GLUTAMATE METABOLISM IN BRUCELLA ABORTUS STRAINS OF LOW AND HIGH VIRULENCE

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ABSTRACT

DASINGER, B. L. (University of Wisconsin, Madison) and J. B. WILSON. Glutamate metabolism in Brucella abortus strains of low and high virulence. J. Bacteriol. 84:911–915. 1962.—Brucella abortus strains of low virulence oxidize glutamate at a high rate, whereas strains of high virulence oxidize glutamate at a relatively low rate. Results indicated that this observation was not related to differences in pathway of glutamate oxidation or to differences in total enzyme activity. Permeability studies showed that the maximal rates of glutamate accumulation were 2.8 moles per 2 min per g (wet wt) for a strain of high virulence and 6.2 moles per 2 min per g for a strain of low virulence, but equal intracellular steady-state concentrations were attained by both types of strains. Evidence is presented which suggests that the site of glutamate oxidation is separate from the pool of glutamate being measured. Unequal rates of permeability at these sites could be the reason for the differences in rate of glutamate oxidation.

Studies by other workers in this laboratory (Gerhardt, Levine, and Wilson, 1950; Olsen, 1951; Kamn, 1956; Grogan, 1957) have shown that Brucella abortus and B. suis strains of high virulence oxidize glutamate at a low rate, whereas strains of low virulence oxidize glutamate at a much higher rate. The study by Grogan (1957) with B. suis is especially significant because these strains were all mutants derived from a single parent strain. This relationship has been observed with a large number of strains, so there may be an underlying relationship between rate of glutamate oxidation and the virulence of the strain. As a first step in understanding this relationship, experiments were conducted to find the basis for this difference.

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MATERIALS AND METHODS

Culture methods and preparation of cell-free extracts. Cultures were maintained on slants of Alibi brucella agar and transferred to new slants every 6 to 8 weeks. Cells were obtained by inoculating 500 ml of Alibi brucella broth in a 2-liter Erlenmeyer flask with cells grown for 24 hr on an Alibi agar slant in a 300-ml prescription bottle. Flasks were shaken on a rotary shaker for 24 hr at 37 C. Cells were harvested by centrifugation and washed twice with 0.9% NaCl containing 0.05 M potassium phosphate buffer (pH 6.8). Cell-free extracts were obtained by treating 6 g of cells (wet wt) suspended in 20 ml of 0.1 M tris(hydroxymethyl)aminomethane (tris) buffer (pH 7.65) for 15 min in a 10-ke Raytheon sonic oscillator. The cells were maintained at 3 C during treatment. Whole cells and debris were removed by centrifugation at 3,000 X g for 30 min. Most of the preparations were dialyzed overnight against 100 volumes of distilled water at 4 C.

Strains used and virulence determination. B. abortus, strains 11 and 2308, were obtained originally from the Bureau of Animal Industry, U.S. Department of Agriculture. Their virulence for guinea pigs was determined with the method used by Tepper and Wilson (1958) except that tissue from spleen, liver, and three lymph nodes was cultured from each animal.

Manometric techniques. Conventional manometric techniques were used (Umbreit, Burris, and Stauffer, 1957). The water bath was maintained at 34 C. Glutamic acid was determined manometrically by decarboxylation with glutamic decarboxylase obtained from Sigma Chemical Co. Recovery of 5-mole quantities was 95 to 105%.

Analytical methods. The total nitrogen content of cell suspensions was determined by a semimicro-Kjeldahl method followed by nesslerization. Protein was measured by the method of Lowry et al. (1951). Dry weights of cells were determined.

Chromatographic procedures. Keto acids were qualitatively determined by paper chromatography of their dinitrophenylhydrazones by the method of Seligson and Shapiro (1952). Reaction mixtures were examined for organic acids by the paper chromatographic method of Palmer (1955), and for amino acids by the paper chromatographic method described by Smith (1958). Butanol-acetic acid-water (60:15:25) and phenol-water were used as solvents in the latter procedure.

Glutamate accumulation experiments. The procedures of Holden and Holman (1959) were employed with some modifications. Experiments on rate of accumulation were carried out in a medium composed of potassium phosphate buffer (2 x 10^{-2} M, pH 6.8), NaCl (7 x 10^{-2} M), MgSO_4 (2 x 10^{-3} M), chloramphenicol (20 µg/ml), and glutamate (concentrations in Results). Uptake medium without cells (17 ml) was dispensed into 125-ml Erlenmeyer flasks and shaken (90 cycles/min) in a water bath at 34 C. After temperature equilibration, 3 ml of a cell suspension containing 1 g (wt wt) of cells were rapidly added to the flasks. In experiments where the steady-state glutamate concentration was measured, the same uptake medium described above was utilized, except chloramphenicol was omitted. An incubation period of 15 min was used because respiration experiments with glutamate as substrate had shown that at this time oxygen uptake was proceeding at a constant rate. To prevent appreciable change in glutamate concentration during incubation, 0.4 to 0.5 g (wt wt) of cells was used in 200 ml of uptake medium. After incubation, the suspensions were rapidly cooled to 0 C by pouring the suspension over ice made of uptake medium with the appropriate glutamate concentration. Cells added to uptake medium having the appropriate glutamate concentration at 0 C and centrifuged immediately in the cold served as unincubated controls. Other procedures of Holden and Holman (1959), including separation of cells from the medium, extraction with ethanol, and preparation of glutamate for assay, were followed exactly. Glutamate was determined manometrically.

RESULTS

Table 1 shows the relationship of glutamate oxidation to virulence in the strains of B. abortus,

<table>
<thead>
<tr>
<th>Strain</th>
<th>Q02(N) (m)</th>
<th>Q02(N) (+)</th>
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<tbody>
<tr>
<td>11</td>
<td>2.2 x 10^6</td>
<td>520</td>
</tr>
<tr>
<td>2308</td>
<td>240</td>
<td>250</td>
</tr>
</tbody>
</table>

* Warburg flasks contained cell suspension, 500 µg of cell nitrogen; glutamate, 7 x 10^{-3} M; potassium phosphate buffer (pH 6.8), 0.0067 M.
† Endogenous rates have been subtracted.

used in this study. To investigate this difference in glutamate oxidation rate, both metabolic and permeability aspects of the problem were considered.

Metabolic studies. To get an over-all comparison of glutamate oxidation in the two strains, it was determined that 1.80 moles of oxygen were taken up per mole of glutamate utilized by strain 11. The corresponding result for strain 2308 was 1.65.

Complete oxidation would require 4.5 moles of oxygen per mole of glutamate. Further work on qualitative and quantitative comparison of internal glutamate oxidation was done with sonically prepared extracts to circumvent permeability barriers. To circumvent permeability barriers, the pH optimum for glutamate oxidation by these extracts was between 7.5 and 7.8; pH 7.65 was chosen for these experiments. Diphenolphosphoryridine nucleotide (DPN) was the only cofactor tried which stimulated oxygen uptake. Concentrations above 2.3 x 10^{-3} M did not increase the oxidative rate. Triphosphopyridine nucleotide, flavin adenine dinucleotide, flavin mononucleotide, thiamine pyrophosphate, adenine dinucleotide, inorganic phosphate, Mg^{2+}, and coenzyme A did not increase the stimulation observed with DPN alone.

To determine whether the difference in rate of glutamate oxidation observed with whole cells still persisted after sonic disruption, the glutamate-oxidizing activity of sonically prepared extracts was measured. Q02(N) values obtained with five different preparations of strain 11 ranged from 33 to 54, with a mean of 42. Those with strain 2308 ranged from 40 to 70, with a mean of 55. In only one instance was the Q02(N) obtained with strain 2308 lower than that of strain 11.

It has been proposed that, in strain 11, glutamate is oxidized via a-ketoglutarate through the tricarboxylic acid cycle (Marr et al., 1953). In-
hibitors of some of the reactions in this sequence were used, and their effects on glutamate oxidation by cell-free extracts are given in Table 2 (van Eys et al., 1958; Sanadi, Langley, and White, 1959; Quastel and Whetman, 1924; Adelstein and Vallee, 1958). Ethylenediaminetetraacetic acid (EDTA) was the only compound tested which showed a significant difference in degree of inhibition. Results on oxidation of proposed intermediates are presented in Table 3. Although pyruvate was not oxidized by the strain 11 preparation, it was oxidized by intact cells of strain 11 with a \( Q_{O_2}(N) \) of 106. \( \alpha \)-Ketoglutarate and pyruvic acids were detected by paper chromatography in reaction mixtures of glutamate oxidation by cell-free extracts of both strains. No other products could be detected.

Another characteristic of the cell-free glutamate-oxidizing systems that could be compared was the Michaelis constant \( (K_m) \). \( K_m \) constants were determined by the method of Lineweaver and Burk (1934) from initial oxygen uptake rates at various limiting glutamate concentrations. Essentially equal values of \( 1.5 \times 10^{-3} \text{M} \) for strain 11 and \( 1.2 \times 10^{-3} \text{M} \) for strain 2308 were found.

Permeability studies. Obviously, studies on permeability of glutamate in \( B. \) abortus are complicated by the rapid metabolism of this substrate. In spite of this difficulty, preliminary results had

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Conc</th>
<th>% inhibition</th>
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<tbody>
<tr>
<td></td>
<td>Strain 11</td>
<td>Strain 2308</td>
</tr>
<tr>
<td>Mercaptoacetic acid</td>
<td>( 1 \times 10^{-3} )</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>( 1 \times 10^{-2} )</td>
<td>58</td>
</tr>
<tr>
<td>Arsenite</td>
<td>( 1 \times 10^{-4} )</td>
<td>80</td>
</tr>
<tr>
<td>Cadmium chloride</td>
<td>( 1 \times 10^{-4} )</td>
<td>90</td>
</tr>
<tr>
<td>Malonate</td>
<td>( 1 \times 10^{-3} )</td>
<td>100</td>
</tr>
<tr>
<td>p-Chloromercuribenzoate</td>
<td>( 5 \times 10^{-3} )</td>
<td>100</td>
</tr>
<tr>
<td>EDTA</td>
<td>( 1 \times 10^{-2} )</td>
<td>53</td>
</tr>
</tbody>
</table>

* Warburg flasks contained, in addition to inhibitors: DPN, \( 2.3 \times 10^{-3} \text{M} \); tris buffer \( \text{pH} 7.65 \), \( 0.1 \text{M} \); glutamate, \( 7 \times 10^{-3} \text{M} \); and cell-free extract, \( 15 \text{mg} \) of protein; in a total volume of \( 3 \text{ml} \). Inhibitors were tipped in with substrate.

\[ \text{aliters } O_2/\text{hr} = \text{aliters } O_2/\text{hr} \text{(inhibitor)} \times 100 \]

\( \alpha \)-Ketoglutarate

<table>
<thead>
<tr>
<th>Substrate</th>
<th>( Q_{O_2}(N) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate</td>
<td>47</td>
</tr>
<tr>
<td>( \alpha )-Ketoglutarate</td>
<td>44</td>
</tr>
<tr>
<td>Succinate</td>
<td>23</td>
</tr>
<tr>
<td>Fumarate</td>
<td>31</td>
</tr>
<tr>
<td>Malate</td>
<td>31</td>
</tr>
<tr>
<td>Oxalacetate</td>
<td>12</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>18</td>
</tr>
<tr>
<td>Citrate</td>
<td>124</td>
</tr>
</tbody>
</table>

|       | 138†           |

Endogenous activity

\( \text{Qo}_2(N) \) determinations, the internal steady-state glutamate concentration is practically equal for shown that quantities of glutamate accumulated in 2 min were well below steady-state values, and these were considered as rates of accumulation. It should be noted that results of this type are not absolute, since part of the glutamate is oxidized. Performance of experiments under the same conditions for each strain permitted comparisons to be made. Rates of accumulation at various extracellular glutamate concentrations are presented as Lineweaver-Burk (1934) plots in Fig. 1. The apparent maximal rate of glutamate accumulation of strain 11 is over twice that of strain 2308, whereas the \( K_m \) values for accumulation are nearly equal. Related to this are the results on the relationship of external glutamate concentration to the rate of glutamate oxidation by intact cells. Initial oxygen uptake rates at glutamate concentrations of 0.25 to \( 1.5 \times 10^{-3} \text{M} \) were used to determine \( K_m \) values. The results formed a straight line when Lineweaver-Burk plots were made. The \( K_m \) values were \( 5.6 \times 10^{-4} \text{M} \) for strain 11 and \( 8 \times 10^{-4} \text{M} \) for strain 2308.

More detailed information on accumulation is provided by comparing intracellular steady-state concentrations at various extracellular concentrations of glutamate. These results (Table 4) show that at the higher extracellular concentrations (e.g., 4 \text{mM} \), which are in the range used in typical \( Q_{O_2}(N) \) determinations, the internal steady-state glutamate concentration is practically equal for
both strains. Only at 0.5 mm is an appreciable difference in internal concentration observed. These results also show that glutamate is accumulated against a concentration gradient.

**DISCUSSION**

According to the studies of Marr et al. (1953) with *B. abortus* strain 11, glutamate is oxidized via α-ketoglutarate through the tricarboxylic acid cycle to oxalacetate, which is decarboxylated to pyruvate. The results of the present study are compatible with this scheme and indicate that the same pathway operates in both strains. The consumption of approximately four atoms of oxygen per molecule of glutamate suggests an oxidation to the level of oxalacetate. The oxidation of proposed intermediates by extracts (Table 3), the results obtained with inhibitors (Table 2), and the detection of α-ketoglutarate and pyruvate indicate that the tricarboxylic acid cycle is involved. The near equality of $K_m$ values for glutamate oxidation by extracts and general similarity in other results obtained with strains 11 and 2308 suggest identity in pathway of glutamate oxidation. EDTA inhibited oxidation unequally in the two strains, but it could be acting on terminal oxidation in addition to steps in glutamate degradation.

Assuming no marked differential destruction of activity, the fact that glutamate oxidation activities obtained with strain 11 extracts were rarely higher than those of strain 2308 indicates that their potential activities before breakage must have been more nearly equal. This tends to rule out differences in enzyme content as the reason for the disparity of whole-cell activities.

The experiments on accumulation show that strain 11 can accumulate glutamate at over twice the rate of strain 2308 (Fig. 1). The similarity in $K_m$ values for accumulation and similarity in $K_m$ values for oxidation of glutamate by whole cells suggest that glutamate enters both strains by the same mechanism. The faster rate of accumulation by strain 11 does not explain the faster rate of oxidation by this strain, since the steady-state internal concentration reached by both strains is approximately equal at external concentrations of 2.0 mM or greater (Table 4). The possibility exists, however, that glutamate occupies more than one pool inside the cells, and that the steady-state concentration based on total water space is not actually the concentration in contact with the oxidizing system. Results suggesting pool compartmentation of amino acids in relation to protein synthesis have been described with yeast (Cowie and McClure, 1959) and mammalian cells (Kipnis, Reiss, and Helmreich, 1961). Vickery and Zeitch (1960) also postulated that citrate accumulates in one pool of tobacco leaves but is rapidly oxidized in another pool. To explain the results on this basis would mean that the capacity for glutamate accumulation in the two strains is essentially equal, but that there is a faster supply of glutamate to the sites of oxidation in strain 11.

The involvement of pool compartmentation in glutamate oxidation is suggested by further consideration of the data. The $K_m$ value for the oxidation of glutamate by extracts is $1.5 \times 10^{-2}$ M and the average $K_m$ for oxidation by whole cells is $6.8 \times 10^{-2}$ M. These constants predict that, at an external concentration of $6.8 \times 10^{-2}$ M, the internal concentration at the site of oxidation should be about $1.5 \times 10^{-2}$ M. Upon determina-
tion of the internal steady-state concentration at an external concentration of $5 \times 10^{-4}$ M (Table 4), the internal concentrations were $6.1 \times 10^{-2}$ M in strain 2308 and $11.0 \times 10^{-4}$ M in strain 11. These values are four to seven times higher than would be expected, suggesting again that measurements of over-all glutamate concentrations inside the cells do not reflect those in contact with the oxidizing system.

ACKNOWLEDGMENT

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LITERATURE CITED


