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Rickettsia prowazekii Transports UMP and GMP, but Not CMP, as Building Blocks for RNA Synthesis

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***Rickettsia prowazekii*, the etiological agent of epidemic typhus, is an obligate intracellular bacterium and is apparently unable to synthesize ribonucleotides de novo. Here, we show that as an alternative, isolated, purified *R. prowazekii* organisms transported exogenous uridyl- and guanylribonucleotides and incorporated these labeled precursors into their RNA in a rifampin-sensitive manner. Transport systems for nucleotides, which we have shown previously and show here are present in rickettsiae, have never been reported in free-living bacteria, and the usual nucleobase and nucleoside transport systems are absent in rickettsiae. There was a clear preference for the monophosphate form of ribonucleotides as the transported substrate. In contrast, rickettsiae did not transport cytidylribonucleotides. The source of rickettsial CTP appears to be the transport of UMP followed by its phosphorylation and the amination of intrarickettsial UTP to CTP by CTP synthetase. A complete schema of nucleotide metabolism in rickettsiae is presented that is based on a combination of biochemical, physiological, and genetic information.**

Rickettsia prowazekii, the etiological agent of epidemic typhus, is an obligate intracellular parasite that is morphologically a typical gram-negative bacterium (14). A reasonable explanation for the fact that rickettsiae can grow only within the cytoplasm of eucaryotic host cells is that they do not have the enzymic systems to synthesize all the nutrients necessary for their growth and replication and consequently transport an unusual and extensive array of building blocks from the eucaryotic cytoplasm into their own cytoplasm. This model is supported by observations that *R. prowazekii* has the ability to transport substrates that are not usually transported, for example, UDP-glucose (15), ATP or ADP (11), and NAD (4). Although there is experimental evidence that exogenous UMP can be incorporated into RNA (13), little is known about the transport of precursors for the synthesis of nucleic acids. The well-characterized transport system for ATP is an obligate exchange system; therefore, it is a means for the rickettsiae to acquire energy, not adenylates. Systems for the transport of AMP and the AMP moiety derived from NAD have been characterized (3, 4) and are not further investigated in this study. Rickettsiae obtain the deoxyribonucleotides needed for DNA synthesis by reduction of ribonucleotides rather than by directly transporting deoxyribonucleotides (6). Since neither uracil phosphoribosyltransferase nor uridine kinase activity could be detected in *R. prowazekii*, it was strongly suggested that rickettsiae transport uridylribonucleotides, that is, a nucleotide rather than the pyrimidine base or the nucleoside (9). However, there was no direct evidence for such transport, and the question of what form of nucleotide (UMP, UDP, or UTP) is the preferred form for transport remained open. Unfortunately, transport activity for these substrates in *R. prowazekii* is insufficient for measurement and characterization of the transport of nucleic acid precursors by a conventional filtration assay. Therefore, in the present study we used RNA synthesis as a trap for radioactive substrate and indirectly measured transport based on the incorporation of the radiolabeled purine nucleotide GXP and the pyrimidine nucleotides UXP and CXP into RNA. We show that the monophosphate is the dominant form of UXP and GXP transported by rickettsiae and that no form of CXP is transported. This lack of CMP transport was confirmed by a microspace assay involving the centrifugation of rickettsiae through silicon oil. The conversion of UTP to CTP in crude rickettsial extracts was demonstrated to be a rickettsial source of CTP in the absence of CXP transport. AMP transport was not included as a part of this investigation because ATP was present in all reactions as an energy source and the AMP transport system had been previously described.

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MATERIALS AND METHODS

Rickettsiae. Rickettsiae were purified from the yolk sacs of embryonated chicken eggs inoculated with *R. prowazekii* Madrid E (yolk sac passage 281) as previously described (11). Only fresh, unfrozen rickettsiae prepared from fresh, unfrozen infected yolk sacs were used. Samples of rickettsiae were suspended in a sucrose-phosphate-glutamate-magnesium solution (SPG-Mg; 0.218 M sucrose, 3.76 mM KH_2PO_4, 7.1 mM K_2HPO_4, 4.9 mM potassium glutamate, and 10 mM $MgCl_2$) (5).

RNA synthesis. For incorporation studies (13), 1 ml of SPG-Mg medium was supplemented with 3 mM ATP; 25 or 100 μM (each) GMP, CMP, and UMP; and 1 to 5 μCi of either [^{32}P]UXP, [^{32}P]CXP, or [^{32}P]GXP. *R. prowazekii* (100 μl of a suspension of 5 mg protein/ml) was added, and the suspension was incubated at 34°C. At the indicated times, 100-μl portions were added to tubes containing 1 ml of ice-cold solution A (10% trichloroacetic acid with 10 mM sodium pyrophosphate) and placed in ice for 30 min. The precipitated RNA was collected on membrane filters (HAWP025; Millipore Corp., Bedford, Mass.) and washed with 20 ml of ice-cold solution A and then with 20 ml of ice-cold 70% ethanol. The filters were dried and counted by liquid scintillation techniques, and the amount incorporated was calculated based on the specific activities of the NXPs.

Microspace measurements. The permeability of the rickettsiae to UMP and CMP was determined by centrifuging concentrated rickettsiae (19 mg/ml) that had been incubated with radioactive water, sucrose (an extracellular space marker), and nucleotides through nonaqueous layers into a perchloric acid solution at the bottom of a tube as previously described (12).

Nucleotides. [α-^{32}P]GTP, [α-^{32}P]CTP, and [α-^{32}P]UTP were purchased from ICN Biochemicals, Irvine, Calif. To prepare ^{32}P-labeled monophosphates and diphosphates, α-^{32}P-ribonucleotide triphosphates (5 mM) were treated with 0.01 U of apyrase for 15 min at 34°C and inactivated at 100°C for 1 min. When only the monophosphate form was needed, α-^{32}P-ribonucleotide triphosphates (5 mM) were treated with 0.5 N HCl at 100°C for 10 min and then immediately

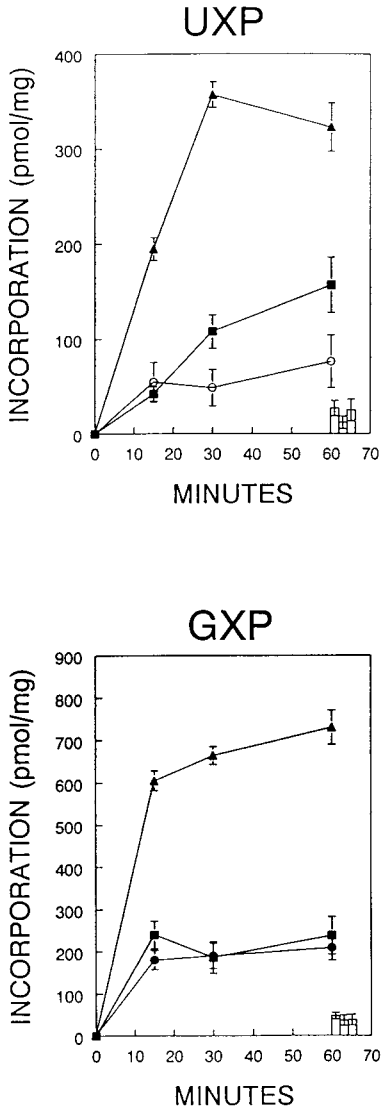

FIG. 1. Time course for the transport of radiolabeled NXP as measured by the incorporation of label into rickettsial RNA. Top, UXP (■, UDP; ○, UTP; ▲, UMP); bottom, GXP (■, GDP; ●, GTP; ▲, GMP). The error bars indicate standard deviations. The open bars indicate incorporation in the presence of rifampin.

neutralized with 0.5 N KOH. The various α-^{32}P-ribonucleotides in the reaction mixtures were analyzed by polyethylenimine thin-layer chromatography (8).

RESULTS AND DISCUSSION

The time courses of UXP and GXP incorporation into RNA by *R. prowazekii* are shown in Fig. 1. The monophosphate forms of both ribonucleotides were incorporated better than the diphosphate or triphosphate forms. Importantly, the incorporation of all UXPs and GXPs into RNA could be blocked by rifampin, a potent inhibitor of rickettsial RNA polymerase (7, 13), which indicated that the sink that we used to measure nucleotide transport was principally RNA synthesis. We measured the incorporation of these six ribonucleotides over a concentration range from 12.5 to 200 μM and established that the preference for monophosphates was exhibited in all cases (data not shown). Obviously, within the rickettsiae it is the triphosphate, not the monophosphate, form of the nucleotides

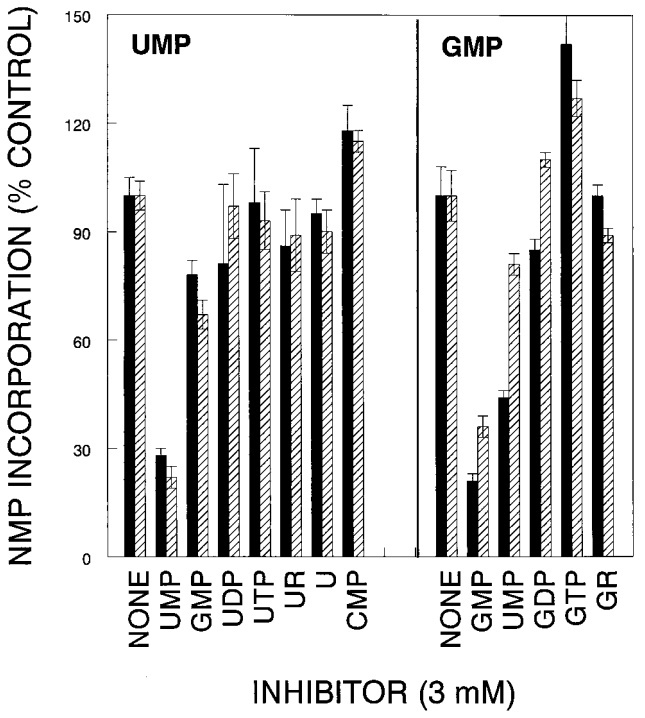

FIG. 2. Specificity of UMP and GMP transport as measured by the effect of excess unlabeled putative inhibitors (3 mM) on the 60-min incorporation of radiolabeled substrate into rickettsial RNA. The control values at 25 and 100 μM for UMP were 170 and 404 pmol mg^{-1} h^{-1}, and for GMP they were 104 and 342 pmol mg^{-1} h^{-1}. Solid bars, 25 μM substrate; hatched bars, 100 μM substrate. Abbreviations: UR, uridine; U, uracil; GR, guanosine. The means and standard errors are shown with four determinations in one rickettsial preparation except for UMP and GMP, where there were 19 to 25 determinations in three rickettsial preparations. The error bars indicate standard errors.

that is polymerized into RNA. Hence, the observed preference for incorporation of the exogenous monophosphates into RNA must be at the level of transport across the rickettsial membrane with the incorporation of the exogenous nucleoside triphosphates into RNA limited by a low level of transport.

The specificity of this unusual transport system for [^{32}P]UMP and [^{32}P]GMP was measured in experiments in which various putative competitive inhibitors were present at 3 mM, a 30- or 120-fold excess (Fig. 2). Marked inhibition of the incorporation of labeled UMP substrate into RNA was observed only with an excess of the same compound. However, the incorporation of 25 μM GMP could be inhibited by 3 mM UMP (although it is statistically significant, the biological significance of a 120-fold excess in an indirect assay is suspect). Importantly, the nucleobases and nucleosides, the usually transported sources of nucleic acid precursors in free-living bacteria, were not inhibitory. The deoxymononucleotides were also not inhibitory (data not shown). These results demonstrate that the transport mechanisms for UMP and GMP are separate and specific, although perhaps with some overlap between UMP and GMP, with the determinants of specificity residing in the nucleobase, phosphate, and ribose moieties.

Exogenous ^{32}P-cytidylribonucleotides (CMP, CDP, and CTP) were not incorporated into the RNA of the isolated rickettsiae. Incorporation of [^{32}P]CXP did not increase from 0 to 60 min, and the background level of radioactivity observed in the precipitates on the filters was insensitive to rifampin. This failure of exogenous nucleotide to be incorporated might be due to the lack of transport of CXP or, less likely, to the

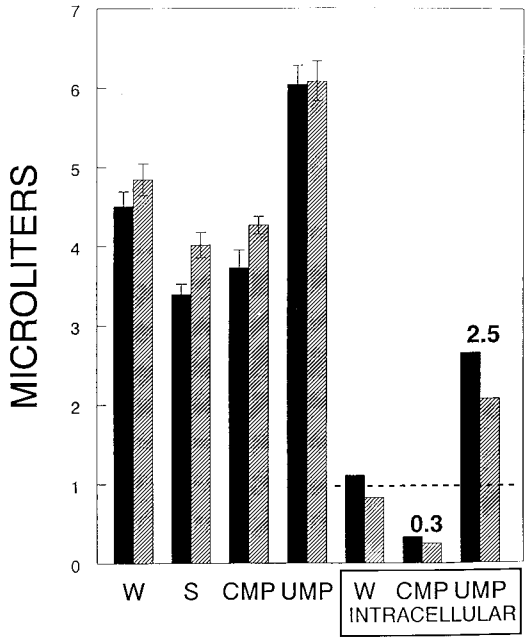

FIG. 3. Microspace assays demonstrate that UMP, but not CMP, is transported by rickettsiae. The virtual volumes (disintegrations per minute in the pellet/disintegrations per minute per microliter of incubation mixture) of water (W), sucrose (S), CMP, and UMP are shown for two experiments, with the standard deviations of 6 to 9 replicates within that experiment. The intracellular virtual volumes of water, CMP, and UMP are shown. The dashed line represents the volume that would be occupied at an intracellular concentration equal to the extracellular concentration. The numbers above the bars represent the ratio of the intracellular concentrations to the extracellular concentrations of nucleotides.

presence of a huge pool of CXP within the rickettsiae. Such a pool could lower the specific activity of transported [^{32}P]CXP to a level where its incorporation into RNA would be unmeasurable. An added complication is that, if CXP cannot be transported, then CTP must be synthesized within the rickettsial cytoplasm.

To determine if there was any transport of CMP, independent of whether or not it was incorporated, the transport of CMP and UMP was measured by the microspace technique under conditions where there was no incorporation of either nucleotide (Fig. 3). In contrast to filtration assays of transport activity, this technique (12) does not require that the rickettsiae be efficiently washed to remove external substrate because it measures the intracellular and extracellular space of the sedimented rickettsiae from the distribution of radiolabeled sucrose and water and allows one to calculate the amount of intracellular substrate. Equally important, small signals can be measured because this method allows large numbers of rickettsiae to be assayed. As predicted from the incorporation assays, the virtual volume (disintegrations per minute of UMP in the pellet/disintegrations per minute of UMP per microliter of incubation mixture) occupied by UMP was greater than the total water volume of the rickettsial pellet. These values indicate that UMP was not only transported but also was concentrated 2.5-fold within the rickettsiae. In contrast, the virtual volume of CMP was less than the total water volume of the rickettsial pellet and was essentially the same as the sucrose volume. These values indicate that CMP was not transported across the rickettsial membrane and was distributed only within the extracellular space of the pellet.

Once it was established that CMP was not transported, the source of CTP for nucleic acid synthesis was a prime question. We determined that an extract of isolated, purified rickettsiae could catalyze the conversion of UTP to CTP. This activity, CTP synthetase, was assayed spectrophotometrically based on the higher molar extinction coefficient of CTP compared to that of UTP at 291 nm (1,520 and 182, respectively) essentially as previously described (1). CTP was formed in this system at a curvilinear rate that was directly proportional to the amount of the crude, clarified, French-pressed extract that was added (90, 180, 288, 360, and 720 μg).

The recently published genomic sequence of *R. prowazekii* (2) provided additional perspective for these data (Fig. 4).

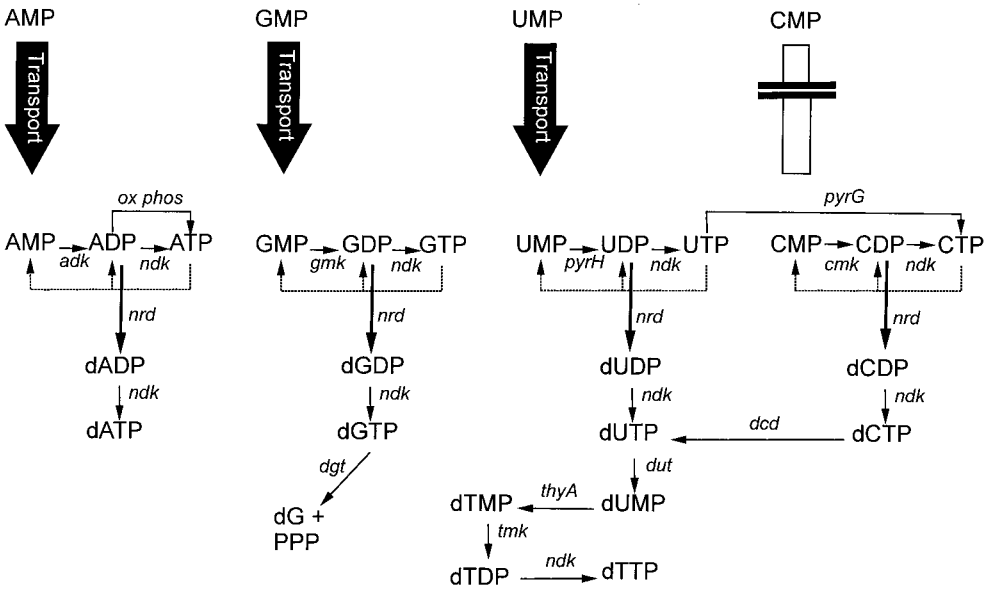

FIG. 4. Schema for nucleotide metabolism in *R. prowazekii* based on genetic and biochemical data.

Previous investigations had strongly suggested that nucleotides were not synthesized de novo by rickettsiae. This is supported by the genome sequence, in which the genes encoding the enzymes of purine and pyrimidine biosynthesis are absent (or at least unannotated). However, there is one curious exception: the rickettsial gene RP220 is annotated as *purC* based on a very significant score when analyzed by the Blast2 program, with matches of amino acid residues throughout the length of the protein. The gene *purC* encodes an enzyme whose role is in the middle of the complex purine biosynthesis pathway, a pathway for which no other genes have been found in the genome and which most likely does not exist in rickettsiae. As was predicted from biochemical investigations (6), the gene encoding the enzyme to reduce ribonucleotides to deoxyribonucleotides is present. In addition, as predicted (9, 10), the genes to encode the enzymes to convert dCTP to dUTP and dUTP to dTTP are present. The genome contains *pyrG*, which encodes a protein whose sequence is 55% identical to the sequence of the CTP synthetase of *Azospirillum brasilense*, which supports the identification of such an activity in this study. Although there are a large number of unannotated putative transport systems in the genome, no genes coding for NMP transport systems described in the present study (or AMP transport described in previous studies [3, 4]) are annotated. Because of the unusual nature of transport systems for nucleotides and their absence from free-living bacteria, it is not surprising that no homologs of such transport systems could be found in GenBank.

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