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Biochemical stratagem for obligate parasitism of eukaryotic cells by *Coxiella burnetii*

(intracellular parasitism/pathogenic mechanisms/phagolysosome/compartmentation)

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ABSTRACT ***Coxiella burnetti*, the etiologic agent of Q fever, is an oligate intracellular parasite of eukaryotes. Unlike the majority of successful bacterial parasites, which escape the bactericidal environment of the phagolysosome by various means, *C. burnetii* multiplies only in the phagolysosome. In view of the relatively harsh environment inhabited by *C. burnetii*, we have examined (*i*) the *in vitro* metabolism of glucose and glutamate by whole cells of *C. burnetii* under conditions designed to approximate the pH within the phagolysosome and (*ii*) the effect of manipulation of the phagolysosomal pH by lysosomotropic amines on the replication of *C. burnetii* in chicken embryo fibroblasts. The transport, catabolism, and incorporation of both glucose and glutamate were found to be highly stimulated by acidic conditions, whereas at pH 7.0 metabolism of these substrates was minimal. The transport processes were shown to be energy dependent and highly sensitive to inhibition by uncouplers of oxidative phosphorylation. Increasing the phagolysosomal pH of infected chicken embryo fibroblasts by use of the lysosomotropic agents chloroquine, methylamine, or ammonium chloride inhibited the multiplication of *C. burnetii*, thus demonstrating the *in vivo* requirement for the acidic conditions of the phagolysosome. This apparent dependence upon phagosome–lysosome fusion to generate pH conditions favorable to *C. burnetii* replication suggests a unique biochemical mechanism of parasite activation. A pathogenic mechanism based on regulation of microbial metabolism by H^+-dependent stimulation of cell function is proposed.**

Coxiella burnetii, a monospecific genus within the family Rickettsiaceae, is an obligate intracellular parasite of eukaryotes and possesses a number of characteristics in common with Gram-negative bacteria (1–3). Intracellular growth of *Coxiella* and members of the genus *Rickettsia* is compartmentalized into the vacuoles or cytoplasmic space, respectively. Thus compartmentalized replication forms a basis for the separation of the genera (1). Although *C. burnetii* shares with members of the genus *Rickettsia* a characteristic of strict intracellular parasitism, they have evolved different biochemical mechanisms for entry and survival in the eukaryotic cell. *C. burnetii* enters cells primarily by phagocytosis and remains within phagocytic vacuoles throughout its life cycle. Fusion of phagosomes with lysosomes has been demonstrated by the presence of the lysosomal marker enzymes acid phosphatase and 5′-nucleotidase in those phagolysosomes containing *C. burnetii* (4, 5). Thus, circumstantial evidence suggests that *C. burnetii* progresses though its developmental cycle in the phagolysosome. The intraphagolysosomal niche occupied by *C. burnetii* is considered a hostile environment to most bacteria due to the number of hydrolytic enzymes (6), a pH of 4.7–4.8 (7), and other microbicidal mechanisms (8). Penetration of eukaryotic cells by typhus rickettsia, however, is an active process that requires expenditure of energy by the bacterium and the participation of receptor components of both the bacterium and host (9). The phagocytic process involves transient location of the typhus rickettsia within phagosomes and destruction of those organisms that do not escape the phagosome prior to lysosomal fusion. For those organisms that escape the phagosome, replication occurs in the cytoplasm (10, *).

Metabolic studies have shown a further dichotomy between the biochemical parasitic mechanisms of the genera *Rickettsia* and *Coxiella*. *Rickettsia typhi* whole cells, at pH 7.0, have been shown to vigorously oxidize glutamate and selected tricarboxylic acid cycle intermediates to carbon dioxide, with the subsequent production of adenosine 5′-triphosphate (11), whereas glucose is not metabolized (12). Furthermore, enzymes of glycolysis have not been detected in typhus rickettsia, nor was glucose transported by these organisms (12). In contrast, *C. burnetii* has been shown to possess a number of enzymes of the tricarboxylic acid cycle and glucose metabolism, including enzymes of both the glycolytic cycle and of the pentose-phosphate shunt (13–17). However, whole cell metabolism studies, at pH 7.0, have demonstrated that glutamate and tricarboxylic acid cycle intermediates are only moderately utilized (18). Glucose was not utilized in these experiments (18, 19). The marked differences in metabolic capabilities between whole cells of *R. typhi* and *C. burnetii* indicated to us that perhaps the proper conditions for substrate transport had not been identified for *C. burnetii*. Because of the intracellular compartment inhabited by *C. burnetii*, we examined the metabolic capabilities of this organism under conditions that more closely reflect those of the phagolysosomal environment.

We report here that *C. burnetii* has evolved a parasitic mechanism that is based on the regulation of microbial metabolism by hydrogen ion concentration-dependent stimulation of cell function. We show that the transport and metabolism of both glucose and glutamate by *C. burnetii* are stimulated by acidic conditions and that lysosomotropic amines prevent the intracellular multiplication of this microbe. This enhancement of generalized metabolism represents a biochemical pathogenic mechanism that is in sharp contrast to the active inhibition of phagosome–lysosome fusion (PLF) by most intracellular pathogens.

MATERIALS AND METHODS

Organism. Plaque-purified *C. burnetii*, 9 mile strain, phase I (clone 7), was propagated in specific pathogen-free type IV,

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Abbreviations: PLF, phagosome–lysosome fusion; CEF, chicken embryo fibroblasts.

* Meyer, W. H., III & Wisseman, C. L. (1980) *Abstr. Annu. Meeting Am. Soc. Microbiol.*, May 11–16, 1980, Miami Beach, FL, D10.

antibiotic-free fertile hens' eggs (H and N Hatchery, Redmond, WA) and purified from 7-day-infected yolk sacs by Renografin density gradient centrifugation (20, 21). All experiments reported here were performed on freshly harvested and purified *C. burnetii* with no prior freezing.

Metabolism Experiments. ^{14}C-Labeled substrates were purchased from New England Nuclear. Unlabeled substrates were obtained from Sigma. The labeled compounds were diluted with the respective unlabeled chemicals to provide the desired substrate concentration to the reaction mixture and 0.2 μCi (1 Ci = 3.7×10^{10} becquerels) in 0.2-ml volumes. Cell pellets were resuspended in 50 mM potassium phosphate/152.5 mM KCl/15 mM NaCl/100 mM glycine (P-25 buffer) at 4°C and at the desired pH.

Production of $^{14}CO_2$ from glutamate and glucose was determined as described (22) in 25-ml Erlenmeyer flasks fitted with plastic cups, containing paper wicks, suspended from rubber stoppers. The reaction mixture contained the following components in a final volume of 2 ml: 0.1 mM uniformly ^{14}C-labeled substrate (1 μCi/μmol), 22.3 mM potassium phosphate, 135.7 mM KCl, 13.4 mM NaCl, 89 mM glycine, 20 μM $MgCl_2$, and 0.25–1.0 mg dry weight of viable *C. burnetii* whole cells (3.7×10^{10} plaque-forming units/mg of dry weight) (21). Components were added to the reaction vessel at room temperature, and the rubber stoppers were placed on each vessel. The reaction was initiated by shaking the flasks at 200 rpm in an Aquatherm water bath shaker (New Brunswick) thermoequilibrated at 36°C. After 2-hr incubation, the reaction was terminated by the addition of 0.2 ml of Hyamine hydroxide (New England Nuclear) to the cups and 0.5 ml of 25% (wt/vol) trichloroacetic acid to the reaction mixture. After an additional 0.5 hr of incubation at 36°C, the cups were transferred to vials to which were added 1 ml of methanol and 7.5 ml of Econofluor (New England Nuclear) and the radioactivity was measured in a liquid scintillation spectrometer equipped with automatic quench compensation (Beckman, LS 9000).

Incorporation of ^{14}C into macromolecules was measured by filtering 0.5-ml volumes of the trichloroacetic acid-precipitated flask contents through 0.45-μm-pore-diameter Millipore filters and washing the filters with approximately 15-ml volumes of 5% trichloroacetic acid, kept at room temperature. The filters were placed in vials with 7.5 ml of Aquasol (New England Nuclear) and their radioactivities were measured after 24 hr as described above.

For the determination of the effect of pH, 12 buffered solutions (P-25) containing 100 mM glycine were prepared by mixing the appropriate proportions of potassium phosphate (monobasic, dibasic, or tribasic) and phosphoric acid. Suspensions of *C. burnetii*, prepared as described (21), were divided into 12 lots and centrifuged, and each pellet was suspended in a different buffer. The cells were used immediately in the reaction mixtures. The pH values given were those obtained at the end of the experiment without ^{14}C-labeled substrates, after 2 hr at 36°C.

All metabolism experiments were done in triplicate. Variation was usually <10%, and the results shown are the means. Radioactivities obtained in control flasks without cells were generally <100 cpm and were subtracted from the corresponding values of the test flasks prior to calculation of metabolic activity. Metabolic activity was expressed as nmol/mg·hr for $^{14}CO_2$ production and ^{14}C incorporation.

Measurement of Glucose and Glutamate Transport. For determination of initial rates of glucose and glutamate transport, freshly harvested *C. burnetii* whole cells were suspended at a concentration of 5 mg (dry wt)/ml in P-25 buffer as described above at the desired pH. The transport medium contained 40 mM potassium phosphate, 122 mM KCl, 12 mM NaCl, 80 mM glycine, 25 μM uniformly ^{14}C-labeled substrate (160 mCi/mmol), and *C. burnetii* at 1 mg (dry wt)/ml. Organisms and components of the transport medium were thermoequilibrated at 36°C for 5 min and transport was initiated by adding prewarmed substrate (125 μl). For glutamate transport, samples (100 μl) were removed at 30, 60, 90, 120, and 240 s from the reaction vessel and immediately filtered through prewetted 0.22-μm-pore-diameter filters (GSWP, Millipore) followed by a 15-ml wash with P-25 buffer, pH 6.0, at room temperature. Filters were placed in 7.5 ml of Aquasol and their radioactivities were measured as described above. Procedures were identical for glucose transport, except samples from the reaction vessel were removed at 30, 60, 120, 300, and 600 s. Rates of transport were calculated from the linear portion of the uptake curve and are reported here as initial rates in pmol/mg·s for each pH.

Cell Culture and Lysosomal Perturbation. Chicken embryo fibroblast (CEF) monolayers were prepared as described by Ormsbee and Peacock (23) in eight-well Lab-Tek slides (2.4×10^5 cells per chamber; Naperville, IL) using medium 199 (GIBCO) plus 5% tryptose phosphate broth (Difco) and 5% fetal calf serum (GIBCO). After 32-hr incubation at 37°C under 5% CO_2/95% air, the monolayers were infected with purified *C. burnetii* at a multiplicity of approximately 50 plaque-forming units per cell. After an additional 24-hr incubation to allow penetration and multiplication of the organisms, the medium was replaced with fresh medium 199 plus 5% tryptose phosphate broth and 5% fetal calf serum containing one of the following: 100 μM chloroquine, 10 mM methylamine, 10 mM NH_4Cl, or no addition. The cultures were then incubated an additional 96 hr, fixed in acetone, stained with rhodamine isothiocyanate-conjugated guinea pig antibodies to phase I and phase II *C. burnetii* (24), and viewed through a Zeiss epifluorescence microscope. Images were recorded on Kodak Tri-X film.

RESULTS

Metabolism and Substrate Transport. Metabolism studies were carried out on viable *C. burnetii* whole cells separated from host components by isopycnic Renografin gradient centrifugation (21). Whole cells were incubated with uniformly ^{14}C-labeled substrates over a pH range of 2 to 9 and $^{14}CO_2$ evolved and ^{14}C incorporated into trichloroacetic acid-insoluble material were determined. An increase in glutamate metabolism was observed above pH 2.0, and a marked decrease occurred above pH 5.5 (Fig. 1*A*). Catabolism of glutamate was maximal at approximately pH 3.5, whereas incorporation of glutamate consistently exhibited a slightly higher optimum of about pH 4.8. Collectively, the effective pH range was between 2.0 and 5.5, with metabolism above pH 6.0 declining to values only slightly greater than background activities. Although incorporation of amino acids into trichloroacetic acid-insoluble products has been described for disrupted cells of *C. burnetii* (25), amino acid incorporation into whole cells of *C. burnetii* in an axenic medium had not been demonstrated previously.

Stimulation of glutamate metabolism was shown to be dependent upon pH activation of substrate transport (Fig. 1*B*). The transport process for glutamate was activated by conditions of low pH, and maximal rates were achieved between pH 3 and 5. The decline in transport activity was consistent with a decreased rate of metabolism of glutamate above pH 5.5 and below pH 3.0.

Metabolism of glucose by whole cells of *C. burnetii* also was examined over a pH range of 2 to 9 (Fig. 2*A*). Glucose metabolism was similarly stimulated by acidic conditions, although the maximal rates of catabolism and incorporation coincided at approximately pH 4.8. Metabolism of glucose was also shown

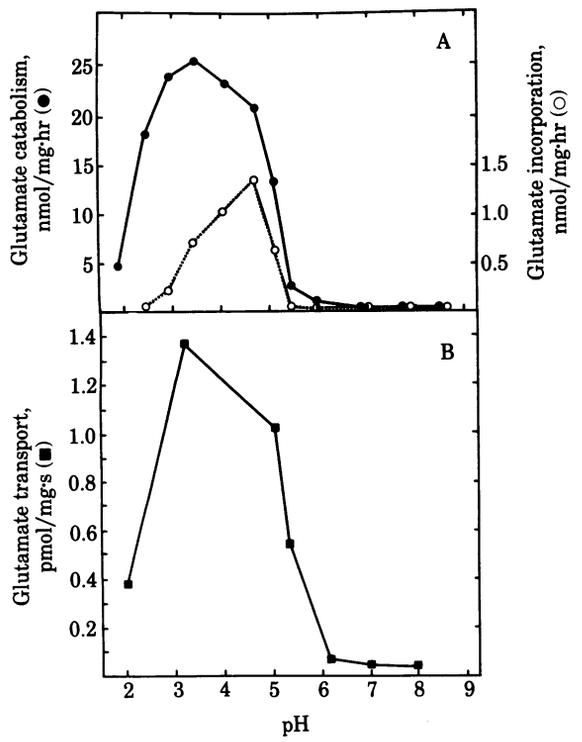

FIG. 1. Effect of pH of the suspending medium on catabolism (●) and incorporation (○) of glutamate (*A*) and transport (■) of glutamate (*B*) by purified suspensions of *C. burnetii*.

to be dependent upon pH activation of glucose transport (Fig. 2*B*). The transport of glucose was activated between pH 2 and 3, whereas metabolism was stimulated at a pH ≥ 3. Although

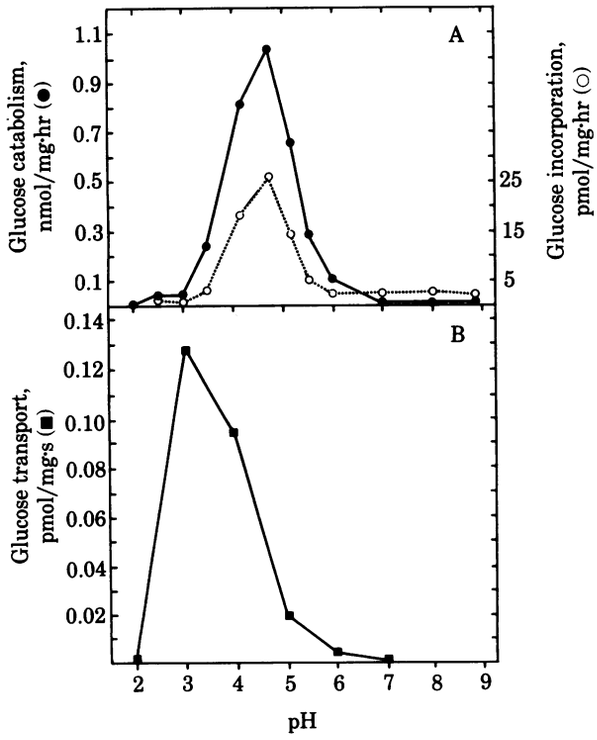

FIG. 2. Effect of pH of the suspending medium on catabolism (●) and incorporation (○) of glucose (*A*) and transport (■) of glucose (*B*) by purified suspensions of *C. burnetii*.

glucose metabolism was maximal at pH 4.8, the transport of this uncharged substrate was about 25% of the maximum recorded at pH 3. Evolution of $^{14}CO_2$ from both [1-^{14}C]glucose and [6-^{14}C]glucose indicates that both the Embden–Meyerhof pathway of glycolysis and the pentose phosphate pathway (26) were active in *C. burnetii* at pH 4.5 (data not shown).

Effect of Metabolic Inhibitors on Glutamate and Glucose Transport. A number of metabolic inhibitors were utilized to examine the energization of transport processes in *C. burnetii* (Table 1). In general, uncouplers of oxidative phosphorylation, such as 2,4-dinitrophenol and carbonyl cyanide-*m*-chlorophenylhydrazone (CCCP), were found most effective in inhibition of transport processes. The adenosine 5′-triphosphate phosphohydrolase inhibitor *N,N′*-dicyclohexylcarbodiimide was a less potent inhibitor of glutamate transport than the proton-conducting ionophore CCCP. This is consistent with observations in other bacterial systems (27) and suggests that the glutamate transport process of *C. burnetii* was energized via a protonmotive force (28).

Effect of Glutamate on Glucose Metabolism. Because metabolism of glucose was markedly less than metabolism of glutamate, we tested the effect of adding unlabeled glutamate to cells that were actively metabolizing [*U*-^{14}C]glucose. The evolution of $^{14}CO_2$ was increased 3.3-fold, and the incorporation of [^{14}C]glucose was enhanced 51.3-fold by the addition of glutamate. These results indicate that an additional energy source such as glutamate facilitates the utilization of glucose by providing another source of adenosine 5′-triphosphate to drive glucose transport. Indeed, as shown in Table 1, the inhibition of oxidative phosphorylation reduced transport of both glucose and glutamate.

Effect of Lysosomal Perturbants. In living cells the pH of lysosomes is maintained at about 4.7–4.8 by an active process (7). Various weakly basic substances are concentrated in lysosomes as a consequence of lysosomal acidity and, as a result, increase the pH of the lysosomal contents (29). To demonstrate the pH dependence of *C. burnetii* metabolism *in vivo*, the ability of *C. burnetii* to replicate in cells exposed to the lysosomal perturbants chloroquine, methylamine, or ammonium chloride (7, 30) was examined. In untreated CEF, extensive growth of *C. burnetii* was apparent (Fig. 3*A*), and the organisms were evident as discrete clusters within the phagolysosome. However, in CEF treated with 100 μM chloroquine, 10 mM meth-

Table 1. Effect of metabolic inhibitors on glutamate and glucose transport by *C. burnetii*

		Transport, % of control*	
Substrate	Inhibitor	5 min†	60 min†
Glutamate	—	100	100
	KCN, 1.0 mM	107.8	74.0
	Dinitrophenol, 1.0 mM	12.1	9.4
	NaF, 1.0 mM	117.4	52.7
	DCCD, 0.5 mM	100.4	—
	CCCP, 0.05 mM	20.8	—
Glucose	—	100	100
	KCN, 1.0 mM	97.2	2.2
	Dinitrophenol, 1.0 mM	>1	2.0
	NaF, 1.0 mM	>1	>1

DCCD, *N,N′*-dicyclohexylcarbodiimide; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

* Percent of initial rates of transport of either substrate in the absence of inhibitors at pH 3.5.

† Exposure of organisms to inhibitor at 37°C prior to addition of substrate.

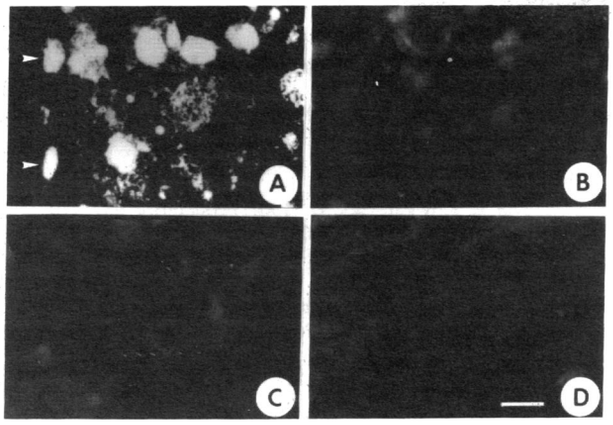

FIG. 3. Effect of perturbation of intralysosomal pH on growth of *C. burnetii*. CEF were infected as described in the text and treated with no addition (*A*), 100 μM chloroquine (*B*), 10 mM methylamine (*C*), or 10 mM ammonium chloride (*D*). When 96 hr had elapsed after addition of the inhibitors, the monolayers were fixed in acetone and labeled with rhodamine isothiocyanate-conjugated guinea pig antibodies to phase I and phase II *C. burnetii*. In untreated cultures extensive growth of *C. burnetii* is evident. Organisms often appear in discrete clusters (arrow), presumably still bound by the phagolysosome. In cultures treated with any of the lysosomal perturbants, only individual *C. burnetii* cells are occasionally seen. Bar indicates 10 μm.

ylamine, or 10 mM NH_4Cl, very few *C. burnetii* cells were observed (Fig. 3 *B*, *C*, and *D*), thus demonstrating the *in vivo* requirement of an acidic pH for metabolism and replication.

DISCUSSION

An insight into the biochemical strategy of obligate intracellular parasitism of phagocytic cells has been achieved by studying the *in vitro* metabolic capabilities of *C. burnetii*. The apparent association of *C. burnetii* with the phagolysosome of eukaryotic cells (4, 5) suggested that a key feature of phagolysosomal growth of *C. burnetii* would be a membrane structurally and functionally compatible with an otherwise harsh environment. The acidic nature of this compartment prompted us to carry out relatively simple *in vitro* metabolic studies employing a pH range of 2 to 9. Our results indicate that previous reports (18, 19) of minimal glutamate metabolism and absence of glucose metabolism by whole cells of *C. burnetii* were the result of incubation at pH values (>7.0) beyond the upper limit at which the organism actively transports and metabolizes these substrates. Several enzymes of glycolysis and the pentose phosphate pathway have been detected in extracts of *C. burnetii* when assayed at approximately pH 7 (14, 15), and $^{14}CO_2$ evolution from radiolabeled glucose by similar extracts has been detected (31). In our studies the evolution of $^{14}CO_2$ from both [1-^{14}C]- and [6-^{14}C]glucose indicated that both the Embden–Meyerhof pathway of glycolysis and the pentose phosphate pathway are active in whole cells of *C. burnetii* at pH 4.5. These results indicate that the active transport and metabolism of exogenously supplied substrates are activated by acidic conditions. Thus, glucose is metabolized by *C. burnetii* whole cells, which demonstrates that this important metabolite may serve as a source of energy and carbon during intraphagolysosomal replication.

Glutamate was metabolized by *C. burnetii* approximately 26-fold greater than glucose under optimal conditions. This was not surprising because glutamate has been shown to be a primary energy source for several microbes (11, 22, 32). Because both glucose transport and glutamate transport were active processes, it seemed that glucose metabolism might be enhanced in the presence of glutamate, which would provide an additional energy source for *C. burnetii* metabolic processes. Indeed, the formation of $^{14}CO_2$ from glucose metabolism in the presence of glutamate was stimulated 3.3-fold and incorporation of glucose was markedly increased by 51.3-fold.

C. burnetii enters cells primarily by phagocytosis (4, 5), whereupon PLF would provide sugars, amino acids, and dipeptides (33) and the proper hydrogen ion concentration for the stimulation of *C. burnetii* metabolism. Furthermore, the acidic environment of the phagolysosome may provide a sufficiently large concentration gradient of hydrogen ions across the bacterial membrane to produce the electrochemical potential necessary as a driving force in such processes as ATP synthesis and active transport (34). Indeed, preliminary studies have shown that rapid addition of sufficient H^+ to decrease the pH of suspensions of *C. burnetii* from 7.0 to 3.3 stimulates an almost instantaneous burst of ATP synthesis (unpublished observations). Thus an artificially imposed protonmotive force appears able to drive ATP synthesis in *C. burnetii*, as has been described in other prokaryotic and eukaryotic systems (35, 36). Therefore, the metabolic capabilities of *C. burnetii* are apparently activated under the harsh conditions of the phagolysosome.

The ingestion of *C. burnetii* by phagocytic cells ultimately results in phagolysosomal replication of the microbe, which eventually leads to the destruction of the host cell. We propose that a biochemical stratagem for *C. burnetii* is the regulation of microbial membrane function by pH activation of substrate transport and metabolism. To test this hypothesis *in vivo*, we employed lipophilic amines (methylamine and chloroquine) and ammonia as effectors of lysosomal pH and as an inhibitor of PLF, respectively. Ammonia, methylamine, and chloroquine raise the intralysosomal pH and inhibit degradation of lysosomal proteins (37–41). Recently, Gordon *et al.* (42) have shown that ammonia prevents PLF, whereas chloroquine and methylamine do not. In our studies, the addition of any of these chemicals to *C. burnetii*-infected tissue culture cells restricted the replication of *C. burnetii*. Therefore, our original *in vitro* observations on the pH activation of metabolism of *C. burnetii* have been verified by *in vivo* experiments designed to prevent phagolysosome replication of *C. burnetii* by increasing the pH of an otherwise acidic compartment.

Other intracellular parasites have been shown to live freely in the cytoplasm (10, 43–45), in vacuoles (46–50, †), and in the phagolysosomes (51–53). *C. burnetii* resembles the phagolysosomal parasites *Mycobacterium lepraemurium* (51) and *Leishmania donovani* (53) in that PLF seems to be required for their intracellular multiplication. However, a requirement for the acidic pH of the phagolysosome to stimulate transport processes and metabolic pathways has not been demonstrated in these organisms. Circumvention of a requirement for mycobactin by some mycobacteria (54, 55) under acidic conditions in an axenic medium suggests a nutritional dependency in these microbes that may be overcome by low pH. Thus adaptive biochemical mechanisms apparently exist for intracellular parasites.

Conceptually, the activation of *C. burnetii* metabolism by the pH of the phagolysosome may represent a general phenomenon that would regulate energy transformation by vectorial metabolism. We therefore examined the metabolic capabilities of *R. typhi* and *C. psittaci* (strain 6BC), which are cytoplasmic (2, 10) and phagosomal pathogens (2, 50, 56) of eukaryotes, respectively. Both of these organisms are representative members of the two orders of rickettsias, and both were shown to metabolize glutamate (2, 57). In contrast to *C. burnetii* but in accord with

† Eissenberg, L. G. & Wyrick, P. B. (1980) *Abstr. Annu. Meeting Am. Soc. Microbiol.*, May 11–16, 1980, Miami Beach, FL, D5.

compartmentalized replication, both *R. typhi* and *C. psittaci* metabolized glutamate optimally at about pH 7.0–7.4, and neither metabolized glucose to a detectable level at any pH examined (58). The pH activation of transport and metabolism of glucose and glutamate by *C. burnetii* should confer selective biological advantage to this phagolysosomal pathogen. The minimal metabolic activity displayed by *C. burnetii* at neutral or alkaline pH suggests a mechanism by which this microbe persists in the environment for prolonged periods of time without a significant loss of viability. The stimulation of metabolism under acidic conditions may, therefore, effect a chemical resistance of *C. burnetii* at neutral pH in extracellular environments (2, 59). Recognition of the acidophilic nature of *C. burnetii* should have a positive impact on future research designed to delineate the mechanisms of pathogenicity of this medically important bacterium.

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