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Estimation of the Cytoplasmic pH of *Coxiella burnetii* and Effect of Substrate Oxidation on Proton Motive Force

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The magnitude of the proton motive force generated during in vitro substrate oxidation by *Coxiella burnetii* was examined. The intracellular pH of *C. burnetii* varied from about 5.1 to 6.95 in resting cells over an extracellular pH range of 2 to 7. Similarly, $\Delta\psi$ varied from about 15 mV to −58 mV over approximately the same range of extracellular pH. Both components of the proton motive force increased during substrate oxidation, resulting in an increase in proton motive force from about −92 mV in resting cells to −153 mV in cells metabolizing glutamate at pH 4.2. The respiration-dependent increase in proton motive force was blocked by respiratory inhibitors, but the ΔpH was not abolished even by the addition of proton ionophores such as carbonyl cyanide-*m*-chlorophenyl hydrazone or 2,4-dinitrophenol. Because of this apparently passive component of ΔpH maintenance, the largest proton motive force was obtained at an extracellular pH too low to permit respiration. *C. burnetii* appears, therefore, to behave in many respects like other acidophilic bacteria. Such responses are proposed to contribute to the extreme resistance of *C. burnetii* to environmental conditions and subsequent activation upon entry into the phagolysosome of eucaryotic cells in which this organism multiplies.

Coxiella burnetii, the rickettsial agent of Q fever, is an obligate intracellular parasite that replicates within the phagolysosome of eucaryotic cells (2, 3). *C. burnetii* has evidently adapted its metabolic processes to the acidic environment of the phagolysosome as the transport and metabolism of a number of substrates are stimulated by hydrogen ion concentrations representative of that compartment (7–9). A requirement for acidic pH to allow growth and multiplication of *C. burnetii* in vivo is implied by the inhibition of its multiplication after the addition of lysosomotropic amines known to raise the pH of lysosomal vacuoles (7).

All acidophilic bacteria examined to date maintain a cytoplasmic pH that is more alkaline than the environment (4, 13, 16, 19, 23). This difference in pH across the cytoplasmic membrane is most extreme in acidophiles, but occurs in virtually all bacteria except alkalophiles (24), and contributes to a number of physiological functions according to Mitchell's chemiosmotic hypothesis (22). This electrochemical gradient of protons consists of an electrical potential, $\Delta\psi$ (inside negative; at low external pH, most acidophilic bacteria maintain a small or slightly positive $\Delta\psi$), and a proton gradient, ΔpH (inside alkaline). Together these components constitute the proton motive force (PMF = $\Delta\psi - 59\Delta$pH; where 59 is a factor for the conversion of ΔpH to mV and equal to [2.3 RT]/F) (11). In neutrophilic bacteria and mitochondria, the PMF is generated concomitantly with electron transport (11, 12) and has been shown to play a role in the energization of transport of some solutes (5) and ATP synthesis (17). Under fermentative conditions, a PMF may be generated by ATP hydrolysis (11, 12).

I demonstrate here that *C. burnetii*, like more extreme acidophilic bacteria, maintains an intracellular pH near neutrality and that maintenance of the intracellular pH is at least partially dependent on active chemiosmotic mechanisms. In an accompanying paper (10), the role of ΔpH in the energization of glutamate transport is examined.

MATERIALS AND METHODS

Organisms. The Nine Mile strain of *C. burnetii*, phase 1 (clone 7) was propagated in specific-pathogen-free fertile hen's eggs (SPF type IV; H and N Hatchery, Redmond, Wash.) and purified from 8-day infected yolk sacs by Renografin density gradient centrifugation as previously described (29).

Internal water volume. Cell volumes were determined as described by Stock et al. (27). For the determination of cytoplasmic water volume, purified *C. burnetii* was incubated at 37°C for 15 min at concentrations of 1 mg of dry weight per ml in 50 mM

potassium phosphate–152.5 mM KCl–15 mM NaCl–100 mM glycine (P-25 buffer) containing 30 μCi of ^3H_2O and 1.1 μCi of [*U*-^{14}C]sucrose (434.6 mCi/mmol). Cells were separated from the suspending medium by centrifugation through 100-μl pads of high-temperature silicon oil (d = 1.05; Aldrich Chemical Co., Inc., Milwaukee, Wis.) in a Microfuge B (Beckman Instruments Inc., Fullerton, Calif.). A 50-μl sample of the supernatant was taken, and the bottom of the microfuge tube was cut off to obtain the cell pellet. Both were placed in scintillation vials containing 7.5 ml of Aquasol (New England Nuclear Corp., Boston, Mass.) and held overnight before scintillation counting in a Beckman LS 9000 liquid scintillation spectrometer programmed for dual channel counting. Cell volumes were calculated as described previously (27). Total cellular volume was similarly determined, except that the labeled sucrose was omitted and replaced with 0.125 μCi of (hydroxy[^{14}C]methyl)inulin (5 mCi/mmol).

Determination of ΔpH and Δψ. The intracellular pH was determined essentially as described by Rottenberg (26). One milligram (dry weight) of *C. burnetii* was incubated in P-25 buffer, unless otherwise noted, containing either 10 μM 5,5-dimethyl[2-^{14}C]oxazolidine-2,4-dione ([^{14}C]DMO; 55 mCi/mmol) or 22.1 μM [7-^{14}C]benzoic acid (22.6 mCi/mmol) plus 0.1 μCi of [^{3}H]sucrose (9.7 mCi/mmol) in a volume of 1 ml. After 15 min of incubation at 37°C, the rickettsiae were separated from the medium by centrifugation through silicone oil, and samples taken for scintillation counting as described above for the determination of intracellular water volume. Cytoplasmic water volumes were determined in parallel and intracellular pH calculated from the following equation:

$$pH_i = pK_a + \log \left\{ \left[\left[\frac{C_t}{C_e} \left(1 + \frac{V_e}{V_i} \right) \right] - \frac{V_e}{V_i} \right] [10^{pH_e - pK_a'} + 1] \right\} - 1$$

where pH_i and pH_e are the intracellular and extracellular pH, respectively, C_t is the disintegrations per minute of ^{14}C-acid per μl of pellet volume, C_e is the disintegrations per minute of ^{14}C-acid per μl of the suspending medium, and V_e and V_i are the extracellular and cytoplasmic water volume of the pellet, respectively (18).

Δψ was similarly determined by incubation of organisms in 50 mM $NaPO_4$, 100 mM glycine, 134 mM NaCl buffer plus 11.2 μM ^{86}RbCl (7.41 mCi/mg) and 100 μg of valinomycin per ml. After 15 min of incubation at 37°C, the rickettsiae were separated from the medium by centrifugation through silicone oil and radioactivity associated with the supernate and pellet fractions determined as described above. Δψ was calculated in mV according to the following equation (26):

$$\Delta\psi = -59 \log \frac{Rb^+_{in}}{Rb^+_{out}}$$

Oxygen uptake. Oxygen consumption was measured in a volume of 2 ml of P-25 buffer with *C. burnetii* at a concentration of 2 mg of dry weight per ml with a Clark-type oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio).

Chemicals and radiochemicals. (Hydroxy[^{14}C]methyl)inulin, 5,5-dimethyl-[2-^{14}C]oxayolidine-2,4-dione, [*U*-^{14}C]sucrose, [^{3}H]inulin, [1-^{14}C]acetic acid, (6,6'-(*n*)[^{3}H]sucrose, L-[*U*-^{14}C]glutamic acid, and KS^{14}CN were obtained from Amersham Corp. (Arlington Heights, Ill.). ^3H_2O, ^{86}RbCl, [^{14}C]methylamine, and [*phenyl*-^{3}H]tetraphenyl phosphonium bromide were from New England Nuclear Corp. Other reagents were from Sigma Chemical Co. (St. Louis, Mo.).

RESULTS

Cytoplasmic water volume. The internal water volume of *C. burnetii* was determined from the distribution of ^3H_2O and either [^{14}C]inulin or [^{14}C]sucrose after centrifugation through silicone oil as described by Stock et al. (27). Since *C. burnetii* has been reported to consist of two morphologically distinct cell types (20, 21, 28) which occur in variable ratios, the cytoplasmic water volume was determined for each experiment in parallel with ΔpH and Δψ determinations. By this method, the cellular water volume of *C. burnetii* purified from 8-day-infected yolk sacs was determined to be 1.85 ± 0.18 μl per mg of dry weight, and the cytoplasmic water volume was determined to be 1.48 ± 0.12 μl per mg of dry weight (mean ± standard error; $n = 11$).

Estimation of intracellular pH. The intracellular pH of *C. burnetii* was similarly determined from the distribution of radiolabeled weak acids by the method of Rottenburg (26) with [^{3}H]sucrose as a marker for extracytoplasmic space. A variety of probe molecules was utilized in initial attempts to determine the intracellular pH of *C. burnetii*. As shown in Table 1, [^{14}C]benzoate was accumulated to a greater extent than was [^{14}C]DMO. This is not unexpected because of the lower pK_a of benzoic acid. When the internal pH was calculated, similar values for pH_i were obtained whether [^{14}C]benzoate or [^{14}C]DMO was used as the probe molecule. The use of [^{14}C]acetate resulted in slightly lower calculated values for pH_i over an external pH range of 2 through 7 (data not shown). A weak base, [^{14}C]methylamine, was excluded by *C. burnetii*. In the presence of a metabolizable energy source, in this case glutamate, the pH_i increased by approximately 0.5 to 0.9 units. The increase in ΔpH resulting from substrate oxidation was consistently greater when calculated from the distribution of [^{14}C]DMO than when calculated from the distribution of [^{14}C]benzoate.

Because DMO has a pK_a of 6.21 (1), its usefulness in determining ΔpH at very low external pH has been questioned (4). At pH 4.5 the differences in accumulation of DMO observed in the presence or absence of substrate were found to be highly reproducible. DMO was therefore

TABLE 1. Determination of intracellular pH from the distribution of various weak acids

Probe	Glut (5 mM)	pH_e	cpm_i[a]	cpm_o[b]	cpm_i/cpm_o	pH_i[c]	ΔpH[d]
DMO	−	4.78	1757	1292	1.36	5.82	1.04
DMO	+	4.64	5455	1296	4.21	6.73	1.92
Benzoate	−	4.61	24650	1987	12.40	5.84	1.23
Benzoate	+	4.56	66258	1841	35.98	6.27	1.71
Acetate[e]	−	4.70	1473	753	1.96	5.16	.46

[a] cpm_i, Counts per minute of ^{14}C-acid per microliter of cytoplasmic volume.
[b] cpm_o, Counts per minute of ^{14}C-acid per microliter of extracellular volume.
[c] Intracellular pH (pH_i) was calculated here by using the following formula (26): $pH_i = pK_a' = \log[[cpm_i/cpm_o](10^{pH_e - pK_a'} + 1)] - 1$.
[d] $\Delta pH = pH_i - pH_e$.
[e] [*U*-^{14}C]acetate distribution was determined as described in the text, except the final concentration of [*U*-^{14}C]acetate was 10 μM (57.8 mCi/mmol).

utilized as a probe molecule in several initial experiments. However, since the use of DMO is possibly inappropriate at external pH values much less than 4.5, [^{14}C]benzoate was used in many of the subsequent experiments and particularly those which required determination of ΔpH at external pH values less than 4.5.

The increase of ΔpH during substrate oxidation took place within 5 min of addition of substrate and did not increase further after up to 1 h of incubation (Table 2). No increase in cytoplasmic volume was observed in the presence or absence of glutamate over the same time frame. In addition, the ΔpH and increase in ΔpH during substrate oxidation were not affected by varying the concentration of cells over a range of from 1 to 5 mg of dry weight per ml.

Effect of external pH on pH_i. The intracellular pH of *C. burnetti*, as determined from the distribution of [^{14}C]benzoate, increased gradually from about 5.1 at an external pH of 2 to 6.95 at pH 7.0 (Fig. 1). Since the intracellular pH was relatively stable, the ΔpH increased accordingly with decrease in pH_e, reaching a maximum value of about 3.1 units at an external pH of 2. In the presence of glutamate, the pH_i and ΔpH increased over the range of external pH required by *C. burnetii* for substrate metabolism. As with other acidophiles (4, 13, 19, 23), the proton ionophore carbonyl cyanide-*m*-chlorophenyl hydrazone (CCCP) decreased the pH_i by up to 0.3 to 0.5 units below that of cells in the absence of substrate, but did not abolish the ΔpH. No effect of CCCP on pH_i was noted at the upper or lower extremes of pH_e examined.

Determination of Δψ. The Δψ was determined from the distribution of $^{86}Rb^+$ in the presence of valinomycin as described previously (26). From $^{86}Rb^+$ distribution data the Δψ calculated for *C.*

TABLE 2. Effect of incubation period on ΔpH and cellular volume

Incubation (min)	5 mM glutamate	ΔpH[a]	V_{cell}[b] (μl/mg)	V_{cyto}[b] (μl/mg)
0	−	0.96	1.94	1.62
0	+	1.02		
5	+	1.64		
15	+	1.59		
60	+	1.72	1.93	1.53
60	−	0.85	1.86	1.50

[a] Calculated from the distribution of [^{14}C]DMO in P-25 buffer at pH 4.8.
[b] Cellular volume (V_{cell}) and cytoplasmic volume (V_{cyto}) were calculated from the distribution of ^3H_2O and [^{14}C]inulin and ^3H_2O and [^{14}C]sucrose, respectively.

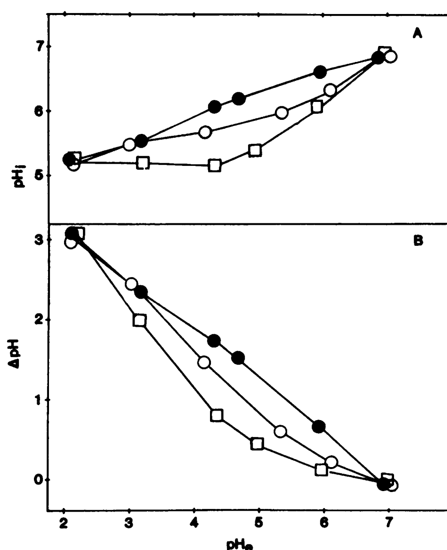

FIG. 1. Effect of extracellular pH on the intracellular pH (A) and ΔpH (B) of *C. burnetii* determined from the distribution of [^{14}C]benzoic acid. Symbols: (○) no substrate; (●) plus 5 mM glutamate; and (□) plus 5 mM glutamate and 50 μM CCCP.

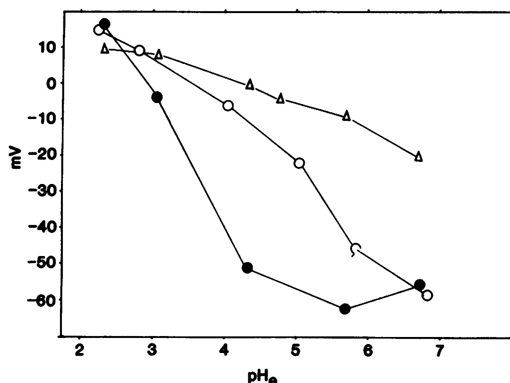

FIG. 2. Determination of $\Delta\psi$ in *C. burnetii* and the effect of extracellular pH. $\Delta\psi$ was calculated from the distribution of ^{86}Rb in the presence of valinomycin. Symbols: (○) no substrate; (●) plus 5 mM glutamate; and (△) plus 5 mM glutamate and 50 μM CCCP.

burnetii in the absence of substrate varied inversely with external pH from about 15 mV at pH 2.2 to −58 mV at pH 6.8 (Fig. 2). Again, $\Delta\psi$ increased (more negative) in the presence of glutamate only over the range of pH where *C. burnetii* metabolizes glutamate. The membrane potential calculated from $^{86}Rb^+$ distribution was greatly reduced, although not completely abolished, by the addition of CCCP.

PMF generated during the oxidation of various substrates. The magnitude of the PMF resulting from the oxidation of a number of substrates was examined (Table 3). Of those substrates examined, glutamate oxidation resulted in the greatest ΔpH and succinate oxidation in the greatest $\Delta\psi$. There was no direct correlation apparent, however, between rates of substrate oxidation, ATP levels (9), and magnitude of the PMF.

Effect of metabolic inhibitors on the PMF. Various metabolic inhibitors known to inhibit ATP synthesis in *C. burnetii* (9) were examined for effect on the generation of a PMF (Table 4). Of those inhibitors, the uncouplers of oxidative phosphorylation, CCCP and 2,4-dinitrophenol, depressed both the pH_i and PMF to the lowest levels. With these uncouplers, both components of the PMF (ΔpH and $\Delta\psi$) were reduced below the values obtained in the absence of any added substrate. Only in the presence of dicyclohexylcarbodiimide, an ATPase inhibitor (11), was the ΔpH resulting from glutamate oxidation equal to or greater than that of control cells. For all inhibitors except the uncouplers, the $\Delta\psi$ did not vary by more than 3 mV from the value of cells in the absence of substrate.

Comparison of PMF with respiration rates. The magnitude of the PMF generated by *C. burnetii* in the presence or absence of glutamate was compared with respiration rates under those same conditions (Fig. 3). As one might expect from the failure of uncouplers to break down the ΔpH, CCCP did not abolish the PMF maintained by *C. burnetii*. Metabolism of glutamate increased the PMF by up to −60 mV over an extracellular pH range of 3 to 6. In comparison, oxygen consumption by *C. burnetii* in the presence of glutamate was maximal at approximately pH 4.8. A low level of oxygen consumption was detected even in the absence of added substrate. Rather than stimulating respiration, CCCP inhibited O_2 consumption by *C. burnetii*. In a separate experiment, O_2 consumption by *C. burnetii* at pH 4.8 without glutamate, with 5 mM glutamate, or with 5 mM glutamate plus 50 μM CCCP was 0.56, 5.16, or 0.45 nmol/min per mg of dry weight, respectively. The mechanism of inhibition of respiration by uncouplers of oxidative phosphorylation is not clear, but the results are similar to those obtained with a more extreme acidophile, *Thiobacillus acidophilus* (19).

DISCUSSION

The results presented here indicate that respiring *C. burnetii* cells, like more extreme acidophilic bacteria, maintain a cytoplasmic pH near neutrality. The intracellular pH appeared relatively stable even in nonmetabolizing cells over a range of external pH of about 2 to 7, but increased by up to 0.9 unit during substrate oxidation. The $\Delta\psi$ was, of necessity, determined

TABLE 3. Magnitude of the PMF generated during the oxidation of various substrates

Substrate[a]	pH_e	ph_i[b]	ΔpH	$\Delta\psi$ (mV)	PMF (mV)
Control	4.84	5.88	1.04	−13.92	−75.28
Glutamate	4.67	6.59	1.92	−34.68	−147.96
Pyruvate	4.71	6.13	1.42	−24.48	−108.26
Succinate	4.73	6.37	1.64	−56.37	−153.13
α-Ketoglutarate	4.76	6.21	1.45	−17.83	−103.38
Glucose	4.75	6.41	1.66	−15.46	−113.40
Citrate	4.76	5.79	1.03	−10.18	−70.95

[a] All substrates were present at a final concentration of 5 mM.
[b] Determined from the distribution of [^{14}C]DMO.

TABLE 4. Effect of metabolic inhibitors on the proton motive force

5 mM glutamate	Inhibitor (mM)	pH_e	pH_i[a]	ΔpH	Δψ (mV)	PMF (mV)
−		4.77	5.85	1.06	−14.16	−76.70
+		4.64	6.14	1.50	−35.51	−124.01
+	CCCP (0.05)	4.69	5.24	0.55	−9.78	−42.23
+	DNP[b] (0.5)	4.63	5.23	0.60	−6.04	−41.44
+	DCCD[c] (2.5)	4.65	6.26	1.61	−14.69	−109.68
+	KCN (10)	4.66	5.52	0.86	−11.51	−62.25
+	NaF (10)	4.81	5.49	0.68	−15.65	−55.77
+	Arsenite (1)	4.68	5.81	1.13	−16.53	−83.20

[a] Determined from the distribution of [^{14}C]benzoate.
[b] DNP, 2,4-Dinitrophenol.
[c] DCCD, Dicyclohexylcarbodiimide.

under slightly different conditions than ΔpH; therefore, a possible source of error in total PMF was introduced. However, the values for Δψ were similar to those of other acidophiles (4, 14, 16, 19, 23) in that the Δψ of *C. burnetii* varied from slightly positive at low external pH to −58 mV at pH 6.8. The Δψ increased concomitantly with ΔpH during substrate oxidation with the result an increase in PMF from approximately −92 mV to −153 mV at pH 4.2.

The validity of radioisotope distribution techniques for determining the intracellular pH of acidophiles has not been confirmed by comparison with techniques such as ^{31}P nuclear magnetic resonances, fluorescence, or direct probes as has been done with neutrophilic bacteria or eucaryotic organelles (26). Radioisotope distribution techniques are, however, widely applied to acidophilic bacteria with results similar to those obtained here with *C. burnetii*. The results presented here clearly demonstrate an increase in PMF during substrate oxidation and, with the above caveat, imply that the intracellular pH of *C. burnetii* is maintained through two mechanisms: (i) a respiration-dependent component inhibitable by either proton ionophores or respiratory inhibitors, and (ii) a passive mechanism that is insensitive to proton ionophores and allows maintenance of the intracellular pH at an apparent baseline level of about 5.1 even at the extremes of low pH tested. Because of this apparent passive component of pH maintenance, the largest values for PMF were obtained at an external pH that did not permit respiration.

The mechanism by which acidophilic bacteria maintain their intracellular pH near neutrality even in extremely acid environments is not generally agreed upon. Both passive and active chemiosmotic mechanisms for pH homeostasis appear to be involved. A passive mechanism for pH maintenance was first suggested by Hsung and Haug (13), who reported that the ΔpH of *T. acidophilus* was not affected by 2,4-dinitrophenol, azide, or even boiling of the cells. They suggested that the major proportion of the ΔpH was maintained by a Donnan potential across the cytoplasmic membrane generated by an internal charged molecule impermeable to the membrane (13). Similar results were obtained by Oshima et al. (23) with *Bacillus acidocaldarius*. Although these authors did not feel that a Donnan potential was the mechanism of ΔpH main-

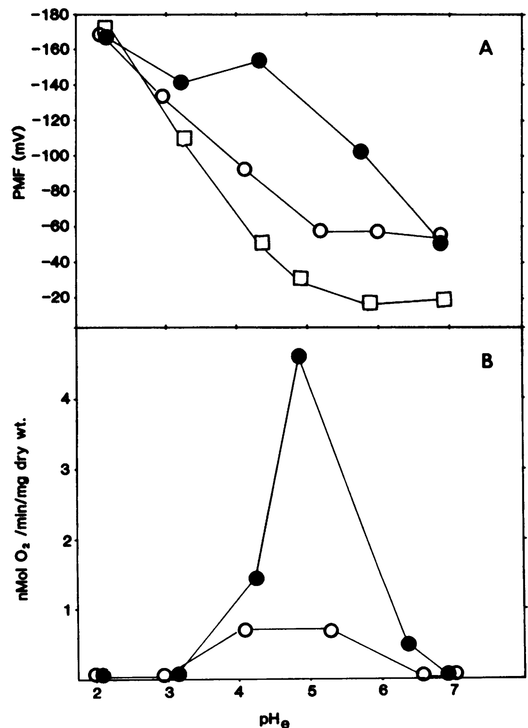

FIG. 3. Effect of extracellular pH on the magnitude of the PMF (A) and rates of respiration (B) by *C. burnetii*. Symbols: (○) no substrate; (●) plus 5 mM glutamate; (□) plus 5 mM glutamate and 50 μM CCCP.

tenance, an alternative mechanism was not proposed. Cox et al. (4) studying *Thiobacillus ferrooxidans*, demonstrated a decrease in intracellular pH of about 0.5 units in the presence of uncouplers of oxidative phosphorylation. A large ΔpH remained, however, even after treatment with these proton ionophores (4). Similarly, Matin et al. found that proton ionophores only partially collapsed the ΔpH of *T. acidophilus* (19). In contrast, Krulwich et al. (16) found that the addition of 2,4-dinitrophenol or nigericin to cultures of *B. acidocaldarius* abolished the ΔpH and that either uncouplers of oxidative phosphorylation or respiratory inhibitors blocked PMF-linked transport processes. These results led them to propose that the ΔpH of *B. acidocaldarius* was maintained through an active process requiring respiration (16). The intracellular pH of *C. burnetii* appears to be maintained through both active and passive mechanisms. It is the actively maintained component, however, that appears to be coupled to performance of cellular functions, as uncouplers of oxidative phosphorylation that do not abolish ΔpH are highly efficient inhibitors of presumably PMF-linked functions such as glutamate transport (10) and ATP synthesis (9) by *C. burnetii*.

It has been proposed (9) that the sensitivity of *C. burnetii* to an acid environment (9, 15) may reflect activation of metabolic processes leading to depletion of endogenous reserves and loss of viability due to starvation. Oxygen consumption by *C. burnetii* shown here in the absence of any added substrate would seem to further imply oxidation of an unidentified endogenous reserve. In view of an apparent necessity of pH homeostasis in bacterial cells (6, 24, 25), it seems reasonable to speculate that at least some of the energy expended at pH 4.5 would be directed toward maintenance of the cytoplasmic pH. The activation of *C. burnetii* metabolism under acidic conditions is believed to represent a survival mechanism whereby the organism exhibits only minimal metabolic activity in neutral or alkaline environments, but is activated by acidification of the ingesting phagosome (7, 9). The lack of demand for expenditure of metabolic energy to maintain intracellular pH in neutral environments shown here would be in keeping with those proposals and may contribute to the extreme resistance of *C. burnetii* to environmental conditions.

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