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# Acquisition of Thymidylate by the Obligate Intracytoplasmic Bacterium *Rickettsia prowazekii*

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**The pathway for the acquisition of thymidylate in the obligate bacterial parasite *Rickettsia prowazekii* was determined. *R. prowazekii* growing in host cells with or without thymidine kinase failed to incorporate into its DNA the [$^3$H]thymidine added to the culture. In the thymidine kinase-negative host cells, the label available to the rickettsiae in the host cell cytoplasm would have been thymidine, and in the thymidine kinase-positive host cells, it would have been both thymidine and TMP. Further support for the inability to utilize thymidine was the lack of thymidine kinase activity in extracts of *R. prowazekii*. However, [$^3$H]uridine incorporation into the DNA of *R. prowazekii* was demonstrable (973 ± 57 dpm/3 × $10^8$ rickettsiae). This labeling of rickettsial DNA suggests the transport of uracil, uridine, uridine phosphates (UXP), or 2′-deoxyuridine phosphates, the conversion of the labeled precursor to thymidylate, and subsequent incorporation into DNA. This is supported by the demonstration of thymidylate synthase activity in extracts of *R. prowazekii*. The enzyme was determined to have a specific activity of 310 ± 40 pmol/min/mg of protein and was inhibited ≥70% by 5-fluoro-dUMP. The inability of *R. prowazekii* to utilize uracil was suggested by undetectable uracil phosphoribosyltransferase activity and by its inability to grow (less than 10% of control) in a uridine-starved mutant cell line (Urd$^-$A) supplemented with 50 μM to 1 mM uracil. In contrast, the rickettsiae were able to grow in Urd$^-$A cells that were uridine starved and supplemented with 20 μM uridine (117% of control). However, no measurable uridine kinase activity could be measured in extracts of *R. prowazekii*. Normal rickettsial growth (92% of control) was observed when the host cell was blocked with thymidine so that the host cell's dUXP pool was depressed to a level inadequate for growth and DNA synthesis in the host cell. Taken together, these data strongly suggest that rickettsiae transport UXP from the host cell's cytoplasm and that they synthesize TTP from UXP.**

Members of the genus *Rickettsia* are obligate intracytoplasmic parasitic bacteria. *Rickettsia prowazekii*, the etiologic agent of epidemic typhus, is a morphologically typical gram-negative bacterium (1, 2, 12). *R. prowazekii* grows free in the cytoplasm of its eucaryotic host cell unbounded by any internal membranes of host cell origin. This rich external milieu, the eucaryotic cytoplasm, offers many intermediate and end products of metabolism to the bacterium for transport. Carrier-mediated membrane transport systems have been described in *R. prowazekii* for lysine (20), proline (29), uridine 5′-diphosphoglucose (30), AMP (3), $K^+$ (28), ATP/ADP (27), and NAD (4).

While transport plays a significant role in the acquisition of many rickettsial metabolites, *R. prowazekii* has the ability to synthesize other metabolites. A prime example of this is the synthesis of polyamines from arginine by *R. prowazekii* (21). Since virtually all eucaryotic cells contain significant amounts of the naturally occurring polyamines (18), one might have expected *R. prowazekii* to have evolved to transport polyamines from the host cell's cytoplasm. However, this organism has retained the biosynthetic machinery necessary to synthesize polyamines.

The pathway by which *R. prowazekii* obtains thymidylate for DNA synthesis is unknown. Several possibilities could exist for such an obligate intracytoplasmic parasite. First, the rickettsiae could transport the deoxy forms, either the ultimate end product TXP or those molecules that require a single additional enzyme: dUMP with thymidylate synthase or thymidine with thymidine kinase. (Throughout the manuscript, the phosphorylation state of the nucleotide will be ignored; for example, TMP, TDP, and TTP will be included in the general term TXP.) Second, the rickettsiae could transport uridine phosphates (UXP) from the host cell's cytoplasm and convert UXP to TXP via a rickettsia-encoded ribonucleotide reductase and a thymidylate synthase (TS). Third, the rickettsiae could transport uracil or uridine from the host cell's cytoplasm and form the nucleotide via a rickettsia-encoded uracil phosphoribosyltransferase or uridine kinase, respectively. Last, the rickettsiae could synthesize TXP de novo from existing amino acids.

In this study, we investigated the various possible pathways for thymidylate acquisition using both rickettsial extracts and the growth and metabolic labeling of *R. prowazekii* in situ (i.e., growing within the host cell's cytoplasm).

## MATERIALS AND METHODS

**Chemicals.** Dulbecco modified Eagle medium and Eagle minimum essential medium were purchased from Mediatech, Washington, D.C. and Serum Plus was purchased from KC Biologicals, Lenexa, Kans. Radioisotopes, [terminal methylenes-$^3$H]thymidine (77 Ci/mmol), and [5,6-$^3$H]uridine (49 Ci/mmol), were purchased from ICN Biomedicals, Costa Mesa, Calif. Although the starting material for the uridine labeling experiments was [5,6-$^3$H]uridine, the fifth carbon atom of the pyrimidine base is lost during the TS reaction and only the $^3$H in the sixth position is incorporated

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into DNA. All other chemicals were of the highest purity obtainable from commercial sources.

**Propagation of rickettsiae in yolk sacs.** *R. prowazekii* Madrid E strain was propagated in 6-day-old embryonated, antibiotic-free hen eggs after inoculation with a dilution of a seed pool (yolk sac passage 280). Heavily infected yolk sacs were harvested 8 days postinoculation, and purified rickettsial suspensions were prepared by a modification (26) of the methods of Bovarnick and Synder (7) and Wisseman et al. (31). Viable rickettsiae were enumerated by the antibody hemolysis method of Walker and Winkler (23).

**Propagation of cell cultures.** Mouse L929 cells were obtained from Flow Laboratories, Inc., McLean, Va. A thymidine kinase mutant [LM(TK$^-$)] (11) of mouse L929 cells was obtained from American Type Culture Collection, Rockville, Md. A uridine-requiring mutant (Urd$^-$A) (17) of the Chinese hamster ovary cell line (CHO-K1) was generously provided by David Patterson, Eleanor Roosevelt Institute for Cancer Research, Denver, Colo.

L929 cells were grown in Eagle minimum essential medium supplemented with 10% Serum Plus. LM(TK$^-$) cells were grown in the same medium containing 30 μg of 5-bromo-2′-deoxyuridine per ml. The Urd$^-$A cell line, which lacked the ability to synthesize orotidine-5′-phosphate from aspartate, was cultured in Dulbecco modified Eagle medium supplemented with 30 μM uridine, nonessential amino acids, and 10% Serum Plus. Cells were cultured as monolayers at 34°C in a humidified atmosphere of 3% $CO_2$ in air except for the Urd$^-$A cell line (10% $CO_2$ in air).

**Measurement of rickettsial growth.** Rickettsiae in Hanks balanced salt solution supplemented with 0.1% gelatin and 4.9 mM L-glutamate (monopotassium salt) were incubated with washed cells at 34°C at a multiplicity of infection that would give two to six rickettsiae per infected cell after 1 h. To prevent host cell division, either 1 μg of emetine per ml, a eucaryotic protein synthesis inhibitor, was added to the culture after the infection or the culture was X-irradiated (5,000 rads) before the infection. At the indicated times, duplicate coverslips were removed and stained by a modification of the method of Gimenez (9). The percentage of infected cells and the average number of rickettsiae per infected cell were determined by light microscopy. A cell containing greater than 100 rickettsiae was assigned a value of 100.

**Determination of rickettsial growth in the presence of inhibitors.** One-eighth confluent 100-mm dishes of L929 cells were cultured in the presence of 10 nM folinic acid and in the presence or absence of 5 μM 5-fluorouracil, 5 μM 5-fluorouridine, or 5 μM 5-fluoro-2′-deoxyuridine for 48 h prior to infection and for 48 h postinfection. LM(TK$^-$) cells were cultured in the presence or absence of 30 μg of 5-bromo-2′-deoxyuridine per ml for 48 h prior to infection and 48 h postinfection. Coverslips were removed and rickettsial growth was determined at 48 h postinfection.

**Incorporation of [$^3$H]uridine or [$^3$H]thymidine into *R. prowazekii* DNA.** One-eighth confluent 100-mm dishes of host cells were incubated in the presence or absence of 5 μM 5-fluoro-2′-deoxyuridine and 10 nM folinic acid for 48 h prior to infection. At 24 h postinfection, 100 μCi of [$^3$H]thymidine (71 Ci/mmol) or 100 μCi of [5,6-$^3$H]uridine (49 Ci/mmol) was added to the culture medium. At 48 h postinfection, the cells were trypsinized from the monolayer and centrifuged at 500 × *g* for 5 min. The infected cell pellet was resuspended in 2 ml of SPG (218 mM sucrose, 3.76 mM $KH_2PO_4$, 7.1 mM $K_2HPO_4$, 5 mM potassium glutamate) and lysed by $N_2$ cavitation (mini-bomb cell disruption chamber; Kontes, Vineland, N.J.) at 300 lb/in$^2$ for 5 min. After centrifugation at 500 × *g* for 5 min to pellet unbroken cells and nuclei, the supernatant fluid was centrifuged at 10,886 × *g* for 20 min at 4°C to pellet the rickettsiae. The rickettsiae were purified from host cellular debris by centrifuging the rickettsial suspension through 30 ml of 25% Renografin (E. R. Squibb & Sons, Princeton, N.J.) at 30,240 × *g* for 60 min at 4°C.

To determine the incorporation of [$^3$H]thymidine into DNA, the purified rickettsial pellet was resuspended in 1 ml of ice-cold 10% trichloroacetic acid (TCA) and incubated on ice for 2 h, and the TCA-insoluble material was pelleted by centrifugation at 10,886 × *g* for 30 min. After centrifugation, this TCA precipitation procedure was repeated. The pellet was resuspended in 0.5 ml of 1% sodium dodecyl sulfate (SDS), a biodegradable liquid scintillation cocktail (2.5 ml) was added, and the radioactivity of the TCA-insoluble material was determined by liquid scintillation spectroscopy.

To determine the incorporation of [$^3$H]uridine into DNA, the purified rickettsial pellet was resuspended in 1 ml of 1% SDS in 1 M Tris-HCl (pH 7.5) and placed at 60°C for 30 min. The nucleic acids were precipitated with 2.5 ml of ice-cold 100% ethanol and 100 μl of 3 M sodium acetate. The tube was placed at −20°C overnight and then centrifuged at 16,000 × *g* for 20 min. NaOH (200 μl of 5 N NaOH) was added to the pellet, and the RNA was hydrolyzed at 50°C for 1 h by the method of Eick et al. (8). The solution was neutralized with 200 μl of 5 N HCl, and the DNA was precipitated by adding 3 ml of ice-cold isopropanol, 1 μg of glycogen, and 140 μl of 3 M sodium acetate, incubating overnight at −20°C, and centrifuging at 16,000 × *g* for 20 min. The precipitated DNA was suspended in 200 μl of 88% formic acid and hydrolyzed to deoxynucleobases at 170°C for 30 min (8). The sealed ampoules were slowly cooled to room temperature, and the solution was transferred to a 1.5-ml microcentrifuge tube, covered with perforated Parafilm, and lyophilized to dryness overnight. The dried residue was resuspended in 50 μl of 50% acetic acid and subjected to high-performance liquid chromatography (HPLC).

The HPLC method used to separate thymine was a modification of the method of Eick et al. (8). A ternary gradient HPLC system was used with a V4 variable wavelength detector (Isco, Inc., Lincoln, Neb.). The column used for the separation was an Adsorbosphere RP-18 (250 by 4.6-mm inner diameter, 5-μm particle size) column from Alltech Associates, Deerfield, Ill. Thymine was eluted from the column isocratically by using 100 mM ammonium acetate (pH 4.25) containing 0.5% acetonitrile at a flow rate of 1 ml/min. The UV absorbance of the effluent was monitored at 264 nm. The peak corresponding to an authentic thymine standard was collected (thymine, retention time of 9.1 ± 0.9 min; uracil, retention time of 4.3 ± 0.7 min), and the radioactivity was determined by liquid scintillation counting.

**Preparation of extracts for enzyme assays.** *R. prowazekii* (4 mg of protein), purified from chick yolk sacs, was suspended in 2 ml of either a kinase reaction buffer (100 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] buffer [pH 7.4], 33 mM NaCl, 13 mM ATP, 6 mM $MgCl_2$) or a TS reaction buffer (50 mM Tris-HCl [pH 7.8], 50 mM 2-mercaptoethanol, 5 mM formaldehyde, 1 mM tetrahydrofolate) and passed through a French pressure cell twice at 20,000 lb/in$^2$. The lysate, clarified at 10,886 × *g* for 20 min, was used as the enzyme source.

For yolk sac contamination controls, purified sham suspensions were prepared from mock-infected eggs by the same procedures used to isolate rickettsiae from infected

yolk sacs. In addition, a crude yolk sac extract was prepared from mock-infected eggs that had only been centrifuged at 600 × *g* for 10 min to remove yolk and unbroken debris. These pellets were suspended in TS reaction buffer and lysates were prepared as described above.

**Determination of kinase activities in *R. prowazekii*.** Uridine and thymidine kinases were assayed by the method of Pfefferkorn (19). The reaction was initiated by the addition of 30 μCi of [$^3$H]uridine or [$^3$H]thymidine to the rickettsial extract in kinase reaction buffer, and the tubes were placed at 37°C. At 15 and 30 min, 1/10th of the sample was removed and spotted onto a Whatman DE-81 ion-exchange filter that had been soaked in 1 mg of unlabeled substrate per ml. After drying, each filter was washed twice with kinase reaction buffer and placed in a 20-ml scintillation vial, and the radioactivity was counted in 5 ml of a toluene-based liquid scintillation fluid.

**Determination of TS activity in *R. prowazekii*.** TS was assayed by the method of Krungkrai et al. (13). The reaction was initiated by adding 25 μl of a dUMP solution (to give a final concentration of 0.1 mM) to 250 μl of rickettsial extract in TS reaction buffer. In some instances, the TS inhibitor 5-fluoro-dUMP (FdUMP; final concentration of 0.1 mM) was preincubated with the rickettsial lysate for 15 min at 37°C before the reaction was initiated by addition of the substrate. After incubation at 37°C for the indicated time, 250 μl of ice-cold 1 M perchloric acid was added to stop the reaction and the reaction mixture was then placed in an ice bath for 30 min. After centrifugation of the mixture at 16,000 × *g* for 5 min, the supernatant fluid was neutralized with 0.1 volume of 10 M potassium hydroxide containing 1 M potassium dihydrogenphosphate. The resultant mixture was then centrifuged at 16,000 × *g* for 5 min, and the supernatant fluid was analyzed by HPLC. An Adsorbosphere RP-18 column (250 by 4.6-mm inner diameter, 5-μm particle size) and isocratic elution with 10 mM potassium phosphate buffer (pH 4.0) at a flow rate of 1.0 ml/min were used to separate the product of TS (TMP) from the substrate (dUMP). The TMP peak, identified by comigration with an authentic standard, was quantitated by its $A_{260}$.

**Determination of host cell TS contamination.** Intact *R. prowazekii* organisms (0.8 mg of protein) purified from infected yolk sacs were incubated with 0.1 mM FdUMP in TS reaction buffer for 30 min at room temperature to inhibit extrarickettsial TS. After the incubation, 15 ml of SPG was added and the tube was centrifuged at 10,886 × *g* for 20 min to pellet the rickettsiae. The rickettsial pellet was suspended in 15 ml of SPG and centrifuged at 10,886 × *g* for 20 min. An extract was prepared from the washed rickettsial pellet, and TS activity was measured as described above.

**Determination of protein concentrations.** Protein concentrations were measured by using the bicinchoninic acid protein assay system from Pierce Chemical Co., Rockford, Ill.

## RESULTS

**Growth of *R. prowazekii* in the presence of halogenated analogs.** Rickettsial growth was inhibited in L929 cells cultured in the presence of 5 μM 5-fluorouridine (only a 3-fold increase in rickettsiae was observed over the 48-h period), while a 15-fold increase in rickettsiae was observed over the same period in L929 cells cultured in the absence of inhibitor (Table 1). However, normal growth of *R. prowazekii* was observed in L929 cells cultured in the presence of 5 μM 5-fluorouracil or 5 μM 5-fluoro-2′-deoxyuridine. L929 cells failed to grow (data not shown) in the presence of these compounds, which, after conversion to FdUMP, are potent irreversible inhibitors of TS (10). Also, normal rickettsial growth was observed in a thymidine kinase mutant of mouse L929 cells [LM(TK$^-$)] cultured in the presence or absence of 30 μg of 5-bromo-2′-deoxyuridine per ml (an analog of thymidine which is lethal when incorporated into DNA [11]).

TABLE 1. Growth of *R. prowazekii* in eucaryotic cells

| Cell line | Inhibitor[a] | Growth of *R. prowazekii*[b] | | | | Doubling time (h) |
|---|---|---|---|---|---|---|
| | | % of cells infected | | Rickettsiae per infected cell | | |
| | | 0 h | 48 h | 0 h | 48 h | |
| L929 | None | 66 ± 7 | 85 ± 9 | 6 ± 2 | 79 ± 5 | 12 |
| L929 | FdUr | 70 ± 13 | 84 ± 11 | 6 ± 2 | 78 ± 9 | 13 |
| L929 | FUr | 52 ± 1 | 60 ± 8 | 3 ± 1 | 11 ± 4 | 23 |
| L929 | FUra | 52 ± 1 | 65 ± 3 | 3 ± 1 | 72 ± 5 | 10 |
| LM(TK$^-$) | None | 73 ± 7 | 79 ± 14 | 3 ± 1 | 67 ± 4 | 11 |
| LM(TK$^-$) | BdUr | 71 ± 4 | 70 ± 5 | 3 ± 1 | 77 ± 4 | 10 |

[a] This column represents the addition of various inhibitors to the culture medium. The following inhibitors were used: 5 μM 5-fluoro-2′-deoxyuridine (FdUr), 5 μM 5-fluorouridine (FUr), 5 μM 5-fluorouracil (FUra), and 30 μg of 5-bromo-2′-deoxyuridine (BdUr) per ml. The 5-fluoro-nucleoside analogs are converted by normal metabolic pathways to the active irreversible inhibitor FdUMP. This substrate, FdUMP, is irreversibly bound to the enzyme, TS, and the cofactor, $N^5$,$N^{10}$-methylenetetrahydrofolate, preventing the formation of TTP needed for DNA synthesis.

[b] Each value represents the mean ± standard deviation of four experiments for L929 cells in the absence of inhibitor, four experiments for L929 cells plus FdUr, two experiments for L929 cells plus FUr, two experiments for L929 cells plus FUra, three experiments for LM(TK$^-$) cells in the absence of inhibitor, and two experiments for LM(TK$^-$) cells plus BdUr (although not formally correct, with two experiments the same statistical treatment was applied for consistency).

**Lack of thymidine incorporation into *R. prowazekii* DNA.** *R. prowazekii* might acquire the TTP needed for DNA synthesis from either the thymidine or thymidine phosphates (TXP) in the host cell's cytoplasm. In LM(TK$^-$) cells, the [$^3$H]thymidine would not be converted to [$^3$H]TXP in the host cell and the [$^3$H]thymidine would be available to the rickettsiae. In contrast, in the Urd$^-$A (TK$^+$) cell line, both thymidine and TXP in the host cell would be labeled; however, the specific activity of the TXP would be lowered by the TXP formed from a functional TS in these cells. To avoid dilution of the label, this cell line was incubated in the presence of 5 μM 5-fluoro-2′-deoxyuridine and 10 nM folinic acid to inhibit host cell TS. Assuming a host cell cytoplasmic TTP pool of 10 μM (6) and complete equilibration of all pools in the system over the 24-h labeling period, then, if the TXP or thymidine is transported by the rickettsiae, 1.1 × $10^8$ dpm of [$^3$H]TXP per 3 × $10^8$ rickettsiae would be expected to be incorporated into rickettsial DNA.

No incorporation of [$^3$H]thymidine and/or [$^3$H]TXP into *R. prowazekii* DNA was demonstrable. Infected LM(TK$^-$) cells incorporated 1,925 ± 825 dpm/5 × $10^6$ cells, while the same number of uninfected control cells incorporated 1,525 ± 425 dpm of [$^3$H]thymidine into the TCA-insoluble fraction (Table 2). In infected Urd$^-$A cells, 1,322 ± 172 dpm/5 × $10^6$ cells was incorporated into the TCA-insoluble material, while 1,300 ± 186 dpm was incorporated into the same number of uninfected control cells.

**Lack of kinase activities in extracts of *R. prowazekii*.** To determine whether *R. prowazekii* could utilize ribonucleosides or 2′-deoxyribonucleosides in the acquisition of thymidylate, we assayed the activities of both uridine and

TABLE 2. In situ incorporation of uridine or thymidine into *R. prowazekii* DNA

| Cell line | $^3$H label | dpm/5 × $10^6$ cells[a] | |
|---|---|---|---|
| | | Uninfected | Infected[b] |
| LM(TK$^-$) | Thymidine | 1,525 ± 425 | 1,925 ± 825 |
| Urd$^-$A | Thymidine | 1,300 ± 186 | 1,322 ± 172 |
| Urd$^-$A | Uridine | 85 ± 12 | 1,022 ± 158 |

[a] Incorporation of [$^3$H]thymidine or [$^3$H]uridine into the DNA of 5 × $10^6$ mock-infected or rickettsia-infected cells (in the presence of 1 μg of emetine per ml or 5,000 rads of X-irradiation) at 48 h postinfection. For the [$^3$H]thymidine incorporation into Urd$^-$A cell experiments, the cells were incubated in the presence of 5 μM 5-fluoro-2'-deoxyuridine and 10 nM folinic acid to inhibit the host cell's TS. Each value represents the mean ± standard deviation; $n$ = 2 experiments for the LM(TK$^-$) cells and $n$ = 3 experiments for the Urd$^-$A cells.

[b] On the average, there were 3 × $10^8$ rickettsiae per 5 × $10^6$ cells (80% of the cells were infected with rickettsiae and had 75 rickettsiae per infected cell at 48 h postinfection).

thymidine kinases in vitro. Neither uridine nor thymidine kinase activities were detectable in extracts of *R. prowazekii* (less than 0.05 pmol/min/mg of protein), while measurable activities were observed in an *Escherichia coli* positive control (58 to 60 pmol/min/mg of protein; $n$ = 2 experiments).

**Growth of *R. prowazekii* in uridine-requiring host cells.** Having eliminated host cell TXP pools, rickettsial uridine kinase, and rickettsial thymidine kinase as components for the acquisition of rickettsial TXP, we investigated whether *R. prowazekii* required either uracil or uridine phosphates (UXP) to synthesize nucleic acids (Table 3). The rickettsiae grew poorly (less than 29% of control) in Urd$^-$A cells cultured with uridine (at a concentration of 10 μM or less) or cultured with uracil at concentrations up to 1 mM uracil. However, rickettsial growth was fully restored in Urd$^-$A cells cultured in the presence of 20 μM uridine (117% of control) or 30 μM uridine (126% of control).

TABLE 3. Growth of *R. prowazekii* in Urd$^-$A cells

| Addition to Dulbecco modified Eagle medium[a] | Growth of *R. prowazekii*[b] | | % of control[c] |
|---|---|---|---|
| | %R | RI | |
| Unstarved | 84 ± 12 | 66 ± 12 | 100 |
| Uridine (0 μM) | 76 ± 5 | 9 ± 1 | 12 |
| Uridine (5 μM) | 71 ± 3 | 8 ± 2 | 10 |
| Uridine (10 μM) | 91 ± 2 | 17 ± 1 | 28 |
| Uridine (20 μM) | 97 ± 4 | 67 ± 9 | 117 |
| Uridine (30 μM) | 87 ± 12 | 80 ± 17 | 126 |
| Uracil (50 μM) | 77 ± 3 | 7 ± 1 | 10 |
| Uracil (250 μM) | 77 ± 1 | 6 ± 1 | 8 |
| Uracil (1 mM) | 71 ± 3 | 6 ± 1 | 8 |
| Thymidine (2.5 mM) | 81 ± 10 | 63 ± 7 | 92 |

[a] This column represents the addition of various compounds to the normal culture medium for the Urd$^-$A cell line as described in Materials and Methods. The cells were either starved or supplemented with 30 μM uridine (unstarved) for 24 h prior to infection. At the time of the infection, the compounds were added to the culture medium and rickettsial growth was measured. Both the unstarved cells and the cells that received 2.5 mM thymidine remained in 30 μM uridine throughout the assay period.

[b] The growth of the rickettsiae is expressed as the percentage of cells infected with rickettsiae (%R) and the number of rickettsiae per infected cells (RI) at 48 h postinfection. The initial infection gave 69% ± 4% of the cells infected with 6 ± 2 rickettsiae per infected cell. Each value represents the mean ± standard deviation for two experiments.

[c] This column is an index of rickettsial growth at 48 h based on the number of rickettsiae per 100 cells in the test conditions relative to that in the unstarved condition.

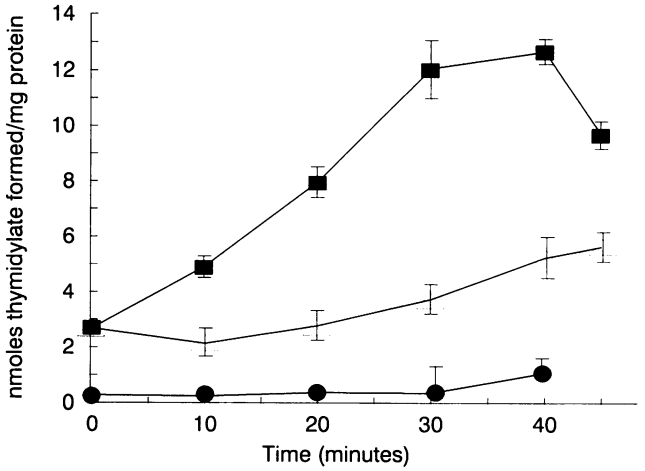


FIG. 1. TS activity of isolated, yolk sac-purified *R. prowazekii*. The nanomoles of thymidylate formed from dUMP at increasing incubation times were determined by HPLC. Each value represents the mean ± standard deviation ($n$ = 2 experiments) for freshly isolated, yolk sac rickettsial extract (■), the rickettsial extract plus 0.1 mM FdUMP (△), and a rickettsial extract that had been isolated from frozen yolk sacs (●).

**Incorporation of [$^3$H]UXP into *R. prowazekii* DNA.** To incorporate host cell uridine into rickettsial DNA, the host cell might convert the uridine to UXP via a uridine kinase and then the rickettsiae could transport the UXP, convert it to TTP, and incorporate the TTP into their DNA. Alternatively, the host cell might convert the uridine to dUXP via host cell-derived uridine kinase and ribonucleotide reductase and then this host cell dUMP could be transported by the rickettsiae and converted to TMP via a rickettsial TS.

Incorporation of [$^3$H]uridine into rickettsial DNA was demonstrable. In infected Urd$^-$A cells, 1,022 ± 158 dpm/3 × $10^8$ rickettsiae was incorporated into rickettsial DNA, while only 85 ± 12 dpm was observed in the DNA of the uninfected control cells (Table 2). Furthermore, normal rickettsial growth (92% of control) was observed in Urd$^-$A cells that had been cultured in the presence of 2.5 mM thymidine (Table 3), which causes a buildup of TXP in the host cell, leading to feedback which inhibits the host cell's ribonucleotide reductase and the conversion of UDP to dUDP in the host cell's cytoplasm (32). Taken altogether, these data strongly support the first alternative, that UXP is transported from the host cell's cytoplasm by the rickettsiae.

**TS activity in isolated, purified *R. prowazekii*.** If UXP is transported by the rickettsiae and incorporated into DNA, the rickettsiae must possess a functional ribonucleotide reductase and a TS. Having already identified a functional ribonucleotide reductase (7a), we assayed TS activity in rickettsial extracts. Synthesis of TMP from dUMP in rickettsial extracts was linear over the first 30 min in freshly purified rickettsiae and then plateaued (Fig. 1). Over the first 30 min, 9.4 ± 1.1 nmol of thymidylate was formed per mg of protein at a substrate concentration of 100 μM with freshly harvested rickettsiae. This corresponds to a specific activity of 310 ± 40 pmol of thymidylate per min per mg of protein for the rickettsial enzyme. This enzymatic activity was inhibited ≥70% by 0.1 mM FdUMP. No activity of the rickettsial enzyme was detectable when the rickettsial ex-

TABLE 4. Requirements of the TS enzyme from *R. prowazekii*

| Exptl condition[a] | *E. coli*[b] | | *R. prowazekii*[c] | |
|---|---|---|---|---|
| | nmol of thymidine/mg of protein/10 min | % of control | nmol of thymidine/mg of protein/10 min | % of control |
| Complete | 16.5 ± 9 | 100 | 3.1 ± 0.7 | 100 |
| FdUMP | 0.2 ± 0.1 | 1 | <0.1 | <3 |
| No extract | <0.1 | <1 | <0.1 | <3 |
| No HCHO | 0.7 ± 0.1 | 4 | 0.4 ± 0.1 | 13 |
| No dUMP | 0.1 ± 0.0 | 1 | <0.1 | <3 |
| No THF | <0.1 | <1 | <0.1 | <3 |

[a] This column represents the additions or omissions from the complete TS reaction buffer described in Materials and Methods. The following abbreviations are used: complete, complete reaction buffer; FdUMP, reaction buffer plus 0.1 mM FdUMP; no extract, no cell extract; no HCHO, reaction buffer minus formaldehyde; no dUMP, reaction buffer minus dUMP; no THF, reaction buffer minus tetrahydrofolate.

[b] These data were determined with an extract of *E. coli* B that was grown to the late logarithmic phase in LB medium. Each value represents the mean ± standard deviation for two experiments. These data are expressed as nanomoles of thymidylate formed per milligram of protein over the 10-min assay period and are normalized to percentage of the complete reaction mixture.

[c] These data were determined with an extract of chick yolk sac-propagated *R. prowazekii*. Each value represents the mean ± standard deviation for two experiments. The values are expressed as nanomoles of thymidylate formed per milligram of protein over the 10-min assay period and normalized to percentage of the complete reaction.

tract, dUMP, or tetrahydrofolate was omitted (Table 4). Only 13% of the activity remained when formaldehyde (which serves as a one-carbon donor molecule to nonenzymatically convert tetrahydrofolate to $N^5$,$N^{10}$-methylenetetrahydrofolate) was omitted from the reaction buffer. Rickettsiae that had been purified from infected yolk sacs that had been stored frozen at −80°C lacked any measurable TS activity in two experiments.

**Lack of host cell TS contamination.** When dealing with intracellular organisms it is essential to discriminate between host cell contamination and the true activity of the organism being studied. By preincubating the final rickettsial suspension with 0.1 mM FdUMP before lysing the rickettsiae, any TS from the host cell that may have copurified with the rickettsiae should be irreversibly inhibited. (The yolk sac enzyme was sensitive since the uninfected crude yolk sac preparation converted 8 nmol of dUMP to TMP per 10 min per mg of protein in the absence of 0.1 mM FdUMP, but only 0.8 nmol was converted in the presence of 0.1 mM FdUMP.) After two washes to remove the inhibitor, the rickettsiae were lysed and any remaining TS activity was measured. No statistically significant difference was observed in the preparation that had been incubated in the absence of 0.1 mM FdUMP (host cell activity plus rickettsial activity), which formed 2.9 ± 0.9 nmol of thymidylate per 10 min per mg of protein, compared with the preparation that had been incubated in the presence of 0.1 mM FdUMP (rickettsial activity only), which formed 2.5 ± 0.5 nmol of thymidylate per 10 min per mg of protein. Furthermore, purified sham suspensions had less than 1% of the rickettsial activity. Only 20 pmol of dUMP was converted to TMP per 10 min per mg of protein in the absence of inhibitor by the purified sham extracts, and <0.1 pmol of dUMP was converted to TMP per 10 min per mg of protein in the presence of 0.1 mM FdUMP.

## DISCUSSION

The pathway by which *R. prowazekii* obtains TTP is unknown. Several possibilities were examined in this study. First, *R. prowazekii* might acquire TTP by transport of the deoxy forms TXP, thymidine, or dUXP. No incorporation of [$^3$H]thymidine or [$^3$H]TXP into rickettsial DNA was observed in situ, which is consistent with the observations of Weiss et al. (24) in *R. typhi*. Also, the enzyme thymidine kinase, which converts thymidine to TMP, was unmeasurable in extracts of *R. prowazekii*. Williams and Peterson (25) published similar findings using *R. typhi*. To analyze dUXP transport by *R. prowazekii* we observed rickettsial growth in the presence of 2.5 mM thymidine (this will decrease the pool of dUXP below the level required by the host cell through feedback inhibition of ribonucleotide reductase and the conversion of UDP to dUDP). *R. prowazekii* grew normally in this thymidine-blocked host cell. Thus, *R. prowazekii* does not appear to acquire thymidylate as a deoxy form.

In a previous study (22), we found a serine requirement for rickettsial growth in a thymidine-depleted, TS-deficient mutant of V79 cells (TS5A [14]). However, when these cells were cultured in their normal growth medium (FAT medium; Eagle minimum essential medium plus 0.1 μM folinic acid, 1 μM aminopterin, and 10 μM thymidine), the rickettsiae grew normally and no serine requirement was observed. The lack of a serine requirement in the FAT-supplemented TS5A cells was construed to suggest that the rickettsiae can transport TXP. However, neither the serine dependence nor the ability to grow in FAT medium were found in a TS-deficient mutant of FM3A cells (FSthy$^-$21 [5]) (data not shown). The exact mechanism(s) that is operating in the TS5A cell line remains unknown, but the deduction made from the TS5A cells does not appear to be valid in other cell lines.

A second possibility for the acquisition of TTP by the rickettsiae is the transport of ribo forms (uracil, uridine, or UXP) and conversion to TTP within the rickettsiae. Rickettsial growth could not be measured in uridine-starved Urd$^-$A cells supplemented with various concentrations of uracil. Also, the enzyme uracil phosphoribosyltransferase, which is involved in the conversion of uracil to UMP, was undetectable in broken-cell extracts of *R. prowazekii* (data not shown). This indicates the lack of utilization of the uracil in the host cell's cytoplasm by the rickettsiae. Incorporation of [$^3$H]uridine into rickettsial DNA in situ was demonstrable. This suggests that *R. prowazekii* transports either uridine or UXP from the host cell's cytoplasm. However, the fact that *R. prowazekii* lacked measurable uridine kinase activity, which is consistent with the data of Williams and Peterson (25) in *R. typhi*, suggests that *R. prowazekii* transports UXP.

If the TTP requirement for DNA synthesis in the rickettsiae is met by the transport of UXP from the host cell's cytoplasm, then the reduction of the ribonucleotide to its corresponding deoxyribonucleotide and the conversion of dUXP to TXP must occur in the rickettsiae, i.e., the rickettsiae must possess both a ribonucleotide reductase and a TS (Fig. 2). We have demonstrated the presence of a ribonucleotide reductase in *R. prowazekii* (7a). In isolated, purified extracts of *R. prowazekii*, TS activity could be detected with a specific activity of 310 pmol/min/mg of protein. Since *R. prowazekii* has a genome size of $1.1 \times 10^9$ Da (16), is 71% A+T, and has a doubling time of 8 to 10 h, the rickettsiae would require 345 pmol of thymidylate per min per mg of protein to completely replicate their genome. The measured activity for the isolated enzyme was approx-

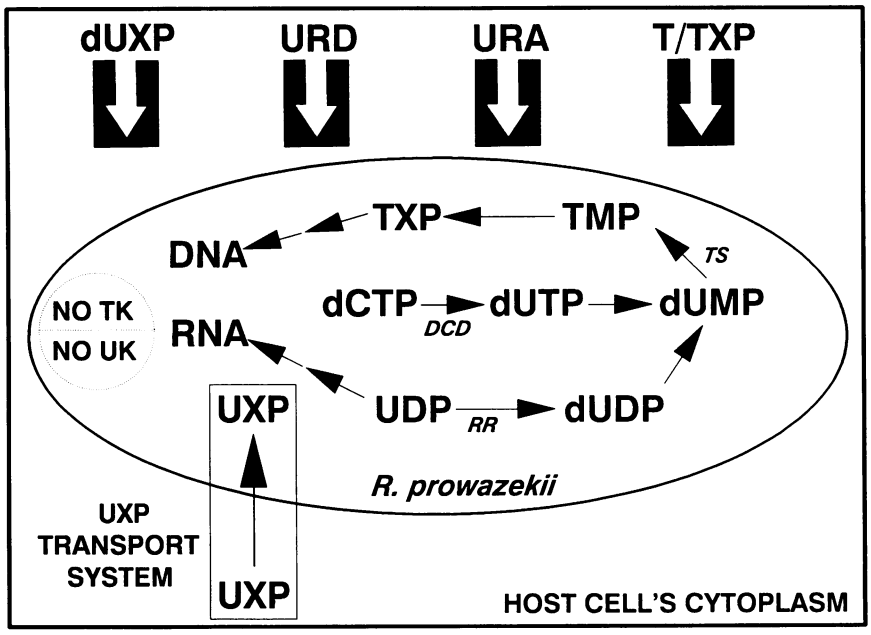


FIG. 2. Proposed model for the acquisition of thymidylate in *R. prowazekii*. The blocked arrows represent the inability of the rickettsiae to transport these compounds from the host cell's cytoplasm, while the transport of uridine phosphates (UXP) by the rickettsiae from the host cell's cytoplasm and the metabolism of this compound within the rickettsiae are indicated. Abbreviations used: dUXP, 2'-deoxyuridine phosphate(s); URD, uridine; URA, uracil; T/TXP, thymidine and/or thymidine phosphates; RR, ribonucleotide reductase; TS, thymidylate synthase; DCD, dCTP deaminase; TK, thymidine kinase; UK, uridine kinase.

imately the amount of activity that would be required in situ. This is one of the few examples in which the activity measured in this isolated, labile organism has been enough (or even close to enough) to allow the organism to divide with an 8-h doubling time.

Over the 24-h labeling period, 973 ± 57 dpm of [$^3$H]uridine per $3 \times 10^8$ rickettsiae was incorporated into rickettsial DNA. Several reasons could account for the low level of [$^3$H]uridine incorporation into rickettsial DNA. Either incomplete equilibration of all the pools in the system or poor recovery of the rickettsial DNA could explain these results. However, the most likely candidate to explain these results is the presence of a second pathway in the rickettsiae for dUXP acquisition (Fig. 2). According to the published literature on *E. coli* and *Salmonella typhimurium* (15), 75% of the dUXP is derived from the conversion of dCTP to dUTP via a dCTP deaminase which would dilute the specific activity of the dUXP. The presence of this pathway in *R. prowazekii* will be the focus of our future studies.

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