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Stability of the Adenosine 5'-Triphosphate Pool in *Coxiella burnetii*: Influence of pH and Substrate

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The ability of *Coxiella burnetii* to couple oxidation of metabolic substrates to adenosine 5'-triphosphate (ATP) synthesis in axenic reaction buffers was examined. Pyruvate, succinate, and glutamate were catabolized and incorporated at the highest rates of 11 substrates tested. Glutamate oxidation, however, resulted in the greatest stability of the ATP pool and highest intracellular ATP levels over a 48-h period. At pH 4.5, the optimum for metabolism by *C. burnetii*, glutamate oxidation resulted in maintenance of the ATP pool at a concentration of approximately 0.7 nmol of ATP per mg of dry weight over a 96-h period. In the absence of substrate, ATP declined by 96 h to less than 0.01 nmol/mg of dry weight. When cells were maintained at pH 7.0 in the presence or absence of glutamate, ATP pools were considerably more stable, presumably due to the minimal metabolic activity displayed by *C. burnetii* at pH 7. The stability of the ATP pool reflected viability as there was greater than an 8-log decrease in viable *C. burnetii* after incubation for 7 days at pH 4.5 in the absence of glutamate. Viability was retained in the presence of glutamate at pH 4.5 or 7.0 in the absence of any added substrate. The stability of the ATP pool was due to endogenous synthesis of ATP coupled to substrate oxidation as shown by depression of ATP levels in the presence of inhibitors of electron transport or oxidative phosphorylation. In addition, the adenylate energy charge increased from an initial value of 0.57 to 0.73 during glutamate oxidation with a concomitant rise in the total adenylate pool size. *C. burnetii* therefore appears able to regulate endogenous ATP levels in response to substrate availability and pH, thus effecting a conservation of metabolic energy in neutral or alkaline environments. Such a mechanism has been proposed to play a role in the resistance of *C. burnetii* to environmental conditions and subsequent activation upon entry into the phagolysosome in which this organism replicates.

Coxiella burnetii, the rickettsial agent of Q fever, is a highly specialized obligate intracellular pathogen with a number of adaptive mechanisms to enhance both its replication within the phagolysosome and survival in the extracellular environment. Unlike other rickettsia, which replicate primarily within the cytoplasm of eucaryotic cells, *C. burnetii* undergoes its entire developmental cycle (9, 10) within the phagolysosome (1, 2) and is apparently dependent upon conditions within the phagolysosome to stimulate growth. This requirement is demonstrated by the pH-dependent activation of metabolic processes by whole cells of *C. burnetii* in axenic media. The transport, catabolism, and incorporation of both glucose and glutamate are stimulated by hydrogen ion concentrations reflective of the intraphagolysosomal space (5). Furthermore, precursors of both DNA and RNA, in the presence of an energy source such as glutamate, are incorporated at pH 4.5 but not at 7.0 (6). The in vivo requirement for acidic conditions may be demonstrated in cultured chicken embryo fibroblasts by increasing the pH of the phagolysosome with the lysosomotropic agents choroquine, methylamine, or ammonium chloride, thus inhibiting the replication of *C. burnetii* (5).

Although the metabolic processes of *C. burnetii* are greatly enhanced by an acidic environment, axenic growth of *C. burnetii* has not yet been achieved. Therefore, additional factors must restrict growth of *C. burnetii* to the phagolysosome.

Because of the apparent correlation of adenylate energy charge (energy charge = [ATP] + ½[ADP]/[ATP] + [ADP] + [AMP]) with growth potential in both procaryotic and eucaryotic cells (3, 4, 18), we have examined the ability of *C. burnetii* to couple ATP synthesis to sub-

strate oxidation and the resultant energy charge. We demonstrate here that oxidation of glutamate, apparently a preferred energy source, results in endogenous ATP synthesis and that an energy charge close to that theoretically sufficient for growth is achieved.

MATERIALS AND METHODS

Organisms. The Nine Mile strain of *C. burnetii*, phase I (clone 7), was propagated in specific-pathogen-free, type IV, antibiotic-free, fertile hen's eggs (H and N Hatchery, Redmond, Wash.) and purified from 7-day infected yolk sacs by Renografin density gradient centrifugation (22). The passage history of the Nine Mile strain of *C. burnetii*, phase I, has been described (22). The Wilmington strain of *Rickettsia typhi* was grown and purified by the same procedures. Plaque assay of *C. burnetii* was as previously described (14).

Metabolism studies. Catabolism of ^{14}C-labeled substrates to CO_2 or incorporation into a trichloroacetic acid-insoluble product was determined as described previously (5). Results are expressed as nanomoles of substrate recovered as CO_2 or incorporated.

ATP determination. Organisms were suspended at a final concentration of 1 mg of dry weight per ml in P-25 buffer (5) and incubated in marble-capped tubes at 37°C. Additional substrates or metabolic inhibitors were made up in P-25 buffer and, when necessary, adjusted to the appropriate pH by the addition of HCl or NaOH. Dicyclohexylcarbodiimide and carbonyl cyanide-*m*-chlorophenyl hydrazone were solubilized in ethanol and diluted 1:10 in P-25 buffer before addition to the reaction mixture. Samples of 200 μl of cell suspension were removed at intervals, and nucleotides were extracted by the addition of 20 μl of 60% perchloric acid. After overnight extraction at 4°C, samples were neutralized by the addition of 7.5 N KOH plus 50 mM EDTA. The potassium perchlorate salt was pelleted by centrifugation, and the supernatant was used for ATP determination. ATP was assayed by the luciferase reaction using a Du Pont 760 luminescence biometer with Du Pont reagents according to attached instructions (E. I. Du Pont de Nemours & Co.). The instrument was calibrated against ATP standards.

Analysis of adenine nucleotide pools. *C. burnetii* was suspended in P-25 buffer at 1 mg of dry weight per ml in the presence or absence of 5 mM glutamate. Incubation was carried out under room atmosphere in sealed flasks with constant shaking at 37°C. At appropriate intervals, 20-ml samples were removed and pelleted by centrifugation (12,000 × *g*) for 10 min at 4°C. The supernatant was removed, the cells were suspended in 1.5 ml of P-25 buffer (pH 4.5), and 100 μl of 60% perchloric acid was added. Nucleotides were extracted overnight at 4°C and neutralized and clarified as described above. Nucleotides were analyzed and quantitated using a Waters high-pressure liquid chromatograph with attached Waters data module and system controller (Waters Associates Inc., Milford, Mass.). Nucleotides were separated on a Whatman Partisil-10 SAX column (Whatman Inc., Clifton, N.J.) by elution with a linear gradient of 0.01 M $NH_4H_2PO_4$ (pH 2.8) to 0.5 M $NH_4H_2PO_4$ (pH 4.8) and identified by coelution with known standards.

Chemicals and radiochemicals. [2,3-^{14}C]succinate (17.5 mCi/mmol), [*U*-^{14}C]pyruvate (13.5 mCi/mmol), [*U*-^{14}C]acetate (57.8 mCi/mmol), L-[*U*-^{14}C]glutamate (285 mCi/mmol), α-[5-^{14}C]ketoglutarate (20.2 mCi/mmol), [*U*-^{14}C]glycerol (171 mCi/mmol), D-[*U*-^{14}C]glucose (260 mCi/mmol), [1,5-^{14}C]citrate (98 mCi/mmol), [*U*-^{14}C]glycine (114 mCi/mmol), L-[*U*-^{14}C]lactate (100 mCi/mmol), and [*U*-^{14}C]fructose (301 mCi/mmol) were obtained from Amersham Corp. (Arlington Heights, Ill.). Other reagents were obtained from Sigma Chemical Co. (St. Louis, Mo.).

RESULTS

Substrate metabolism. In preliminary attempts to determine preferred carbon and energy sources for *C. burnetii*, the metabolism of a number of substrates was examined (Table 1). Of those substrates examined, pyruvate was both catabolized and incorporated maximally, followed by succinate and glutamate. Although the number of substrates is by no means complete, tricarboxylic acid cycle intermediates were readily metabolized by *C. burnetii*. It is notable that virtually all substrates were metabolized considerably better at pH 4.5 than at pH 7. Although metabolism of some substrates appeared to be minimal, it has been shown that the presence of a substrate (i.e., glutamate) which is more readily utilized may enhance the catabolism or incorporation or both of a second substrate (i.e., glucose, thymidine, uridine, guanosine) which is less readily metabolized (6). Since pyruvate appeared to be utilized best of those substrates examined, the pH dependence of pyruvate utilization was determined (Fig. 1).

TABLE 1. *Metabolism of glycolytic and tricarboxylic acid intermediates and selected amino acids by C. burnetii*

Substrate[a]	Catabolism (nmol/h per mg)		Incorporation (nmol/h per mg)	
	pH 4.5	pH 7.0	pH 4.5	pH 7.0
Pyruvate	488.7	28.5	10.56	0.93
Succinate	282.6	0.15	9.47	0.17[b]
Glutamate	163.1	0.50	6.20	0.12[b]
α-Ketoglutarate	7.0	0.82	0.35[b]	0.28[b]
Glucose	4.1	0.07	0.08	0.05[b]
Glycerol	0.55	0.42	0.12	0.22
Citrate	0.30	—[b]	0.10[b]	—[b]
Glycine	0.15	—[b]	0.09	0.07[b]
Acetate	0.08[b]	—[b]	0.14[b]	—[b]
Fructose	0.02	—[b]	0.01[b]	0.01[b]
Lactate	0.01[b]	—[b]	—[b]	—[b]

[a] Pyruvate, succinate, glutamate, and α-ketoglutarate were at 1.0 mM final concentration (1 μCi/μmol); the remaining substrates were at 0.1 mM final concentration (10 μCi/μmol). Results are the mean of triplicate determinations.

[b] Not significantly different from background at a level of $P \leq 0.05$ by Student's *t* test, or less than background (—).

Although the optimal pH for pyruvate catabolism and incorporation was slightly higher than for glutamate or glucose (5), pyruvate metabolism was markedly enhanced by acidic conditions.

Effect of pH and substrate oxidation on the ATP pool. The ability of *C. burnetii* to couple glutamate oxidation to ATP synthesis was examined and compared with energy coupling as described for typhus rickettsiae (23). The effect of pH on endogenous ATP synthesis from glutamate oxidation by whole cells of *C. burnetii* is depicted in Fig. 2A. Similarly, the effects of providing glutamate, glucose, or no substrate at pH 4.5 were examined (Fig. 2B). Minor fluctuations in the endogenous ATP content were evident during the initial time points, yet despite repeated attempts, no consistent patterns of ATP synthesis or hydrolysis in response to substrate oxidation or starvation could be demonstrated over a 1-h period. As a control, the ability of *R. typhi*, another obligately intracellular bacterium, to couple glutamate oxidation to ATP synthesis was examined under identical conditions (Fig. 2C). Glutamate oxidation at pH 7, the optimum for glutamate metabolism by *R. typhi* (6), resulted in a rapid increase in intracellular ATP concentrations as had been demonstrated previously (23). In the absence of glutamate, or when ATP synthesis from glutamate oxidation was blocked by an ATPase inhibitor such as dicyclohexylcarbodiimide (8), or an inhibitor of oxidative phosphorylation such as carbonyl cyanide-*m*-chlorophenyl hydrazone (8), there was a rapid decline in intracellular ATP levels. It appeared, from these initial experiments, that endogenous ATP pools in *C. burnetii* were much more stable than those of *R. typhi*.

Because of the remarkable stability of the ATP pool in *C. burnetii*, the observation period was extended. Endogenous ATP levels were monitored over a 96-h period at either pH 4.5 or 7 in the presence or absence of glutamate as an energy source (Fig. 3). At pH 7, after an initial decline in ATP concentration either in the presence or absence of substrate, the ATP pools remained comparatively stable. In the presence of glutamate at pH 7, the ATP level, in fact, increased by 12 h, and by 24 h it was slightly greater than that of cells at pH 4.5 with glutamate. Such a result is in keeping with the metabolism of glutamate at a reduced rate at pH 7 (5). At pH 4.5, in the presence of glutamate, ATP levels increased slightly or remained stable over the 96-h incubation period. However, in the absence of substrate at pH 4.5, ATP levels declined markedly. Addition of glutamate (final concentration, 5 mM) to these starved cells after 96 h of incubation did not result in an increase in ATP over at least a 1-h period (data not shown). Plaque assay of the *C. burnetii* after 7 days under the above conditions indicated that the decline in ATP pool at pH 4.5 without substrate corresponded to loss of viability. Cells remained viable at pH 7 in the absence of substrate or with glutamate at pH 4.5 (Table 2).

An examination of glutamate metabolism by

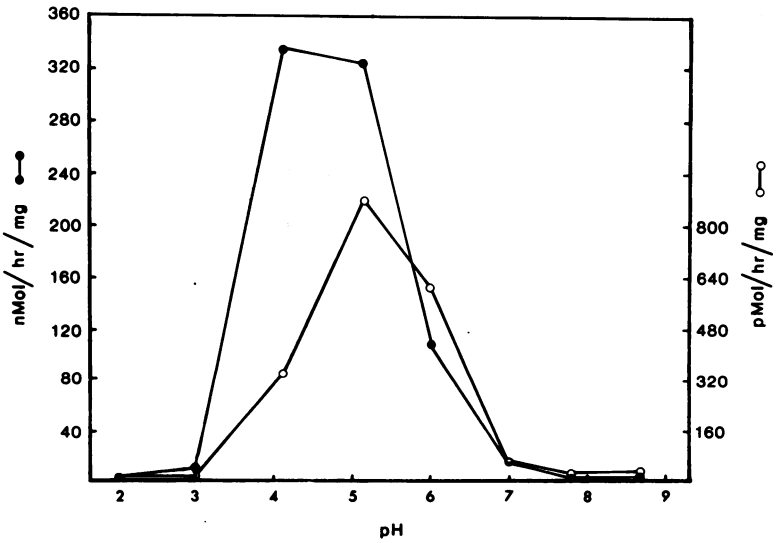

FIG. 1. *Hydrogen ion-dependent stimulation of pyruvate metabolism by C. burnetii. Effect of pH on the catabolism of pyruvate to CO_2 (●) or incorporation (○) by purified suspensions of C. burnetii. Each point represents the mean of triplicate determinations.*

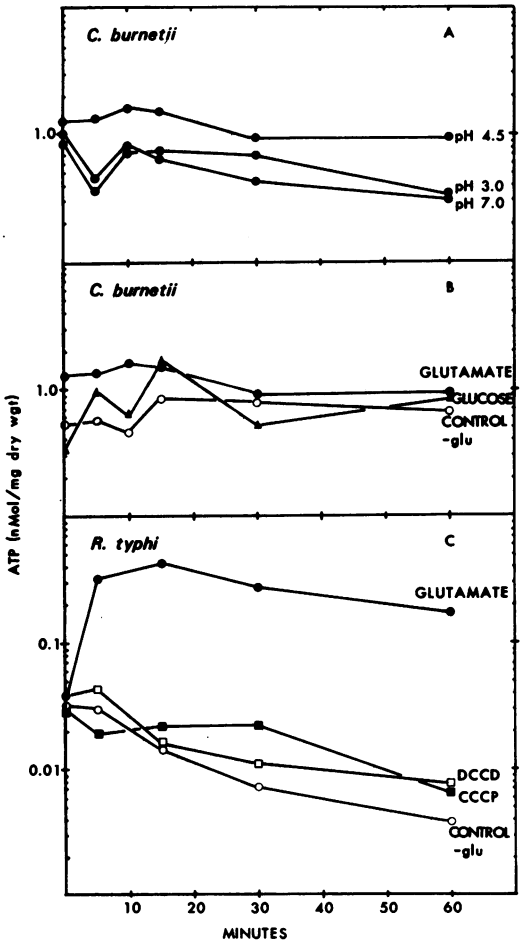

FIG. 2. *Comparison of ATP synthesis by C. burnetii or R. typhi in response to substrate oxidation. (A) Effect of pH on ATP synthesis from glutamate utilization by C. burnetii; (B) effect of glutamate or glucose on ATP synthesis by C. burnetii at pH 4.5; (C) ATP synthesis by R. typhi at pH 7.0 during glutamate metabolism. Symbols: (●) 5 mM glutamate; (○) no added substrate; (▲) 5 mM glucose; (□) 5 mM glutamate + 0.5 mM dicyclohexylcarbodiimide (DCCD); (■) 5 mM glutamate + 0.05 mM cyanide-m-chlorophenyl hydrazone (CCCP).*

C. burnetii over the 96-h time period under the conditions of the above experiments demonstrated that glutamate was both catabolized and incorporated by *C. burnetii* for at least 48 h in the axenic reaction buffer, after which a plateau was reached (data not shown).

Substrate utilization and maintenance of ATP pools. The efficiency of energy coupling to oxidation of various metabolizable substrates was also examined (Fig. 4). A complex response of ATP synthesis to initial metabolism of the various substrates was noted; however, metabolism of any of the substrates resulted in markedly greater stability of the ATP pool than was observed without substrate. Of those substrates examined, glutamate utilization resulted in the maintenance of the greatest ATP levels over the 48-h incubation period. Oxidation of pyruvate, which as shown above (Table 1) was metabolized most rapidly of these substrates, did not result in generation of the highest ATP levels. By 48 h in pyruvate-fed cells, ATP levels were, in fact, lowest of any of the substrates tested. Succinate

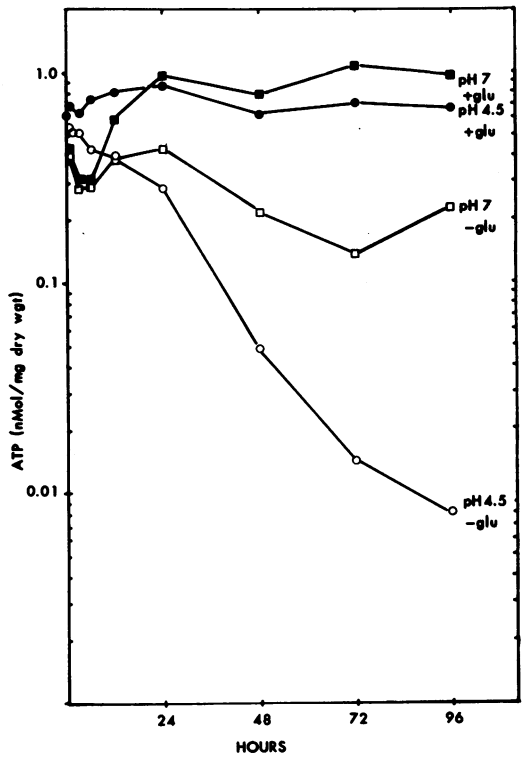

FIG. 3. *Maintenance of endogenous ATP levels by C. burnetii in the presence or absence of 5 mM glutamate. Each point represents the mean of duplicate assays. Symbols: (●) pH 4.5 + glutamate; (○) pH 4.5 − glutamate; (■) pH 7.0 + glutamate; (□) pH 7.0 − glutamate.*

TABLE 2. *Effect of pH and glutamate on the viability of C. burnetii in axenic media*

Conditions[a]	PFU/ml
pH 4.5, − glutamate	<10
pH 4.5, + glutamate	1.7×10^9
pH 7.0, − glutamate	1.8×10^8

[a] Renografin-purified *C. burnetii* were suspended at 1 mg of dry weight per ml (1 mg of dry weight = 3.7×10^{10} plaque-forming units [PFU]; 22) in P-25 buffer (5) at the desired pH in the presence or absence of glutamate and incubated for 7 days at 37°C in marble-capped tubes.

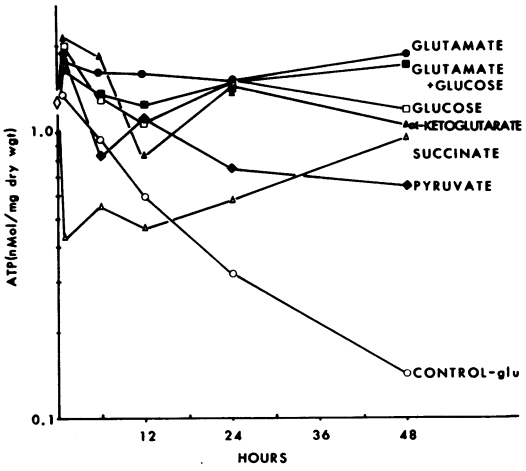

FIG. 4. *Efficacy of various metabolizable substrates in the maintenance of endogenous ATP pools in C. burnetii at pH 4.5. Each point represents the mean of duplicate determinations. The ATP concentration at T = 0 was 1.28 ± 0.13 nmol/mg of dry weight with a range of 1.08 to 1.43 nmol/mg of dry weight. Symbols:* (●) *5 mM glutamate;* (□) *5 mM glucose;* (▲) *5 mM α-ketoglutarate;* (△) *5 mM succinate;* (◆) *5 mM pyruvate;* (■) *5 mM glutamate + 5 mM glucose;* (○) *no substrate.*

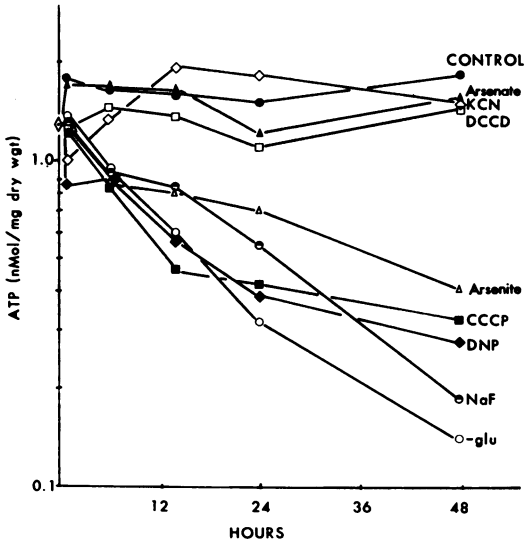

FIG. 5. *Effect of metabolic inhibitors on maintenance of ATP pools in C. burnetii under conditions of glutamate oxidation. Glutamate was present in each at a concentration of 5 mM. Each point represents the mean of duplicate determinations. The ATP concentration at T = 0 was 1.26 ± 0.15 nmol/mg of dry weight with a range of 1.08 to 1.49 nmol/mg of dry weight. Symbols:* (●) *control, 5 mM glutamate;* (◓) *0.5 mM NaF;* (◆) *0.5 mM 2,4-dinitrophenol (DNP);* (■) *0.05 mM carbonyl cyanide-m-chlorophenyl hydrazone (CCCP);* (△) *0.5 mM arsenite;* (□) *0.5 mM dicyclohexylcarbodiimide (DCCD);* (◇) *0.5 mM KCN;* (▲) *0.5 mM arsenate; and* (○) *control, no glutamate.*

utilization resulted in a rapid decline in ATP levels, but these levels were restored to near normal by 48 h.

Effect of metabolic inhibitors on ATP synthesis. To gain some insight into the energy coupling systems of *C. burnetii*, the sensitivity of ATP synthesis resulting from the oxidation of glutamate to various metabolic inhibitors was examined (Fig. 5). Uncouplers of oxidative phosphorylation (8) such as carbonyl cyanide-*m*-chlorophenyl hydrazone and 2,4-dinitrophenol caused a depression in ATP levels, as did arsenite, an inhibitor of dihydrolipoyl dehydrogenase (8), and sodium fluoride, an inhibitor of enolase (8) and possibly adenylate kinase (21). Of those inhibitors which were ineffective in reducing ATP levels under these conditions, trivial mechanisms may explain some of the anomalous results. Potassium cyanide appeared to be unstable at pH 4.5, since KCN indeed initially depressed ATP levels but normal levels were obtained after a period of time dependent upon the initial concentration of KCN (data not shown). *N,N'*-Dicyclohexylcarbodiimide, an ATPase inhibitor (8), did not affect ATP synthesis at pH 4.5 at concentrations typically employed. At higher concentrations (5 mM) ATP synthesis was inhibited (data not shown); however, solubility was poor, and the inhibitor did not remain in solution throughout the incubation period. Arsenate at concentrations up to 25 mM was ineffective in reducing endogenous ATP levels in *C. burnetii*. None of the inhibitors reduced ATP levels below those resulting from substrate starvation.

Adenine nucleotide pools and adenylate energy charge. The adenylate energy charge resulting from glutamate oxidation or from starvation of *C. burnetii* was determined (Table 3). After 3 h of incubation at 37°C in the presence of glutamate, the energy charge increased from an initial value of 0.57 to about 0.73. The increased energy charge was accompanied by an increase in the total adenylate pool size, thus indicating endogenous ATP synthesis. Under conditions of glutamate starvation, however, the energy charge declined slowly, with a concomitant slight decline in total adenylates.

DISCUSSION

The recent recognition of the acidophilic nature of *C. burnetii* (5) now provides the opportunity for physiological studies on intact *C. burnetii* in a eucaryote-free environment. The extracellular metabolic capabilities of *C. burnetii* have been shown to be much greater than pre-

TABLE 3. *Adenine nucleotide pools in C. burnetii during glutamate metabolism and starvation*

Time (h) at 37°C	Glutamate (5 mM)	Pool concn (nmol/mg [dry wt])[a]				Energy charge[b]
		ATP	ADP	AMP	Σ	
0	−	1.31	1.05	0.85	3.21	0.57
3	−	1.01	0.85	1.07	2.93	0.49
3	+	2.45	2.00	0.31	4.76	0.73
12	−	0.55	1.73	0.65	2.93	0.48
12	+	1.32	1.85	0.19	3.36	0.67

[a] Data from a single representative experiment.
[b] Energy charge = [ATP] + ½ [ADP]/[ATP] + [ATP] + [AMP].

viously believed (5, 6), but axenic cultivation has not yet been achieved. We continued here to probe the metabolic potential of purified *C. burnetii* and made the following observations. (i) Tricarboxylic acid cycle intermediates appeared to be readily metabolized by *C. burnetii*; however, glutamate, which may enter the tricarboxylic acid cycle, appeared to best sustain enhanced ATP levels. (ii) ATP pools declined rapidly at pH 4.5 in the absence of glutamate but remained relatively stable in the presence of a metabolizable substrate or at pH 7.0. (iii) The decline in ATP levels at pH 4.5 in the absence of glutamate corresponded to a loss of cell viability as judged by reduction in plaque count.

Previous studies have shown that tricarboxylic acid cycle intermediates are preferentially metabolized by purified *C. burnetii* (13); however, these studies were carried out in a reaction buffer of approximately pH 7.25, a value much greater than the optimum pH at which this organism actively transports and metabolizes these substrates (5). In a similar study (15), glucose was reported not to be utilized by intact *C. burnetii*. The present study demonstrates, under pH conditions which more closely reflect that of the intracellular compartment occupied by this organism, that tricarboxylic acid cycle intermediates indeed appear to be primary carbon and energy sources for *C. burnetii*. Furthermore, virtually all substrates examined were both catabolized and incorporated better at pH 4.5 than at pH 7.0. It should be emphasized that the catabolism or incorporation, or both, of those substrates only minimally metabolized here may be enhanced by the presence of a more readily metabolized energy source (5, 6).

The stability of the ATP pool during substrate oxidation appears to be due to endogenous synthesis of ATP, as opposed to inhibition of degradation, since inhibitors of electron transport or oxidative phosphorylation blocked ATP synthesis resulting from glutamate oxidation. Furthermore, both the ATP and total adenylate pools increased during glutamate metabolism, while there was a decline in both in the absence of substrate.

The adenylate energy charge is generally assumed to reflect the metabolic potential of the cell. It is considered that, in bacteria, an adenylate energy charge of <0.5 results in loss of viability, a charge of 0.5 to 0.8 is necessary for survival, and an energy charge of >0.75 is required for growth (3, 23). At pH 4.5 in the presence of glutamate, an energy charge of 0.73 was obtained, a value approaching that theoretically sufficient for growth. Growth has not, however, been demonstrated in these axenic reaction mixtures.

Under conditions of starvation, there is in most bacteria a decrease in total adenylates, due primarily to removal of AMP from the adenylate pool by the enzymes adenosine monophosphate nucleosidase (17) or adenylate deaminase (11) or both. These mechanisms allow for the stabilization of the adenylate energy charge during unfavorable conditions. As in *Escherichia coli* and other bacteria (3, 4, 18), nutrient deprivation in *C. burnetii* appears to result in a decrease in the adenylate pool with a more moderate decrease in energy charge. In this respect, *C. burnetii* differs from typhus rickettsia, which do not appear to have a mechanism for removal of AMP from the adenylate pool in response to glutamate starvation (20). The adenylate pool in *R. typhi*, therefore, remains constant, or even increases, under conditions of starvation, with a dramatic increase in AMP and a resultant decrease in energy charge (23). The inability of *R. typhi* to effectively stabilize its energy charge outside of living cells may be one factor in the relative instability of *R. typhi* in extracellular environments.

The relative lack of metabolic activity and stability of energy charge by *C. burnetii* at pH 7 would seem to explain, in part, the extreme resistance of this organism in neutral or alkaline environments (7, 12, 16, 19). The sensitivity of *C. burnetii* to an acid environment (7) may, in view of the results here, be best explained by activation of the metabolic processes of the organism, leading to depletion of endogenous reserves and loss of viability due to starvation. We have previously proposed that the pH dependence of *C. burnetii* may represent a survival mechanism by which the organism exhibits only minimal metabolic activity in neutral environments but is activated upon fusion of lysosomes to the ingesting phagosome (5). The results presented here appear to support that proposal and substantiate the concept that intracellular parasites have evolved metabolic programs complementary to the cellular compartment in which they reside.

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