by following the formation of tritiated water. There is a good correlation between in situ TS activity and DNA synthesis activity during the chlamydial growth cycle. In addition, both *C. trachomatis*-specific DNA synthesis and ^3H_2O release are inhibited by exogenously added 5-fluorouridine but not by 5-fluorodeoxyuridine. Finally, we demonstrated in vitro TS activity in crude extracts prepared from highly purified *C. trachomatis* reticulate bodies. The activity is dependent on the presence of methylenetetrahydrofolic acid and can be inhibited with 5-fluoro-dUMP. Taken together, these results indicate that *C. trachomatis* contains a TS for the synthesis of dTMP.

Chlamydia is a unique genus of gram-negative bacteria consisting of three species, *Chlamydia trachomatis*, *C. psittaci*, and *C. pneumoniae*. These organisms are important pathogens which have been linked to an expanding spectrum of human and animal diseases (4, 20). Chlamydiae have evolved a highly specialized life cycle characterized by obligatory intracellular parasitism of eucaryotic cells. Two different cell types, the infectious elementary body (EB) and the replicating noninfectious reticulate body (RB), alternate within the life cycle (14, 20, 21). Chlamydiae require the eucaryotic intracellular environment in order to obtain ATP and other metabolic intermediates for growth and replication (14, 21, 31). Our laboratory is particularly interested in the question of how chlamydiae obtain the four deoxyribonucleoside triphosphate (dNTP) precursors for DNA synthesis. Results accumulated from several studies indicate that host-derived thymidine or thymidine nucleotides or both are either not incorporated or incorporated poorly into the DNA of intracellular chlamydiae (1, 6, 11, 24, 29). In addition, the work of Hatch (6) has shown that *C. psittaci* lacks detectable thymidine kinase activity.

Recently, we reported that intracellular *C. trachomatis* readily incorporates medium-supplied ribonucleosides into DNA but that deoxyribonucleosides were not utilized (13). These findings led us to hypothesize that *C. trachomatis* lacks a transport system(s) for dNTPs and, as a result, must synthesize DNA precursors directly from host-supplied ribonucleotide triphosphate (NTP). In support of this hypothesis, we have recently presented evidence that *C. trachomatis* does contain a ribonucleotide reductase which reduces the four NTPs to their corresponding dNTPs (27). Ribonucleotide reductase alone is capable of directly supplying three (dATP, dGTP, and dCTP) of the four dNTPs required (16, 26). However, to meet the requirement for dTTP, the deoxyuridine phosphate generated by ribonucleotide reductase must be converted to thymidine phosphate. In all cells studied to date, a single enzyme, thymidylate synthase (TS; EC 2.1.1.45), catalyzes the synthesis of dTMP from dUMP (8, 12).

TS catalyzes the conversion of dUMP and 5,10-methylenetetrahydrofolate (CH_2-H_4folate) to dTMP and 7,8-dihydrofolate (H_2folate). Dihydrofolate is reduced to 5,6,7,8-tetrahydrofolate (H_4folate) by dihydrofolate reductase (DHFR). The third reaction of the dTMP synthesis cycle is catalyzed by serine hydroxymethyltransferase, which converts H_4folate and serine to CH_2-H_4folate and glycine. Blockade of the TS cycle causes depletion of dTMP, cessation of DNA synthesis, and resultant "thymineless death" of cells (7). Because of the central role of TS in the synthesis of thymidine and its importance as a potential chemotherapeutic target, the enzyme has been studied in detail (8, 12). In this report, we present biochemical evidence for the existence of TS in *C. trachomatis*.

MATERIALS AND METHODS

Chemicals. Eagle's minimum essential medium and RPMI 1640 were purchased from GIBCO (Grand Island, N.Y.). [2-^{3}H]adenine (28 Ci/mmol), 5-[methyl-^{3}H]thymidine (65 Ci/mmol), [6-^{3}H]uridine (20 Ci/mmol), [5-^{3}H]uridine (21 Ci/mmol), and [6-^{3}H]dUMP (15 Ci/mmol) were purchased from Moravek Biochemicals (Brea, Calif.). Other chemicals were purchased from Sigma Chemical Company (St. Louis, Mo.).

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Cell lines. The mutant CHO K_1 subline deficient in DHFR cells was kindly provided by R. Johnson (30). The wild-type (GC_3C_1) and thymidine kinase-deficient human (GC_3 TK^-) cell lines were generously supplied by P. Houghton (18).

Mouse L cells were used as host cells for preparing both EB stock and logarithmically growing RBs. The mouse L cells were routinely cultured in suspension with minimum essential medium supplemented with 10% fetal bovine serum and 0.2 mM L-glutamine. CHO $DHFR^-$ cells were maintained as monolayer cultures in the same medium containing 10% fetal bovine serum, 0.3 mM proline, 0.3 mM glycine, 30 μM hypoxanthine, and 30 μM thymidine. Human GC_3C_1 and GC_3 TK^- cells were grown as monolayers in RPMI 1640 medium supplemented with 10% fetal bovine serum. For all precursor incorporation studies and assays of TS activity in situ, dialyzed serum was used, and, in the case of CHO $DHFR^-$ cells, thymidine was omitted from the medium unless otherwise indicated.

Preparation of chlamydial EB stock. The lymphogranuloma venereum L_2/454/Bu strain of *C. trachomatis* was grown in suspension culture of mouse L cells, as previously described (28). *C. trachomatis* EBs were harvested at 44 h postinoculation. EB stocks were titrated in mouse L-cell monolayers grown on 1-cm^2 coverslips, which were then fixed at 40 h postinoculation and stained with fluorescent-labelled monoclonal antibody (Syva Company, Palo Alto, Calif.). Inclusions were counted under a fluorescent microscope and inclusion-forming units were calculated. EB aliquots were stored at −70°C.

In situ incorporation of radiolabelled precursors into *C. trachomatis* nucleic acids. CHO K_1 cells, CHO $DHFR^-$ cells, human wild-type cells, or human TK^- cells were seeded into 60-mm-diameter disposable tissue culture dishes (Corning Glass Works) and cultured at 37°C in a humidified atmosphere of 5% CO_2 in air. Confluent monolayers formed within 24 to 48 h. Monolayers were inoculated with L_2 EBs at a multiplicity of infection of 3 inclusion-forming units per cell. After 1.5 h of adsorption, 5 ml of medium containing 1 μg of cycloheximide per ml was added into each dish. Dishes containing infected cells were incubated at 37°C as above. At specified times postinoculation, all but 2 ml of medium was removed. Radiolabelled precursor was added, without dilution, to yield a final concentration of 0.3 μM. Incubation in the presence of the isotope was continued for 2 h. In all cases, incorporation of label into nucleic acid remained linear beyond 2 h, and at no time was the isotope limiting in the experiments. To terminate incubations, the dishes were transferred immediately to an ice bath, medium was aspirated, and monolayers were rinsed 3 times with ice-cold phosphate-buffered saline (PBS). The cell monolayer was dissolved in 2 ml of 0.3 N NaOH and then incubated at 37°C for 16 h to degrade RNA. To determine radiolabel incorporation into DNA, the DNA was precipitated from the NaOH-solubilized sample by adding 5 ml of 10% trichloroacetic acid (TCA) prepared in 0.1 M tetrasodium pyrophosphate, and then the samples were incubated at 4°C for 2 h. The precipitate was collected by filtration through Whatman GF/B glass microfiber filters, and, after washing with 10% TCA and then with ethanol, the radioactivity in the dried filters was counted in 5 ml of scintillation cocktail with a liquid scintillation counter (Beckman LS 5000). In all cases, the values obtained for mock-infected control cultures were subtracted from the values obtained for *C. trachomatis*-infected cultures. All analyses were made with duplicate dishes, with results varying by less than 10%. Unless otherwise indicated, values are normalized to 10^6 cells. For *C. trachomatis*-infected cultures, the percent infection was monitored by light microscopy and experiments were not performed unless 90 to 100% of the cells were infected. Under these circumstances, the term 10^6 cells is taken to mean approximately 10^6 infected cells.

Acid hydrolysis of DNA and subsequent nucleobase analysis. In order to determine which nucleobases were labelled in DNA isolated from cultures pulsed with [5-^{3}H]uridine or [6-^{3}H]uridine, the following procedure was carried out. CHO $DHFR^-$ cell monolayers were prepared and infected as described above. At 22 h postinoculation, [5-^{3}H]uridine or [6-^{3}H]uridine was added without dilution to achieve a final concentration of 0.3 μM, and incubation was continued at 37°C for 2 h. Reactions were terminated on ice, medium was aspirated, and the cell monolayer was washed 3 times with ice-cold PBS. DNA was isolated as previously described (22). Briefly, protein was digested with proteinase K, samples were phenol extracted 3 times, and then nucleic acid was precipitated with ethanol. RNA was degraded with 0.3 N NaOH, and the DNA was precipitated with 10% TCA. The resulting DNA was hydrolyzed to free bases by boiling in 11.3 N perchloric acid for 1 h. The acid-hydrolyzed samples were neutralized with NaOH and subjected to high-performance liquid chromatography (HPLC) analysis. Isotope incorporation into nucleobases was measured by on-line radioactive flow detection (Beckman 171 radiodetector) after separation of the nucleobases by HPLC on a 12.5-cm μBondapak C18 column (Whatman) under isocratic conditions (flow rate, 1 ml/min with 100 mM ammonium acetate [pH 4.25]–0.5% acetonitrile buffer) (3). The identity of the radioactive peaks was confirmed by simultaneously monitoring the A_{254} (Shimadzu SPD 2A UV wavelength detector) of known nucleobase standards. All data were plotted and processed with an IBM PC50 and Beckman System Gold software.

Assay of TS activity in situ. Intracellular *C. trachomatis*-specific TS activity was measured in infected CHO $DHFR^-$ cells by monitoring the amount of tritium transferred to water when uridine labelled in the 5 position of the pyrimidine ring was used as a precursor (15, 17). The amount of isotope that transferred to water was used to measure the synthesis of dTMP from uridine phosphates via dUMP. CHO $DHFR^-$ cell monolayers were prepared and infected as described above. At specific times postinoculation, [5-^{3}H]uridine was added, without dilution, into 2 ml of medium to yield a final concentration of 0.3 μM. After incubation at 37°C for the specified times, 100 μl of the medium was removed and treated with a 500-μl suspension of 10% (wt/vol) activated charcoal to remove the radiolabelled uridine and its derivatives (15, 17). The mixture was kept at room temperature for 1 h and then centrifuged to pellet the charcoal. The radioactivity in a 120-μl sample of the supernatant was counted after 5 ml of liquid scintillation cocktail was added. TS activity was expressed as disintegrations per minute of ^3H_2O released per 10^6 host cells.

Effect of 5-fluorouridine and 5-fluorodeoxyuridine on intracellular *C. trachomatis* DNA synthesis and TS activity. To determine the effects of 5-fluorouridine (5-FUR) and 5-fluorodeoxyuridine (5-FdUrd) on in situ *C. trachomatis* DNA synthesis and TS activity, various concentrations of the inhibitor were added to cell culture medium at 22 h postinoculation. Then, the plates were returned to 37°C and, 2 h later, [5-^{3}H]uridine or [6-^{3}H]uridine was added to monitor in situ TS activity and DNA synthesis, respectively. After a further 2 h of incubation at 37°C, incubations were termi-

nated and in situ TS activity and DNA synthesis were measured as described above.

Preparation of RB extracts. Suspension cultures of mouse L cells were infected as previously described (28). At 24 h postinfection, cells were collected by centrifugation (500 × *g* for 10 min) and resuspended in cold Hanks' balanced salt solution. Cell suspensions, bathed in ice water, were disrupted by sonication with one 1-min pulse at an intensity setting of 3 (sonifier; Branson). The sonicate was clarified by centrifugation (500 × *g* for 10 min) at 4°C. The resulting supernatant was layered onto a Renografin density gradient consisting of 15 ml of 30% and 10 ml of 44% Renografin in 100 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [pH 7.0]) and then centrifuged for 60 min at 43,000 × *g* in a Beckman SW28 rotor. RBs at the 30 and 44% Renografin interface were collected, diluted in 3 volumes of cold 10 mM potassium phosphate buffer (pH 7.0) containing 0.1 M KCl, and then pelleted by centrifugation at 33,000 × *g* for 30 min. The RB pellet was resuspended in 10 mM potassium phosphate buffer (pH 7.0) containing 0.1 M KCl and 2 mM dithiothreitol, and then the suspension was sonicated in an ice-water bath with 10 10-s bursts, with a microprobe at intensity 5. The resulting sonicate was clarified by centrifugation at 18,000 × *g* for 15 min at 4°C. The RB lysate was immediately used as the crude enzyme preparation for TS assays or was stored at −70°C for later use.

Purified sham extracts were prepared from mock-infected mouse L cells by the same procedure used to purify RBs from infected mouse L cells.

Assay of TS activity in vitro. TS was assayed by using previously described methods (10) with the following modifications. CH_2-H_4folate was prepared prior to each experiment by dissolving H_4folate, final concentration 1 mM, in 100 mM Tris-HCl buffer (pH 7.8) containing 100 mM 2-mercaptoethanol and 10 mM formaldehyde. The CH_2-H_4folate synthesis reaction was allowed to proceed in the dark at room temperature for 20 min. The final TS assay mixture contained, in a total volume of 100 μl, 50 μl of CH_2-H_4folate preparation and a predetermined amount of crude RB extract. The reaction was initiated by the addition of dUMP (final concentration, 50 μM with 40 μCi of [6-^{3}H]dUMP per ml) and then allowed to proceed at 37°C for the specified time. The reaction was terminated by adding ice-cold TCA (final concentration, 10%), and then the tubes were placed on ice for 30 min. After centrifugation to remove precipitated material, the supernatant was neutralized by extraction with 1.1 volumes of tri-n-octylamine–freon (9). The neutralized extract was analyzed by HPLC. The radioactive dTMP product was measured by on-line radioactive flow detection after separation of dTMP product from dUMP substrate by HPLC on a 12.5-cm μBondapak C18 column under isocratic conditions (flow rate, 1 ml/min, with 10 mM potassium phosphate buffer [pH 4.0]). The identity of the radioactive peaks were confirmed by simultaneously monitoring the A_{254} of known dTMP and dUMP standards. Data were plotted and analyzed with Beckman System Gold software. TS assay background controls included boiled RB extracts and mock-infected mouse L cell extracts.

RESULTS

Failure of medium-supplied thymine or thymidine to serve as a precursor for *C. trachomatis* DNA. Results of experiments to measure the incorporation of exogenously added [^{3}H]thymidine into the DNA of logarithmically growing, mock- and *C. trachomatis*-infected wild-type and TK$^-$ human cells are shown in Table 1. Thymidine was readily incorporated into the DNA of logarithmically growing wild-type cells; however, in keeping with the phenotype, [^{3}H]thymidine did not label TK$^-$ cell DNA. Mock-infected cultures (confluent monolayers in the presence of 1 μg of cycloheximide per ml) are not synthesizing DNA (1, 13, 25) and, as a result, do not incorporate significant amounts of [^{3}H]thymidine. *C. trachomatis*-infected cultures incorporate essentially the same amount of [^{3}H]thymidine as do mock-infected controls. [^{3}H]thymine was not utilized to any significant extent by logarithmically growing, mock- or *C. trachomatis*-infected wild-type or TK$^-$ cells. To verify that the cultures are infected and that *C. trachomatis* is growing, we monitored [^{3}H]adenine incorporation into DNA. *C. trachomatis*-infected cultures, both wild type and TK$^-$, incorporate approximately 18 and 10 times more adenine, respectively, than do mock-infected controls.

TABLE 1. Incorporation of thymine, thymidine, and adenine into host and *C. trachomatis* DNA in wild-type and TK$^-$ cells

Cell line	Status[a]	^{3}H-labelled precursor[b]		
		Thymine	Thymidine	Adenine
GC_3C_1 (TK$^+$)	Log growing	5.1	2912	365
	Mock infected	1.7	81	10
	Infected	1.7	69	180
GC_3 TK$^-$	Log growing	0.7	5.9	260
	Mock infected	0.8	6.1	11
	Infected	0.8	4.8	120

[a] Wild-type human GC_3C_1 (TK$^+$) and mutant TK$^-$ cells were either logarithmically growing (1.0 × 10^6 cells per plate cultured in the absence of cycloheximide) or mock- or *C. trachomatis*-infected confluent monolayers (3.0 × 10^6 cells per plate, cultured in the presence of 1 μg of cycloheximide per ml). For details, see Materials and Methods.

[b] The various ^{3}H-labelled precursors were added to achieve a final concentration of 0.3 μM. Then, incubation was continued for 2 h, and the incorporation of precursor into DNA was determined as described in Materials and Methods. *C. trachomatis*-infected cultures were labelled at 24 h postinfection. All analyses were made with duplicate dishes, and results varied less than 10%. Results are expressed in 10^3 dpm per 10^6 cells.

Growth of *C. trachomatis* in DHFR$^-$ CHO cells. The DHFR$^-$ cell line is genetically deficient in the DHFR gene and, as a result, cannot reduce H_2folate to H_4folate (30). Since CH_2-H_4folate is a required cofactor for TS, DHFR$^-$ cells are unable to synthesize dTMP from dUMP and, consequently, are auxotrophic for thymidine. In order to determine if exogenously added thymidine had an effect on *C. trachomatis* growth in DHFR$^-$ cells, we monitored chlamydial replication by two parameters, inclusion development and *C. trachomatis*-specific DNA synthesis activity. In one case, DHFR$^-$ cells were infected with *C. trachomatis* EBs and then incubated with minimum essential medium supplemented with 10% fetal bovine serum, 30 μM thymidine, 30 μM hypoxanthine, 0.3 mM glycine, 0.3 mM proline, and 1 μg of cycloheximide per ml (i.e., complete medium). Under the second condition, DHFR$^-$ cells were starved for thymidine for 24 h prior to infection and then, following inoculation with EBs, the cultures were incubated in complete medium minus thymidine. For measurements of DNA synthesis activity, cultures were labelled with [^{3}H]adenine for 2 h at 24 h postinfection. Chlamydial growth was also assessed at 40 h postinfection by staining infected monolayers for the presence of *C. trachomatis* inclusions. Results from these experiments clearly show that exogenous thymi-

TABLE 2. Effect of exogenous thymidine on the growth of *C. trachomatis* in DHFR$^-$ cells

Medium supplemented with thymidine[a]	DNA synthesis[b]		Inclusions/ coverslip[c]
	Mock infected	Infected	
Yes	1.3	105	20,100
No	1.0	113	19,750

[a] CHO DHFR$^-$ cells were cultured in either the presence or absence of 30 μM thymidine for 24 h prior to and during infection. See text for details.

[b] The effect of exogenous thymidine on DNA synthesis was assessed by measuring [^{3}H]adenine (final concentration of 0.3 μM) incorporation into DNA. CHO DHFR$^-$ cells were either mock- or *C. trachomatis*-infected confluent monolayers (3.0×10^6 cells per plate, cultured in the presence of 1 μg of cycloheximide per ml). For details, see Materials and Methods. All analyses were made with duplicate dishes, and results varied less than 10%. Results are expressed in 10^3 dpm per 10^6 cells.

[c] The effect of exogenous thymidine on *C. trachomatis* inclusion formation was assessed at 40 h postinfection by fluorescent staining. For details, see Materials and Methods. All analyses were made with duplicate dishes, and results varied less than 10%.

dine has little or no effect on *C. trachomatis* growth, as assessed by either parameter (Table 2).

Incorporation of radiolabelled uridine into *C. trachomatis* DNA. The results presented above indicate that *C. trachomatis* does not utilize host-supplied thymidine or thymidine nucleotides and, therefore, suggest that the parasite must synthesize thymidine de novo from uridine. To address this question, we used [5-^{3}H]uridine or [6-^{3}H]uridine of approximately equal specific activity as precursor to label DNA of *C. trachomatis*. For these experiments, we again employed the CHO DHFR$^-$ cells as a host. The isotope was introduced as shown schematically in Fig. 1. Inside the cell, the nucleoside is rapidly phosphorylated to uridine phosphates and, subsequently, cytidine phosphates can be formed. Pyrimidine deoxyribonucleotides are formed by reduction of UDP and CDP. When [5-^{3}H]uridine is used as a precursor, deoxycytidine phosphates are labelled but thymidine phosphates are not, since isotope is lost from the pyrimidine ring and recovered as ^3H_2O in the medium during the formation of dTMP. In contrast, when [6-^{3}H]uridine is used as a

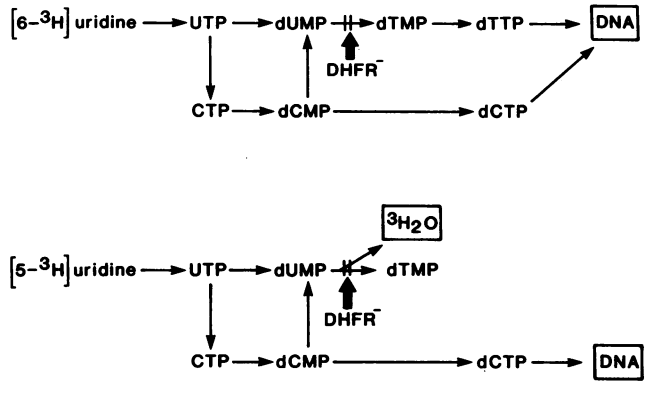

FIG. 1. Schematic diagram of the metabolism of [6-^{3}H]uridine and [5-^{3}H]uridine in *C. trachomatis*-infected CHO DHFR$^-$ cells. End products that accumulate the isotope are shown in boxes. Radiolabel is incorporated into DNA from [5-^{3}H]uridine via dCTP and from [6-^{3}H]uridine via both dCTP and dTTP. CHO DHFR$^-$ cells cannot convert dUMP to dTMP because they lack DHFR, which is required for the generation of CH_2-FH_4folate, the necessary cofactor for the TS reaction. This metabolic deficiency is indicated by a heavy arrow.

TABLE 3. Incorporation of radiolabelled uridine into *C. trachomatis* DNA in DHFR$^-$ cells

CHO cell line	Status[a]	Radiolabelled precursor[b]	
		[5-^{3}H]uridine	[6-^{3}H]uridine
K_1	Log growing	71	200
DHFR$^-$	Log growing	150	142
	Mock infected	0.3	0.7
	Infected	41	120

[a] CHO K_1 cells were logarithmically growing (1.0×10^6 cells per plate cultured in the absence of cycloheximide). DHFR$^-$ cells were either logarithmically growing (1.0×10^6 cells per plate cultured in the absence of cycloheximide) or mock- or *C. trachomatis*-infected confluent monolayers (3.0×10^6 cells per plate cultured in the presence of 1 μg of cycloheximide per ml). For details, see Materials and Methods.

[b] [5-^{3}H]uridine or [6-^{3}H]uridine was added to culture medium to achieve a final concentration of 0.3 μM, and then incorporation into DNA was determined as described in Materials and Methods. All analyses were made with duplicated dishes, and results varied less than 10%. Results are expressed in 10^3 dpm per 10^6 cells.

precursor, all pyrimidine deoxyribotides are labelled, because the isotope is retained with the pyrimidine ring. Logarithmically growing wild-type CHO K_1 cells readily incorporate both [5-^{3}H]uridine and [6-^{3}H]uridine into DNA, with [6-^{3}H]uridine labelling more efficiently. Since DHFR$^-$ cells lack H_4folate and its derivatives (CH_2-H_4folate, CH_3-H_4folate, CHO-H_4folate, etc.), they are unable to synthesize thymidine from uridine. As a result, both [5-^{3}H]uridine and [6-^{3}H]uridine label the DNA of logarithmically growing DHFR$^-$ cells to about the same extent (Table 3). High levels (100 to 200 times that of mock-infected controls) of incorporation occurred when *C. trachomatis*-infected cultures were pulsed with [5-^{3}H]uridine or [6-^{3}H]uridine, with [6-^{3}H]uridine being more effective at labelling DNA. A possible explanation for this result is that *C. trachomatis* contains a TS, and, as a result, [6-^{3}H]uridine gives rise to both labelled deoxycytidine and thymidine phosphates, whereas [5-^{3}H]uridine yields only labelled deoxycytidine phosphates.

We addressed this question by examining the base content of DNA isolated from infected and mock-infected DHFR$^-$ cells that had been labelled with either [6-^{3}H]uridine or [5-^{3}H]uridine. The isolated DNA was acid hydrolyzed, and then the resulting free bases were separated by HPLC. The HPLC eluent was simultaneously monitored for UV absorbance and radioactivity. No radioactive peaks were detected from acid-hydrolyzed DNA isolated from [6-^{3}H]uridine- or [5-^{3}H]uridine-labelled mock-infected DHFR$^-$ cells (Fig. 2). When [6-^{3}H]uridine was used as a precursor to label *C. trachomatis*-infected DHFR$^-$ cells, both radioactive cytosine and thymine were detected in the acid-hydrolyzed DNA samples. In contrast, when [5-^{3}H]uridine was used as a precursor, cytosine was the only radioactive nucleobase detected (Fig. 2).

We also assessed the effects of 5-FUR and 5-FdUrd on *C. trachomatis* DNA synthesis activity. Both 5-FUR and 5-FdUrd can be converted by host enzyme activities to 5-FdUMP, a specific inhibitor of TS (2, 19). Our results indicate that 5-FUR is an effective inhibitor of *C. trachomatis* DNA synthesis (50% effective dose, 0.2 μM), whereas 5-FdUrd had little or no effect, even at very high (>1 mM) concentrations (data not shown).

Demonstration of *C. trachomatis* TS activity in situ. The presence of radioactive thymine in DNA of *C. trachomatis*-infected DHFR$^-$ cells labelled with [6-^{3}H]uridine certainly

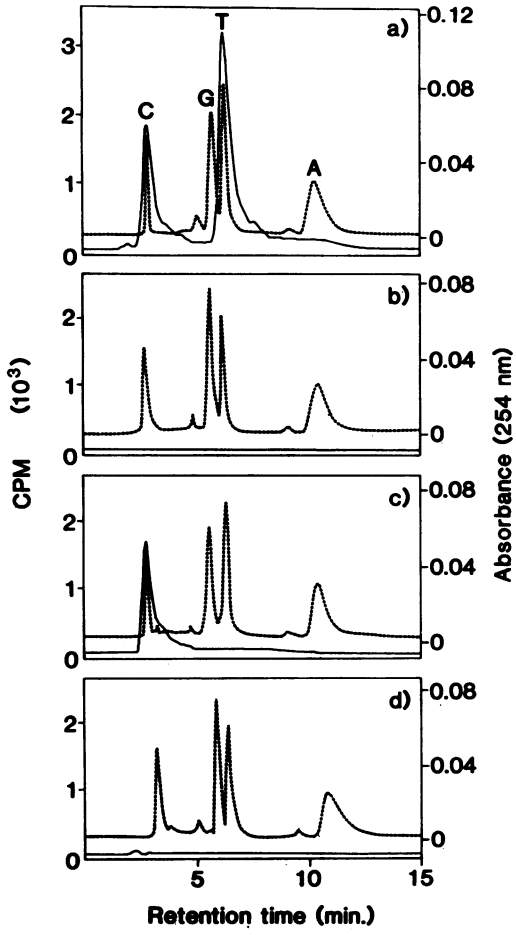

FIG. 2. Incorporation of [5-^{3}H]uridine and [6-^{3}H]uridine into DNA of mock-infected confluent monolayers (3.0×10^6 cells per plate, cultured in the presence of 1 μg of cycloheximide per ml) and *C. trachomatis*-infected CHO DHFR$^-$ cells (3.0×10^6 cells per plate, cultured in the presence of 1 μg of cycloheximide per ml). Duplicate dishes of DHFR$^-$ cell monolayers were infected with *C. trachomatis* L_2 EBs. At 24 h postinfection, [6-^{3}H]uridine (a) or [5-^{3}H]uridine (c) (final concentration, 0.3 μM) was added and incubation was continued for 2 h. DNA was isolated and hydrolyzed to free bases, which were separated and analyzed by HPLC. For details, see Materials and Methods. Chromatograms obtained from acid-hydrolyzed DNA of mock-infected DHFR$^-$ control cultures labelled with [6-^{3}H]uridine (b) or [5-^{3}H]uridine (d) are also shown. The identity of the radioactive peaks was confirmed by simultaneously monitoring the A_{254} of known cytosine and thymine standards. The position of the free bases are indicated by arrows. A, adenine; G, guanine; C, cytosine; T, thymine. A_{254} is shown by the dotted line; the radioactive detection of the ^{3}H label is shown by the solid line.

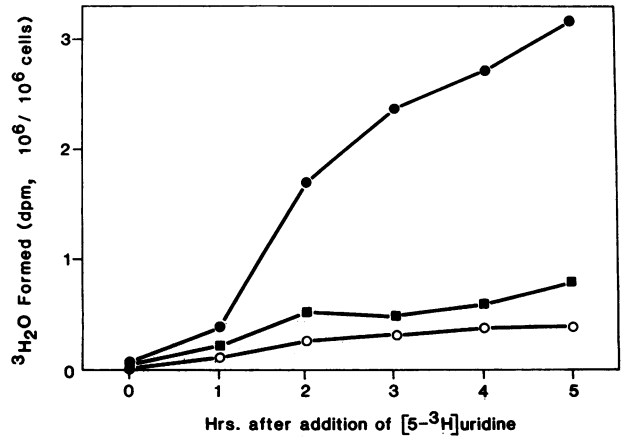

FIG. 3. In situ TS activity in *C. trachomatis*-infected CHO DHFR$^-$ cells, determined by measuring the amount of ^3H_2O released into the medium from cells labelled with [5-^{3}H]uridine. Mock-infected DHFR$^-$ cells (○), *C. trachomatis*-infected DHFR$^-$ cells (●), and *C. trachomatis*-infected DHFR$^-$ cells (all at 3.0×10^6 cells per plate, cultured in the presence of 1 μg of cycloheximide per ml) pretreated with 0.5 μM 5-FUR (■) were labelled for 2 h with [5-^{3}H]uridine (final concentration, 0.3 μM) at 24 h postinfection. At the times indicated, an aliquot of the medium was removed and the amount of ^3H_2O present was determined. For details, see Materials and Methods. For infected cells treated with 0.5 μM 5-FUR, the inhibitor was added to the medium 2 h prior to the addition of the isotope. All analyses were made with duplicate samples, and values varied less than 10%.

provides strong suggestive evidence for the presence of a parasite-specific TS. As a more direct test of this hypothesis, we conducted in situ assays for TS. [5-^{3}H]deoxyuridine has been used to assay in situ TS activity in mammalian cells (15, 17). Since we have previously shown that deoxyribonucleosides, including deoxyuridine, are very poorly incorporated into *C. trachomatis* DNA (13), we had to use [5-^{3}H]uridine to monitor in situ TS activity in *C. trachomatis*-infected DHFR$^-$ cultures. The isotope was introduced from the medium as [5-^{3}H]uridine, as shown schematically in Fig. 1. Inside the cells, the nucleoside follows the metabolic pathway described above for labelling DNA with uridine. The basis of the in situ TS assay is the formation of ^3H_2O, with the isotope lost from the pyrimidine ring during dUMP conversion to dTMP. Thus, the appearance of ^3H_2O in the medium is used to measure the TS reaction. Results from a time course in situ TS assay with [5-^{3}H]uridine-labelled mock- and *C. trachomatis*-infected DHFR$^-$ cells are shown in Fig. 3. After a 5-h labelling period, *C. trachomatis*-infected cultures had formed substantially more ^3H_2O than had mock-infected control cultures. As expected, addition of 0.5 μM 5-FUR 2 h prior to addition of [5-^{3}H]uridine caused almost complete inhibition of ^3H_2O formation (Fig. 3). Under similar conditions, 5-FdUrd had no effect on isotope release (data not shown).

In order to determine whether in situ TS activity correlates with DNA synthesis activity during the chlamydial growth cycle, we monitored both activities at various times over the 48-h developmental cycle (Fig. 4). In situ TS activity was monitored by measuring ^3H_2O formation from [5-^{3}H]uridine, and DNA synthesis was monitored by determining incorporation of [6-^{3}H]uridine into DNA. At the indicated times, [5-^{3}H]uridine was added to the medium of *C. trachomatis*-infected DHFR$^-$ cells. Then after 2 h, the ^3H_2O formed and the amount of [6-^{3}H]uridine incorporated into DNA was determined. There is a good correlation between TS and DNA synthesis activities. Both activities first appear approximately 16 h postinfection, peak at 24 to 32 h postinfection, and rapidly decline thereafter.

Detection of in vitro TS activity in extracts prepared from highly purified *C. trachomatis* RBs. In order to conclusively show that *C. trachomatis* contains TS, we prepared extract from highly purified RBs and assayed for TS activity in vitro. TS activity was measured by following the formation of [6-^{3}H]dTMP from [6-^{3}H]dUMP. We consistently detected

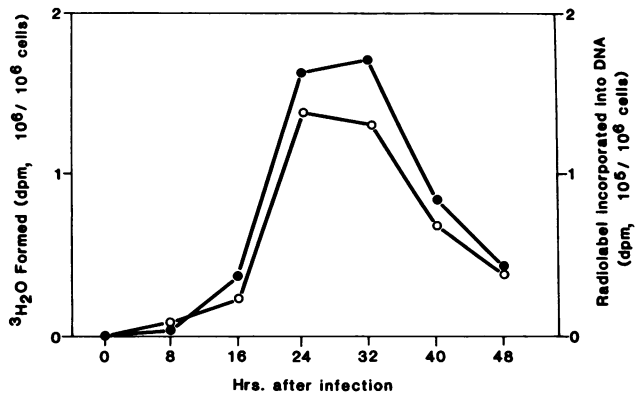

FIG. 4. In situ TS activity and DNA synthesis activity during the *C. trachomatis* growth cycle. Parallel dishes of DHFR$^-$ cells were infected with *C. trachomatis* L_2 EBs. At each of the indicated times, one dish was labelled with [5-^{3}H]uridine for measurement of in situ TS activity (○) and the other with [6-^{3}H]uridine for measurement of DNA synthesis activity (●). Incubation in the presence of the isotope was continued for 2 h, and then the amount of ^3H_2O present in the medium and the amount of radiolabel incorporated into DNA were determined. For details, see Materials and Methods. Values obtained, at each time, for mock-infected DHFR$^-$ control cultures (3.0×10^6 cells per plate, cultured in the presence of 1 μg of cycloheximide per ml) were subtracted from the values obtained for *C. trachomatis*-infected cultures (3.0×10^6 cells per plate cultured in the presence of 1 μg of cycloheximide per ml). All analyses were made with duplicate samples, and values varied less than 10%.

dTMP formation from dUMP by using RB extracts as a source of the enzyme (Table 4). Under our assay conditions, the TS activity remained linear for about 30 min (data not shown). The formation of dTMP was dependent on the presence of the RB extract of both formaldehyde and H_4folate (the two compounds nonenzymatically form CH_2-H_4folate, the required cofactor for the TS reaction) and was inhibited by 5-FdUMP. Mock-infected mouse L-cell extract and boiled RB extract had no measurable TS activities.

TABLE 4. TS activity in crude extracts prepared from purified *C. trachomatis* RBs

Enzyme source	Cofactor or inhibitor[a]	Mean ± SD of dTMP (pmol/mg of protein/min)[b]	Activity (%)
RB extract	CH_2-H_4folate	15.6 ± 2.1	100
	CH_2-H_4folate + 5-FdUMP	0.3 ± 0.1	2
	H_4folate	<0.1	>1
	5-CH_3-H_4folate	<0.1	>1
	5-CHO-H_4folate	<0.1	>1
Boiled RB extract	CH_2-H_4folate	<0.1	>1
Mock-infected L-cell extract	CH_2-H_4folate	<0.1	<1

[a] 5-CH_3-H_4folate, 5-methyltetrahydrofolate; 5-CHO-H_4folate, 5-formyltetrahydrofolate. CH_2-H_4folate was prepared fresh prior to use by treating H_4folate with formaldehyde as described in Materials and Methods. To determine the effect of 5-FdUMP on TS activity, a complete reaction mix minus substrate was incubated in the presence of 100 μM 5-FdUMP for 5 min at room temperature. The reaction was initiated by addition of dUMP substrate and incubation was at 37°C.

[b] Reactions were carried out at 37°C for 20 min. Each value represents the mean ± standard deviation from two experiments.

DISCUSSION

Our laboratory is interested in nucleotide metabolism in the obligate intracellular bacterial parasites, the chlamydiae. Recently, we reported that *C. trachomatis* does not utilize host cell dNTP pools as its source of DNA precursors (13). As an alternative, we suggested and have since shown that *C. trachomatis* obtains DNA precursors from the host cell as ribonucleotides, with subsequent reduction to deoxyribonucleotides being catalyzed by a chlamydia-specific ribonucleotide reductase (27). Since ribonucleotide reductase is capable of supplying three (dATP, dGTP, and dCTP) of the four precursors required, it left us with the question of how chlamydiae obtain the dTTP also required for DNA replication. This question was addressed in the present communication.

The nonincorporation of medium-supplied thymidine by intracellular chlamydiae has been thoroughly documented (1, 6, 11, 24, 29). Hatch (6) showed that *C. psittaci* growing in either thymidine kinase (TK) containing or thymidine kinase-deficient mouse L cells is unable to incorporate medium-supplied thymidine into DNA. Our results confirm this finding for *C. trachomatis* (Table 1). In addition, our results show that the free-base thymine is also not utilized by *C. trachomatis*. The nonutilization of exogenous thymine and thymidine in both TK$^+$ and TK$^-$ cells indicates that chlamydiae are unable to salvage any preformed thymine derivative from the host cell. The most likely explanation for the nonutilization of host cell dTTP is that chlamydiae lack a suitable transport system for the deoxyribonucleotide. This finding is in agreement with our previous results which indicated that *C. trachomatis* does not efficiently transport any purine or pyrimidine deoxyribonucleotides (13). The inability to use host-supplied thymine or thymidine may result from a lack of transport or from the fact that the parasite lacks the enzymes required to raise the precursors to the nucleotide level. In support of the latter, Hatch (6) previously reported that extracts prepared from *C. psittaci* RBs lack thymidine kinase activity.

In this study, we made use of a mutant CHO cell line deficient in DHFR to assist our studies on thymidine metabolism in *C. trachomatis*. As a result of the DHFR deficiency, this cell line is unable to regenerate H_4folate from H_2folate and consequently loses the capacity to synthesize one-carbon H_4folate derivatives, which are required for many biochemical reactions, including thymidylate synthesis (30). Using this mutant cell line, we were able to demonstrate that *C. trachomatis* growth, as monitored by DNA synthesis activity and inclusion development, is essentially the same in the presence and absence of exogenous thymidine (Table 2). The combined findings that *C. trachomatis* is unable to salvage thymidine or thymine and is capable of normal growth in a thymidine-deficient host provide strong suggestive evidence that it is capable of de novo thymidine synthesis. TS is the only enzyme known to be capable of de novo thymidine synthesis (8, 12, 16). It is a ubiquitous enzyme present in almost all wild-type organisms. TS catalyzes an unusual reaction, wherein CH_2-H_4folate serves as both a one-carbon donor and a reductant in the conversion of dUMP to dTMP (8, 12).

Further support for the presence of TS in chlamydiae comes from our results which show that intracellular *C. trachomatis* readily incorporates exogenously supplied uri-

dine into DNA (Table 3). Uridine enters the host cell and is metabolized by the pathways shown schematically in Fig. 1. Uridine can label all pyrimidine ribonucleotides and deoxyribonucleotides, and, as a result, there are several pathways which the isotope could follow before finally being incorporated into chlamydial DNA. We believe that our results support the following pathway for uridine metabolism. Uridine enters the host cell and is sequentially phosphorylated to uridine phosphates by cellular uridine-cytidine kinase, uridylate kinase, and nucleoside diphosphate kinase. Some of the UTP formed will be converted to CTP by host cell CTP synthetase. The UTP and CTP can then be taken into *C. trachomatis* by specific but as yet undefined transport system(s). It is also possible that *C. trachomatis* could convert UTP to CTP; however, we have no evidence to support the existence of a chlamydia-specific CTP synthetase. Inside the RB, the two pyrimidine ribonucleotides are reduced to pyrimidine deoxyribonucleotides by a chlamydial ribonucleotide reductase. The dCTP thus formed is utilized directly by DNA polymerase. The deoxyuridine phosphate is further processed by chlamydial TS to thymidine phosphates, which are, in turn, incorporated into DNA.

This proposed pathway is supported by several independent lines of evidence. First, there would be few, if any, pyrimidine deoxyribonucleotides formed by the infected $DHFR^-$ host cell, since cellular ribonucleotide reductase levels are negligible in the presence of cycloheximide (27) and the $DHFR^-$ cell cannot synthesize thymidine phosphates (30). Furthermore, even if some exogenously added uridine is converted to pyrimidine deoxyribonucleotides by host cell enzymes, these dNTPs cannot enter chlamydiae (13). Secondly, the works of Hatch (5) using *C. psittaci* and ourselves (13) using *C. trachomatis* have shown that chlamydiae can and do draw on host cell ribonucleoside triphosphate pools as a source of precursors. Thirdly, we have demonstrated that *C. trachomatis* does contain a ribonucleotide reductase (27). Fourth, *C. trachomatis* DNA synthesis activity and in situ TS activity are inhibited by 5-FUR but not 5-FdUrd. 5-FdUrd is converted to 5-FdUMP by cellular thymidine kinase. The presence of 5-FdUMP in the host cell has no effect on *C. trachomatis* growth because it is a deoxyribonucleotide derivative and, as a result, it is not transported by chlamydiae. In contrast, 5-FUR is converted to 5-FUTP by host cell enzymes and then it enters chlamydiae. Once inside the RB, the 5-FUTP is converted to 5-FdUMP by chlamydial ribonucleotide reductase. The resulting 5-FdUMP inhibits *C. trachomatis* TS activity and, as a result, the parasite is starved for thymidine nucleotides and DNA synthesis ceases. Fifth, the results presented in Fig. 2 indicate that exogenous [5-^{3}H]uridine gives rise to radiolabelled cytosine in *C. trachomatis* DNA and that exogenous [6-^{3}H]uridine yields both radiolabelled cytosine and thymine. In contrast, there is no radiolabel present in pyrimidine bases isolated from mock-infected $DHFR^-$ cells pulsed with either [5-^{3}H]uridine or [6-^{3}H]uridine. The only way to generate radioactive thymine under these conditions is by way of a *C. trachomatis*-specific TS.

Further evidence for the existence of TS in *C. trachomatis* comes from the demonstration of in situ TS activity when [5-^{3}H]uridine is used as precursor (Fig. 3). These studies were aided by the use of the $DHFR^-$ cell line, which has low background ^3H_2O formation. Similar to DNA synthesis activity, in situ TS activity was inhibited by 5-FUR but not by 5-FdUrd. This result clearly shows that *C. trachomatis* TS is an eventual target for 5-FUR by way of 5-FdUMP. Even though TS is believed to be the primary target of 5-FUR, the drug is also known to be cytotoxic in mammalian cells, as a result of 5-FUTP incorporation into RNA where it prevents correct processing of rRNA (23). We are uncertain whether a similar mechanism occurs in chlamydiae. The in situ TS activity correlated very well with DNA synthesis activity, peaking at approximately 24 to 32 h postinfection. Chlamydiae possess a unique life cycle represented by two forms, the infectious metabolically inert EB and the noninfectious metabolically active RB. The peaks of DNA synthesis and TS activity also correlate with maximal RNA and protein synthesis (data not shown) and likely represent the times when the maximum number of RBs are undergoing binary fission. After this time, all metabolic activities rapidly decrease as RBs differentiate to EBs.

As a final line of evidence supporting the existence of TS, we were able to demonstrate TS activity in vitro by using extracts prepared from highly purified RBs as a source of enzyme (Table 4). Similar to TS from other sources (8, 12), the *C. trachomatis* TS activity was dependent on CH_2-H_4folate as a cofactor and was inhibited by 5-FdUMP. A more detailed characterization of the enzyme awaits further purification.

In summary, studies from our laboratory have answered several questions regarding deoxyribonucleotide metabolism in chlamydiae. The results presented in this communication add to our overall understanding of this process by indicating that *C. trachomatis* meets its requirement for dTTP by encoding a TS. In vivo, the proper functioning of TS requires functional DHFR, serine hydroxymethyltransferase, and a supply of folate cofactor. These other components of the thymidylate cycle are the focus of our current studies.

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