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Acquisition of Polyamines by the Obligate Intracytoplasmic Bacterium *Rickettsia prowazekii*

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Both the polyamine content and the route of acquisition of polyamines by *Rickettsia prowazekii*, an obligate intracellular parasitic bacterium, were determined. The rickettsiae grew normally in an ornithine decarboxylase mutant of the Chinese hamster ovary (C55.7) cell line whether or not putrescine, which this host cell required in order to grow, was present. The rickettsiae contained approximately 6 mM putrescine, 5 mM spermidine, and 3 mM spermine when cultured in the presence or absence of putrescine. Neither the transport of putrescine and spermidine by the rickettsiae nor a measurable rickettsial ornithine decarboxylase activity could be demonstrated. However, we demonstrated the de novo synthesis of polyamines from arginine by the rickettsiae. Arginine decarboxylase activity (29 pmol of $^{14}CO_2$ released per h per 10^8 rickettsiae) was measured in the rickettsiae growing within their host cell. A markedly lower level of this enzymatic activity was observed in cell extracts of *R. prowazekii* and could be completely inhibited with 1 mM difluoromethylarginine, an irreversible inhibitor of the enzyme. *R. prowazekii* failed to grow in C55.7 cells that had been cultured in the presence of 1 mM difluoromethylarginine. After rickettsiae were grown in C55.7 cells in the presence of labeled arginine, the specific activities of arginine in the host cell cytoplasm and polyamines in the rickettsiae were measured; these measurements indicated that 100% of the total polyamine content of *R. prowazekii* was derived from arginine.

Rickettsia prowazekii, the etiological agent of epidemic typhus, is an obligate intracytoplasmic bacterium. *R. prowazekii* grows free in the cytoplasm of its eucaryotic host cell unbounded by any internal membranes of host cell origin. If the rickettsiae have evolved appropriate transport systems, then they would be able to scavenge from this rich external milieu many of the end products and intermediate metabolites needed for growth rather than expending energy synthesizing these metabolites via complex pathways. For example, the enzymes of the glycolytic and hexose monophosphate pathways are absent in typhus group rickettsiae (9) and the essential metabolites generated by these pathways must be obtained from the cytoplasm of the host cell. Rickettsiae can also employ a dual strategy for the acquisition of metabolites: they can both transport and synthesize the same compound. This can be seen most clearly in the acquisition of ATP. The enzymes of the tricarboxylic acid cycle (9), oxidative phosphorylation (7, 12), and an ATPase (37) are present in the rickettsiae to synthesize ATP. However, the rickettsiae also have a transport system to obtain ATP from the cytoplasm of the host cell (32).

Virtually all cells contain significant amounts of some or all of the polyamines putrescine, spermidine, and spermine. Proposed functions for these highly basic amines include, but are not limited to, the stabilization of nucleic acids and the stimulation of protein biosynthesis in both eucaryotic and procaryotic organisms (3, 27, 36). For an obligate intracytoplasmic bacterium such as *R. prowazekii*, three possible pathways for the acquisition of polyamines from the host cell are possible (assuming the rickettsiae contain polyamines). First, the rickettsiae may transport ornithine or arginine from the host cell and via a rickettsial ornithine or arginine decarboxylase synthesize putrescine with the subsequent formation of spermidine and spermine. Second, the rickettsiae may transport putrescine from the host cell and convert the host-derived putrescine to spermidine and spermine via rickettsial spermidine and spermine synthases. Finally, the rickettsiae may satisfy all of their polyamine requirements by transporting these compounds directly from the cytoplasm of the host cell.

In this report, we demonstrate the presence of putrescine, spermidine, and spermine in the rickettsiae. Neither the transport of putrescine and spermidine nor the decarboxylation of ornithine could be measured either in isolated *R. prowazekii* or in situ (i.e., in the growing rickettsiae within the host cell cytoplasm). We found that *R. prowazekii* synthesizes polyamines exclusively from arginine. It is pertinent to the study of rickettsial metabolism to note that the rate of biosynthetic reactions observed in isolated rickettsiae often is not adequate to support an 8-h doubling time (33). These in vitro assays are performed on rickettsiae that are not growing and may be biosynthetically compromised because of the extensive time required to purify rickettsiae from chick yolk sacs. This necessitates the development of methods to study the metabolism of rickettsiae while they are growing in their host cell. Accordingly, an in situ approach to rickettsial metabolic studies was begun recently by Austin and co-workers (4, 5). Herein, we describe the acquisition of polyamines by *R. prowazekii* growing inside an ornithine decarboxylase mutant cell line.

MATERIALS AND METHODS

Chemicals. Dulbecco modified Eagle medium was purchased from Mediatech (Washington, D.C.). Serum Plus was purchased from KC Biologicals (Lenexa, Kans.), and the MEM Select-Amine kit was purchased from GIBCO Life Technologies, Inc. (Grand Island, N.Y.). L-[U-^{14}C]arginine (334.4 mCi/mmol) and [1,4-^{14}C]putrescine dihydrochloride (120 mCi/mmol) were purchased from Dupont, NEN Re-

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search Products (Boston, Mass.). DL-[5-^{14}C]ornithine hydrochloride (61 mCi/mmol) was purchased from Amersham Corp. (Arlington Heights, Ill.). All other chemicals were of the highest obtainable purity 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 (31) of the methods of Bovarnick and Synder (8) and Wisseman et al. (34). Viable rickettsiae were enumerated by the antibody hemolysis method of Walker and Winkler (29).

Propagation of cell cultures. An ornithine decarboxylase mutant of Chinese hamster ovary cells (C55.7) (23) was generously provided by Carolyn Steglich, East Carolina University, and grown in Dulbecco modified Eagle medium supplemented with 10% Serum Plus, 5×10^{-4} M putrescine, and nonessential amino acids. Chinese hamster ovary cells (CHO-K1) were purchased from the American Type Culture Collection (Rockville, Md.) and grown in Dulbecco modified Eagle medium supplemented with 10% Serum Plus and nonessential amino acids. The cells were cultured as monolayers at 34°C in a humidified atmosphere of 10% CO_2 in air. C55.7 cells were starved of putrescine by the removal of putrescine from the medium 48 h before infection.

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 for 1 h at 34°C at a multiplicity of infection that would give 2 to 4 rickettsiae per infected cell at time zero of the infection and 80 to 160 rickettsiae per cell at the time of harvest (48 h). After the infection, 1 μg of emetine per ml, a eucaryotic protein synthesis inhibitor, was added to the culture. At the indicated times, duplicate cover slips were removed and stained by a modification of the method of Gimenez (11). Rickettsiae present in each of 100 cells were counted microscopically as previously described (28). A total of 4 to 11% of the cells contained more than 100 rickettsiae and were assigned a value of 100. Growth was calculated as the average number of rickettsiae per infected cell at a given time since, in the presence of emetine, the percentage of cells infected remained essentially constant.

Determination of polyamine content in *R. prowazekii*. C55.7 cells, either putrescine starved for 48 h or supplemented with 0.5 mM putrescine, were infected with rickettsiae, and the rickettsiae were allowed to grow for another 48 h. The infected cells were removed from the monolayer and centrifuged at 500 × *g* for 5 min to pellet the cells. The infected cell pellet was then suspended in 2 ml of a sucrose buffer (218 mM sucrose, 3.76 mM KH_2PO_4, 7.1 mM K_2HPO_4, 5 mM potassium glutamate), and the host cells were lysed by N_2 cavitation (mini-bomb cell disruption chamber; Kontes Glass Company, 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. Purification of the rickettsiae from host cellular debris was done 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.

The purified rickettsial pellet was extracted in 930 μl of glass-distilled H_2O and 70 μl of 70% perchloric acid. After 60 min on ice, the insoluble proteins and nucleic acids were pelleted by centrifugation at 10,886 × *g* for 30 min at 4°C. The polyamines were benzoylated by the method of Redmond and Tseng (20). Briefly, 1 ml of 2 N NaOH, 1.25 nmol of 1,3-diaminopropane (to monitor for recovery), and 5 μl of benzoyl chloride were added to the supernatant fluid. The contents of the tubes were mixed well to emulsify the benzoyl chloride and placed at room temperature for 20 min, and then 2 ml of a saturated NaCl solution was added and the contents of the tubes were mixed well. The benzoylated polyamines were extracted by the addition of 2 ml of diethyl ether to the tubes. After centrifugation at 10,886 × *g* for 1 min to separate the layers, the upper organic phase was removed and the ether was evaporated under a stream of N_2. The dried residue was suspended in 100 μl of methanol and passed through a 0.2-μm-pore-size filter. The benzoylated polyamines were separated by high-performance liquid chromatography (HPLC).

Benzoylated putrescine, benzoylated spermidine, and benzoylated spermine were separated by the method of Slocum and co-workers (22). A ternary gradient HPLC system was used with a V4 variable wavelength detector (Isco, Inc., Lincoln, Nebr.). The column used for the separation was an Adsorbosphere RP-18 column (250 by 4.6 mm inner diameter, 5-μm particle size) from Alltech Associates, Inc. (Deerfield, Ill.). The benzoylated polyamines were eluted by using a linear gradient program from 0 to 80% methanol at a flow rate of 0.75 ml/min, and the absorbance of the effluent was monitored at 254 nm.

Determination of polyamine transport by *R. prowazekii* in situ. C55.7 cells were cultured in the presence or absence of 0.5 mM putrescine for 48 h before infection. At 24 h postinfection, 1 μCi of [1,4-^{14}C]putrescine was added to each dish, and at 48 h postinfection, the rickettsiae were isolated by N_2 cavitation and Renografin. The rickettsial polyamines were benzoylated and separated by HPLC, and the radioactivity of each peak was determined.

Determination of decarboxylase activities in isolated *R. prowazekii*. Radiorespirametric assays for decarboxylase activities were performed with intact rickettsiae purified from chick yolk sacs or with extracts prepared from these rickettsiae. For preparation of cell extracts, the rickettsiae were suspended in 1 ml of a buffer containing 50 mM KH_2PO_4, 1 mM dithiothreitol, and 5 mM Mg_2SO_4 and passed through a French pressure cell twice at 20,000 lb/in^2. The lysate was clarified by centrifugation at 10,886 × *g* for 20 min.

The rickettsial preparations (4 mg of protein) were incubated at 34°C for 60 min with 1 μCi of L-[U-^{14}C]arginine (334.4 mCi/mmol) or 1 μCi of DL-[5-^{14}C]ornithine (61 mCi/mmol) and with or without 40 μM pyridoxal-5-phosphate. After incubation of the preparations in a 20-ml glass scintillation vial containing a CO_2 cup and stopper, 200 μl of hyamine hydroxide was added to the cup and 100 μl of concentrated HCl was added to the incubation mixture. The vials were then incubated for 30 min at 34°C. Sodium bicarbonate (100 μl of a 500 mM solution) was added to the incubation mixture, and the vials were incubated at 34°C for 15 min. The CO_2 cup was removed, washed on the outside with 95% ethanol, and placed in a clean 20-ml glass scintillation vial containing 10 ml of a toluene-based scintillation fluid. The radioactivity was determined with a liquid scintillation counter (model 1219 Rackbeta; Pharmacia LKB Biotechnology, Piscataway, N.J.).

Determination of in situ decarboxylase activities of *R. prowazekii*. For the arginine decarboxylase measurements, C55.7 cells were passaged twice in an MEM Select-Amine arginine-deficient medium (containing 1/10th normal arginine) with nonessential amino acids, 5×10^{-4} M putrescine, and 10% Serum Plus to reduce the arginine pool in these

TABLE 1. Polyamine content of *R. prowazekii*

Polyamine	C55.7 cells − 0.5 mM putrescine				C55.7 cells + 0.5 mM putrescine			
	pmol	No. of rickettsiae[a] (10^8)	pmol/10^8 rickettsiae	Concn (mM)[b]	pmol	No. of rickettsiae[a] (10^8)	pmol/10^8 rickettsiae	Concn (mM)[b]
Putrescine	92 ± 9	2.7	34 ± 3	5.4 ± 0.5	88 ± 8	2.3	38 ± 4	6.0 ± 0.7
Spermidine	87 ± 10	2.7	33 ± 4	5.2 ± 0.7	74 ± 6	2.3	32 ± 3	5.1 ± 0.5
Spermine	46 ± 5	2.7	17 ± 2	2.7 ± 0.3	47 ± 4	2.3	20 ± 2	3.2 ± 0.3

[a] The number of rickettsiae was determined by Gimenez staining (11) as described in Materials and Methods. Host cell number was determined by measuring the amount of cellular protein per culture (1 mg of protein per 5×10^6 cells).

[b] These values were calculated by using a volume of 0.09 μm^3 per rickettsia (5). The intracytoplasmic water space was determined to be 6.3×10^{-17} liter per rickettsiae assuming 70% of the total volume is water (18). Each value represents the mean ± standard error for three experiments.

cells. For the ornithine decarboxylase measurements, the C55.7 cells were passaged in Dulbecco modified Eagle medium containing nonessential amino acids, 5×10^{-4} M putrescine, and 10% Serum Plus. The cells were then plated in 25-cm^2 flasks containing 5 ml of the same medium and infected with rickettsiae. L-[U-^{14}C]arginine (1 μCi at 334.4 mCi/mmol) or DL-[5-^{14}C]ornithine (1 μCi at 61 mCi/mmol) was added to the flask containing a CO_2 cup and stopper as previously described (16). The infected cells were incubated for another 24 or 48 h, and the $^{14}CO_2$ released was determined as above.

Incorporation of L-[U-^{14}C]arginine into rickettsial polyamines. C55.7 cells were cultured for 48 h in 100-mm tissue culture dishes containing MEM Select-Amine arginine-deficient medium (containing 1/10th normal arginine) with nonessential amino acids and 10% Serum Plus, in either the presence or absence of 0.5 mM putrescine. At 24 h postinfection, 1 μCi of L-[U-^{14}C]arginine (334.4 mCi/mmol) was added to each of the dishes. At 48 h postinfection in some experiments, the total polyamines (host cell plus rickettsial) were isolated, benzoylated, and separated by HPLC and the radioactivity corresponding to each of the polyamines was determined. In other experiments, the rickettsiae were isolated and their polyamines were derivatized and separated by HPLC. The polyamine peaks were identified and quantitated both chemically and radioactively.

To determine the specific activity of the arginine in C55.7 cells, the supernatant fluid, after host cell lysis and differential centrifugation to pellet the rickettsiae, was lyophilized to dryness. This dried residue (host cell cytoplasm) was suspended in 800 μl of 40 mM Li_2CO_3. The amino acids were dansylated by the method of De Jong et al. (10). Dansylated amino acids were separated by HPLC with an Adsorbosphere RP-18 column (250 by 4.6 mm inner diameter, 5-μm particle size) by the method of Oray et al. (19). The dansyl-arginine peak was identified and quantitated both chemically and radioactively.

Determination of protein concentrations. Protein concentrations were measured with the bichinchoninic acid (BCA) protein assay system from Pierce Chemical Company (Rockford, Ill.).

RESULTS

Polyamine content of *R. prowazekii*. *R. prowazekii* isolated from C55.7 cells cultured in the absence of putrescine contained a putrescine concentration of 34 pmol/10^8 rickettsiae, a spermidine concentration of 33 pmol/10^8 rickettsiae, and a spermine concentration of 17 pmol/10^8 rickettsiae (Table 1). This corresponds to approximately 5 mM putrescine, 5 mM spermidine, and 3 mM spermine. The total polyamine content of *R. prowazekii* was 84 pmol/10^8 rickettsiae (13 mM). The rickettsiae would need, on the average, 11 pmol of polyamine per h per 10^8 rickettsiae to maintain their normal polyamine pool and double in number in 8 h. The polyamine content of rickettsiae cultured in C55.7 cells with putrescine was not significantly different.

Lack of transport of putrescine and spermidine by *R. prowazekii*. Measurement of the transport of radiolabeled putrescine or spermidine was attempted in isolated rickettsiae, but because of the nonspecific binding of these molecules to the rickettsiae and the limited assay time possible for these labile organisms, interpretation of the data was not possible (data not shown). Greater sensitivity was sought by measuring the transport activity in situ over a 24-h period. Rickettsial uptake of labeled putrescine (or labeled spermidine formed by the C55.7 cells) was determined in cultures maintained in the presence or absence of putrescine. No detectable transport of radiolabeled putrescine, spermidine, or spermine was observed in rickettsiae that had been grown in C55.7 cells in the presence of putrescine. In C55.7 cells that had been cultured in the absence of putrescine, the rickettsiae (2.1×10^8 organisms) contained 6 ± 1 dpm of putrescine, 10 ± 2 dpm of spermidine, and 0 dpm of spermine. Assuming a specific activity of 256 dpm/pmol for putrescine (using 250 pmol/mg of protein for the putrescine concentration in C55.7 cells [24] and the specific activity of the [^{14}C]putrescine added to the medium), the rickettsiae contained 23 ± 2 fmol of putrescine per 10^8 rickettsiae, 19 ± 3 fmol of spermidine per 10^8 rickettsiae, and undetectable spermine. Thus, the rickettsial polyamines derived from the labeled putrescine correspond to only 0.03% of the total polyamine content of the rickettsiae.

TABLE 2. Growth of *R. prowazekii* in Chinese hamster cells

Cell line	Addition[a]	Growth of *R. prowazekii*[b]	
		0 h	48 h
CHO-K1		2.1 ± 0.1	72.7 ± 2.4
C55.7		2.2 ± 0.2	70.6 ± 5.7
C55.7	0.5 mM put	2.2 ± 0.3	67.6 ± 5.5
C55.7	1 mM DFMO	3.1 ± 0.2	66.8 ± 3.6
C55.7	0.5 mM put + 1 mM DFMO	3.5 ± 1.2	64.3 ± 7.5
C55.7	0.1 mM DFMA	2.2 ± 0.2	71.6 ± 4.5
C55.7	1 mM DFMA	2.2 ± 0.2	7.5 ± 0.2
C55.7	0.5 mM put + 0.1 mM DFMA	2.3 ± 0.1	71.8 ± 2.3
C55.7	0.5 mM put + 1 mM DFMA	2.3 ± 0.1	6.1 ± 1.1

[a] This column represents the addition of 0.5 mM putrescine (put), 1 mM DFMO, 0.1 mM DFMA, or 1 mM DFMA to the growth medium. 1 mM DFMO was added in two experiments; seven experiments were used for all other conditions.

[b] These values are expressed as the number of rickettsiae per infected cell at 0 and 48 h postinfection. Approximately 70% of the cells were infected at 0 h, and this percentage remained constant over the 48-h period. Each value represents the mean ± standard error.

TABLE 3. Decarboxylase activities of isolated *R. prowazekii*

Incubation components[a]	Ornithine decarboxylase						Arginine decarboxylase in rickettsial extract		
	Intact rickettsiae			Rickettsial extract					
	dpm	pmol of CO_2/mg of protein[b]	pmol of CO_2/h per 10^8 rickettsiae[c]	dpm	pmol of CO_2/mg of protein[b]	pmol of CO_2/h per 10^8 rickettsiae[c]	dpm	pmol of CO_2/mg of protein[d]	pmol of CO_2/h per 10^8 rickettsiae[c]
R. prowazekii + pyridoxal-5-phosphate	1,777 ± 342	80 ± 15	2.1 ± 0.4	1,929 ± 333	87 ± 14	2.3 ± 0.4	3,843 ± 167	31 ± 2	0.8 ± 0.1
R. prowazekii	1,861 ± 414	82 ± 19	2.2 ± 0.5	1,816 ± 134	81 ± 6	2.1 ± 0.2	1,283 ± 586	10 ± 5	0.3 ± 0.1
R. prowazekii + pyridoxal-5-phosphate + DFMO	1,888 ± 342	85 ± 15	2.2 ± 0.4	1,819 ± 495	82 ± 22	2.2 ± 0.5	ND	ND	ND
R. prowazekii + pyridoxal-5-phosphate + DFMA	ND[e]	ND	ND	ND	ND	ND	1,471 ± 435	12 ± 4	0.3 ± 0.1
R. prowazekii (heat killed)	1,801 ± 516	81 ± 23	2.1 ± 0.6	1,901 ± 147	85 ± 7	2.2 ± 0.2	1,231 ± 153	10 ± 1	0.3 ± 0.01

[a] *R. prowazekii* (0.4 mg) was incubated in the presence or absence of 40 μM pyridoxal-5-phosphate, 1 mM DFMO, or 1 mM DFMA, as indicated.
[b] The final specific activity of the ornithine was 134 dpm/pmol of ornithine or 22.3 dpm of CO_2 per pmol. Each value represents the mean ± standard error for at least two experiments.
[c] These values were estimated by using 3.8×10^9 rickettsiae per mg of protein.
[d] The final specific activity of the arginine was 736 dpm/pmol of arginine or 122.7 dpm of CO_2 per pmol. Each value represents the mean ± standard error for at least two experiments.
[e] ND, Not determined.

R. prowazekii grew normally in C55.7 cells even in the absence of added putrescine. About a 30-fold increase in the number of rickettsiae was observed both in C55.7 cells with a normal polyamine content and in host cells cultured without putrescine, in which putrescine and spermidine were reduced by more than 90% (24) (Table 2). A similar increase in the number of rickettsiae was observed in CHO-K1 cells over the same period.

Lack of ornithine decarboxylase activity of *R. prowazekii*. To test the hypothesis that the synthesis of putrescine occurs via a rickettsial ornithine decarboxylase, we examined the decarboxylation of ornithine in isolated rickettsiae (Table 3). A whole-cell preparation of *R. prowazekii* incubated with 40 μM pyridoxal-5-phosphate (a required cofactor for ornithine decarboxylase) liberated 2.1 pmol of $^{14}CO_2$ per h per 10^8 rickettsiae. However, the same amount of CO_2 was released from rickettsiae that had been heated at 100°C for 15 min, incubated in the absence of pyridoxal-5-phosphate, or incubated with difluoromethylornithine (DFMO) (an irreversible inhibitor of some ornithine decarboxylases [17]). To eliminate the potential problem of the lack of ornithine transport, we measured ornithine decarboxylase activity in cell extracts of *R. prowazekii*. A cell extract of *R. prowazekii* incubated with 40 μM pyridoxal-5-phosphate released 2.3 pmol of $^{14}CO_2$ per h per 10^8 rickettsiae, a value that, as in the intact cells, was the same as the negative controls.

There could be several explanations of these results. (i) The rickettsial ornithine decarboxylase is extremely labile, as is the eucaryotic enzyme (21), and was degraded during the 4-h purification from the chick yolk sac; (ii) the rickettsial enzymatic activity is so low as to be unmeasurable in a 1-h assay; and finally, (iii) the rickettsiae lack this enzymatic activity. To distinguish these possibilities, we assayed ornithine decarboxylase in uninfected controls and infected C55.7 cells over a 48-h period (Table 4). No rickettsial activity was found. The uninfected C55.7 control cells released 1.3 ± 0.2 pmol of $^{14}CO_2$ per h per mg of protein (1,082 ± 164 dpm of $^{14}CO_2$ per 48 h per mg of protein), while the infected cells released 1.1 ± 0.2 pmol of $^{14}CO_2$ per h per mg of protein (953 ± 128 dpm of $^{14}CO_2$ per 48 h per mg of protein). Also, normal rickettsial growth was observed in C55.7 cells cultured in the presence of 1 mM DFMO with or without added putrescine (Table 2). These data provide strong evidence to indicate that rickettsiae lack measurable ornithine decarboxylase activity.

Arginine decarboxylase activity of *R. prowazekii*. To inves-

TABLE 4. Decarboxylase activities of *R. prowazekii* measured in situ

Cell	Ornithine decarboxylase				Arginine decarboxylase			
	CO_2 evolved		No. of rickettsiae[b]	pmol of putrescine/h per 10^8 rickettsiae	CO_2 evolved		No. of rickettsiae[b]	pmol of putrescine/h per 10^8 rickettsiae
	dpm	pmol/h per mg of protein[a]			dpm	pmol/h per mg of protein[c]		
Uninfected	1,082 ± 164	1.3 ± 0.2	NA[d]	NA	559 ± 19	27 ± 0.7	NA	NA
Infected	953 ± 128	1.1 ± 0.2	1.3×10^8	0 ± 0	1,281 ± 21	59 ± 0.1	1.1×10^8	28.8 ± 1.0

[a] These values were determined by using an intracellular ornithine concentration of 2 nmol/mg of protein (13). The specific activity of the ornithine was calculated to be 88 dpm/pmol of ornithine or 17.6 dpm of CO_2 per pmol. Each value represents the mean ± standard error for three experiments.
[b] The number of rickettsiae was determined by Gimenez staining (11) as described in Materials and Methods. Host cell number was determined by measuring the amount of cellular protein per culture (1 mg of protein per 5×10^6 cells).
[c] These values were determined by measuring the specific activity of the arginine in the cytoplasm of the host cell by HPLC. The final specific activity of the arginine was determined to be 5.1 dpm/pmol of arginine or 0.87 dpm of CO_2 per pmol. Each value represents the mean ± standard error for four experiments.
[d] NA, Not applicable.

TABLE 5. In situ incorporation of arginine into *R. prowazekii* polyamines

Polyamine	C55.7 cells − 0.5 mM putrescine[a]					C55.7 cells + 0.5 mM putrescine[a]				
	dpm	pmol	Sp act		Arginine-derived polyamine (%)[b]	dpm	pmol	Sp act		Arginine-derived polyamine (%)[b]
			dpm/pmol	Avg ± SD				dpm/pmol	Avg ± SD	
Putrescine	125	34	3.7[c]	3.5 ± 0.2	103 ± 5	93	38	2.4[c]	3.1 ± 0.4	90 ± 12
	122	34	3.6[c]			114	38	3.0[c]		
	119	37	3.2			133	42	3.2		
	109	31	3.5			127	35	3.6		
Spermidine	110	33[c]	3.3	3.2 ± 0.2	96 ± 7	76	32[c]	2.4	3.2 ± 0.5	93 ± 14
	105	33[c]	3.2			106	32[c]	3.3		
	107	37	2.9			118	35	3.4		
	107	30	3.6			108	30	3.6		
Spermine	63	17[c]	3.7	4.6 ± 0.7	135 ± 20	67	20[c]	3.4	3.8 ± 0.5	112 ± 14
	95	17[c]	5.6			91	20[c]	4.6		
	83	19	4.4			77	22	3.5		
	75	16	4.7			76	20	3.8		
Total polyamine				3.8 ± 0.4	111 ± 11				3.3 ± 0.5	98 ± 13

[a] All values are expressed per 10^8 rickettsiae as determined by Gimenez staining (11) as described in Materials and Methods.

[b] These values are expressed as (experimental specific activity/theoretical specific activity) × 100. The theoretical specific activity was calculated to be 3.4 dpm/pmol assuming 100% of the polyamines were derived from arginine.

[c] Chemical concentrations in these two experiments are the average of the three experiments in Table 1; the radioactivity in the total polyamines was determined. The chemical concentrations and radioactivity in the other two experiments were measured within the individual experiments from the purified rickettsial fraction.

tigate the possibility that *R. prowazekii* synthesizes putrescine via a rickettsial arginine decarboxylase-agmatine ureohydrolase, we examined the growth of *R. prowazekii* in C55.7 cells cultured with difluoromethylarginine (DFMA) (an irreversible inhibitor of arginine decarboxylase [14]). Only a threefold increase in the number of rickettsiae was observed over the 48-h period in C55.7 cells cultured with or without putrescine in the presence of 1 mM DFMA (Table 2). This is in marked contrast to the more than 30-fold increase in rickettsial numbers seen in these cells without DFMA or with 0.1 mM DFMA over the same period.

Based on the striking effects of DFMA, arginine decarboxylase was assayed in infected C55.7 cells. Minimal background was expected in uninfected C55.7 cells because the enzyme arginine decarboxylase is found only in procaryotic organisms (26) and the ornithine decarboxylase mutation in the C55.7 cells prevents the decarboxylation of ornithine formed from the labeled arginine via serum arginase. Maximum sensitivity was again obtained with an in situ assay. In uninfected C55.7 cells, the background release of $^{14}CO_2$ from arginine was 559 ± 19 dpm/24 h per mg of protein, while 1,218 ± 21 dpm of $^{14}CO_2$ per 24 h per mg of protein was detected in infected C55.7 cells (Table 4). The difference between the infected cells and the uninfected controls, the rickettsial arginine decarboxylase activity, corresponds to 28.8 pmol of $^{14}CO_2$ released per h per 10^8 rickettsiae, which is 2.6 times greater than the activity needed to synthesize the required amount of rickettsial polyamines.

The rickettsial arginine decarboxylase activity was also measured in vitro (isolated, purified rickettsiae) with rickettsiae from chick yolk sacs, which allowed the acquisition of larger quantities of organisms. French press extracts of rickettsiae had measurable arginine decarboxylase activity; they released 3,843 dpm of $^{14}CO_2$ per h per 3.8×10^9 rickettsiae (0.8 pmol of $^{14}CO_2$ per h per 1×10^8 rickettsiae) above the buffer background (Table 3). Cell extracts of rickettsiae that had been incubated in the absence of pyridoxal-5-phosphate, heated at 100°C for 15 min, or incubated with 1 mM DFMA evolved 0.3 pmol of $^{14}CO_2$ per h per 10^8 rickettsiae. No arginine decarboxylase activity was observed in isolated, intact rickettsiae (data not shown). One possible explanation for these results is that the rate of transport of arginine by the rickettsiae is too slow to measure any activity in a 1-h assay.

Incorporation of [U-^{14}C]arginine into the polyamines of *R. prowazekii*. The incorporation of radiolabeled arginine into rickettsial polyamines was demonstrable (Table 5). If the rickettsial polyamines were derived exclusively from arginine, then the specific activity of the polyamines should be four/sixths of that of uniformly labeled arginine (since two carbons are removed during the synthesis). The experimentally determined specific activity of arginine was 5.1 ± 0.2 dpm/pmol, so the arginine-derived polyamines would have a specific activity of 3.4 dpm/pmol. Rickettsiae that had been propagated in C55.7 cells in the absence of putrescine contained an average of 119 dpm of putrescine per 10^8 rickettsiae (34 pmol/10^8 rickettsiae), 107 dpm of spermidine per 10^8 rickettsiae (33 pmol/10^8 rickettsiae), and 79 dpm of spermine per 10^8 rickettsiae (17 pmol/10^8 rickettsiae) (Table 5). From these values, the final specific activity of the polyamines was determined to be 3.5 dpm/pmol for putrescine (103% of the theoretical value), 3.2 dpm/pmol for spermidine (96% of the theoretical value), and 4.6 dpm/pmol for spermine (135% of the theoretical value). In C55.7 cells cultured in the presence of putrescine, the values obtained were not significantly different.

DISCUSSION

Some or all of the naturally occurring polyamines, putrescine, spermidine, and spermine, have been found in virtually every living organism examined with one exception, the extremely halophilic archaebacteria (15). The biosynthetic pathway(s) for polyamines in both eucaryotes and procaryotes has been studied in great detail (26). The ability

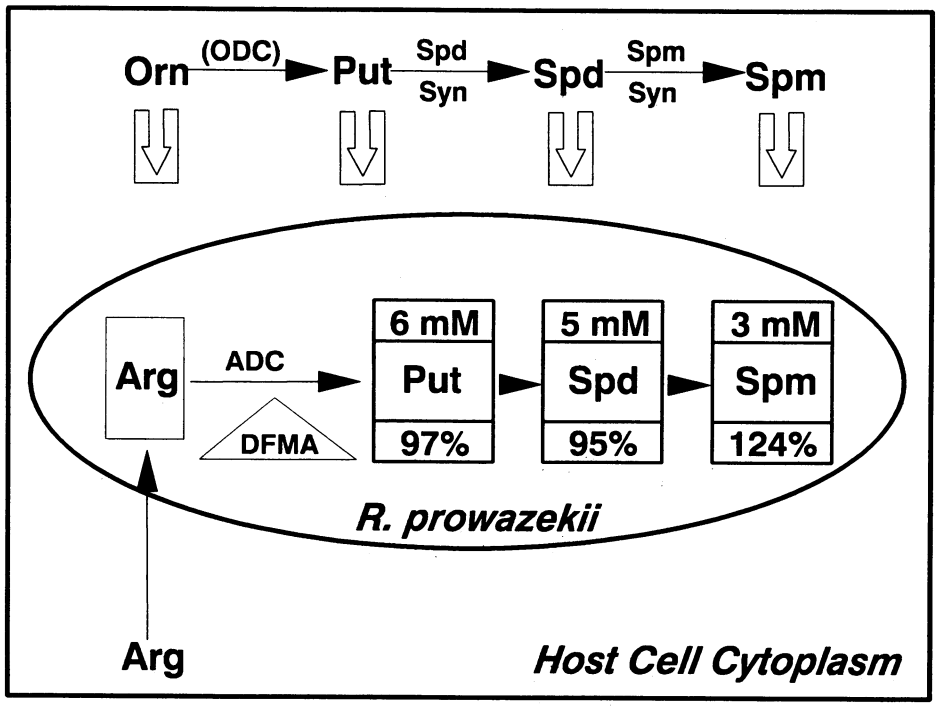

FIG. 1. Acquisition of polyamines by *R. prowazekii*. The blocked arrows represent the lack of conversion of host cell ornithine (Orn) to rickettsial putrescine and the lack of transport of putrescine (Put), spermidine (Spd), and spermine (Spm) from the cytoplasm of the host cell. The upper rectangles represent the individual polyamine concentrations in the rickettsiae, while the lower rectangles represent the percentages of the individual polyamines that are derived from arginine (Arg). The triangle represents the site of action of the irreversible inhibitor DFMA. ADC, Arginine decarboxylase; AUH, agmatine ureohydrolase; ODC, ornithine decarboxylase; Spd Syn, spermidine synthase; Spm Syn, spermine synthase.

of *Escherichia coli*, a gram-negative free-living bacterium, to synthesize and transport polyamines has been studied extensively.

In the present study, we found that *R. prowazekii* contained polyamines (putrescine [6 mM], spermidine [5 mM], and spermine [3 mM]) and lacked modified polyamines (data not shown). These pools are similar to those measured in *E. coli* (putrescine, 19 mM; and spermidine, 6 mM) except that *E. coli* has no spermine when cultured on minimal medium, since it lacks spermine synthase, and contains acetylated polyamines (27).

The characterization of the growth of *R. prowazekii* in C55.7 cells indicates that arginine decarboxylase, but not ornithine decarboxylase, was present and functional in rickettsiae. *R. prowazekii* failed to grow in the presence of 1 mM DFMA (an inhibitor of arginine decarboxylase) but grew normally in the presence of 1 mM DFMO (an inhibitor of some ornithine decarboxylases) and was not inhibited by putrescine and spermidine depletion. In putrescine-starved C55.7 cells, the putrescine and spermidine concentrations fall to 3 and 8% of those in supplemented cells, respectively (24). Furthermore, based on measurements in vitro and in situ, *R. prowazekii* lacked any measurable ornithine decarboxylase activity. Weiss et al. (30), in a 1987 review, also stated that no measurable ornithine decarboxylase activity could be detected in *R. prowazekii*. *E. coli*, on the other hand, contains both constitutive and inducible enzymes for both ornithine decarboxylation (1, 2) and arginine decarboxylation (6, 35), and these have been extensively characterized.

We demonstrated the incorporation of labeled arginine into polyamines of growing rickettsiae but were unable to demonstrate incorporation of labeled host cell putrescine into the rickettsiae. Since the specific activity of the arginine in the cytoplasm of the host cell was 5.1 dpm/pmol, if the arginine pool of the host cell equilibrates with the rickettsial arginine pool, the theoretical specific activity of the arginine-derived putrescine would be 3.4 dpm/pmol. The experimentally derived values for the specific activity of all the rickettsial polyamines indicated that 100% of the total polyamine content of the rickettsiae was derived from arginine (Fig. 1).

Arginine decarboxylase activity was demonstrated in *R. prowazekii* growing in C55.7 cells as well as in cell extracts of isolated rickettsiae. The amount of activity in situ was 2.6 times greater than that needed to synthesize the required amount of polyamine. It seems likely that arginine was converted to glutamate in the rickettsiae and metabolized to $^{14}CO_2$ via the tricarboxylic acid cycle. The activity observed in situ was approximately 36-fold greater than that observed in cell extracts of isolated rickettsiae. Had the enzymatic activity only been determined in isolated rickettsiae, proper interpretation of this low activity would have been confused by the lability of the rickettsiae during extensive purification and the fact that they were not growing. This underscores the importance of performing in situ metabolic studies since the cell is the only natural environment where rickettsiae are able to grow and metabolize.

Evolutionarily, it is very interesting that *R. prowazekii* synthesized its polyamines from arginine exclusively. Why would an obligate intracytoplasmic bacterium such as *R. prowazekii* evolve to synthesize polyamines from arginine and not transport preformed polyamines from the large pool of polyamines in the cytoplasm of its host cell? In fact,

transport systems for all three polyamines have been found in *E. coli* (25). Perhaps, because of the highly charged nature of polyamines, most, if not all, of the polyamines in the cytoplasm of the host cell are bound to RNA and other negatively charged molecules. If this were the case, very little free polyamine would be available for transport by the rickettsiae. The rickettsiae would need to either synthesize their polyamines or evolve a system (analogous to the bacterial siderophores) to out-compete the host cell for the bound polyamines.

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LITERATURE CITED

1. **Applebaum, D. M., J. C. Dunlap, and D. R. Morris.** 1977. Comparison of the biosynthetic and biodegradative ornithine decarboxylases of *Escherichia coli*. Biochemistry **16**:1580–1584.
2. **Applebaum, D. M., D. L. Sabo, E. H. Fischer, and D. R. Morris.** 1975. Biodegradative ornithine decarboxylase of *Escherichia coli*. Purification, properties, and pyridoxal 5'-phosphate binding site. Biochemistry **14**:3675–3681.
3. **Atkins, J. F., J. B. Lewis, C. W. Anderson, and R. F. Gestland.** 1975. Enhanced differential synthesis of proteins in a mammalian cell-free system by addition of polyamines. J. Biol. Chem. **250**:5688–5695.
4. **Austin, F. E., J. Turco, and H. H. Winkler.** 1987. *Rickettsia prowäzekii* requires host cell serine and glycine for growth. Infect. Immun. **55**:240–244.
5. **Austin, F. E., and H. H. Winkler.** 1988. Proline incorporation into protein by *Rickettsia prowazekii* during growth in Chinese hamster ovary (CHO-K1) cells. Infect. Immun. **56**:3167–3172.
6. **Boeker, E. A., E. H. Fischer, and E. E. Snell.** 1969. Arginine decarboxylase from *Escherichia coli*. III. Subunit structure. J. Biol. Chem. **244**:5239–5245.
7. **Bovarnick, M. R.** 1956. Phosphorylation accompanying the oxidation of glutamate by the Madrid E strain of typhus rickettsiae. J. Biol. Chem. **220**:353–361.
8. **Bovarnick, M. R., and J. C. Synder.** 1949. Respiration of typhus rickettsiae. J. Exp. Med. **89**:561–565.
9. **Coolbaugh, J. C., J. J. Progar, and E. Weiss.** 1976. Enzymatic activities of cell-free extracts of *Rickettsia typhi*. Infect. Immun. **14**:298–305.
10. **De Jong, C., G. J. Hughes, E. Van Wieringen, and K. J. Wilson.** 1982. Amino acid analyses by high-performance liquid chromatography. An evaluation of the usefulness of pre-column Dns derivatization. J. Chromatogr. **241**:345–359.
11. **Gimenez, D. F.** 1964. Staining rickettsiae in yolk sac culture. Stain Technol. **39**:135–140.
12. **Hayes, J. E., F. E. Hahn, Z. A. Cohn, G. B. Jackson, and J. E. Smadel.** 1957. Metabolic studies of rickettsiae. IV. Terminal respiratory enzymes in *Rickettsia mooseri*. Biochim. Biophys. Acta **26**:571–576.
13. **Kabus, P., and G. Koch.** 1982. Quantitative determination of amino acids in tissue culture cells by high performance liquid chromatography. Biochem. Biophys. Res. Commun. **108**:783–790.
14. **Kallio, A., P. P. McCann, and P. Bey.** 1981. DL-alpha-(Difluoromethyl)arginine: a potent enzyme-activated irreversible inhibitor of bacterial decarboxylases. Biochemistry **20**:3163–3168.
15. **Kamekura, M., K. Hamana, and S. Matsuzaki.** 1987. Polyamine contents and amino acid decarboxylation activities of extremely halophilic archaebacteria and some eubacteria. FEMS Microbiol. Lett. **43**:301–305.
16. **Keysary, A., T. F. McCaul, and H. H. Winkler.** 1989. Roles of the Fc receptor and respiratory burst in killing of *Rickettsia prowazekii* by macrophagelike cell lines. Infect. Immun. **57**:2390–2396.
17. **Metcalf, B. W., P. Bey, C. Danzin, M. J. Jung, P. Casara, and J. P. Verert.** 1978. Catalytic irreversible inhibition of mammalian ornithine decarboxylase by substrate and product analogues. J. Am. Chem. Soc. **100**:2551–2553.
18. **Neidhardt, F. C.** 1987. Chemical composition of *Escherichia coli*, p. 3–7. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol. 1. American Society for Microbiology, Washington, D.C.
19. **Oray, B., H. S. Lu, and R. W. Gracy.** 1983. High-performance liquid chromatographic separation of Dns-amino acid derivatives and applications to peptide and protein structural studies. J. Chromatogr. **270**:253–266.
20. **Redmond, J. W., and A. Tseng.** 1979. High-pressure liquid chromatographic determination of putrescine, cadaverine, spermidine, and spermine. J. Chromatogr. **170**:479–481.
21. **Russell, D. H., and S. H. Snyder.** 1969. Amine synthesis in regenerating rat liver: extremely rapid turnover of ornithine decarboxylase. Mol. Pharmacol. **5**:253–262.
22. **Slocum, R. D., H. E. Flores, A. W. Galston, and L. H. Weinstein.** 1989. Improved method for HPLC analysis of polyamines, agmatine and aromatic monoamines in plant tissue. Plant Physiol. **89**:512–517.
23. **Steglich, C., A. Grens, and I. Scheffler.** 1985. Chinese hamster cells deficient in ornithine decarboxylase activity: reversion by gene amplification and by azacytidine treatment. Somatic Cell Mol. Genet. **11**:11–23.
24. **Steglich, C., and I. E. Scheffler.** 1982. An ornithine decarboxylase-deficient mutant of Chinese hamster ovary cells. J. Biol. Chem. **257**:4603–4609.
25. **Tabor, C. W., and H. Tabor.** 1966. Transport systems for 1,4-diaminobutane, spermidine, and spermine in *Escherichia coli*. J. Biol. Chem. **241**:3714–3723.
26. **Tabor, C. W., and H. Tabor.** 1976. 1,4-Diaminobutane (putrescine), spermidine, and spermine. Annu. Rev. Biochem. **45**:285–306.
27. **Tabor, C. W., and H. Tabor.** 1985. Polyamines in microorganisms. Microbiol. Rev. **49**:81–99.
28. **Turco, J., and H. H. Winkler.** 1983. Inhibition of the growth of *Rickettsia prowazekii* in cultured fibroblasts by lymphokines. J. Exp. Med. **157**:974–986.
29. **Walker, T. S., and H. H. Winkler.** 1979. Rickettsial hemolysis: rapid method for enumeration of metabolically active typhus rickettsiae. J. Clin. Microbiol. **9**:645–647.
30. **Weiss, E., M. E. Dobson, and G. A. Dasch.** 1987. Biochemistry of rickettsiae: recent advances. Acta Virol. **31**:271–286.
31. **Winkler, H. H.** 1974. Inhibitory and restorative effects of adenine nucleotides on rickettsial adsorption and hemolysis. Infect. Immun. **9**:119–126.
32. **Winkler, H. H.** 1976. Rickettsial permeability: an ADP-ATP transport system. J. Biol. Chem. **251**:389–396.
33. **Winkler, H. H., and R. M. Daugherty.** 1984. Proline transport and metabolism in *Rickettsia prowazekii*. J. Bacteriol. **158**:460–463.
34. **Wisseman, C. L., E. B. Jackson, and F. E. Hahn.** 1951. Metabolic studies of rickettsia. I. The effects of antimicrobial substances and enzyme inhibitors on the oxidation of glutamate by purified rickettsiae. J. Immunol. **67**:123–136.
35. **Wu, W. H., and D. R. Morris.** 1973. Biosynthetic arginine decarboxylase from *Escherichia coli*: purification and properties. J. Biol. Chem. **248**:1687–1695.
36. **Young, D. V., and P. R. Srinivasan.** 1972. Regulation of macromolecular synthesis by putrescine in a conditional *Escherichia coli* putrescine auxotroph. J. Bacteriol. **112**:30–39.
37. **Zahorchak, R. J., and H. H. Winkler.** 1981. Hydrolysis and synthesis of ATP by *Rickettsia prowazekii*, p. 401–410. *In* W. Burgdorfer and R. Anacker (ed.), Rickettsiae and rickettsial diseases. Academic Press, Inc., Orlando, Fla.