

## The Metabolism of *Brucellae*: The Nature of the Effects of pH and Concentration on the Rate of Oxidation of Succinate<sup>1</sup>

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### INTRODUCTION

A dramatic increase in the rate of oxidation of an organic acid by bacterial cells often occurs when the concentration of hydrogen ions or of the substrate is grossly increased. As an explanation, the effect may be due to a permeability response at the cell surface or, alternatively, to an enzymatic change internally. The present experiments were designed to distinguish between these possibilities in the oxidation of succinate by *Brucella abortus*, in which the rate of the reaction is markedly altered with adjustment of pH and substrate concentration, and to analyze the effect in terms of substrate ionization.

For want of direct methods of measuring the penetration process in whole cells, a comparison of the relative changes in the rates of oxygen and substrate uptake with adjustment of pH and concentration first was made. The effect of pH and concentration changes on oxidation, uptake, and intermediate accumulation with succinate as compared to a reported metabolic precursor, glutamate, gave substantiating information. Further investigation of the two variables revealed an interdependency and a possible basis of their action. Finally, the changes that accompanied physical disruption of the cells were studied. The results generally favored the explanation that the test variables exert an external influence on the dissociation of succinate, upon which utilization by the cell is primarily but not exclusively dependent.

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## METHODS

Smooth *Brucella abortus*, strain 19, was grown on glucose-tryptose agar for 24 hr., harvested, and washed with 0.67 *M* potassium phosphate-saline at pH 6.8, and finally diluted to a concentration of approximately 0.4 mg. cellular nitrogen/ml. Adjustment of the cell suspensions to other pH levels was made just before use by addition of either 4 *N* KOH or concentrated  $H_3PO_4$ ; in doing this, care must be taken to add the reagents dropwise and with rapid stirring as the enzyme activity is rapidly lost with abrupt change of pH. Substrates were dissolved in the buffer, and the pH was altered as above.

Enzyme preparations were made by freezing-thawing and by sonic disintegration, essentially as described previously (1). In the latter method, the succinoxidase was separated from the cells by centrifugation; this could be accomplished best when 0.3 *M* sucrose was used as a diluent. Grinding with glass beads (2) also gave active preparations, although the method was impractical from the standpoint of safety.

The manometric procedures have been described before (3). For analyses, reactions were stopped by immersing the Warburg flask in boiling water for 10 min. Rates were expressed as the number of microliters ( $\mu$ l.), or micromoles ( $\mu$ mole) of reactant used or produced/mg. cellular nitrogen/hr. in an air atmosphere, abbreviated as  $Q_{O_2}(N)$  or  $MQ_{O_2}(N)$  and  $MQ_s(N)$  for oxygen and substrate, respectively. The endogenous values were subtracted.

The reactants were estimated by the following analyses: nitrogen by nesslerization (4); succinate by succinoxidase from pig heart (4); L-glutamate by decarboxylase from *Escherichia coli* (5); and pyruvate (6) and total alanine (7) by colorimetry.

## RESULTS

If limited permeability to succinate accounts for the apparent inability of the cells to oxidize it (i.e., if the penetration process is rate limiting, the metabolic pathway remaining constant) then it follows that, when the rate of oxidation is increased by an environmental variable, there should occur concomitantly an equal or greater degree of increase in the rate of uptake of substrate. This hypothesis was tested first by following concurrently  $MQ_{O_2}(N)$  and  $MQ_s(N)$ , with the pH lowered from the reference of 6.8 to 5.5, the concentration of substrate increased from the reference of 0.0033 to 0.033 *M*, and the two adjustments made in combination. These adjusted levels were consistent with previous comparisons (1) and subsequently were found to approximate optima for the variables; pH 6.8 approximates the optimum for growth of the organism. The results are included in Table I. A considerably greater increase in  $MQ_s(N)$  than in  $MQ_{O_2}(N)$  was found in every case, when compared to the reference conditions, supporting the premise of limiting permeability. For example, the change from pH 6.8 to 5.5 at

TABLE I  
*Uptake, Oxidation and Pyruvate Formation as a Function of  
 pH and Concentration of Succinate and L-Glutamate*

[Substrate]			Observed rate		Moles O <sub>2</sub> /mole substrate consumed	Terminal $\mu$ mole/mg. N <sup>c</sup>	
Compound	pH	Molarity <sup>b</sup>	$MQ_{O_2}(N)$	$MQ_s(N)$		O <sub>2</sub> consumed	Pyruvate produced
Succinate <sup>a</sup>	6.8	0.0033	11.2	5.4	2.07	25.01	0.58
	5.5	0.0033	22.3	20.4	1.09	38.56	3.93
	6.8	0.033	32.4	31.4	1.03	56.00	28.45
	5.5	0.033	31.2	32.2	0.97	52.80	35.31
L-Glutamate	6.8	0.0033	22.5	10.0	2.25	32.7	0.84
	5.5	0.0033	22.3	9.98	2.24	29.5	0.59
	6.8	0.033	22.3	9.98	2.24	35.8	0.93
	5.5	0.033	24.4	10.7	2.28	30.6	0.73

<sup>a</sup> Rate data are the means of three comparable experiments.

<sup>b</sup> Final molarity in 3.0 ml. of total flask contents, or 10 and 100  $\mu$ mole, respectively.

<sup>c</sup> Experiments were conducted for 100 min. No alanine was detected in any of the samples; the analysis procedure used was insensitive below approximately 0.3  $\mu$ mole.

0.0033 *M* succinate resulted in an increase in  $MQ_s(N)$  of 278% and in  $MQ_{O_2}(N)$  of 99%.

This disparity of change between  $MQ_s(N)$  and  $MQ_{O_2}(N)$  with increasing concentration of hydrogen ions or the substrate was further investigated in other experiments (8) and was found to be accounted for predominantly in greater accumulation of pyruvate with greater uptake of succinate. The respiratory quotients and the product-substrate ratios remained relatively constant, further indicating that the effect of the variables was not explainable by a shift in metabolic pathway.

The oxidation of L-glutamate through succinate with the accumulation of pyruvate and alanine (9), the relative constancy of  $MQ_{O_2}(N)$  of glutamate with similar pH and concentration changes (1), and the marked increase of pyruvate accumulation from succinate oxidation when the substrate uptake was increased by lowered pH (8) suggested a comparison between glutamate and succinate in the effect of pH on

$MQ_{O_2}(N)$ ,  $MQ_s(N)$ , and the accumulation of intermediates with these substrates. If the pH change primarily affects the oxidation of succinate internally, then there should result comparable changes in the accumulation of the common intermediates. However, as shown by the data in Table I, the degree of change of product formation from glutamate was small as compared with that from succinate, suggesting an external effect. However, the unpredicted quantitative difference (i.e. tenfold less pyruvate from glutamate) in the presumably common pathways lessened the usefulness of the data for the point in question, although they were of considerable interest metabolically (8). Alanine formation from glutamate (9) was expected to account for this difference, but no alanine was found.

The common influence of the selected pH and concentration changes on the ionic concentration of succinate suggested a further investigation of the two variables together. A family of pH curves obtained with four concentrations of succinate vs. oxidation rate is given in Fig. 1. The optimal pH for succinoxidase activity was found to become strikingly higher with increased concentration. The  $MQ_{O_2}(N)$  at the optima also varied; direct comparisons between curves were made possible by the use of the same cell suspension throughout. A possible nonspecific effect due to variable amounts of potassium and phosphate ions was examined by comparing  $MQ_{O_2}(N)$  values with 0.0033 *M* succinate at pH 6.8 in the presence of 0.13, 0.067, and 0.033 *M* potassium phosphate buffer; no appreciable differences occurred. Moreover, adjustment of the succinic acid substrate to pH 5.5 to 6.8 and then back to 5.5 gave results identical to substrate adjusted only to pH 5.5. The independence of the pH response from a buffer effect was suggested by the similar optima at pH 5.5 for 0.0033 *M* succinate that were obtained with either ortho- or pyrophosphate.

The succinoxidase response of intact *B. abortus* to the substrate concentration was found to differ from the usual enzyme saturation curve in that clear optima occurred. Moreover, these optimal concentrations were found to vary with pH, becoming higher (and the optima more narrow) with increased pH in the range studied. Data showing the magnitude of change in the optimal concentration of total succinate with pH are given in Fig. 2. The concentration vs.  $MQ_{O_2}(N)$  curves, from which the data of Fig. 2 were derived, showed that the  $MQ_{O_2}(N)$  at the concentration optima also varied with pH, although marked reduction occurred only at the extremes of pH.

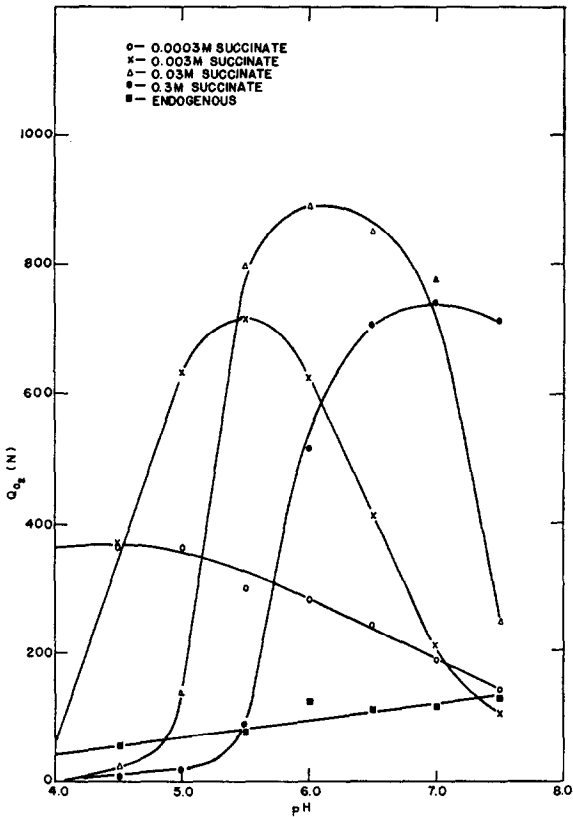


FIG. 1. The rate of oxidation of succinate as a function of pH and concentration.

The data of Fig. 2 include a test of the hypothesis that only the undissociated molecule is active. If so, the optimal concentration of undissociated molecules should remain constant with changes in pH (10). The optimal concentration of undissociated succinic acid did not remain constant over a broad range of pH. However, the comparatively small changes on the acid side of neutrality suggested that in this range the activity was largely dependent on the concentration of undissociated succinic acid in the external medium.

Distinction between an external or internal influence of pH also was attempted by comparing the pH curves obtained with intact *B. abortus* to those obtained with physically disrupted cells. If pH changes pri-

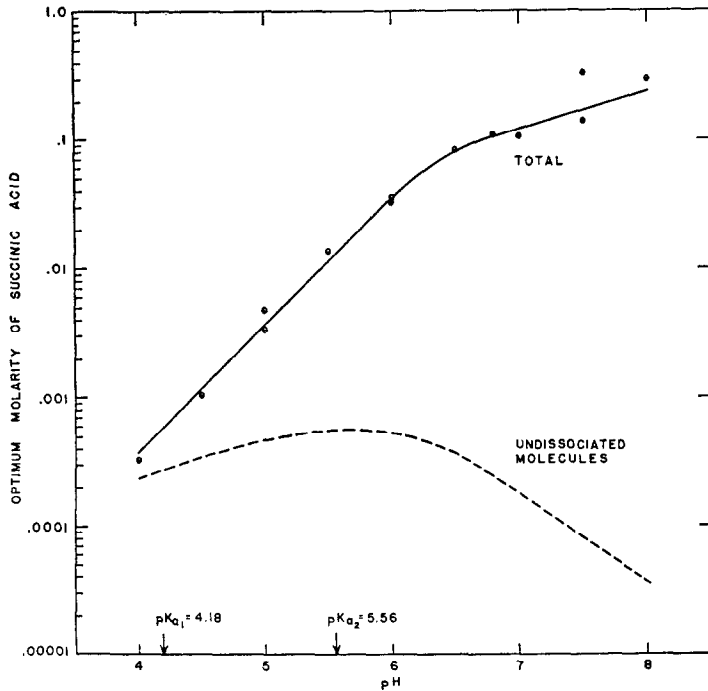


FIG. 2. The effect of pH on the concentration of succinate required for optimal rate of oxidation. The plot of undissociated acid was calculated from the Henderson-Hasselbalch equation using  $pK_{a1}$ .

marily affect the oxidation of succinate extracellularly, it follows that the exposed enzyme system should remain relatively unaffected by such changes. In contrast to the change in pH optima seen with whole cells in varying concentrations of succinate (Fig. 1), optima at pH's 7.2, 7.2, and 7.0 were found for a sonic extract with 0.033 or 0.33 *M* succinate, and for a frozen-thawed preparation with 0.0033 *M* succinate, respectively. Although these results did not permit an analysis as detailed as that for intact cells, a markedly different pH-concentration relationship at the enzyme locus was indicated.

#### DISCUSSION

Several lines of evidence bear on whether the effect of pH and concentration on the oxidation of succinate by *B. abortus* is due to a surface effect or to an internal enzymatic response:

1. Earlier work (1) has indicated that each of a number of methods presumably having a common effect of influencing permeability stimulated the oxidation of succinate.

2. The present experiments showed that the increase in  $MQ_{O_2}(N)$  for succinate with pH and concentration alteration could be correlated with a *greater* degree of increase in  $MQ_s(N)$ .

3. The degree of change of  $MQ_{O_2}(N)$ ,  $MQ_s(N)$ , and pyruvate formation from glutamate, a reported precursor, was minimal in comparison with succinate.

4. An analysis of the combined effect of pH and concentration changes on  $MQ_{O_2}(N)$  for succinate suggested a partial dependency on the concentration of undissociated molecules.

5. Contrary to the findings with intact cells, crude succinoxidase preparations exhibited a pH optimum approximately at neutrality, which remained the same with changes in substrate concentration. These data mutually favor a conclusion that the test variables exert primarily an external influence on succinate utilization. In the sense of being at least partially dependent on the concentration of undissociated succinic acid molecules, permeability appears to be rate limiting. This conclusion is in accord with that of Terui and Mochizuki (11), who studied concentration effects of succinate and acetate on intact and dried yeast.

Effects of pH on the rate of utilization of organic acids have been recorded for a wide variety of tissues and substrates, representative cases of which have been reviewed (10). In the brucellae (12, 1) and in other bacteria (13, 14) this effect of pH has been attributed to permeability. However enzymatic changes internal to the surface membranes frequently have been demonstrated to result from external pH changes, a typical example of which is in the shift in product formation by the lactic acid bacteria (15); such modifications of enzymatic activity might very well account for the pH-concentration effects on respiration rate, so that observation of the effect in itself does not necessarily implicate the surface membranes.

The possible influence of ionization in the activity of weak organic acids on bacteria was recognized early (16). Recently Simon and Beevers (10) have analyzed and constructed a generalized plot of 90 different pH experiments, many in themselves incomplete, and have come to the conclusion that usually "the effect of pH on the degree of dissociation of the acid or base in the external medium is responsible for a large part of the effect of pH on activity, but it cannot be held that only the undis-

sociated molecules are active." The present data are confirmatory with the additional inference that the ionization effects observed here are upon permeability.

#### SUMMARY

The sharp rise in the rate of oxidation of succinate by *Brucella abortus* that occurs with gross increases in hydrogen ion or the substrate concentration was accompanied by an even greater degree of increase in the rate of substrate uptake. The degree of change in these rates and of pyruvate formation with glutamate, a reported precursor of succinate, was minimal in comparison to that with succinate. The optimal pH for oxidation of succinate became strikingly higher with increased concentration of substrate; similarly, concentration optima were observed and they rose with increased pH. An analysis of these data indicated that the activity was largely but not exclusively dependent on the concentration of undissociated molecules. Contrary to these findings with intact cells, crude succinoxidase preparations exhibited a constant pH optimum at neutrality with changes in substrate concentration. The results favored a conclusion that permeability is rate limiting in the oxidation of succinate by the organism.

#### REFERENCES

1. GERHARDT, P., MACGREGOR, D. R., MARR, A. G., OLSEN, C. B., AND WILSON, J. B., *J. Bacteriol.* **65**, 581 (1953).
2. LAMANNA, C., AND MALLETTE, M. F., *J. Bacteriol.* **67**, 503 (1953).
3. GERHARDT, P., LEVINE, H. B., AND WILSON, J. B., *J. Bacteriol.* **60**, 459 (1950).
4. UMBREIT, W. W., BURRIS, R. H., AND STAUFFER, J. P., "Manometric Techniques and Tissue Metabolism." Burgess Publishing Co., Minneapolis, Minn., 1949.
5. UMBREIT, W. W., AND GUNSALUS, I. C., *J. Biol. Chem.*, **159**, 333 (1945).
6. FRIEDEMANN, T. E., AND HAUGEN, G. E., *J. Biol. Chem.* **147**, 415 (1942).
7. WOOD, W. A., AND GUNSALUS, I. C., *J. Biol. Chem.* **190**, 403 (1951).
8. MACDONALD, R. E., ERLANDSON, A. L., JR., AND GERHARDT, P., *Arch. Biochem. and Biophys.* **64**, 382 (1956).
9. MARR, A. G., OLSEN, C. B., UNGER, H. S., AND WILSON, J. B., *J. Bacteriol.* **66**, 606 (1953).
10. SIMON, E. W., AND BEEVERS, H., *New Phytologist* **51**, 163 (1952).
11. TERUI, G., AND MOCHIZUKI, T., *Tech. Repts. Osaka Univ.* **3**, 397 (1953).
12. ALTENBERN, R. A., AND HOUSEWRIGHT, R. D., *Arch. Biochem. and Biophys.* **36**, 345 (1952).
13. BARRON, E. S. G., *Arch. Biochem.* **29**, 130 (1950).
14. GANGULY, S., AND ROY, S. C., *Arch. Biochem. and Biophys.* **59**, 45 (1955).
15. GUNSALUS, I. C., AND NIVEN, C. F., *J. Biol. Chem.* **145**, 131 (1942).
16. VAN DAM, W., *Biochem. Z.* **87**, 107 (1918).