METABOLIC ACTIVITY IN *COXIELLA BURNETII*

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ABSTRACT

ORMSBEE, RICHARD A. (Rocky Mountain Laboratory, Hamilton, Mont.), AND MARIUS G. PEACOCK. Metabolic activity in *Coxiella burnetii*. J. Bacteriol. **88**:1205–1210. 1964.—Purified suspensions of *Coxiella burnetii* were shown to utilize α-ketoglutarate, succinate, fumarate, malate, oxaloacetate, pyruvate, glutamate, and serine. The addition of nicotinamide adenine dinucleotide[+] was necessary to elicit the maximal rate of oxygen uptake with L-glutamate as substrate, but was unnecessary when other substrates were employed. It was concluded that the Krebs cycle of intermediary carbohydrate metabolism probably operates within *C. burnetii*, and that pyruvate is the chief energy source.

The existence of an independent metabolic capacity in rickettsiae was demonstrated first by Bovarnick and Snyder (1949), who reported that purified suspensions of epidemic and murine typhus took up oxygen if incubated in the presence of glutamate, pyruvate, or succinate. Bovarnick and Miller (1950) showed that α-ketoglutarate also was oxidized, and that typhus rickettsiae possessed the ability to transaminate glutamate. Wisseman et al. (1952) demonstrated oxidative activity by *Rickettsia mooseri* in the presence of all the substrates involved in the Krebs (1948) cycle except the tricarboxylic acids. These data, considered together with the finding of Bovarnick and Miller (1950) that 1 to 2 moles of oxygen were utilized per mole of pyruvate in a reaction with a RQ of 1.3 to 1.4, suggested that the Krebs cycle or something very similar to it operated in *R. mooseri* and *R. prowazekii*.

Price (1953) reported that *R. rickettsii* could utilize glutamate, pyruvate, α-ketoglutarate, succinate, fumarate, malate, and oxaloacetate, and that this rickettsia could synthesize citrate from pyruvate and oxaloacetate. Paretsky et al. (1958) demonstrated that *Coxiella burnetii* could synthesize citrate from acetate or acetylphosphate. Undocumented observations by Ormsbee,

reported by Bell and Philip (1952), indicated that *C. burnetii* could utilize the Krebs cycle intermediate succinate, pyruvate, α-keto-glutarate, and oxaloacetate. The purpose of this communication is to document the preliminary observations, and to present additional data bearing on the Krebs cycle in *C. burnetii*.

MATERIALS AND METHODS

The fifth egg passage of the Ohio 314 (phase I) strain of *C. burnetii* was used. It was grown in the yolk sacs of embryonated eggs which were killed when approximately half of the embryos had died, usually on the 6th day after inoculation. Yolk sacs of live embryos were harvested aseptically, rinsed in cold solution X (0.20 M sucrose, 0.1 M KCl, 0.02 M potassium phosphate buffer, pH 7.25), and ground in a Waring Blendor for 1 min in enough cold solution X to yield a 20% (w/v) yolk-sac suspension. All purification procedures were performed at 4 to 6 C unless otherwise specified. The suspension was centrifuged at 2,200 × g for 60 min or at 20,000 × g for 20 min, and the supernatant fluid was discarded. The pellet was resuspended in solution X to give a volume equivalent to 50% (w/v) suspension of original yolk sac. Dry diethylaminoethyl (DEAE) cellulose (Peterson and Sober, 1956) was added in a concentration of 1 g per 50 g of yolk sac, thoroughly mixed, and then centrifuged at 650 × g for 5 min. The supernatant fluid was treated with Trypsin (Difco) in 0.5% concentration for 20 min at 37 C, and was then centrifuged at 20,000 × g for 20 min. The pellet of rickettsiae was resuspended in solution X containing 6% bovine albumin (fraction V; Armour and Co.), and was allowed to stand for 5 min. The suspension was then centrifuged at 650 × g for 5 min, and the sediment was discarded. The supernatant fluid was centrifuged at 20,000 × g for 20 min; the pellet of rickettsiae was resuspended in solution X, mixed with 1 g of Celite (Johns-Manville, New York, N.Y.) per 20 g of

original yolk sac, and was then centrifuged at 650 × g for 5 min; the supernatant fluid was harvested. The rickettsial suspension was then centrifuged a final time at 20,000 × g for 20 min, and the resulting supernatant fluid was discarded. The pellet of *C. burnetii* was resuspended to the desired concentration. This final preparation was a dense, white suspension of *C. burnetii*. Contaminating material could not be detected in these suspensions by direct microscopic examination, by the sucrose density-gradient technique (Ribi and Hoyer, 1960), by complement-fixation (CF) tests with antiyolk-sac serum, or by the formation of a visible precipitate upon the addition to the suspension of antiserum containing antibodies to normal yolk-sac tissue (Karp, 1954). The yield, which was estimated turbidimetrically (Ormsbee, 1964), was 200 to 600 μg of rickettsial cells per gram of yolk sac. The final volume of suspension was governed by the amount of starting material, but usually was such that the final concentration of purified organisms was 3 to 10 mg/ml.

The choice of diluent for initial suspension of infected yolk sacs depended upon whether the material was to be processed without delay, in which case solution X was employed, or whether the material was to be shell-frozen and stored at −60 C, in which case sucrose-phosphate-glutamate solution (SPG) (Bovarnick, Miller, and Snyder, 1950) was used.

Total nitrogen determinations were made by the usual micro-Kjeldahl technique, followed by nesslerization and absorption measurement at 540 mμ. Total protein determinations were done by the method of Lowry et al. (1951) and CF titrations were done by the method of Welsh, Jensen, and Lennette (1959). A complement-fixing unit (CFU) was defined as the amount of antigen necessary to fix 2 full units of complement in the presence of 8 units of antiserum. Because 0.2 ml of antigen per tube was used in the titrations, the number of CFU per milliliter was calculated by multiplying by 5 the highest dilution of antigen giving complete fixation.

Infectious suspensions were titrated in female guinea pigs which weighed 300 to 450 g. The animals in groups of six were given 1-ml intraperitoneal injections of decimal dilutions of the infectious suspension, held for 30 days, and bled by intracardiac puncture. Infectious end points based on the presence of specific CF antibodies

in the sera were calculated by the method of Reed and Muench (1938). Standard errors (SE) were estimated by the method of Pizzi (1950).

Oxygen uptake measurements were made in a conventional Warburg apparatus with 15-ml flasks, in air, at 37 C and with a total fluid volume of 3.0 ml, of which 0.2 ml was 20% KOH in the center well. Compounds employed as substrates and accessory factors were of highest purity available. In all cases, substrates and buffers were used as the potassium salts, and suspensions and solutions were made up in solution X. The reaction mixtures of 2.8 ml contained 1 ml of rickettsial suspension, 7.0 mM substrate and, when used, accessory factors in the following amounts: cytochrome *c* (horse heart), 0.1 mM; nicotinamide adenine dinulceotide (NAD), 1.5 × 10^{-3} mM; $MgCl_2$, 1.2 × 10^{-2} mM; and $MnCl_2$, 1.2 × 10^{-3} mM.

Experiments with C^{14}-labeled compounds were performed in Warburg flasks under the conditions specified above. Approximately 4.5 mg of rickettsiae per flask were used, and were allowed to metabolize for 1 hr. $C^{14}O_2$ was trapped in KOH, converted to $BaC^{14}O_3$, and counted as an infinitely thick layer with an end-window Geiger tube having an efficiency of 3.1%.

RESULTS

Analytical data on representative purified suspensions of live *C. burnetii* are presented in Table 1. These data indicate an average nitrogen content of 8.5%, which is somewhat lower than the value of 10% found previously for formalin-killed organisms purified by a high salt-ether method (Ormsbee, 1962). Total protein averaged 17.5% of the total dry weight of rickettsiae, although this is only an approximate value, because no absolute protein standard for rickettsial protein exists. The low protein value suggests that a significant amount of the nitrogen present is incorporated in compounds other than protein, or that the protein is deficient in aromatic amino acids which give high color values in the Lowry et al. (1951) method, and which are present in relatively high concentration in bovine serum albumin which was used as the reference standard. The CF titers of the various suspensions were consistent within the usual limits of the test, and gave an average value of 217 CFU per milligram (dry weight) as calculated from turbidity measurements. The constancy of the

various ratios which may be calculated from the data in Table 1 suggests that the different preparations were of fairly consistent composition.

Results of manometric studies with substrates presumably involved in the Krebs (1948) cycle are presented in Table 2. *C. burnetii* is apparently not very active metabolically; consequently, oxygen uptakes were low in comparison to values ordinarily obtained with bacteria or yeast. The values for *C. burneti* are of the same order of magnitude as those which were found for epidemic and murine typhus organisms, however (Bovarnick and Snyder, 1949; Bovarnick and Miller, 1950; Wisseman et al., 1952). Table 2 presents the results of experiments in which substrates were tested under conditions which permitted the effect of accessory factors to be observed. The data represent averages of four determinations each, and are representative of many previous experiments. Endogenous oxygen uptake varied from 1 to 10 μliters of O_2 per hour per milligram of nitrogen in various suspensions of purified *C. burnetii*.

In the absence of supplementary factors, oxygen uptake significantly greater than the endogenous rate was obtained only by the addition of succinate, α-ketoglutarate, fumarate, malate, oxaloacetate, and pyruvate. The fumarate and malate values are of borderline significance in terms of the accuracy of manometric determinations. However, confirmatory experiments with the corresponding C^{14}-labeled compounds demonstrated the formation of $C^{14}O_2$ from them, and thus conclusively confirmed the oxidation of

TABLE 1. *Analytical and serological data on representative purified suspensions of living Coxiella burnetii*

Suspension no.	Weight* of rickettsiae	Total nitrogen	Total protein	CFU†per ml
	mg/ml	*mg/ml*	*mg/ml*	
1	3.8	0.28	0.55	800
2	5.5	0.48	0.92	1,120
3	8.7	0.74	1.40	2,880
4	8.3	0.68	1.60	1,440
5	6.2	0.49	1.16	720
6	6.1	0.58	1.22	1,080
7	3.0	0.29	0.53	1,080
Avg	6.0	0.51	1.05	1,300

* Estimated turbidometrically at 420 mμ in a Klett colorimeter (Ormsbee, 1964).

† Complement-fixing unit.

TABLE 2. *Oxygen uptake of suspensions of Coxiella burnetii in the presence of Krebs cycle intermediates and other compounds as substrates*

Substrate*	Substrate alone	NAD, Mn^{++} + Mg^{++} added†	Cytochrome c + NAD + Mn^{++} + Mg^{++} added
Citrate	0	0	0
Cis-aconitate	0	0	0
D,L-*iso*-citrate	0	0	0
α-Ketoglutarate	5	4	?
Succinate	10	8	10
Fumarate	3	6	2
Malate	3	4	6
Oxaloacetate	18	16	18
Pyruvate	35	35	34
Aspartate	0	0	2
L-Glutamate	1	8	8
L-Serine	12	11	15
L-Glutamine	1	4	4

* All substrates added as potassium salts.

† All values listed are net figures from which endogenous values have been subtracted. NAD = nicotinamide adenine dinucleotide. Results expressed as microliters of O_2 per hour per milligram of nitrogen.

these substrates by *C. burnetii* (Table 3). The manometric data are unquestionably valid.

Addition of NAD, Mg^{++}, and Mn^{++} in the absence of cytochrome c resulted in no increase in activity with any of the Krebs cycle substrates. Further addition of cytochrome c resulted in a small and probably insignificant increase in oxygen uptake in the presence of malate.

The rate of oxygen uptake by suspensions of *C. burnetii* was stimulated also by addition of L-glutamate, L-serine, and L-glutamine, but not by L-aspartate (Table 2). Again, the effect of supplementary factors was of doubtful significance, except in the case of glutamate in which the addition of NAD clearly was stimulatory. Other compounds which did not cause oxygen uptake when added to suspensions of *C. burnetii* included lactate, glucose, glucose-6-phosphate, glycerol, asparagine, and ethyl alcohol.

One unexpected result was obtained when α-ketoglutarate was tested as a substrate. Initial tests with suspensions of *C. burnetii* gave oxygen uptakes of 65 to 131 μliters of O_2 per hour per milligram of nitrogen of suspension when cyto-

TABLE 3. *Formation of $C^{14}O_2$ by Coxiella burnetii from malate, fumarate, and α-ketoglutarate labeled with carbon 14*

Substrate	Amount added		Recovered as $C^{14}O_2$	
	μM	Total counts per min	μM	Total counts per min
1-Malate-C^{14}-Ul	4.44×10^{-1}	522,000	1.21×10^{-1}	144,000
Fumarate-1,4-C^{14}.	1.11×10^{-1}	82,000	5.61×10^{-2}	41,000
α-Ketoglutarate-5-C^{14}.	1.20×10^{-1}	96,000	1.55×10^{-2}	12,000

chrome c, NAD, Mn^{++}, and Mg^{++} all were present, whereas the absence of cytochrome c or Mn^{++} and Mg^{++} gave values of 4 to 5 μliters of O_2 per hour per milligram of nitrogen. It was then discovered that almost identical results could be obtained in the absence of *C. burnetii*, but in the presence of α-ketoglutarate and Mn^{++} only. A similar finding was reported by Kalnitsky (1953), who determined that 1 mole of O_2 was taken up, and approximately 0.7 mole of CO_2 was liberated, per mole of α-ketoglutarate utilized. However, he reported that only small amounts of succinate, equivalent to 9 to 19% of the α-ketoglutarate, were formed as a result of this nonenzymatic oxidation. This effect made it possible to test the effect of these accessory factors together on the oxygen uptake of *C. burnetii* with α-ketoglutarate as substrate. It should be mentioned that endogenous activity of purified suspensions of *C. burnetii* varied from 1 to 10 μliters per hour per milligram of nitrogen.

The effect of freezing and thawing on the metabolic activity of *C. burnetii* was examined in experiments in which succinate served as substrate. A suspension of purified *C. burnetii* in solution X was prepared from freshly harvested infected yolk sacs. One portion of the suspension was held at 4 C, and a second portion was shell-frozen in an alcohol-Dry Ice bath, and was then quickly thawed in a 37 C water bath. After a sample was removed for testing, the remainder was frozen and thawed twice more, but much more slowly than before. Metabolic rates of the three suspensions were then compared in a Warburg apparatus with succinate as substrate. The rates of oxygen uptake with the three suspensions were indistinguishable. This finding differs from that of Bobarnick and Snyder (1949), who found that freezing and thawing increased oxygen uptake of suspensions of typhus rickett-

siae in the presence of succinate, presumably by increasing cell permeability.

The possibility that the purification process itself might have damaged the rickettsiae sufficiently to reduce their infectiousness was examined. Purified suspensions of *C. burnetii* were titrated in guinea pigs, and the ID_{50} points were compared with those obtained by similar titrations of samples of the infected yolk sacs which had been frozen in SPG and from which the purified suspensions had been derived. The purified preparations were made from freshly harvested yolk sacs, as well as from yolk sacs which had been shell-frozen and stored at −60 C as a slurry in SPG.

Guinea pigs in groups of six were given intraperitoneal inoculations of 1 ml each of decimal dilutions in solution X of the preparations under test. The animals were held for 30 days, and were bled by intracardiac puncture. The infectious end points were calculated on the basis of serological conversion as measured by CF and agglutination-resuspension (AR) (Ormsbee, 1964) tests. In all cases, the calculations were based on straight yolk sac, and thus the ID_{50} values are directly comparable.

With infected material which had been frozen and held at −60 C before titration in guinea pigs, the ID_{50} of the initial infectious material, by CF test of the sera, was $10^{11.00} \pm$ SE 0.42. The corresponding ID_{50} value for the purified preparation was $10^{10.43} \pm$ SE 0.47. By AR test, the ID_{50} of the crude yolk sac was $10^{11.67} \pm$ SE 0.47, and that of the purified material was $10^{12.00} \pm$ SE 0.47. Similar results were obtained in the second set of titrations, with a different purified preparation made from another harvest of infected yolk sacs. It was concluded from the foregoing data that no detectable loss of infectiousness had occurred during purification, and

that the critical leaching out of NAD which Bovarnick and Allen (1957) had demonstrated for typhus rickettsiae had not occurred in *C. burnetii*.

DISCUSSION

Fundamental to any discussion of the results is an estimate of the purity of the suspensions of *C. burnetii* used to obtain those results. The rickettsial suspensions employed in this study were examined microscopically, serologically, by density-gradient, and by analyses for protein and total nitrogen. Suspensions were subjected to combinations of different purification steps, and were then examined for metabolic activity. Normal yolk-sac preparations were subjected to the same purification procedures, and were then tested for metabolic activity. The conclusion was reached that the purified suspensions of *C. burnetii* contained small amounts of impurities, but that the enzymatic capabilities of these impurities was negligible and did not significantly influence the results obtained.

Oxygen uptake by *C. burnetii* occurred in response to addition of the postulated substrates of the Krebs cycle except for citrate, *cis*-aconitate, and *iso*-citrate (Table 2). This metabolic activity appeared to be independent of added supplementary factors known to be necessary in at least some of these reactions. These findings strongly suggest the presence of an operative Krebs cycle in this organism, and thus further document the idea that this organism is similar in its enzymatic mechanisms to *R. prowazekii*, *R. mooseri*, and *R. rickettsii* (Bovarnick and Snyder, 1949; Bovarnick and Miller, 1950; Wisseman et al., 1952; Price, 1953), as well as to most bacteria (Delwiche, 1955), rather than to the larger viruses.

Although the substrates of the Krebs cycle which are utilized by *C. burnetii* are the same ones which were found to stimulate respiration of typhus and spotted fever rickettsiae, there are obvious quantitative differences between these organisms and *C. burnetii*. For example, from the data on *R. mooseri* reported by Wisseman et al. (1952), the substrate causing the greatest oxygen uptake was glutamate; succinate gave about one-third that of glutamate, and α-ketoglutarate and pyruvate about one-tenth. This is markedly different from the results for *C. burnetii* shown in Table 2. With *C. burnetii*, the greatest activity was elicited by pyruvate, followed by oxaloace-

tate and succinate. The rate of oxygen uptake elicited by glutamate is about equal to that caused by succinate, and is about one-quarter of the activity resulting from the use of pyruvate as substrate. Thus, although it appears that glutamate may serve as the most important energy source for *R. mooseri* and *R. prowazekii*, pyruvate appears to be most important for *C. burnetii*. On the other hand, the rate of oxidation of a substrate and its actual use as an energy source by a cell are not necessarily synonymous.

The rates of oxidation observed may well have been influenced by permeability factors and, without much more extensive data, can only be said to indicate the presence of specific enzymes and, therefore, the potential of *C. burnetii* cells to oxidize certain substrates. The demonstration by Paretsky et al. (1958) that citrate can be synthesized by *C. burnetii* from acetate or acetyl-coenzyme A in the presence of oxaloacetate suggests that this area of the Krebs cycle also is operative in *C. burnetii* and, as Bovarnick and Miller (1950) suggested in the case of typhus rickettsiae, the lack of response to addition of the tricarboxylic acids of the citrate group is due simply to impermeability of *C. burnetii* to them. Thus, we were not able to detect any oxygen uptake due to oxidation of any of the tricarboxylic acids, even in the presence of NAD, nicotinamide adenine dinucleotide phosphate, coenzyme A, or adenosine triphosphate as supplementary factors, although Consigli and Paretsky (1962) demonstrated the oxidation of *iso*-citrate by cell-free extracts of *C. burnetii*.

Added weight is lent to the possibility that cell permeability may be an important factor in assessing the metabolic capabilities of *C. burnetii* by the findings with glucose-6-phosphate. We found that the addition of this compound to a suspension of *C. burnetii* does not stimulate oxygen uptake, whereas Consigli and Paretsky (1962) showed that cell-free extracts of this rickettsia will oxidize it. These differing data are most reasonably explained by the existence of a cell membrane which, in the intact organism, prevents the entry of glucose-6-phosphate. If this explanation is true, it raises some doubt about the importance of glucose as a major energy source for *C. burnetii*, because unphosphorylated glucose was shown also not to be metabolized by this rickettsia (Ormsbee and Weiss, 1963).

Mammalian cytochrome c, which will function efficiently as an electron transporter in the presence of normal yolk-sac tissue and succinate as substrate, had no detectable stimulatory effect. Hayes et al. (1957) found spectroscopic evidence for cytochromes a or a_1 and b (but not for c) in purified suspensions of R. mooseri. Because cytochrome c is present in normal yolk-sac tissue, and is the most easily soluble of the cytochromes, it was thought that it might play a role in electron transport in rickettsiae dwelling within yolk-sac cells. No evidence for this hypothesis could be adduced.

ACKNOWLEDGMENTS

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