

## Reduction of Ribonucleotides by the Obligate Intracytoplasmic Bacterium *Rickettsia prowazekii*

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*Rickettsia prowazekii*, an obligate intracellular parasitic bacterium, was shown to have a ribonucleotide reductase that would allow the rickettsiae to obtain the deoxyribonucleotides needed for DNA synthesis from rickettsial ribonucleotides rather than from transport. In the presence of hydroxyurea, *R. prowazekii* failed to grow in mouse L929 cells or SC2 cells (a hydroxyurea-resistant cell line), which suggested that *R. prowazekii* contains a functional ribonucleotide reductase. This enzymatic activity was demonstrated by the conversion of ADP to dADP and CDP to dCDP, using (i) a crude extract of Renografin-purified *R. prowazekii* that had been harvested from infected yolk sacs and (ii) high-performance liquid chromatographic analysis. The rickettsial ribonucleotide reductase utilized ribonucleoside diphosphates as substrates, required magnesium and a reducing agent, and was inhibited by hydroxyurea. ADP reduction was stimulated by dGTP and inhibited by dATP. CDP reduction was stimulated by ATP and adenylylimido-diphosphate and inhibited by dATP and dGTP. These characteristics provided strong evidence that the rickettsial enzyme is a nonheme iron-containing enzyme similar to those found in mammalian cells and aerobic *Escherichia coli*.

*Rickettsia prowazekii*, the etiological agent of epidemic typhus in humans, is an obligate intracellular parasitic bacterium. The rickettsiae grow directly in the host cell's cytoplasm and are not bounded by phagosomal or phagolysosomal membranes (24). This unusual external milieu provides the rickettsiae with a rich source of preformed metabolites.

Knowledge of the metabolism and transport activities of rickettsiae for nucleotides and related metabolites is limited. The route of acquisition of the deoxyribonucleotides needed for DNA synthesis is unknown. Two distinct possibilities exist in such an obligate intracytoplasmic bacterium. The rickettsiae could transport deoxyribonucleotides from the host cell's cytoplasm and incorporate them into their DNA directly. Alternatively, the rickettsiae could transport ribonucleotides and then synthesize deoxyribonucleotides by employing a rickettsial ribonucleotide reductase. The ability of *R. prowazekii* to transport large, charged metabolites from the host cell's cytoplasm is well documented (26). For example, carrier-mediated membrane transport systems that provide for exchange of intra- and extracellular adenine nucleotides have been described in *R. prowazekii* for both AMP (1) and ATP/ADP (25). However, no system for the net transport of ribonucleotides, ribonucleosides, ribonucleobases, or the corresponding deoxy forms has been described.

Ribonucleotide reductase (ribonucleoside diphosphate reductase [EC 1.17.4.1] and ribonucleoside triphosphate reductase [EC 1.17.4.2]) catalyzes the conversion of ribonucleotides to their corresponding deoxyribonucleotides (10, 18, 22). The activity of this enzymatic system is controlled by ribo- and deoxyribonucleotides, dithiols, and additional metal ions in complex ways. Because the pool size of deoxyribonucleoside triphosphates is small compared with the rate of DNA synthesis, the reduction of ribonucleotides is considered to be a rate-limiting step in the formation of

deoxyribonucleotides needed for DNA synthesis (10, 18, 22). Ribonucleotide reductase from various procaryotic and eucaryotic sources has been extensively studied, but no data have been reported for intracellular bacteria.

To investigate the existence of a ribonucleotide reductase in *R. prowazekii*, we observed that *R. prowazekii* failed to grow in mammalian cells cultured in the presence of hydroxyurea (14), whereas normal growth was observed in the absence of hydroxyurea. Furthermore, the existence of a ribonucleotide reductase in *R. prowazekii* was confirmed by demonstrating the conversion of labeled ADP to dADP and CDP to dCDP in a crude extract of purified *R. prowazekii*. Regulation of the rickettsial enzyme by allosteric effectors was similar to that observed in aerobically grown *Escherichia coli*.

### MATERIALS AND METHODS

**Reagents and supplies.** Adenylylimido-diphosphate ( $\beta,\gamma$ -imidoadenosine 5'-triphosphate; AMP-PNP), adenosine, deoxyadenosine, cytosine, cytidine, deoxycytidine, ADP, CDP, ATP, AMP, CTP, snake (*Crotalus adamanteus*) venom, coenzyme B<sub>12</sub>, and hydroxyurea were purchased from Sigma Chemical Co., St. Louis, Mo. dATP and dGTP were purchased from Calbiochem, San Diego, Calif. [2,8-<sup>3</sup>H]ADP (30 Ci/mmol) was purchased from Mallinckrodt Co., St. Louis, Mo. [5-<sup>3</sup>H]CDP (25.3 Ci/mmol) was purchased from New England Nuclear Corp., Boston, Mass. [5-<sup>3</sup>H]CTP (31 Ci/mmol) and [2,8-<sup>3</sup>H]ATP (30 Ci/mmol) were purchased from ICN Biomedicals, Inc., Irvine, Calif.

**Cell cultures.** Mouse L929 cells were purchased from Flow Laboratories, Inc., McLean, Va., and grown in Eagle minimal essential medium (MEM; Mediatech, Washington, D.C.) supplemented with 10% Serum Plus (KC Biologicals, Lenexa, Kans.). Mouse SC2 cells, generously provided by Jim A. Wright, University of Manitoba, Winnipeg, Manitoba, Canada, were grown in MEM supplemented with 10% heat-inactivated fetal calf serum (Flow Laboratories) and 5

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mM hydroxyurea. All cells were grown as monolayers in a humidified atmosphere of 3% CO<sub>2</sub> in air at 34°C.

**Measurement of rickettsial growth.** The cell monolayers, in 12-well plates from which medium had been aspirated, were infected with rickettsiae which were diluted with Hanks balanced salt solution supplemented with 5 mM L-glutamic acid (monopotassium salt) and 0.1% gelatin at multiplicities of infection ranging from 100 to 200. After a 1-h adsorption period at 34°C, the medium was removed and the cells were washed twice with MEM plus 10% serum, and then MEM plus 10% serum, with or without hydroxyurea, was added. After the infection, 1 µg of emetine, a eucaryotic protein synthesis inhibitor, was added per ml of culture. At the indicated times, duplicate coverslips were removed and stained by a modification of the method of Gimenez (7). Rickettsiae present in each of 100 cells were counted microscopically as previously described (23). A cell containing more than 100 rickettsiae was assigned a value of 100.

**Preparation of extracts for ribonucleotide reductase assay.** Six-day-old embryonated, antibiotic-free hen eggs (Truslow Farms, Chestertown, Md.) were inoculated with a seed pool of *R. prowazekii* Madrid E (yolk sac passage 280). Eight days later, the rickettsiae were harvested and purified as described previously (25). The preparation of *R. prowazekii* was further purified by a variation of Renografin density gradient centrifugation (5, 9). The rickettsiae were suspended in SPG (218 mM sucrose, 3.76 mM KH<sub>2</sub>PO<sub>4</sub>, 7.1 mM K<sub>2</sub>HPO<sub>4</sub>, 5 mM glutamic acid, pH 7.0), layered onto 25% Renografin (E. R. Squibb, Inc., Princeton, N.J.), and then centrifuged at 30,240 × g for 60 min at 4°C in an SA-600 rotor (Dupont Sorvall, Inc., Norwalk, Conn.). Rickettsiae purified in this manner are referred to herein as Renografin-purified rickettsiae. In preparations used for enzyme assays, only freshly harvested rickettsiae were used.

For control purposes, homogenates containing no active rickettsial enzymes were prepared by adding purified heat-killed *R. prowazekii* to suspensions of yolk sacs from mock-infected eggs. This homogenate, after removal of yolk and unbroken cells, was referred to as the crude sham preparation. After Renografin purification, it was referred to as the Renografin-purified sham preparation.

The rickettsiae, crude sham preparations, and Renografin-purified sham preparations were suspended in a solution containing 40 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.2), 2 mM dithiothreitol (DTT), 2 mM magnesium acetate, and 20% glycerol. The suspensions were lysed by passage through a French pressure cell at 20,000 lb/in<sup>2</sup> twice, and the lysates were clarified by centrifugation at 10,886 × g for 20 min in an SA-600 rotor to remove cellular debris. These crude extracts were stored at -80°C in aliquots as the enzyme sources and were used within 2 weeks.

**Ribonucleotide reductase assay.** Ribonucleotide reductase activity was assayed by measuring the product, dADP or dCDP, formed from the substrate, ADP or CDP. Assays were carried out in a total volume of 40 µl, of which 20 µl (85 to 120 µg of protein) was the crude extract. The reaction system for ADP reduction contained 40 mM HEPES (pH 7.2), 10 mM DTT, 2 mM magnesium acetate, 1 mM dGTP, 20 µM ADP, and [2,8-<sup>3</sup>H]ADP (0.01 µCi). The reaction system for CDP reduction contained 40 mM HEPES (pH 7.2), 10 mM DTT, 2 mM magnesium acetate, 5 mM AMP-PNP, 20 µM CDP, and [5-<sup>3</sup>H]CDP (0.05 µCi).

The reactions were initiated by the addition of the crude extract of *R. prowazekii*, incubated for 20 min at 37°C, and terminated by heating at 100°C for 2 min. The nucleotides

were converted to nucleosides by treatment of the reaction mixture with crude snake (*C. adamanteus*) venom containing phosphodiesterase and 5'-nucleotidase according to the method of Cory et al. (4). At the end of the treatment, the reactions were stopped by heating at 100°C for 2 min and the precipitated material was discarded after centrifugation at 16,000 × g for 3 min. The ribonucleosides were separated from the deoxyribonucleosides by high-performance liquid chromatography (HPLC).

The HPLC system consisted of a model 6000A solvent delivery system (Waters Associates, Inc., Milford, Mass.), a model 7125 injector (20 µl; Rheodyne, Inc., Cotati, Calif.), a model 788 dual-wavelength variable detector (Micromeritics, Inc., Norcross, Ga.), and an SP4270 computing integrator (Spectra-Physics, San Jose, Calif.). Cytosine, cytidine, and deoxycytidine were separated by a modification of the method of Olivares and Verdys (15), using an Adsorbosphere RP-18 column (250-mm length by 4.6-mm inner diameter, 5-µm particle size; Alltech Associates, Deerfield, Ill.) at room temperature with isocratic elution at a flow rate of 1.0 ml/min. The mobile phase was 10 mM boric acid adjusted to pH 7.6 with 10 mM sodium borate. UV absorbance of the effluent was monitored at 260 nm. The cytosine, cytidine, and deoxycytidine peaks were collected, and the radioactivity of each peak was determined by liquid scintillation counting. Separation of deoxyadenosine from adenosine was accomplished by a similar method employing a Versapak RP-18 column (250-mm length by 4.1-mm inner diameter, 10-µm particle size; Alltech) and a mobile phase of 50 mM ammonium dihydrogen phosphate (pH 3.5) at a flow rate of 1.5 ml/min.

The amount of the products formed was calculated by using the percentage of radioactivity in the product and the total amount of substrate added to the reaction mixture. The actual value may be slightly higher than the calculated value because there was a small amount of endogenous ADP and CDP in the rickettsial crude extract.

When Dowex-1-borate chromatography (one of the most widely used methods for the determination of ribonucleotide reductase activity [4, 21]) and HPLC were compared for assay of the ADP reduction activity of the crude extract of *R. prowazekii*, a twofold increase in sensitivity was obtained with HPLC. Similarly, CDP reduction was detected by HPLC but could not be detected with Dowex-1-borate chromatography. The decreased sensitivity of Dowex-1-borate chromatography was due to the presence of labeled adenine and cytosine as contaminants of the commercial [2,8-<sup>3</sup>H]ADP and [5-<sup>3</sup>H]CDP, respectively. Coelution of the labeled nucleobases with the deoxyribonucleosides made the control background artificially high. When working with an intracellular parasite of which limited quantities of organisms were available, such high noise-to-signal ratios were intolerable. Elimination of this high background was accomplished by using HPLC, with which the separation of nucleobases, deoxyribonucleosides, and ribonucleosides was easily obtained.

**Other methods.** Protein concentrations were determined by the method of Bradford as described previously (8). Ribonucleoside triphosphates, ribonucleoside diphosphates, and ribonucleoside monophosphates were separated from each other by polyethyleneimine-cellulose thin-layer chromatography, using a solvent system of 1 N acetic acid-4 M LiCl (4:1) (16).

TABLE 1. Properties of ribonucleotide reductase from *R. prowazekii*

Experimental conditions <sup>a</sup>	Sp act of enzyme (pmol/min/mg of protein) <sup>b</sup>	
	ADP reduction	CDP reduction
Complete reaction mixture	14.7 ± 1.0	192.1 ± 16.3
Low magnesium (1 mM)	3.2 ± 0.5	47.4 ± 12.6
Low dithiothreitol (1 mM)	2.0 ± 1.1	64.5 ± 27.8
+ Coenzyme B <sub>12</sub> (10 μM)	8.0 ± 2.8	132.4 ± 34.9
+ Coenzyme B <sub>12</sub> (100 μM)	7.4 ± 0.9	105.3 ± 74.6
- NDP + NTP	3.3 ± 0.4	1.8 ± 1.4
- NDP + NMP	0.1 ± 0.1	ND
Crude sham preparation	0.8 ± 1.1	0.9 ± 1.3
Renografin-purified sham preparation	0.0	0.0

<sup>a</sup> The complete reaction mixture contained 40 mM HEPES (pH 7.2), 10 mM DTT, 2 mM magnesium acetate, 1 mM dGTP or 5 mM AMP-PNP, and 20 μM substrate. Abbreviations: NDP, nucleoside diphosphate; NTP, nucleoside triphosphate; NMP, nucleoside monophosphate.

<sup>b</sup> Each value represents the mean ± standard deviation for two experiments. ND, Not determined.

## RESULTS

**Effect of hydroxyurea on the growth of *R. prowazekii* in mouse L929 and SC2 cells.** *R. prowazekii* failed to grow in mouse L929 cells in the presence of 0.65 mM hydroxyurea, a potent inhibitor of ribonucleoside diphosphate reductase in eucaryotic cells and some procaryotic cells. Since the L929 cells were sensitive to the inhibitory effect of hydroxyurea and were unable to grow, the failure of *R. prowazekii* to grow could have been due to the depletion of the deoxyribonucleotide pools in the host cell (below the level needed by *R. prowazekii* as transportable substrates) rather than the inhibition of a rickettsial ribonucleotide reductase by hydroxyurea. Therefore, similar experiments were performed in SC2 cells, a hydroxyurea-resistant mutant of mouse L929 cells (13). The SC2 cells grew normally in 0.5 mM hydroxyurea (doubling time of 20 to 22 h) and still grew well in 5 mM hydroxyurea (doubling time of 31 h). *R. prowazekii* grew well in mouse SC2 cells but failed to grow in the presence of 5 mM hydroxyurea. At 0.5 and 2 mM hydroxyurea, the rickettsial morphology was altered: the rickettsiae failed to septate and could not be enumerated. These data suggested that *R. prowazekii* contained a hydroxyurea-sensitive ribonucleotide reductase.

**Reduction of ADP and CDP by the crude extract of *R. prowazekii*.** To directly determine whether *R. prowazekii* contained detectable ribonucleotide reductase activity, a crude extract of Renografin-purified *R. prowazekii* was incubated with substrate (labeled ADP or CDP) and the corresponding deoxyribonucleotide product was measured by HPLC. Radioactive product peaks coinciding with standard deoxyadenosine (retention time of 19.9 ± 1.6 min) and standard deoxycytidine (retention time of 15.7 ± 0.9 min) were observed. Little or no ribonucleotide reductase activity was observed after incubation of either crude or Renografin-purified sham preparations with the substrates under the same conditions as those used with the crude extracts of *R. prowazekii* (Table 1).

**Characterization of the ribonucleotide reductase from *R. prowazekii*.** The ribonucleotide reductase activity of *R. prowazekii* was dependent on the concentration of the crude extract, the concentration of substrates, and the incubation time. A time course of both ADP and CDP reductions showed that the CDP reduction activity was linear for 80

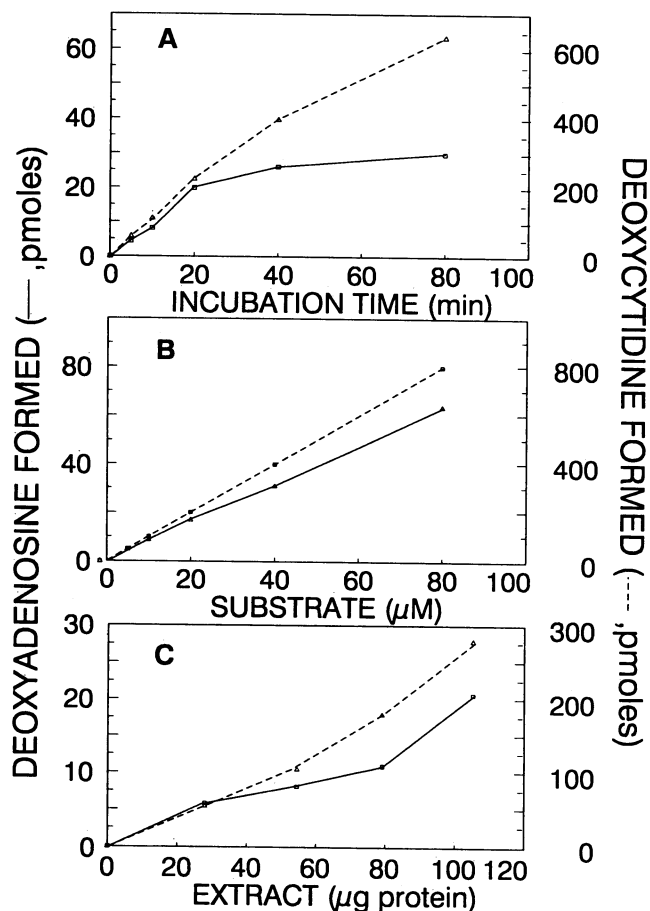


FIG. 1. Ribonucleotide reductase activity in *R. prowazekii*. (A) Dependence of ADP and CDP reductions on the incubation time at a substrate concentration of 20 μM and 100 μg of protein. (B) Dependence of ADP and CDP reductions on the substrate concentration at 20-min incubation time and 100 μg of protein. (C) Dependence of ADP and CDP reductions on the protein concentration of the crude extract at a substrate concentration of 20 μM and at 20-min incubation time. Each value represents the average of duplicate points in a typical experiment.

min, whereas ADP reduction activity was linear for only 20 min and then plateaued under our experimental conditions (Fig. 1A). In our standard procedure, we chose 20 min as the incubation time. The reaction velocity was linear with respect to substrate concentration from 0 to 80 μM for both ADP and CDP reductions (Fig. 1B). The reaction velocity almost paralleled the amount of crude extract from 0 to 100 μg of protein for both ADP and CDP reductions (Fig. 1C). CDP reduction was greater than ADP reduction. At a substrate concentration of 20 μM, the specific enzymatic activity was 15 ± 1 pmol/min/mg of protein for ADP reduction and 192 ± 16 pmol/min/mg of protein for CDP reduction.

To characterize the ribonucleotide reductase of *R. prowazekii*, several properties of the rickettsial ribonucleotide reductase were compared with the properties of ribonucleotide reductases described for other organisms. Three types of ribonucleotide reductases have been described in other organisms: the adenosylcobalamine-dependent ribonucleotide reductase (B<sub>12</sub>-RR), the nonheme iron-containing ribonucleotide reductase (Fe-RR), and the manganese-dependent ribonucleotide reductase (Mn-RR) (10, 18, 22). All

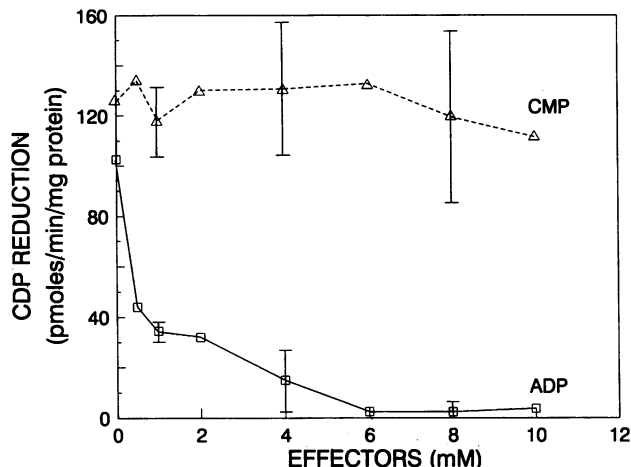


FIG. 2. Effects of CMP and ADP on CDP reduction in *R. prowazekii*. The concentration of each effector represents the final concentration in the reaction mixture. Symbols: ---, addition of CMP to the standard reaction mixture; —, addition of ADP to the standard reaction mixture. Each value represents the mean and standard deviation of two independent experiments.

three enzymes require a reducing agent, such as DTT, to function. The Fe-RR and B<sub>12</sub>-RR enzymes require Mg<sup>2+</sup>, and the activity of the B<sub>12</sub>-RR enzyme is stimulated by deoxyadenosylcobalamin. The properties of the ribonucleotide reductase of *R. prowazekii* are presented in Table 1. Lowering the level of Mg<sup>2+</sup> in the reaction mixture to that present in the extract resulted in a 78% decrease in ADP reduction and a 75% decrease in CDP reduction. Lowering the level of DTT in the reaction mixture to that present in the extract resulted in an 86% decrease in ADP reduction and a 66% decrease in CDP reduction. Adding 10 or 100 μM coenzyme B<sub>12</sub> to the reaction mixture failed to stimulate either CDP reduction or ADP reduction.

To further define the type of ribonucleotide reductase in *R. prowazekii*, substrate specificity was investigated, since the Fe-RR and Mn-RR utilize ribonucleoside diphosphates as substrates, whereas the B<sub>12</sub>-RR utilizes ribonucleoside triphosphates as substrates (10, 18, 22). Ribonucleoside diphosphates were the preferred substrates for the rickettsial enzyme (Table 1). With ATP and CTP (20 μM) as substrates, the specific enzyme activities were only 3 ± 0.4 and 2 ± 1.4 pmol/min/mg of protein, respectively; these values were equal to about 22% of ADP reduction activity and 1% of CDP reduction activity. Similar low levels of enzyme activity with ribonucleoside triphosphates have been observed with the partially purified ribonucleotide reductase of *E. coli* (17, 19). These observations can be explained by the hydrolysis of ribonucleoside triphosphates to ribonucleoside diphosphates during the reaction (see below). Using 20 μM AMP as the substrate, no reduction was observed. Although CMP was not directly tested as a substrate, the inability of CMP to inhibit CDP reduction (Fig. 2) suggested that CMP was not a substrate for the enzyme. The ability of ADP to inhibit CDP reduction (Fig. 2) suggested that the reductions of purines and pyrimidines are catalyzed by the same enzyme.

Hydroxyurea inhibits Fe-RR and Mn-RR but has no effect on the activity of B<sub>12</sub>-RR. As shown in Fig. 3, 50% inhibition of the rickettsial CDP and ADP reduction activities was observed at 10 μM hydroxyurea.

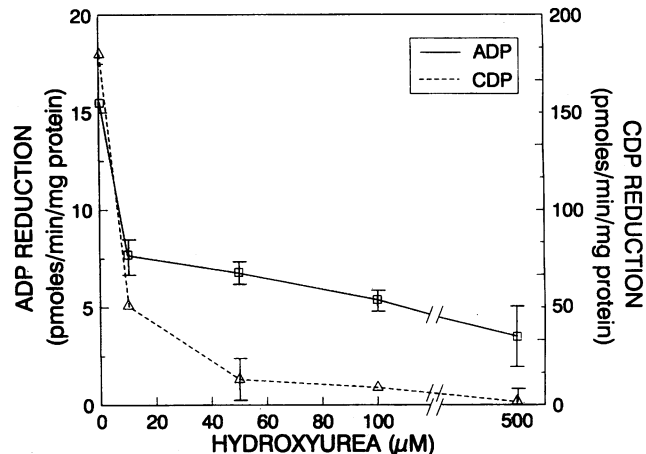


FIG. 3. Effect of hydroxyurea on the specific activities of ADP (—) and CDP (---) reductions. The concentration of hydroxyurea represents the final concentration in the reaction mixture. Each value represents the mean and standard deviation of two independent experiments.

**Degradation of the ribonucleotides.** The crude extract of *R. prowazekii* contained various enzymes, such as phosphatases, kinases, and nucleotidases, which could catalyze the depletion of substrates and effectors. To investigate the degree of degradation of nucleotides by the crude extract of *R. prowazekii*, labeled ATP, ADP, AMP, CTP, and CDP were incubated with the crude extract of *R. prowazekii* under standard reaction conditions. After a 20-min incubation period, the reaction mixture was sampled and the distribution of radioactivity corresponding to ribonucleoside triphosphate, ribonucleoside diphosphate, and ribonucleoside monophosphate was determined by polyethyleneimine-cellulose thin-layer chromatography. At the end of the incubation, only 4% of the labeled ATP remained as ATP; the rest had been dephosphorylated to ADP (31%) and AMP (65%). With labeled CTP, 6% of the label remained as CTP; the other 94% of the label had been dephosphorylated to CDP (7%) and CMP (87%). With labeled ADP, only 19% of the label remained as ADP; the rest was phosphorylated to ATP (7%) and dephosphorylated to AMP (74%). With labeled CDP, 78% of the label remained as CDP, 5% was phosphorylated to CTP, and 17% was dephosphorylated to CMP. When labeled AMP was used, 89% of the label remained as AMP. This high rate of nucleotide degradation in the crude extract of *R. prowazekii* made it impossible to analyze quantitatively the enzyme kinetics, substrate specificity, and allosteric regulation of the enzyme. Attempts to purify the rickettsial ribonucleotide reductase with very limited quantities of starting material have been unsuccessful.

**Allosteric regulation of the ribonucleotide reductase of *R. prowazekii*.** The regulatory pattern of the rickettsial ribonucleotide reductase was examined to compare it with the regulatory patterns described for ribonucleotide reductases from other organisms (10, 18, 22). Other researchers showed that with Fe-RR, CDP reduction is stimulated by ATP and inhibited by both dATP and dGTP; in addition, ADP reduction is stimulated by dGTP, inhibited by dATP, and insensitive to ATP. However, dATP stimulates CTP reduction by the B<sub>12</sub>-RR and CDP reduction by the Mn-RR. With the rickettsial ribonucleotide reductase, dGTP stimulated the reduction of ADP, with a maximal effect observed at 0.5 mM

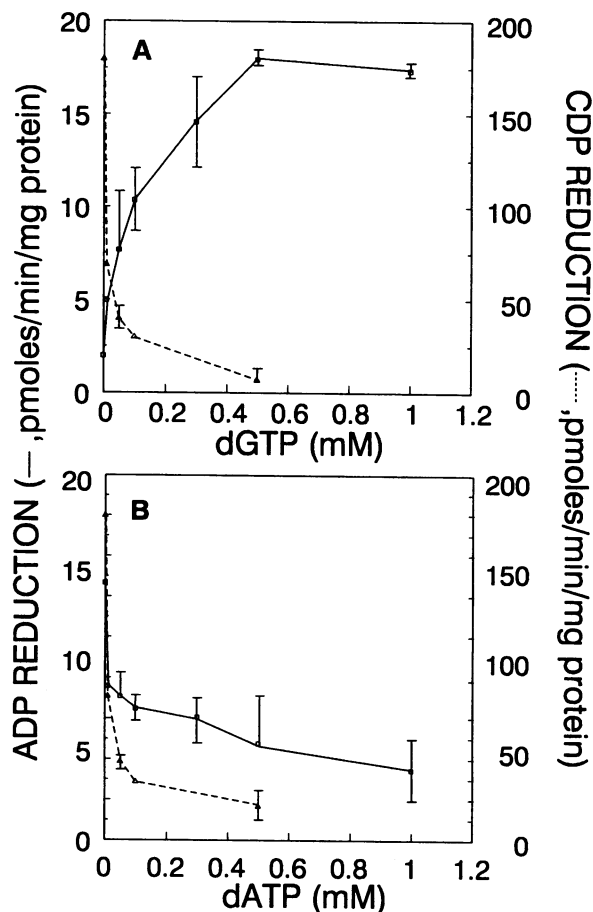


FIG. 4. Effects of dGTP and dATP on the specific activities of ADP and CDP reductions. The indicated concentrations represent the final concentrations of dGTP and dATP in the reaction mixture. (A) Effect of dGTP on ADP (—) and CDP (---) reductions. (B) Effect of dATP on ADP (—) and CDP (---) reductions. Each value represents the mean and standard deviation of two independent experiments.

dGTP (Fig. 4A). In contrast, dGTP strongly inhibited CDP reduction (Fig. 4A). However, both ADP and CDP reductions were inhibited by dATP (Fig. 4B). Although the addition of ATP had an inhibitory effect on ADP reduction (Fig. 5A), it is probable that this inhibition was due to the ADP formed from the hydrolysis of ATP, which diluted the specific activity of the substrate. Accordingly, AMP-PNP (11, 20), a nonhydrolyzable analog of ATP, showed little inhibition of ADP reduction, even at 10 mM AMP-PNP (Fig. 5A). Although ATP only slightly stimulated CDP reduction (Fig. 5B), the stimulation was probably limited because of the inhibitory effect of the ADP (formed from the dephosphorylation of ATP) (Fig. 2). Predictably, the addition of AMP-PNP resulted in a much greater stimulation of CDP reduction than did the addition of ATP (Fig. 5B). This regulatory pattern was consistent with that seen in the Fe-RR enzyme.

#### DISCUSSION

The existence of a ribonucleotide reductase in *R. prowazekii* was first suggested by in situ experiments with SC2 cells, which are hydroxyurea-resistant mutants of

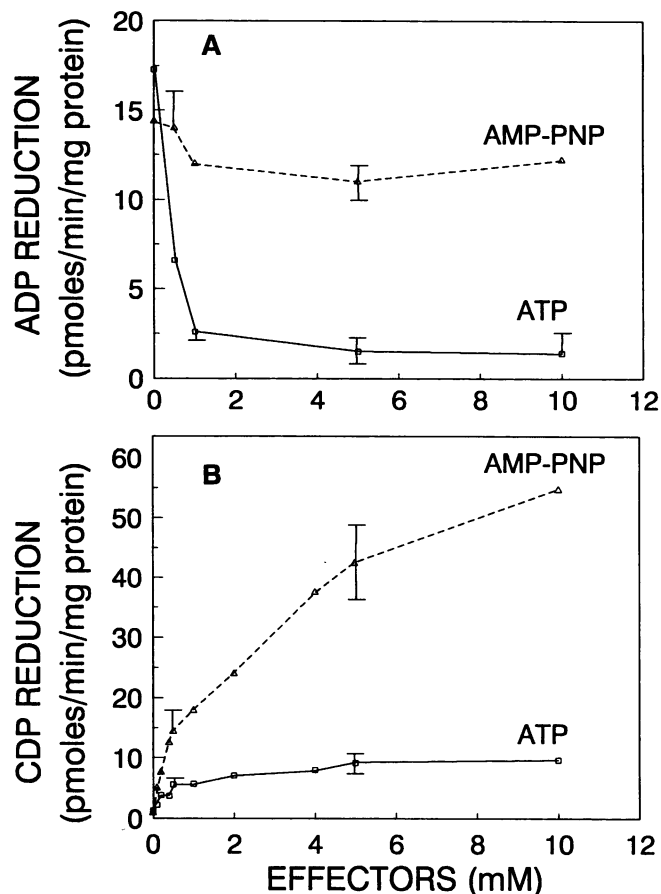


FIG. 5. Effects of ATP and AMP-PNP on the specific activities of ADP and CDP reductions. Concentrations of effectors (ATP and AMP-PNP) are expressed as final concentrations in the reaction mixture. (A) Effect of ATP (—) and AMP-PNP (---) on ADP reduction. (B) Effect of ATP (—) and AMP-PNP (---) on CDP reduction. Each value represents the mean and standard deviation of two independent experiments.

mouse L929 cells and are able to grow in the presence of 5 mM hydroxyurea. *R. prowazekii* grew well in SC2 cells but was unable to grow when 5 mM hydroxyurea was included in the medium. Because the deoxyribonucleotide pools in the cytoplasm of SC2 cells remained adequate for host cell growth in the presence of hydroxyurea, the most likely explanation for these results is that *R. prowazekii* must synthesize its own deoxyribonucleotides by a ribonucleotide reductase and this enzymatic activity was inhibited by hydroxyurea. The rickettsial ribonucleotide reductase was further investigated by in vitro experiments. With crude extracts of *R. prowazekii* as the enzyme source, we demonstrated the ability of *R. prowazekii* to reduce purine nucleotides (ADP) and pyrimidine nucleotides (CDP) to their corresponding deoxyribonucleotides. To confirm the rickettsial origin of the measured ribonucleotide reductase activity, sham preparations were made from mock-infected yolk sacs; little ribonucleotide reductase activity was observed in the crude sham preparation and no activity was observed in the Renografin-purified sham preparation.

In a crude extract of *R. prowazekii*, CDP reductase activity was 10-fold higher than ADP reductase activity. A similar phenomenon was also observed with the Fe-RR-type enzyme from mammalian cells (3) and *E. coli* (12). Since the

ribonucleotide reductase of *R. prowazekii* probably consists of only one enzyme system as demonstrated in *E. coli* (10, 18, 22), which can reduce all four ribonucleotides, the differences in the measured enzymatic activities could be due to a more pronounced degradation of the effectors and substrates for ADP reduction than for CDP reduction or due to kinetic differences between the two substrates.

Ribonucleotide reductase provides the only de novo pathway for the synthesis of the deoxyribonucleotides required for DNA replication in both eucaryotic and procaryotic cells and is a complex enzyme from a structural and regulatory point of view. Three types of ribonucleotide reductases which differ in their coenzyme or metal requirements have been described (10, 18, 22): B<sub>12</sub>-RR, predominant in procaryotes; Fe-RR, found in eucaryotes, aerobically grown *E. coli*, and viruses; and Mn-RR, described in *Brevibacterium*, *Micrococcus*, and *Arthrobacter* species. All three enzymes require a reducing agent for their activities. Both Fe-RR and Mn-RR are heterodimeric proteins and use ribonucleoside diphosphates as substrates, and their enzymatic activities are inhibited by hydroxyurea. The B<sub>12</sub>-RR is a monomeric protein, uses ribonucleoside triphosphates as substrates, and is insensitive to hydroxyurea. Recently, an enzyme from anaerobically growth *E. coli* that does not conform to the above classes of ribonucleotide reductases has been discovered (2, 6). This enzyme is different in that it utilizes ribonucleoside triphosphates as substrates, is resistant to the effect of hydroxyurea, and cannot be stimulated by adenosylcobalamine.

To analyze the characteristics of the rickettsial ribonucleotide reductase, some of the properties of the enzyme, including Mg<sup>2+</sup> and DTT requirements, sensitivity to hydroxyurea, substrate specificity, and the pattern of allosteric regulation, were assayed. The rickettsial ribonucleotide reductase activities showed a requirement for Mg<sup>2+</sup> and DTT and could be inhibited by hydroxyurea. The preferred substrates were ribonucleoside diphosphates. Although small amounts of dADP and dCDP were observed with ATP and CTP as substrates, this finding could be explained by degradation of some of the ATP to ADP and CTP to CDP during the 20-min incubation. Nucleoside monophosphates were not utilized as substrates. The ribonucleotide reductase activities of *R. prowazekii* were also subject to allosteric regulation, and this regulatory behavior was similar to that of Fe-RR (10, 18, 22); that is, allosteric activation of CDP and ADP reduction was exerted by ATP and dGTP, respectively. Moreover, allosteric inhibition of ADP reduction was observed with dATP and allosteric inhibition of CDP reduction was observed with dATP or dGTP.

As expected, ATP stimulated CDP reduction but, surprisingly, inhibited ADP reduction. According to the literature, ATP is not an allosteric inhibitor of ADP reduction in either the Fe-RR or the Mn-RR system. However, as mentioned above, during the 20-min incubation period, ATP was converted to ADP and the ADP formed could compete with the labeled substrate. This hypothesis was confirmed by using AMP-PNP as the effector. The noncleavable ATP analog AMP-PNP, which has been shown to activate L-cell and vaccinia virus-induced ribonucleotide reductases (11, 20), had no effect on ADP reduction, even at concentrations as high as 10 mM, and was much more effective than ATP at stimulating CDP reduction.

In conclusion, the results of both the in vitro and the in situ experiments demonstrated that *R. prowazekii* contains its own functional ribonucleotide reductase and that the ribonucleotide reductase of *R. prowazekii* belongs to the

nonheme iron-containing ribonucleotide reductases and is a highly regulated enzyme.

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