THE METABOLISM OF BRUCELLAE: THE ROLE OF CELLULAR PERMEABILITY[1]

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Intact cells of *Brucella abortus* show inconsistencies in their specificity for the oxidation of amino acids, their comparative rates of oxidation of related organic acids, and their lack of response to certain competitive inhibitors (Gerhardt *et al.*, 1950). For example, glutamate and asparagine are oxidized rapidly whereas glutamine and aspartate are not; compounds such as α-ketoglutarate and succinate that may be predicted as intermediates in the dissimilation of glutamate are oxidized at markedly lower rates; and malonate fails to inhibit the oxidation of succinate. Each can be explained logically in the failure of a reactant to penetrate into the cell. This premise was tested by a number of indirect methods having a common effect of influencing cellular permeability; taken together, they not only reasonably proved permeability as a limiting factor in the test reactions but also provided experimental methods for making the barrier ineffective.

To these ends, the following techniques were compared for representative critical reactions: adjustment of pH and substrate concentration in the reaction mixture; esterification of reactants; chemical treatment of cells with acetone, toluene, and *trans*-1,2-cyclopentanedicarboxylic acid; and physical disruption of the cell wall by freezing-and-thawing, sonic radiation, and alumina trituration.

EXPERIMENTAL METHODS

Conventional manometric techniques were used. With the modifications noted below, the experimental methods were generally the same as those described for previous work (Gerhardt *et al.*, 1950).

Strains 19 and 11 of *B. abortus* usually were employed; both are of a relatively low order of virulence for the guinea pig, and they are closely related metabolically. In some cases, other strains also were used. Precautions were taken to maintain the cultures of strains 19, 11, and 2308 in the smooth phase. Two comparable media (bacto tryptose and albimi) were used.

After harvesting and washing the cells in buffered saline, several methods were used to alter their permeability. pH adjustments of the reaction mixtures were confined to a phosphate buffer system; unless otherwise stated, the pH was adjusted to 6.8. *trans*-1,2-Cyclopentanedicarboxylic acid was tipped into the suspension of cells at the same time as the substrate; the compound was synthesized by the method of Fuson and Cole (1938). The acetone treatment was accomplished by washing the chilled cells with cold acetone for five minutes, centrifuging in the cold, and decanting; after the third such treatment, the cells were dried under vacuum and then resuspended in buffered saline for use. The toluene treatment was done by shaking the chilled suspension of cells with an equal volume of cold toluene for five minutes, allowing the mixture to stand in the cold for one hour, and then withdrawing the layer of cells for use. Freezing-and-thawing of cells was carried out in a stainless steel, double-boiler pan, the lower half of which contained a mixture of alcohol and dry ice; a small amount of the suspension of cells was alternately (and quickly) shell frozen and thawed 10 times. Sonic disintegration of suspensions of cells was done under refrigeration for 15 to 30 minutes with a Raytheon 10 KC magnetostrictive sonic oscillator; it was followed by centrifugation for 20 minutes at 8,000 to 10,000 G and decantation of the metabolically active supernatant solution which contained 60 to 80 per cent of the cellular nitrogen. Alumina trituration of cells was accomplished

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rapidly and with adequate chilling by the method of McIlwain (1948); after refrigerated centrifugation for 10 minutes at 20,000 G, the supernatant appeared as an opalescent solution. Of the three methods for physical disruption of the cell wall, sonic disintegration appeared the most satisfactory for general use with brucellae.

RESULTS

Adjustment of pH. Lowering the pH of the reaction mixture below that normally used for intact cells of brucellae markedly increased the rate of oxidation of several organic acids. A comparison of rates at two pH levels for strain 19 is given in table 1. The data demonstrate the marked stimulation of the adjustment of pH upon the oxidation of α-ketoglutarate, succinate, and other possible intermediates, excepting citrate, to rates comparable to that for L-glutamate. Some stimulation of L-aspartate oxidation partially resolved the asparagine-aspartate anomaly. In contrast, L-glutamate and L-asparagine were oxidized more rapidly at pH 6.8 than at pH 5.5, consistent with previous results (Gerhardt *et al.*, 1950). Using strain 11, a comparable reduction of pH resulted in an increased oxidation of succinate but essentially no oxidation of citrate, confirming the data obtained with strain 19.

TABLE 1

The effect of pH on the rate of oxidation of various compounds by strain 19 of Brucella abortus

0.0033 M SUBSTRATE	Q_{O_2} (N)*	
	pH 6.8	pH 5.5
L-Aspartate	65(55)	107(48)
L-Asparagine	180(55)	55(48)
L-Glutamate	552(67)	521(62)
α-Ketoglutarate	278(67)	665(62)
Succinate	205(62)	487(62)
Fumarate	96(82)	498(67)
L-Malate	406(62)	622(98)
Oxalacetate	119(82)	264(98)
Pyruvate	116(79)	415(98)
Acetate	200(79)	402(65)
Citrate	98(98)	98(98)

* The value in parenthesis is the endogenous Q_{O_2}(N); this designation will be followed in the subsequent text and tables.

Essentially no effect on the succinate-malonate reaction was obtained by the adjustment of pH alone, as shown by the data of table 2.

Adjustment of substrate concentration. When the concentration of the substrate in the reaction mixture was increased considerably in excess of that normally used, several compounds that otherwise were metabolized slowly now were oxidized rapidly. This response to high concentrations of substrate is illustrated by data obtained with α-ketoglutarate and succinate, using strain 19 (table 3). In both cases, the optimal response was attained at 0.167 M substrate. Similar data were obtained using strains 11, 2308, 6980, and 25-25-R of *B. abortus* with α-ketoglutarate, confirming in detail our previous generalization of the concentration effect with this compound (Gerhardt *et al.*, 1950). Pyruvate and oxalacetate, using strain 6980, and fumarate, L-aspartate, L-glutamine, and L-alanine, using strain 19, similarly were metabolized more readily at relatively high concentrations. The technique was essentially unsuccessful in demonstrating the oxidation of citrate by strain 19. Little, if any, change in the rate of oxidation by strain 19 has been found to occur through a concentration range of 0.00033 M to 0.0067 M L-glutamate or 0.00033 M to 0.033 M L-asparagine (Gerhardt *et al.*, 1950).

TABLE 2

The effect of pH and concentration on malonate inhibition of the oxidation of succinate by strain 19 of Brucella abortus

SUBSTRATE	pH 5.5		pH 6.8	
	Q_{O_2}(N)	Percentage of inhibition*	Q_{O_2}(N)	Percentage of inhibition*
0.0033 M succinate	483(62)		243(52)	
0.0033 M succinate + 0.0067 M malonate	483(62)	0	248(52)	−2.6
0.0067 M malonate	62(62)	0	55†(52)	−5.8
0.0033 M succinate	538(50)		243(52)	
0.0033 M succinate + 0.033 M malonate	398(50)	28.6	230(52)	6.8
0.033 M malonate	50(50)	0	55†(52)	−5.8

* In this and subsequent tables, the calculations of inhibition are corrected for the endogenous respiration; e.g., 100 per cent inhibition reaches the endogenous rate.

† 0.0033 M malonate.

The data of table 2 partially resolved the previous attempts to demonstrate a malonate inhibition reaction with succinate. Although an increase in inhibitor concentration (or lowering of the pH) alone failed to influence the reaction significantly, increasing the inhibitor:substrate ratio to 10:1 and lowering the pH to 5.5 together resulted in a moderate degree of inhibition. It may be of interest to note that, under the usual malonate to pass through the cell membrane as a nonionic substance, thus eliminating the permeability barrier to the malonate ion. Presumably, the ester is hydrolyzed once inside the cell, releasing the free malonate ion.

In the present study, diethyl malonate was tested as an inhibitor of the oxidation of succinate by intact cells of strain 11 of *B. abortus*. Diethyl malonate at a concentration of 0.0167 M did not

TABLE 3

The effect of concentration of substrate on the oxidation of succinate and α-ketoglutarate by strain 19 of Brucella abortus

SUBSTRATE	Q_{O_2} (N)					
	0.33 M	0.167 M	0.033 M	0.0167 M	0.0033 M	0.00033 M
Succinate	602(120)	636(120)	591(120)	541(120)	268(120)	122(120)
α-Ketoglutarate	757(57)	800(57)	688(57)	578(57)	252(57)	121(57)

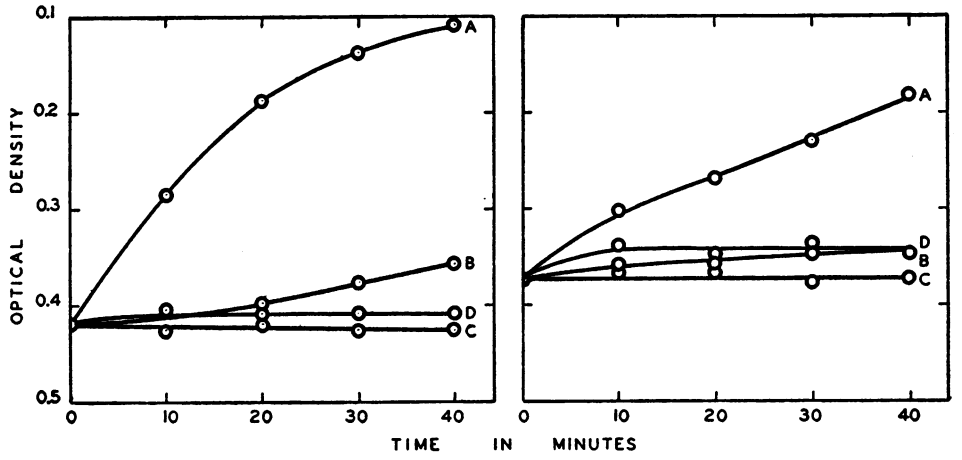

Figure 1. The inhibition of succinic dehydrogenase by malonate using toluene (left) and acetone treated (right) cells of strain 6980 of *Brucella abortus*. A, succinate; B, succinate plus malonate; C, malonate; D, endogenous. Each tube contained 2 ml of 0.02 M substrate, 2 ml of 0.02 M inhibitor, 2 ml of cells (approximately 900 μg cellular nitrogen), and 1 ml of 1:10,000 methylene blue and was read in a colorimeter at 550 mμ.

experimental conditions, other competitive inhibition reactions (fumarate-succinate, oxalacetate-succinate, maleate-succinate, oxalate-succinate) similarly were not demonstrable with *B. abortus*; nor did these reactions (e.g., malonate-succinate) seem to be strain specific.

Esterification of reactants. Beevers, Goldschmidt, and Koffler (1952) have found that diethyl malonate inhibits the oxidation of acetate by *Penicillium chrysogenum* although malonic acid itself is not effective. Esterification permits inhibit the oxidation of 0.00167 M succinate, nor was the ester itself oxidized. Since this strain rapidly oxidizes ethanol, either the diethyl malonate did not enter the cell or, on entering, the ester was not hydrolyzed.

Treatment of cells with acetone and toluene. The malonate-succinate system was studied further by treating the cells of strain 6980 with toluene or by drying the cells with acetone. The conventional Thunberg technique using methylene blue was employed. With either

preparation succinic dehydrogenase was demonstrated, and its activity was inhibited by the addition of malonate; the dehydrogenation of succinate by untreated cells was not inhibited by malonate. With neither preparation was oxidation of the substrates demonstrable in Warburg flasks. Representative results are shown in figure 1. Comparable results were obtained with strain 11.

trans-1,2-Cyclopentanedicarboxylic acid. This compound, a cyclic analogue of succinic acid, has been reported (Seaman and Houlihan, 1950) to increase the permeability of intact cells of *Tetrahymena geleii* to acetate, pyruvate, and succinate, which compounds otherwise are not oxidized by the protozoa. With cell homogenates, however, these compounds not only are oxidized rapidly, but the reactions (each involving succinate) are inhibited competitively by *trans*-1,2-cyclopentanedicarboxylic acid.

TABLE 4

Comparative rates of oxidation of various compounds by sonic extracts of strain 11 of Brucella abortus

0.00167 M SUBSTRATE*	Q_{O_2} (N)†	0.00167 M SUBSTRATE*	Q_{O_2} (N)†
L-Aspartate....	0	L-Malate.....	15
L-Asparagine...	0	Pyruvate.....	19
L-Glutamate...	19	Acetate.....	0
α-Keto-glutarate.....	52	Citrate.....	42
Succinate.....	7	DL-Lactate...	30
Fumarate.....	12	Glucose.....	0

* Supplemented with 0.5 mg diphosphopyridine nucleotide per cup.

† Endogenous respiration was negligible.

With intact cells of strain 19 of *B. abortus*, essentially no effect to increase permeability occurred with the addition of *trans*-1,2-cyclopentanedicarboxylic acid. The effect of 0.04 M and 0.008 M *trans*-1,2-cyclopentanedicarboxylic acid upon the oxidation of 0.0033 M succinate, α-ketoglutarate, L-aspartate, L-asparagine, and L-glutamate was studied at pH 6.8 and 5.5. Each substrate and both levels of *trans*-1,2-cyclopentanedicarboxylic acid were tested alone and in combination. The oxidations of succinate, α-ketoglutarate, and L-aspartate are limited by permeability, and the inhibition of succinate was of interest in the dissimilation of all of the test substrates. Of the substrates, only L-asparagine was oxidized more rapidly in the presence of *trans*-1,2-cyclopentanedicarboxylic acid (an increase of 27.2 per cent with 0.003 M L-asparagine plus 0.04 M *trans*-1,2-cyclopentanedicarboxylic acid at pH 6.8 only). The remaining reactions were inhibited by *trans*-1,2-cyclopentanedicarboxylic acid, more so at pH 5.5 than at 6.8. Indeed, the data indicated that permeability to *trans*-1,2-cyclopentanedicarboxylic acid itself is a function of pH with this organism.

Freezing and thawing of cells. A simple and effective method for disrupting cellular membranes is afforded by alternately freezing and thawing the suspension of cells. When applied in the present study (strain 19, pH 6.8, 0.0033 M substrate concentration), α-ketoglutarate, succinate, fumarate, L-malate, and acetate were oxidized at rates comparable to that for L-glutamate; for example, the average Q_{O_2}(N) for four different preparations with L-glutamate was 145(55) and with succinate, 238(55). Citrate and oxalacetate were oxidized at rates only slightly greater than that for the endogenous respiration. Minimal inhibition by 0.017 M malonate of the oxidation of 0.0033 M succinate (14.3 per cent), L-malate (3.9 per cent), α-ketoglutarate (9.2 per cent), and L-glutamate (7.1 per cent) was obtained with this method.

Cell-free preparations. The complete removal of the limiting effects of the cellular membranes is attainable by physically disrupting the cells and extracting the component enzymes. This was accomplished in the present study by sonic radiation and, to a limited extent, by alumina trituration.

A spectrum of the oxidative activity of sonic preparations of strain 11, supplemented with diphosphopyridine nucleotide, is given in table 4. Rates were decreased without diphosphopyridine nucleotide. Cytochrome C, methylene blue, pyridoxal, or adenosine triphosphate did not affect the rates. The rates of oxidation of α-ketoglutarate, citrate, and lactate were prominently highest. Intermediate activity was found with pyruvate, L-glutamate, L-malate, fumarate, and succinate. L-Aspartate, L-asparagine, acetate, and glucose were not oxidized. Altenbern and Housewright (1952) have reported temporary malonate inhibition of succinate oxidation by sonic preparations of strain 19 at pH 6.6 but not at pH 7.4.

Qualitative conditions for obtaining active, cell-free preparations by alumina grinding were

fairly well defined. The technique was found to have limited use with brucellae because of the hazards involved. With a typical preparation, good but diminishing activity with succinate—a $Q_{O_2}(N)$ of 114(19) in the initial 20 minute period—was obtained; α-ketoglutarate—46(19)—and citrate—28(19)—were oxidized steadily and at significant rates.

DISCUSSION

The effect of extracellular adjustment of pH or of substrate concentration upon intracellular enzyme reactions is explained by organic acids permeating the cell more readily when undissociated than when ionized. A greater concentration of the molecule and thus greater penetration into the cell occurs either by lowering the pH to a level consistent with the dissociation constant (pK_{a_1} succinic acid at 25 C is 4.18) or by greatly increasing the concentration of substrate with the pH at neutrality.

The present observations on the effect of pH are consistent with the concurrent findings of Altenbern and Housewright (1952), using strain 19 with α-ketoglutarate, succinate, and acetate. They have found some oxidation of citrate at pH 5.4 (approximately one-quarter of that with succinate) in contrast to the inactivity obtained with citrate in the present case and the similar finding by Barron *et al.* (1950) with *Corynebacterium creatinovorans*. The difference possibly may be attributed to Altenbern's practice of aerating cells to reduce the endogenous respiration, which may have masked the response to citrate in the present case. The alteration of pH to enhance the malonate-succinate reaction has been applied previously (e.g., Barron *et al.*, 1950; Gray, 1952). Dolin and Gunsalus (1949) have observed the effect of concentration on the oxidation of α-ketoglutarate by *Streptococcus faecalis*.

Treatment of cells with toluene or acetone is presumed to alter chemically the cell membranes. Although direct proof of such action is lacking, the results that have been obtained using these techniques with a number of organisms lead to this conclusion. A similar and more specific example is that of *trans*-1,2-cyclopentanedicarboxylic acid and its effect, presumably chemical, to increase permeability of *Tetrahymena geleii* (Seaman and Houlihan, 1950). With intact protozoa, *trans*-1,2-cyclopentanedicarboxylic acid itself is not oxidized. Compared with those obtained with cell homogenates, the results lead to the logical, yet indirect, conclusion that *trans*-1,2-cyclopentanedicarboxylic acid enhances penetration of the succinate. The mechanism of this action is not known, nor is the apparent inactivity of *trans*-1,2-cyclopentanedicarboxylic acid in the present case with brucellae, where permeability to succinate and other compounds was similarly (although not as absolutely) limiting. However, the inhibition of oxidation even at pH 6.8 proved some penetration of the *trans*-1,2-cyclopentanecarboxylic acid; it is possible that permeability to the compounds actually was enhanced (as apparently occurred with asparagine) but was negated by the concomitant inhibitory effect of *trans*-1,2-cyclopentanedicarboxylic acid.

Physical disruption of the cell surfaces has a more direct effect, apparent in the case of the freezing and thawing technique and obvious in the case of the fragmentation by sonic radiation or alumina grinding. The differences in activity between intact cells and cell-free preparations of bacteria have been emphasized by Rydon (1948). Disruption of cells by sonic radiation has been used successfully with brucellae by Roessler *et al.* (1952) and Altenbern and Housewright (1952). The latter have reported the oxidation of succinate, fumarate, citrate, acetate, and α-ketoglutarate in decreasing order of activity, using sonic preparations of strain 19 at pH 7.4. The present results are generally consistent with these observations. The differences in activity between intact cells and cell-free preparations sometimes are dramatic (e.g., with citrate) and sometimes disappointing (e.g., with L-aspartate). The lability of enzymes and the elution of coenzymes probably account for the limitations of these methods, direct though they are for eliminating permeability effects.

Where each of the above techniques has been applied individually in other work, the observed effects have been attributed to permeability. Taken together in the present study, the methods were in general mutually confirmatory and thereby supplied reasonable proof that permeability is a limiting factor in the test reactions. As such, they allow (but do not prove) certain pathways of intermediate metabolism that previously were untenable (e.g., α-ketoglutarate as an intermediate in glutamate oxidation) and rationalize certain reactions of brucellae that previously appeared inconsistent (e.g., oxidation of asparagine but not aspartate). Apparent exceptions do not negate the general pattern. However, the

difficulty in demonstrating malonate inhibition, under conditions where other reactions proceed unimpeded by permeability effects, indicates that permeability alone may not account entirely for the usual failure to demonstrate the reaction. The general problem of malonate inhibition in bacteria has been studied further by Gray (1952).

The role of permeability demonstrated in these few reactions may apply to other metabolic reactions of the brucellae and possibly may be extended further to many other bacteria as well. The significance of this cytological factor in bacterial metabolism is becoming increasingly apparent, as evidenced by the number of investigations in the past few years in which permeability has received major consideration.

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SUMMARY

Adjustment of pH and concentration of substrate in the reaction mixture, esterification of reactants, chemical treatment of cells with acetone, toluene, and *trans*-1,2-cyclopentanedicarboxylic acid, and physical disruption of the cell wall by freezing-and-thawing, sonic radiation, and alumina trituration were compared as experimental methods to increase the permeability of *Brucella abortus* to metabolites. While each test reaction was not always markedly affected by every method, with the exception of esterification and the pentane acid, the methods generally were mutually confirmatory and thereby supplied reasonable, although indirect, proof that permeability is a limiting factor in these reactions. The methods were used to elicit the oxidation of certain key metabolites (e.g., aspartate, α-ketoglutarate, citrate, etc.) that normally are oxidized slowly or not at all by intact cells and to demonstrate to a limited degree the inhibition by malonate of the metabolism of succinate.

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