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).

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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 veal 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% trypaflavine 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-alanine, 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 aerobiosis. 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 4C, until the extraction of the formazan produced.

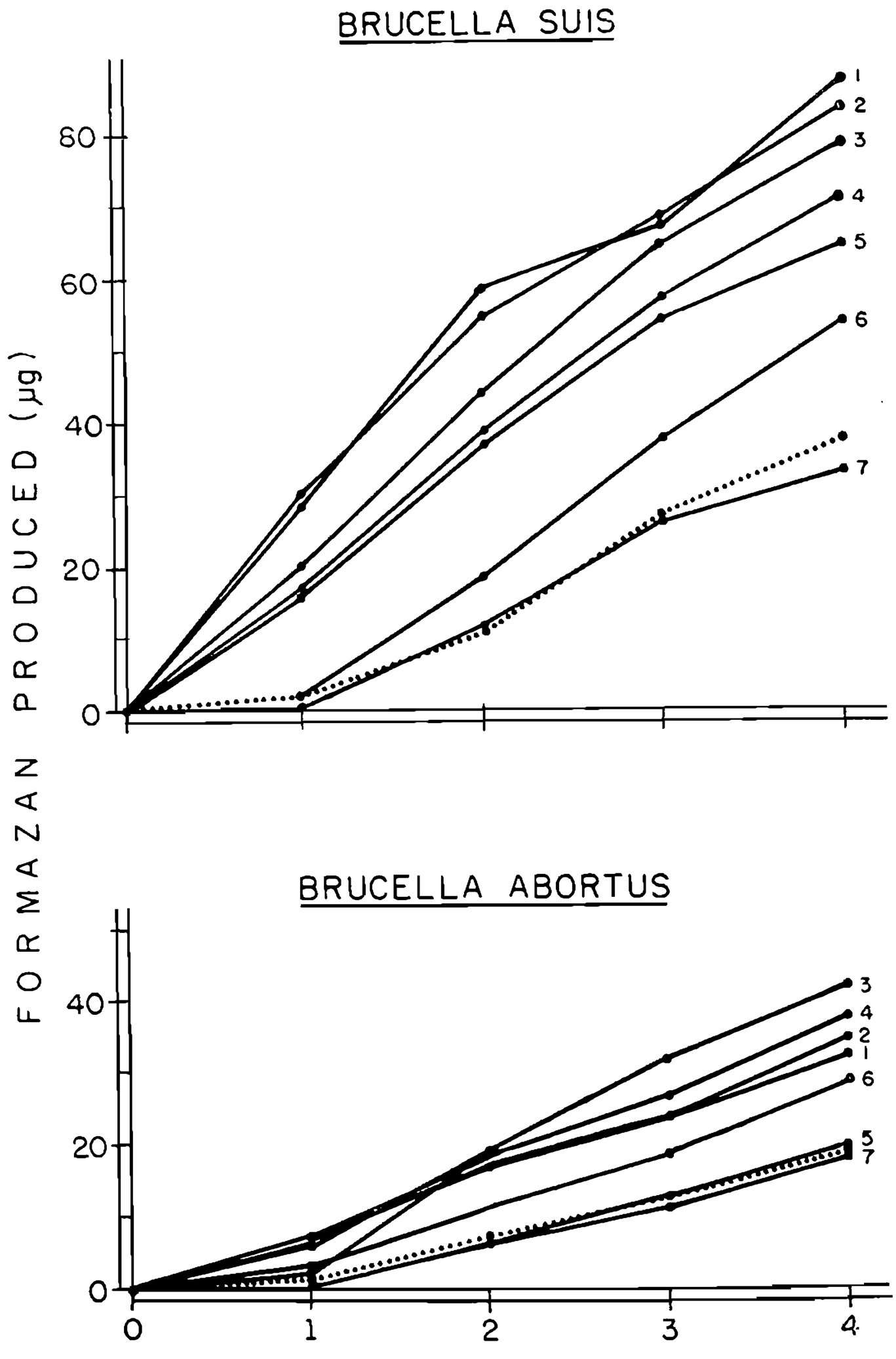

Fig. 1 — Formazan produced by washed cells of *Br. suis* and *Br. abortus* in the presence of: 1 — D-xylose; 2 — D-glucose; 3 — L-arabinose; 4 — D-galactose; 5 — maltose; 6 — D-fructose; 7 — D-lactose.
.....: endogenous activity.

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**

SUBSTRATE	μg FORMAZAN PRODUCED*							
	Brucella abortus				Brucella suis			
	1 hr	2 hr	3 hr	4 hr	1 h	2 hr	3 hr	4 hr
L—arabinose	2 2	19	31 4	41.6	20	43 8	64.3	78 5
D—fructose	3 1	11.1	18.6	28.5	2	18 7	37.6	53 9
D—galactose	6.4	18 2	26 6	37.7	16.6	38.6	56 8	70 9
D—glucose	6.2	17.1	23.7	34.4	29.8	54.8	68.2	83 2
D—lactose	0.4	7.3	11.7	18.3	0	11.2	26.6	32.9
Maltose	0.6	6.7	12.6	19.3	16.3	37	54.1	64.7
D—xylose	6.9	16.7	23.9	32.4	28.5	58.3	67.5	87.1
None**	1.1	7.2	12	19.2	2.5	10.8	27	37.5

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 uncontrolable differences were observed. This is in agreement with observations reported by Bodine *et al.* (1949) and Nordmann *et al.* (1951).

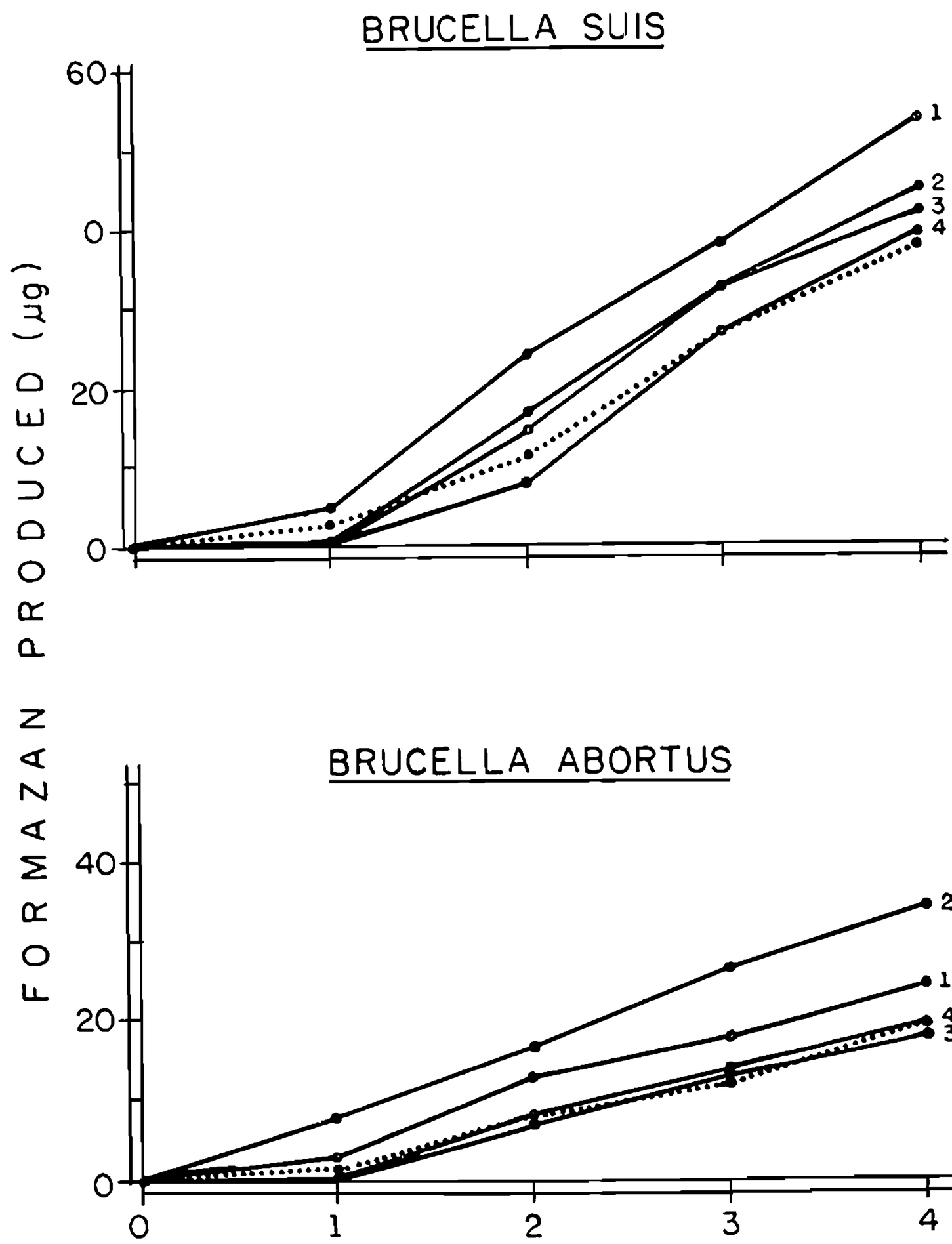

Fig. 2 — Formazan produced by washed cells of *Br. suis* and *Br. abortus* in the presence of: 1 — D-sorbitol; 2 — glycerol; 3 — D-mannitol; 4 — i-inositol.
.....: endogenous activity.

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, Dr-mannitol, and D-sorbitol**

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

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**

SUBSTRATE	μg FORMAZAN PRODUCED*							
	Brucella abortus				Brucella suis			
	1 hr	2 hr	3 hr	4 hr	1 hr	2 hr	3 hr	4 hr
DL—alanine	6.3	14.3	20.6	29.7	9.9	32.6	52.5	68
D—arginine	0.4	7.2	11.6	18.6	0.8	14.8	34	43
L—asparagine	1.9	9.5	13.8	20.8	0.9	11.4	28.6	40
D—glutamic acid	1.8	10.7	11.9	18.9	3	16.8	29	39.4
Glycine	0	7.7	12.9	20.8	0.4	17.4	35.5	54.3
None**	1.1	7.2	12	19.2	2.5	10.8	27	37.5

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-xylose, L-arabinose, D-glucose, D-galactose, and maltose were actively oxidized;

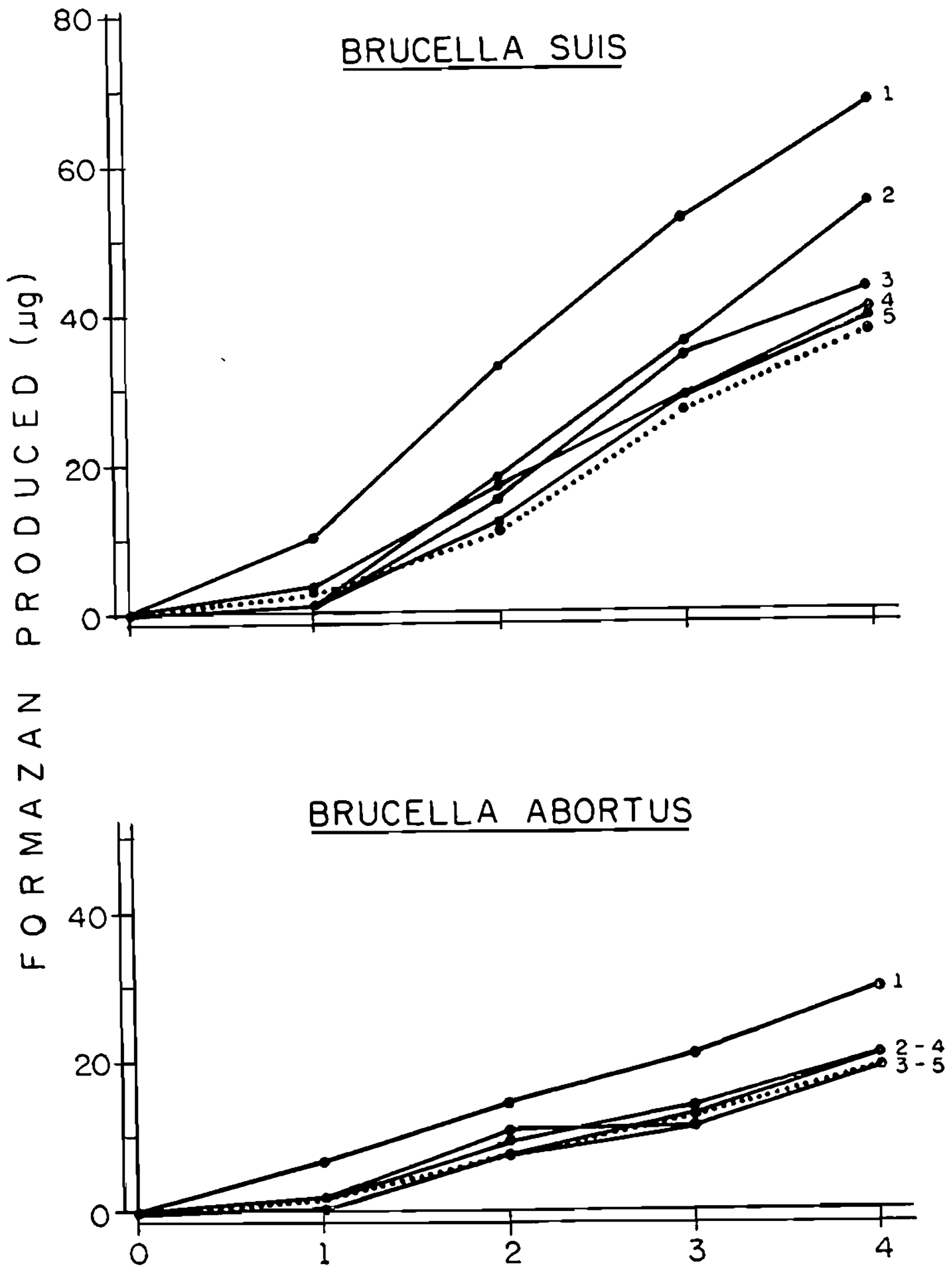

Fig. 3 — Formazan produced by washed cells of *Br. suis* and *Br. abortus* in the presence of: 1 — DL-alanine; 2 — glycine; 3 — D-arginine; 4 — L-asparagine; 5 — D-glutamic acid.
.....: endogenous activity.

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*.

Alchools: *Brucella suis* was not very active in oxidizing the alchools 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).

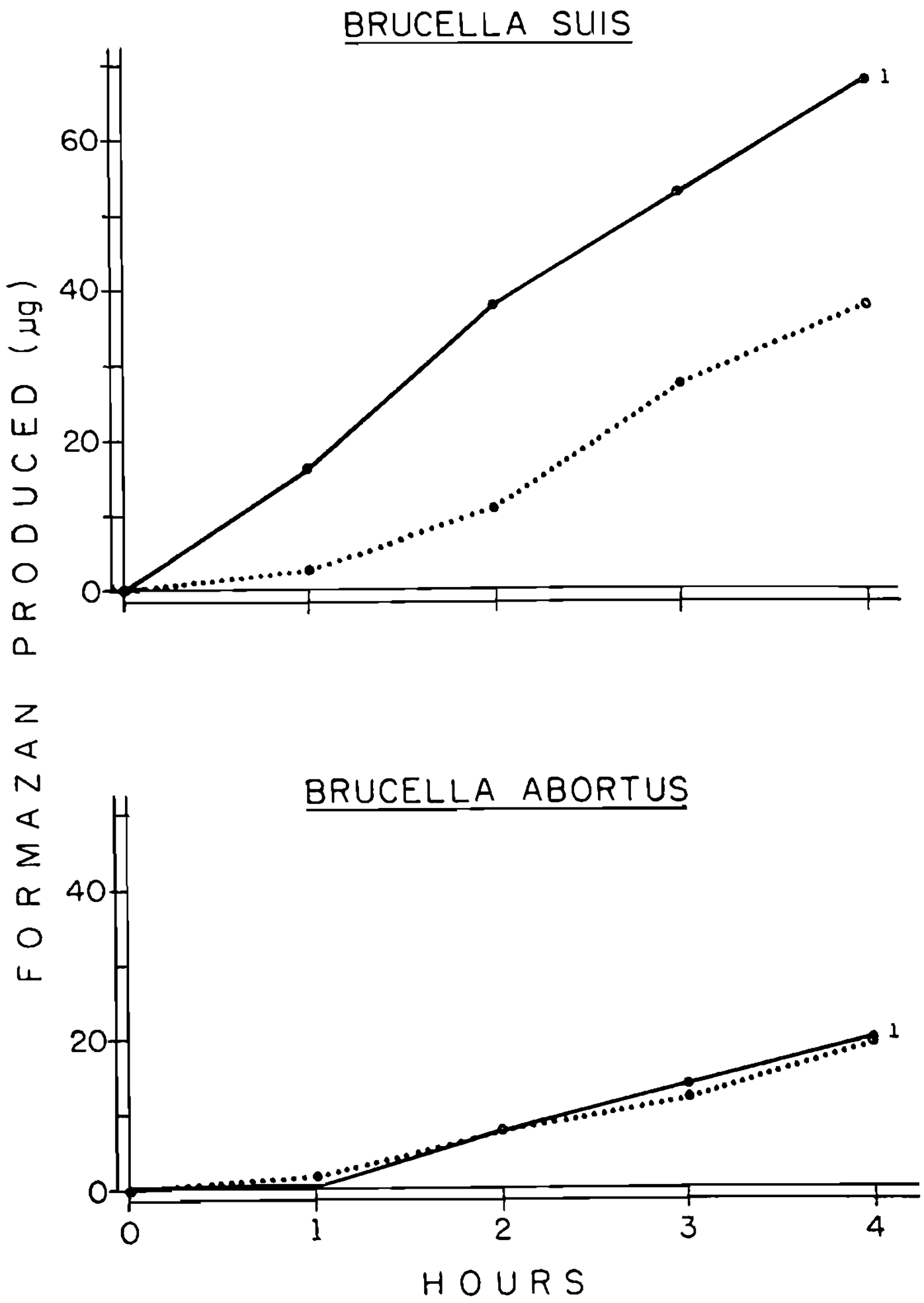

Fig. 4 — Formazan produced by washed cells of *Br. suis* and *Br. abortus* in the presence of: 1 — sodium acetate.
.....: endogenous activity.

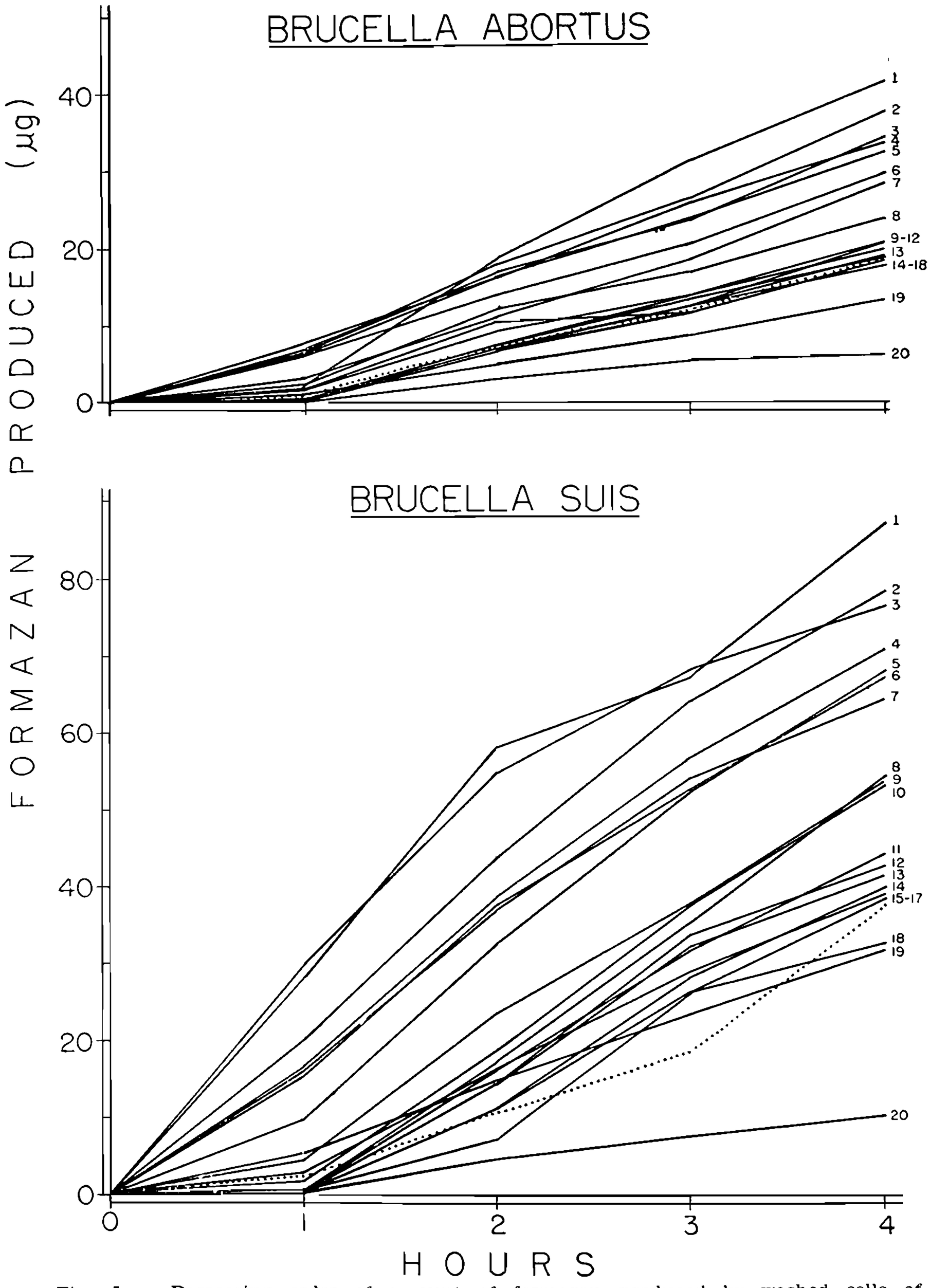

Fig. 5 — Decreasing order of amount of formazan produced by washed cells of *Br. suis* and *Br. abortus* in the presence of 19 single substrates: *Brucella abortus*: 1 — L-arabinose; 2 — D-galactose; 3 — D-glucose; 4 — glycerol; 5 — D-xylose; 6 — DL-alanine; 7 — D-fructose; 8 — D-sorbitol; 9 — glycine; 10 — L-asparagine; 11 — sodium acetate; 12 — maltose; 13 — control; 14 — i-inositol; 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 — i-inositol; — 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**

SUBSTRATE	μg FORMAZAN PRODUCED*							
	Brucella abortus				Brucella suis			
	1 hr	2 hr	3 hr	4 hr	1 hr	2 hr	3 hr	4 hr
Sodium acetate	0.6	7.5	13.8	19.9	15.8	37.6	52.7	67.2
None**	1.1	7.2	12	19.2	2.5	10.8	27	37.5

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).

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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-

drogênio. Os substratos (em solução aquosa M 50) foram os seguintes: Hidratos de carbono: L-arabinose, D-frutose, D-galactose, D-glucose, D--lactose, maltose e D-xilose; alcoóis: glicerol, L-inositol, D-manitol e D-sorbitol; ácidos aminados: ácido D-glutâmico, D-arginina, DL-alanina, L-asparagina e glicina; acetato de sódio.

Empregamos suspensões de culturas de 48 horas de duas amostras típicas: *Brucella abortus* (aeróbia, n.º 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 vêzes 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 prèviamente determinada com formazana pura. Também foram observados os pontos de maior atividade enzimática (provàvelmente mitocôndrias) colocando-se as brucelas, antes da extração da formazana, entre lâmina e lamínula 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.

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