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The Metabolism of Brucellae: The Oxidation of Succinate as a Function of pH and Concentration[1]

R. E. MacDonald, A. L. Erlandson, Jr. and Philipp Gerhardt

From the Department of Bacteriology, University of Michigan, Ann Arbor, Michigan

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Introduction

A markedly disparate increase in the rate of uptake over the rate of oxidation of succinate in *Brucella abortus* results when the concentration of hydrogen ions or the substrate is grossly increased. The relation of this and other findings to possible alteration of the permeability of the cells has been considered in another paper (1). The present experiments are concerned specifically with the metabolic fate of the excess succinate that is consumed with these environmental changes and generally with the terminal oxidative mechanisms in this organism.

Methods

Unless otherwise stated, the experimental methods were generally the same as those described previously (1, 2). Smooth *Brucella abortus*, strain 19, again was employed.

Rates were expressed as the number of micromoles of reactant exchanged per milligram of cellular nitrogen per hour in an air atmosphere, abbreviated as $MQ_{O_2}(N)$ and $MQ_s(N)$ for oxygen and substrate, respectively. The endogenous values were subtracted.

For analyses, reactions usually were stopped by immersing the Warburg flask in boiling water for 10 min. The reactants were estimated by the following analyses: nitrogen by nesslerization (3); succinate by succinoxidase from pig heart (3); L-glutamate by decarboxylase from *Escherichia coli* (4); pyruvate (5) and total alanine (6) by colorimetry; and other amino and organic acids by conventional paper chromatography.

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The isotopic data were obtained with either a flowing-gas or a thin end-window Geiger-Müller counter and the usual scaler circuit. The use of samples having minimal thickness (<0.3 mg./sq. cm.) made correction for self-absorption unnecessary. The samples were prepared by micropipetting 0.1-ml. aliquots to duplicate planchets. For each sample, 12,800 (Fig. 1) or 6400 (Table II) counts were timed.

The internal accumulation ("pooling") of acids was determined essentially by the procedures of Gale (7). A suspension of the cells was exposed as usual to the substrate for 4 hr. After washing three times, approximately 2 nitrogen-mg. of cells was analyzed for substrate adsorbed or in the medium. An equal portion of cells was disintegrated, either by boiling for 10 min. or by triturating with alumina, and then analyzed. The difference in analyses was taken as pooled substrate. Both washings and analyses for glutamate were made with pH 4.5, 0.2 *M* acetate; for pyruvate with pH 6.8, 0.067 *M* phosphate; and for succinate with pH 7.4, 0.067 *M* phosphate. Since the intact cells reacted with the succinoxidase preparation, it was impossible to determine the amount of adsorbed succinate; however, this and consequently the pool had to be the same or less than the minimal 0.6 μmole of succinate/mg. cellular nitrogen found after disruption of the cells.

Results

Accumulation of Intermediates

It seemed most likely that the greatly increased uptake of succinate would result in its partial oxidation and the release of accumulated intermediates into the external environment. The possibility of such a shunt mechanism was approached by a dimensionless comparison of the rate of uptake of succinate-2-C^{14} as determined by isotopic and by succinoxidase analyses. The accumulation with time of intermediates that contained the 2-C^{14} label was indicated by the divergency between the two analyses when plotted on this directly comparable basis. Figure 1 gives these data together with $MQ_{O_2}(N)$ and $MQ_s(N)$ values when the pH was lowered to 5.5 from the reference of 6.8, the concentration of substrate increased to 0.033 from 0.0033 *M*, and the two adjustments made in combination. These levels were selected from previous knowledge (1). The data suggested that accumulation of the intermediates occurs only slightly in the reference conditions (pH 6.8, 0.0033 *M*), more rapidly but with subsequent depletion at lowered pH at the same concentration, and much more rapidly at increased substrate concentration.

The biosynthesis of alanine and pyruvate in brucellae has been demonstrated by other workers (8–10). Accordingly, a direct time study of pyruvate formation was made. These data, shown in Fig. 2, confirmed the isotopic analysis at pH 5.5 and 0.0033 *M* succinate.

Carbon balances then were obtained by reactant and product analyses

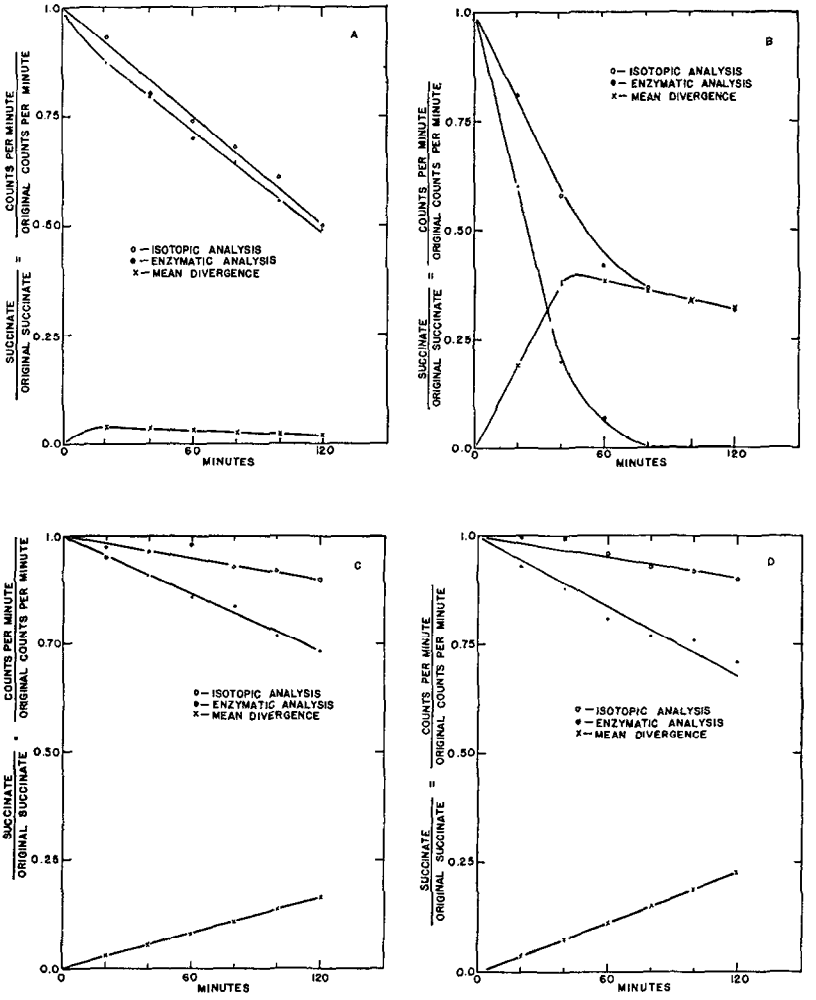

FIG. 1. Dimensionless comparisons of the rates of uptake of succinate-2-C^{14} as determined by isotopic and enzymatic analyses. The substrate disappearance ordinates in *C* and *D* are fractions of an initial succinate concentration which was tenfold greater than that in *A* and *B*, and therefore the slopes of the plots of *C* and *D* require correction to a correspondingly greater value.

A. 0.0033 *M*, pH 6.8. $MQ_{O_2}(N) = 9.20$, $MQ_s(N) = 4.54$.
B. 0.0033 *M*, pH 5.5. $MQ_{O_2}(N) = 16.9$, $MQ_s(N) = 21.8$.
C. 0.033 *M*, pH 6.8. $MQ_{O_2}(N) = 26.9$, $MQ_s(N) = 26.6$.
D. 0.033 *M*, pH 5.5. $MQ_{O_2}(N) = 26.5$, $MQ_s(N) = 34.4$.

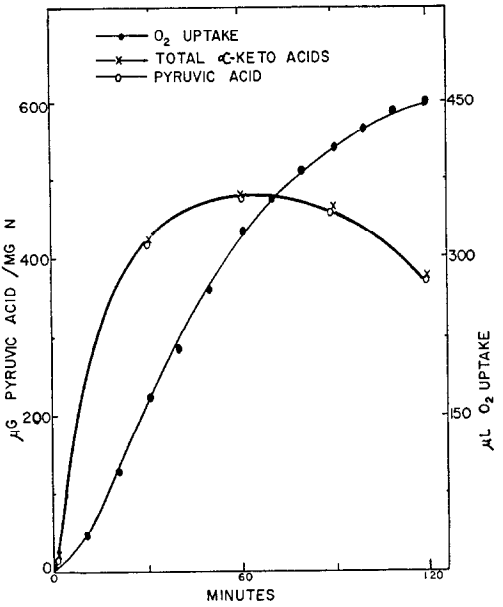

FIG. 2. Accumulation of pyruvate during the oxidation of 0.0033 *M* succinate at pH 5.5.

TABLE I

Stoichiometry of Oxidation of Succinate as a Function of pH and Concentration[a]

Substrate		Terminal μmoles/mg. N				R.Q.	Moles/mole succinate consumed			Terminal % recovery of succinate carbon consumed
pH	Molarity	O_2 consumed	Succinate consumed	Pyruvate produced	CO_2 produced		O_2	CO_2	Pyruvate	
6.8	0.0033	13.4	5.8	0.57	18.1	1.35	2.31	3.12	0.10	85
5.5	0.0033	28.7	11.0	1.69	34.2	1.19	2.61	3.10	0.15	89
6.8	0.033	47.4	29.4	12.90	52.4	1.11	1.61	1.78	0.44	75
5.5	0.033	55.3	52.2	30.10	60.4	1.09	1.06	1.16	0.58	72

[a] Experiment conducted for 120 min. Calculations are based on terminal analyses.

in the four test systems. The data are given in Table I. Pyruvate and carbon dioxide accounted for 72–89 % of the succinate consumed. Direct analyses (Fig. 2) and paper chromatograms failed to reveal the accumulation of other keto acids, amino acids, or citrate. A search for other prod-

ucts is being made. A possible shift in metabolic pathway with the changes in pH was not apparent from the data (Table I), as indicated by the relative constancy of the mole/mole ratios with the change of pH at either of the two concentration levels. The accumulation of pyruvate increasingly with increased substrate uptake suggested that the oxidation of pyruvate might be a secondary limiting reaction in the oxidation of succinate. The maximal oxidative rate for pyruvate was found to be consistent with this viewpoint. Thus, the maximal $MQ_{O_2}(N)$ of 10.3 observed with pyruvate was comparable to the $MQ_{O_2}(N)$ of 11.2 found with succinate under conditions where minimal accumulation of pyruvate occurred. As with succinate (1), the optimal pH for the oxidation of pyruvate became higher with increased concentration (pH 5.1 at 0.0033 *M*, pH 6.2 at 0.033 *M*). Contrary to the findings with strain 11 of *Brucella abortus* (10), relatively little pyruvate and no detectable alanine was formed from L-glutamate by strain 19, with the four conditions of pH and concentration used as above [cf.(1)].

Assimilation

The further possibility existed that the excess succinate was directly assimilated. The optimal concentration of 2,4-dinitrophenol (DNP) that was required for maximal (92% of theory) oxygen consumption with limiting amounts (0.0008 *M*) of succinate was found to be 0.00003 *M*. When this amount of DNP was included in each of the four conditions of pH and concentration, both the rates and mole ratios of oxygen were increased and the rates of succinate uptake were decreased. Percentages of assimilation varying from 0.3 to 13.0% were calculated, depending on the assumptions made of the action of DNP. These data indicated the probable order of magnitude of assimilation prevailing.

The degree of assimilation in the four conditions of pH and concentration of succinate was measured isotopically. The data given in Table II indicated that the amount of assimilation was relatively small and decreased with increasing uptake of succinate-2-C^{14}. The results possibly underestimated the total carbon assimilated, due to the use of incompletely labeled substrate. When applied to the balances of Table I, these data increased the carbon recoveries to 98, 94, 78, and 73%, respectively.

Pooling

It also was questioned whether some succinate might be accumulated intracellularly in a "pool" of free acid; this possibility also was pertinent

TABLE II

Assimilation of Succinate-2-C^{14} as a Function of pH and Concentration[a]

Substrate		Total μmoles succinate		Total observed radioactivity as corrected counts/min.			Mole % C^{14} assimilated of substrate C^{14} consumed	Moles C^{14} assimilated/mole succinate consumed
pH	Molarity	Added	Consumed	Substrate added	Substrate consumed	Cells		
6.8	0.0033	10.0	6.83	42,000	28,650	3,775	13.2	0.528
5.5	0.0033	10.0	10.0	38,600	38,600	2,125	5.51	0.220
6.8	0.033	100.0	42.8	420,000	180,000	5,335	2.97	0.119
5.5	0.033	100.0	33.6	386,000	129,700	1,348	1.04	0.042

[a] Experiment conducted for 120 min. Calculations are based on terminal analyses.

to the uptake of L-glutamate and to the formation of pyruvate from succinate oxidation. Moreover, in the methodology of the preceding kinetic studies, the vessels were heated to stop the reactions; if free substrate were contained in the internal environment of the cell, it would be liberated by the heating and contribute erroneously to the determinations of substrate disappearance. The experiments failed to show measurable pooling of added succinate or glutamate, or of formed pyruvate; that is, the small residues found with whole and alumina- or heat-disrupted washed cells were essentially identical.

Discussion

The sharp rise in the rate of oxidation of succinate by *Brucella* with changes of pH and concentration has been found to be accompanied by an even greater degree of increase in the rate of succinate uptake, probably a permeability effect primarily associated with the concentration of undissociated molecules (1). The large dissimilarity of increases in the substrate uptake and oxidation here was shown to be accounted for mostly in an increased accumulation of pyruvate. This, together with the production of carbon dioxide and assimilation, represented most of the succinate carbon consumed. At higher concentrations of succinate, approximately 20–30% of the substrate carbon remained undetected, probably as an unknown intermediate. The general pattern was that, as the substrate uptake increased with the environmental changes, intermediates accumulated to an even greater degree; or, the more substrate

available, the less efficient its oxidation. This not unusual biological economy of energy was reversed in the assimilatory process.

Both the isotopic comparisons and the pyruvate analyses with time revealed maximal accumulation and subsequent depletion of pyruvate well before the break in the rate of oxygen consumption for succinate. The slower oxidation of pyruvate concomitant with that of succinate was confirmed by rate comparisons. This, together with the observed decrease in oxidation rate with decreased concentration of substrate, probably accounts for the nonlinear curves usually observed with succinate and similar substrates. Consistent with earlier findings that transaminases of strain 19 are inactive aerobically (2, 8) but inconsistent with the positive observations (10) with strain 11, we did not find measurable amounts of alanine or other amino acids and observed very little formation of pyruvate aerobically from L-glutamate. Our finding of assimilation of succinate also differs from the negative results that have been reported with strain 11 and glutamate (10); however the isotopic method is considerably more sensitive at the low degree of assimilation prevailing, and there may be strain or substrate differences. The pooling of amino acids has not been found in gram-negative bacteria in general (11) nor here. Our failure to observe pooling of succinate or pyruvate differs from the recent report (12) of such acids in two gram-negative bacteria; however, our procedures would not likely detect the very low levels so reported. There remains also the possibility of a diffusible pool, lost in washing.

These [cf.(1)] and other studies, notably those of Altenbern *et al.* (8, 9) and of Marr *et al.* (10), permit the formulation of a provisional model of the metabolism of succinate by *Brucella abortus:* Succinate is oxidized via the tricarboxylic acid cycle, from which a shunt favors the accumulation of pyruvate or derivatives of pyruvate. When an increased concentration of succinate occurs in the cell, more pyruvate or a derivative in turn accumulates and is released into the surrounding medium. The increased concentration of succinate within the cell, however, is primarily dependent on the penetration of the undissociated molecule through the cell membranes, which process therefore varies with pH and concentration. The penetration of succinate in turn is rate-limiting on the oxidative steps preceding the formation of pyruvate or its derivatives.

SUMMARY

The marked difference between the rates of succinate uptake and oxidation by *Brucella abortus* that occurs when the concentration of hydro-

gen ions or the substrate is grossly increased was accounted for predominantly in greater accumulation of pyruvate with greater uptake of the substrate. Lesser amounts of other intermediates probably occur. Succinate-2-C^{14} was found to be assimilated at low levels but decreasingly with increased uptake of the substrate. Pyruvate, succinate, or glutamate was not found to be concentrated internally by the organism.

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