The use of triphenyltetrazolium chloride in the study of dehydrogenase activity of Brucellae

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(With 5 text-figures)

The metabolism of Brucellae has been investigated in some instances but a general agreement on many aspects of the problem has not yet been reached. In relation to enzymatic activity in presence of single added substrates, for instance, the data are scarce and somewhat discrepant as was pointed out in Hoyer's review (1950).

Since the publication of the papers of Kuhn et al. (1941) and Kun et al. (1949), tetrazolium salts have had their use generalized as indicators of dehydrogenase activity in many enzymatic systems. The use of these oxidation-reduction indicators in bacteriology is very ample in the present moment. Some papers have been published concerning the use of those salts in work with Brucellae, for the preparation of ring test antigens (Bendtsen, 1949, 1950; Moulin, 1951; Wood, 1950) or serum agglutination antigens (Mello, 1951), and for the differentiation of species and variation within them (Huddleson et al., 1950).

Most of the enzymatic activities in cells seem to be located in mitochondria (for a review see Green, 1951; Mudd et al., 1951c) and these structures are indicated by the reduction of tetrazolium salts to its insoluble colored formazan inside them (Goddard et al., 1952; Nordmann et al., 1951; Shelton et al., 1952; Winterscheid et al., 1952). In bacteria and other microorganisms much evidence is being accumulated that they have mitochondria (Mudd et al., 1951 a, b, c, d; 1952 Winkler, 1950; Winterscheid et al., 1952). Recent investigations have evidenced sites of intense reduction of tetrazolium salts as intracellular inclusions in many bacteria and fungi (Antopol et al., 1948; Bielig et al., 1949; Canizares et al., 1951; Fred et al., 1949; Lederberg, 1948; Mudd et al., 1951 a, c, d; Narahara et al., 1950; Winkler, 1950, Winterscheid et al., 1952).

* This investigation was supported in part by a research grant from Dr. Guilherme Guinle to the senior author. Presented at the Annual Meeting of “Sociedade Brasileira para o Progresso da Ciência”, Porto Alegre, November, 1952.
The present paper is part of a work planned for the study of dehydrogenase activity of Brucellae using tetrazolium salts as hydrogen ions acceptors.

MATERIALS AND METHODS

Cultures — Brucella abortus strain 1868 (aerobic strain B-99, from Dr. A. W. Stableforth, Weybridge, England) and Br. suis strain 1598 (strain SIG carried on agar, from Dr. S. S. Elberg, University of California, U.S.A.), were used throughout. The dye reactions and urease test were typical of the assigned species.

Cell suspensions — The 48-hour growth of cells on real infusion agar in Roux flasks was removed with 0.9% sodium chloride solution, filtered through cotton and gauze and washed 5 times in the NaCl solution (3000 rpm during 20 minutes each washing). The final sediment was resuspended in the NaCl solution until a turbidity that gave a reading of 100 when diluted 1:20 (Klett-Summerson photoelectric colorimeter, filter green 54, 13 x 100 Kimble matched test tubes. NaCl solution as blank). The final suspension was tested with 0.1% trypafline solution: it was kept at — 4 C to + 2 C and used in the same day.

Solutions — a) Buffer: M 15 phosphate buffer, pH 7.0. b) Saline solution: sterile 0.9% NaCl solution. c) TTC: 0.1% solution of 2, 3, 5 — triphenyltetrazolium chloride. (Practical grade, Synthetical Labs., Inc.). d) Substrates: M 50 solutions of the following representatives of groups of compounds known as important in bacterial metabolism: L-arabinose, D-fructose, D-galactose, D-glucose, D-lactose, maltose, D-xylose, i-inositol, D-mannitol, D-sorbitol, DL-ala-nine, L-asparagine, and glycine (Pfanstiehl); glycerol (City Chem., Co.); sodium acetate (Mallinckrodt); D-glutamic acid, and D-arginine (Eastman Kodak). Succinic acid and citric acid were utilized but owing to the low pH of the final systems, the results were not recorded in this paper; further investigations are in course on this subject.

All reagents were considered pure compounds and the solutions made in glass redistilled water. Substrates and TTC solutions were maintained in frozen state and thawed prior to use.

Measurement of dehydrogenase activity — The techniques of Kun et al. (1949) and Hanks (1951) were adapted to the experiments. The tests were made in aerobicosis. The reagents were dispensed in 13 x 100 Pyrex or Kimble test tubes kept at — 4 C to + 2 C, in the following order: 0.2 ml of buffer, 0.1 ml of TTC, 0.2 ml of substrate, and 0.5 ml of cell suspension. Since the tests were conducted in the same day and in relatively short times, strict sterile measures were not taken. Each run for each incubation period consisted of a series of 17 tubes for the different substrates and one control for endogenous activity with 0.2 ml of redistilled water instead of substrate. In addition, another tube (blank for the extraction) had 0.3 ml of buffer, 0.2 ml of redistilled
water, and 0.5 ml of cell suspension; with four periods of incubation, for instance, 17 x 4 tubes were utilized plus the controls. The reagents were mixed gently and the tubes transferred from the cold to a precision water bath at 37°C; the incubation time was measured from this moment on. After 1, 2, 3, and 4 hours the tubes of each run were removed from the bath to the cold and one drop of 30% formaldehyde solution was added to each; then they were rubber stoppered and kept in the dark in the refrigerator at 4°C, until the extraction of the formazan produced.

**BRUCELLA SUIS**

![Graph showing formazan production by Brucella suis](image)

**BRUCELLA ABORTUS**

![Graph showing formazan production by Brucella abortus](image)

Fig. 1 — Formazan produced by washed cells of Br. suis and Br. abortus in the presence of: 1 — D-xylene; 2 — D-glucose; 3 — L-arabinose; 4 — D-galactose; 5 — maltose; 6 — D-fructose; 7 — D-lactose. 

----------: endogenous activity.
Subjective readings were made after the removal of the tubes from the water bath. This procedure, however, permitted only the observation of gross differences, so formazan extractions were made one or two days after, because it was not possible to do so in the same day of the tests. Each tube received 4 ml of pure acetone and was vigorously shaken and centrifuged at 3000 rpm for 20 minutes; part of the clear supernatant was transferred to matched 13 x 100 Kimble test tubes. Readings were made in the Klett-Summerson photoelectric colorimeter with filter violet 42; the blank was the supernatant of the second control tube without TTC. Not less than two readings were made; the average was recorded and compared with readings obtained with standard formazan solutions.

For the measurement of dehydrogenase activity in micrograms of formazan produced, graded amounts of pure formazan were diluted in 80% acetone and the readings in the colorimeter plotted as a linear function of the concentration (Kun et al., 1949; Nordmann et al., 1951; Fahmy et al., 1952). To obtain the formazan an arbitrary amount of 2, 3, 5 — triphenyltetrazolium chloride was diluted in water and an excess of ascorbic acid and normal sodium hydroxide solutions was

### TABLE 1
Formazan production by washed cells of *Brucella abortus* and *Brucella suis* in the presence of various carbohydrates

<table>
<thead>
<tr>
<th>SUBSTRATE</th>
<th>µg FORMAZAN PRODUCED*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 hr</td>
</tr>
<tr>
<td>L-arabinose</td>
<td>2.2</td>
</tr>
<tr>
<td>D-fructose</td>
<td>3.1</td>
</tr>
<tr>
<td>D-galactose</td>
<td>6.4</td>
</tr>
<tr>
<td>D-glucose</td>
<td>6.2</td>
</tr>
<tr>
<td>D-lactose</td>
<td>0.9</td>
</tr>
<tr>
<td>Maltose</td>
<td>0.6</td>
</tr>
<tr>
<td>D-xylose</td>
<td>6.9</td>
</tr>
<tr>
<td>None**</td>
<td>1.1</td>
</tr>
</tbody>
</table>

Composition of test systems: 0.5 ml of cell suspension + 0.2 ml of M/50 substrate + 0.1 ml of 0.1% TTC + 0.2 ml of M/15 potassium phosphate buffer, pH 7.0.

* Each value given is the average of four independent experiments for *Brucella abortus* and of three for *Brucella suis*.
** Endogenous activity.
added; after about 10 minutes the dark crystals of formazan were separated by centrifugation, washed twice in distilled water, and diluted in a mixture of equal parts of acetone and methanol; the solvent was evaporated and the dried formazan kept in the dark. Attempts for obtaining a total reduction of graded amounts of tetrazolium with crystals or saturated solution of sodium hydrosulfide, which could be used as standards (Gots et al., 1952; Kun et al., 1949) were unsuccessful because each time gross uncontrollable differences were observed. This is in agreement with observations reported by Bodine et al. (1949) and Nordmann et al. (1951).

**BRUCELLA SUIS**

![Graph of BRUCELLA SUIS]

**BRUCELLA ABORTUS**

![Graph of BRUCELLA ABORTUS]

Fig. 2 — Formazan produced by washed cells of *Br. suis* and *Br. abortus* in the presence of: 1 — D-sorbitol; 2 — glycerol; 3 — D-mannitol; 4 — 1-inositol.

.............. endogenous activity.
Observation of the sites of more intense enzymatic activity — The same colored cell suspension of the tests was examined microscopically for the sites of more intense enzymatic activity. Sometimes a loopful of 48-hour cultures of Br. abortus, Br. suis, or Br. melitensis was suspended in buffered solution of 0.1 or 1.0°. TTC and incubated in the water bath until intense reduction occurred. The microscopy of the cells was made directly with the colored suspension or with a mixture of it and melted agar, between microscopic slide and cover slip. A Zeiss microscope with 10 or 15 X eyepiece and 90 X oil immersion objective of n.a. 1.25 with built-in diaphragm was used. Illumination was provided by a Bausch and Lomb research microscope lamp with a green filter interposed.

### TABLE 2

Formazan production by washed cells of Brucella abortus and Brucella suis in the presence of glycerol, i-inositol, D-mannitol, and D-sorbitol

<table>
<thead>
<tr>
<th>SUBSTRATE</th>
<th>µM FORMAZAN PRODUCED*</th>
<th>Brucella abortus</th>
<th>1 hr</th>
<th>2 hr</th>
<th>3 hr</th>
<th>4 hr</th>
<th>Brucella suis</th>
<th>1 hr</th>
<th>2 hr</th>
<th>3 hr</th>
<th>4 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>8</td>
<td>16.5</td>
<td>26</td>
<td>33.7</td>
<td>0.6</td>
<td>16.2</td>
<td>32</td>
<td>44.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i-inositol</td>
<td>0.5</td>
<td>7.7</td>
<td>13.5</td>
<td>19</td>
<td>0.7</td>
<td>7.4</td>
<td>26.1</td>
<td>38.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-mannitol</td>
<td>0</td>
<td>7</td>
<td>12.6</td>
<td>17.9</td>
<td>0</td>
<td>14.2</td>
<td>32.1</td>
<td>41.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-sorbitol</td>
<td>2.6</td>
<td>12.4</td>
<td>17.1</td>
<td>24</td>
<td>4.5</td>
<td>23.7</td>
<td>37.8</td>
<td>53.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None**</td>
<td>1.1</td>
<td>7.2</td>
<td>12</td>
<td>19.2</td>
<td>2.5</td>
<td>10.8</td>
<td>27</td>
<td>37.5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

See footnotes of Table 1.

### TABLE 3

Formazan production by washed cells of Brucella abortus and Brucella suis in the presence of DL-alanine, D-arginine, L-asparagine, D-glutamic acid, and glycine

<table>
<thead>
<tr>
<th>SUBSTRATE</th>
<th>µM FORMAZAN PRODUCED*</th>
<th>Brucella abortus</th>
<th>1 hr</th>
<th>2 hr</th>
<th>3 hr</th>
<th>4 hr</th>
<th>Brucella suis</th>
<th>1 hr</th>
<th>2 hr</th>
<th>3 hr</th>
<th>4 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>DL-alanine</td>
<td>6.3</td>
<td>14.3</td>
<td>20.6</td>
<td>29.7</td>
<td>9.9</td>
<td>32.6</td>
<td>52.5</td>
<td>68</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-arginine</td>
<td>0.4</td>
<td>7.2</td>
<td>11.6</td>
<td>18.6</td>
<td>0.9</td>
<td>14.8</td>
<td>34</td>
<td>43</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-asparagine</td>
<td>1.9</td>
<td>9.5</td>
<td>13.8</td>
<td>20.8</td>
<td>0.9</td>
<td>11.4</td>
<td>28.6</td>
<td>40</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-glutamic acid</td>
<td>1.8</td>
<td>10.7</td>
<td>11.9</td>
<td>18.9</td>
<td>3</td>
<td>16.8</td>
<td>29</td>
<td>39.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>0</td>
<td>7.7</td>
<td>12.9</td>
<td>20.8</td>
<td>0.4</td>
<td>17.4</td>
<td>35.5</td>
<td>54.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None**</td>
<td>1.1</td>
<td>7.2</td>
<td>12</td>
<td>19.2</td>
<td>2.5</td>
<td>10.8</td>
<td>27</td>
<td>37.5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

See footnotes of Table 1.
RESULTS

Dehydrogenase activity — This activity was measured as the amount of formazan obtained in 1, 2, 3, and 4 hours with Br. abortus and Br. suis, in 4 and 3 independent series of tests, respectively. The results were compared with those obtained as a consequence of the endogenous activity of the same cell suspension.

Carbohydrates: In general, the compounds were more intensely oxidized by Br. suis than by Br. abortus (Table 1 and fig. 1). D-xylene, L-arabinose, D-glucose, D-galactose, and maltose were actively oxidized;

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Fig. 3 — Formazan produced by washed cells of Br. suis and Br. abortus in the presence of: 1 — Dl-alanine; 2 — glycine; 3 — D-arginine; 4 — L-asparagine; 5 — D-glutamic acid.

............... endogenous activity.
D-fructose was slightly oxidized, and D-lactose was not oxidized by *Br. suis*. L-arabinose, D-galactose, D-glucose, D-xylose, and D-fructose were oxidized, and maltose and D-lactose were not oxidized by *Br. abortus*.

Alcohols: *Brucella suis* was not very active in oxidizing the alcohols employed; only D-sorbitol was slightly oxidized. *Brucella abortus*, however, oxidized intensely glycerol and slightly D-sorbitol (Table 2 and fig. 2).

Amino acids: Only DL-alanine was intensely oxidized by *Br. suis* and *Br. abortus*. Glycine was slightly oxidized by *Br. suis*. The other, amino acids were not oxidized (Table 3 and fig. 3).

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**BRUCELLA SUIS**

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**BRUCELLA ABORTUS**

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Fig. 4 — Formazan produced by washed cells of *Br. suis* and *Br. abortus* in the presence of: 1 — sodium acetate. 

..............: endogenous activity.
Fig. 5 — Decreasing order of amount of formazan produced by washed cells of *Br. suis* and *Br. abortus* in the presence of 19 single substrates: *Brucella abortus*: 1 — L-arabinose; 2 — D-galactose; 3 — D-glucose; 4 — glycerol; 5 — D-xylose; 6 — DL-alanine; 7 — D-fructose; 8 — D-sorbitol; 9 — glycine; 10 — L-asparagine; 11 — sodium acetate; 12 — maltose; 13 — control; 14 — l-malitol; 15 — D-glutamic acid; 16 — D-arginine; 17 — D-lactose; 18 — D-mannitol; 19 — succinic acid; 20 — citric acid. *Brucella suis*: 1 — D-xylose; 2 — L-arabinose; 3 — D-glucose; 4 — D-galactose; 5 — DL-alanine; 6 — sodium acetate; 7 — maltose; 8 — glycine; 9 — D-fructose; 10 — D-sorbitol; 11 — glycerol; 12 — D-arginine; 13 — D-mannitol; 14 — L-asparagine; 15 — D-glutamic acid; 16 — l-malitol; 17 — control; 18 — D-lactose; 19 — succinic acid; 20 — citric acid.
Sodium acetate was intensely oxidized by *Br. suis* but not by *Br. abortus* (Table 4 and fig. 4).

**TABLE 4**

Formazan production by washed cells of *Brucella abortus* and *Brucella suis* in the presence of sodium acetate

<table>
<thead>
<tr>
<th>SUBSTRATE</th>
<th><em>µg FORMAZAN PRODUCED</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Brucella abortus</em></td>
</tr>
<tr>
<td></td>
<td>1 hr</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>0.6</td>
</tr>
<tr>
<td>None*</td>
<td>1.1</td>
</tr>
</tbody>
</table>

See footnotes of Table 1.

In a general manner the dehydrogenase activity of *Br. suis* was much more intense than that of *Br. abortus* (fig. 5).

_Sites of more intense enzymatic activity_ — The microscopic observation of the cells showed in each of them a small, round and subterminal granule. Some cells did not bear a granule and the largest ones sometimes showed a granule in each end; these were, perhaps, in division. The granules are red and can be easily seen when a green filter is interposed.

**DISCUSSION**

In our experiments some results were in agreement and others disagreed with recent reports on enzymatic activity of *Brucellae*. The differences are perhaps related to the different techniques employed, since manometric methods were used by the authors of those papers.

Our results agree with those obtained by McCullough & Beal (1951, 1952) who studied the oxidation of 9 carbohydrates by twelve different strains of the three species of *Brucella* and its inhibition by certain antibiotics. In a general manner carbohydrates are more intensely oxidized by *Br. suis* than by *Br. abortus*. It is interesting to mention that the carbohydrates most intensely oxidized by both strains tested by us were those already known as being utilized by *Brucellae*, v.g. arabinose, galactose, glucose, fructose, and xylose (Coleman et al., 1930; Mallardo, 1930; Mc Nutt et al., 1931; Mc Alpine et al., 1928).

Altenbern _et al._ (1952) observed the same rate of oxidation of glucose and sodium acetate at pH 7.4 by *Br. abortus* strain 19. In our
experiments, however, sodium acetate was not oxidized by Br. abortus, in contrast with its intense oxidation in presence of Br. suis.

Our results are also in disagreement with those of Marr et al. (1952) who observed that oxidation of glutamic acid by Br. abortus strain 19 was the most rapid metabolic reaction thus far studied in that species. In our experiments D-glutamic acid was not oxidized by neither of the strains used, a fact that confirms observations made by others (Hoyer, 1950).

As to the red stained granules within the cells they may possibly be interpreted as mitochondria similar to identical structures observed by some investigators in other microorganisms (Mudd et al., 1951 a, b, c, d; 1952; Winkler, 1950; Winterscheid et al., 1952).

Acknowledgements — The authors are indebted to Dr. H. Moussatché and Dr. A. Curé for the many suggestions during the course of this investigation, and to Dr. A. C. Perissé for the synthesis of 2, 3, 5—triphenyltetrazolium chloride utilized in the early stages of the experiments.

SUMMARY

Experiments for the investigation of dehydrogenase activity of washed cells of a strain of Br. abortus and another of Br. suis in presence of different single added substrates are reported. The activity was measured as the amount of formazan produced by the reduction of 2, 3, 5—triphenyltetrazolium chloride acting as a hydrogen ions acceptor, at pH 7.0.

In a general manner the dehydrogenase activity of Br. suis was much more intense than that of Br. abortus (fig. 5).

In the conditions of the experiments Br. abortus oxidized L-arabinose, D-galactose, D-glucose, glycerol, D-xylose, DL-alanine, D-fructose, and D-sorbitol. Brucella suis oxidized D-xylose, L-arabinose, D-glucose, D-galactose, DL-alanine, sodium acetate, maltose, glycine, D-fructose, and D-sorbitol.

Glycerol was oxidized by Br. abortus but its oxidation by Br. suis was very slight. Sodium acetate and maltose were intensely oxidized by Br. suis but not by Br. abortus.

The sites of more intense enzymatic activity were seen as small red colored round granules located in one pole of the cells.

SUMÁRIO

O EMPRÊGO DO CLORETO DE TRIFENILTETRAZÓLIO NO ESTUDO DA ATIVIDADE DEHIDROGENÁSICA DE BRUCELAS

Com a finalidade de observar a atividade dehidrogenásica de brucelas, em presença de diversos substratos isolados, empregamos o cloreto de trifeniltetrazólio (em solução aquosa a 0,1%) como receptor de hi-

Empregamos suspensões de culturas de 48 horas de duas amostras típicas: Brucella abortus (aeróbia, n." 1 868, amostra B-99, Weybridge) e Br. suis (n.º 1 568, amostra SIG do Dr. S. S. Elberg, da Universidade de Califórnia). As culturas em agar, lavadas 5 vezes em solução de cloreto de sódio a 0,9% ("resting cells") foram suspensas nessa solução salina de maneira a dar uma leitura de 100 na escala do colorímetro fotoelétrico de Klett-Summerson, quando diluídas a 1:20. O tampão utilizado nas provas era de fosfatos em solução M 15, a pH 7,0. Cada tubo de prova continha 0,2 ml de tampão, 0,1 ml de tetrazólio, 0,2 ml de substrato e 0,5 ml de suspensão de brucelas; um testemunho levava 0,2 ml de água destilada em vez de substrato e outro, mais 0,1 de tampão, em vez de tetrazólio ("blank" para a extração). Incubavam-se os tubos em banho-maria a 37°C retirando-se no fim de 1, 2, 3 e 4 horas; a reação enzimática era estabilizada com uma gota de formol a 30%; e os tubos guardados na geladeira, arrolhados.

A formazana resultante da redução do tetrazólio era extraída 1 ou 2 dias depois, com acetona e dosada no colorímetro fotoelétrico K-S, em relação a uma reta padrão previamente determinada com formazana pura. Também foram observados os pontos de maior atividade enzimática (provávelmente mitocôndrias) colocando-se as brucelas, antes da extração da formazana, entre lâmina e laminula e observando-se ao microscópio com filtro verde.

Os resultados permitiram-nos chegar às seguintes conclusões:

a) De um modo geral Br. suis possui atividade dehidrogenásica mais acentuada do que Br. abortus (fig. 5).

b) Br. abortus oxida mais intensamente os seguintes substratos: arabinose e galactose (muito intensamente), glucose, glicerol, xilose, alanina, frutose e sorbitol (que foi o menos oxidado).

c) Br. suis oxida mais intensamente os seguintes substratos, em ordem decrescente: xilose, arabinose e glucose (muito intensamente), galactose, alanina, acetato de sódio, maltose, glicina, frutose e sorbitol.

d) Glicerol não aumenta a atividade dehidrogenásica endógena de Br. suis enquanto o acetato de sódio não aumenta esta atividade em Br. abortus.

e) Os pontos de maior atividade enzimática são arredondados, muito pequenos, e estão situados numa das extremidades do germe, raramente nas duas.

BIBLIOGRAFIA


