ALANINE SYNTHESIS AND CARBOHYDRATE OXIDATION BY SMOOTH BRUCELLA ABORTUS

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Although the metabolism of many bacterial species has been studied intensively, adequate knowledge of the metabolic activity of pathogenic organisms, including *Brucella*, is lacking. The biochemistry and physiology of *Brucella* recently have been summarized by Hoyer (1950).

Goodlow *et al.* (1950) have demonstrated a direct correlation between alanine production and the establishment of nonsmooth variants in originally smooth populations of *Brucella* spp. This finding has led to a study of the mechanism by which alanine is synthesized in *Brucella*. The elucidation of these metabolic processes is of considerable importance in relation to population phenomena involving changes in colony type, antigen production, and loss of virulence.

MATERIALS AND METHODS

A smooth culture of *Brucella abortus*, strain 19, was employed exclusively in this study. Stock cultures were maintained on tryptose agar fortified with 10 g dextrose, 10 mg FeSO₄, and 0.1 mg thiamine HCl per liter and adjusted to pH 7.0 to 7.4.

All experiments were performed on cells that were grown for 48 hours at 37 C on Albimi *Brucella* agar, harvested, washed with and resuspended in 0.1 M phosphate buffer, pH 7.4. Total nitrogen of the suspensions was determined by the (semimicro) method of Johnson (1941). Solutions of all substrates were adjusted to pH 7.4 before use. The incubation temperature for all the experiments was 37 C. Anaerobic incubations were done in evacuated Thunberg tubes. Amino acids were determined quantitatively by filter paper partition chromatography using a phenol-water solvent system (Housewright and Thorne, 1950).

EXPERIMENTAL RESULTS

Two well recognized methods by which alanine may arise are transamination and reductive amination. It was found that *B. abortus*, strain 19, contains a highly active glutamic-alanine transaminase (table 1) thus confirming the work of Cutinelli (1945).

Direct amination also occurs but only to a limited extent. The exact site of incorporation of ammonium ions is unknown; however, utilization does occur and is reflected in increased alanine production (table 2).

The rate of transamination has been determined for resting cell suspensions. Over the first eight hours of incubation the $Q_{\text{transaminase}}$ (N) was 6.54, a value which is commensurate with the observed growth rate of this organism but which is low when compared with the results obtained from whole cells of some saprophytic bacteria (Lichstein and Cohen, 1945).

Several attempts to demonstrate direct amination failed when the suspensions were shaken at 37 C. A study of the relationship of aeration to alanine production showed that neither transamination nor direct amination could be demonstrated under conditions of vigorous aeration (table 3). Both glutamic acid and pyruvic acid are rapidly oxidized. In addition, aerated suspensions of *B. abortus* were able to oxidize 1 mg of DL-alanine per ml within 18 to 24 hours, substantiating the work of Gerhardt (1949).

TABLE 1
Glutamic-alanine transaminase in smooth Brucella abortus

SUBSTRATE IN MICROMOLES	PRODUCTS IN MICROMOLES	
	Alanine	Glutamic acid
L(+)-glutamic acid—500 + Na pyruvate—500.	167	359
L(+)-glutamic acid—500.....	19.7	344
Na pyruvate—500.....	62.6	0
No substrate.....	trace	0

1.73 mg nitrogen per ml. 10 ml total volume. pH 7.4. Incubated anaerobically in Thunberg tubes for 24 hours at 37 C. In this and subsequent tables, amounts of amino acids recovered are calculated for 10 ml of filtrate. Removal of cells by filtration reduces total volume below 10 ml and often results in recoveries greater than 100 per cent of theoretical.

TABLE 2
Direct amination in smooth Brucella abortus

SUBSTRATE IN MICROMOLES	MICROMOLES OF ALANINE SYNTHESIZED
Na pyruvate—500 + $(\text{NH}_4)_2\text{SO}_4$ —500.....	35.7
Na pyruvate—500.....	18.5
No substrate.....	trace

1.8 mg nitrogen per ml. 10 ml total volume. pH 7.4. Each bottle contained: 200 mg MgSO_4 , 1 mg FeSO_4 , 1 mg MnSO_4 , 1 mg thiamin, 1 mg niacin, 0.1 mg Ca pantothenate, 0.01 mg biotin. pH 7.4. Incubated aerobically, without shaking, for 24 hours at 37 C.

Data have appeared which indicate that pyrophosphate salts suppress the establishment of nonsmooth variants in originally smooth *Brucella* populations (Cole and Braun, 1950). It also appears that manganous ion is capable of antagonizing the suppressive effect of pyrophosphate ion. However, these ions were found to have no detectable effect on the production of alanine by transamination (table 4).

The experiments of Goodlow *et al.* (1950) were conducted in Gerhardt-Wilson synthetic medium. Exclusive of vitamins, the sole organic substances present are DL-asparagine, lactic acid, and glycerol.

Aerobically, with L(+)-glutamic acid as a source of amino groups, only traces of alanine are formed from lactic acid or glycerol. Evidently, neither lactic acid nor glycerol is rapidly converted to pyruvate and thence to alanine under these

conditions. Permeability and other factors involved are unknown and prevent any further interpretation of these data.

TABLE 3

The relation of degree of aeration to alanine synthesis by transamination and reductive amination

CONDITIONS OF AERATION	SUBSTRATES	MG OF NITROGEN PER ML OF CELL SUSPENSION	MICROMOLES OF ALANINE SYNTHESIZED
1. Aerobic-shaken	L-glutamic acid + Na pyruvate	1.05	0
	(NH ₄) ₂ SO ₄ + Na pyruvate	1.80	0
2. Aerobic-static	L-glutamic acid + Na pyruvate	1.05	104
	(NH ₄) ₂ SO ₄ + Na pyruvate	1.80	35.8
3. Anaerobic	L-glutamic acid + Na pyruvate	1.77	166.0

0.5 millimole of each substrate in a total volume of 10 ml. pH 7.4. Incubation at 37 C for 24 hours. Experiments with Na pyruvate and (NH₄)₂SO₄ as substrate contained the same vitamins and salts in the same amounts as in table 2.

TABLE 4

Effect of manganous and pyrophosphate ions on transamination in smooth Brucella abortus

SUBSTRATE	MICROMOLES OF ALANINE SYNTHESIZED
L(+)glutamic acid—500 μ M Na pyruvate—500 μ M	146.6
L(+)glutamic acid—500 μ M Na pyruvate—500 μ M Na ₄ P ₂ O ₇ —0.3 mg	150.8
L(+)glutamic acid—500 μ M Na pyruvate—500 μ M Na ₄ P ₂ O ₇ —0.3 mg MnSO ₄ —0.2 mg	144.1

1.62 mg nitrogen per ml. 10 ml total volume. pH 7.4. Na₄P₂O₇ and MnSO₄ are ionic equivalents. Incubated anaerobically in Thunberg tubes for 24 hours at 37 C.

L-Asparagine alone can give rise to alanine. When both L-asparagine and α -ketoglutarate are used as substrates, large amounts of glutamic acid, aspartic acid, and alanine are formed. A system containing L-asparagine and α -ketoglutaric acid seems to be more favorable for alanine production than a system containing α -ketoglutaric acid and an equivalent amount of nitrogen as L-aspartic acid plus either ammonium sulfate or ammonium chloride. The total amount of primary amino nitrogen recovered as ninhydrin positive material

was equal, within probable experimental error, to the primary amino nitrogen added regardless of the amount of nitrogen present as amide or as ammonium ion. A catalytic amount of α -ketoglutarate does not serve as a "sparker" to initiate the synthetic mechanism. In addition, the production of alanine from L-asparagine is unaffected by manganous or pyrophosphate ions. The data supporting these conclusions are given in table 5. It may be seen from this table that there is present an active aspartic-glutamic transaminase as well as the

TABLE 5
Synthesis of alanine by smooth Brucella abortus from various substrates

SUBSTRATE	PRODUCTS IN MICROMOLES		
	Alanine	Glutamic acid	Aspartic acid
L(+)-asparagine—500 μ M	21.9	0	360
L(+)-asparagine—500 μ M α -ketoglutaric acid—500 μ M	181.5	354	42.7
L-aspartic acid—500 μ M α -ketoglutaric acid—500 μ M (NH ₄) ₂ SO ₄ —250 μ M	136.0	354	71.5
L-aspartic acid—500 μ M (NH ₄) ₂ SO ₄ —250 μ M	27.4	0	457
L-asparagine—500 μ M α -ketoglutaric acid—200 μ g	25.8	0	400
L-asparagine—500 μ M Na ₄ P ₂ O ₇ —0.3 mg	25.9	0	428
L-asparagine—500 μ M Na ₄ P ₂ O ₇ —0.3 mg MnSO ₄ —0.2 mg	24.7	0	469

1.45 mg nitrogen per ml. 10 ml total volume. pH 7.4. Incubated anaerobically in Thunberg tubes for 72 hours at 37 C.

glutamic-alanine transamination system. The failures of another investigator (Gerhardt, 1949) to detect aspartic-glutamic transaminase in this organism may stem from the vigorous oxygenation of the test suspensions. Under such conditions, α -ketoglutarate would probably be removed by oxidation at such a rate as to prevent its participation in transamination.

The sequence of appearance of amino acids during alanine synthesis was determined in a system containing L-asparagine and α -ketoglutaric acid. The initial reactions of (1) hydrolysis of asparagine to aspartic acid and (2) transamination between aspartate and α -ketoglutarate are extremely rapid. Both aspartate and glutamate appear after 30 to 60 minutes of incubation. Quantitative data on the sequence of appearance of these metabolites are presented in

table 6. These results show that glutamate builds up rapidly and that alanine formation lags behind glutamate formation. The concentration of aspartate remains relatively constant throughout. Although quantitative data for asparagine are not available, visual inspection of the chromatograms showed a

TABLE 6
Sequence of appearance of amino acids synthesized by smooth Brucella abortus

AMINO ACID	MICROMOLES PER ML PRODUCED IN				
	2 hr	4 hr	8 hr	16 hr	24 hr
Alanine.....	2.92	3.34	8.61	14.0	16.4
Glutamic acid.....	6.5	9.4	18.4	25.9	36.8
Aspartic acid.....	6.7	5.7	7.9	6.7	9.3

1.34 mg nitrogen per ml. 20 ml total volume. pH 7.4. The substrates were 1 millimole each of L-asparagine and α -ketoglutaric acid. Incubated anaerobically in Thunberg tubes at 37 C.

TABLE 7
Production of amino acids from various substrates by smooth Brucella abortus

SUBSTRATE	HOURS OF INCUBATION	MICROMOLES OF PRODUCTS		
		Alanine	Glutamic acid	Aspartic acid
L(+)glutamic acid—500 μ M	24	34.4	408	0
	48	63.7	505	0
	72	53.2	553	0
L(+)glutamic acid—500 μ M succinic acid—500 μ M	24	44	426	trace
	48	42.9	515	trace
	72	trace	576	trace
L(+)glutamic acid—500 μ M fumaric acid—500 μ M	24	55.7	456	trace
	48	96.7	505	trace
	72	76.8	560	trace
L(+)glutamic acid—500 μ M L-malic acid—500 μ M	24	trace	398	trace
	48	51	461	trace
	72	trace	492	trace

1.5 mg nitrogen per ml. 10 ml total volume. pH 7.4. Incubated aerobically, without shaking, at 37 C in milk dilution bottles.

marked decrease in asparagine concentration as time progressed, reaching zero after 24 hours of incubation.

Several intermediates of the oxidative cycle were used in conjunction with an amino donor, either glutamate or aspartate, to test the ability of this organism to oxidize such intermediates to compounds which could participate in transamination. These data are shown in table 7. The appearance of aspartic acid whenever substrates consisted of glutamic acid and a C₄ dicarboxylic acid

indicates that the C₄ compound has been oxidized to oxalacetate and that transamination or direct amination had occurred to form aspartate. However,

TABLE 8
Oxidation of carbohydrates and production of alanine by Brucella abortus

INITIAL SUBSTRATE	PRODUCTS IN MICROMOLES		
	Alanine	Glutamic acid	Aspartic acid
<i>Cis</i> -aconitic acid—250 μ M Na ₂ HAsO ₄ —M/30	58.4	54.1	400
<i>Cis</i> -aconitic acid—250 μ M	17.5	52.3	434
Na ₂ HAsO ₄ —M/30	48.4	0	431
None	0	0	448
Succinic acid—500 μ M Na ₂ HAsO ₄ —M/30	trace	0	536
Succinic acid—500 μ M	0	0	536
Fumaric acid—500 μ M Na ₂ HAsO ₄ —M/30	91.0	0	495
Fumaric acid—500 μ M	46.2	0	495
L-malic acid—500 μ M Na ₂ HAsO ₄ —M/30	88.1	0	495
L-malic acid—500 μ M	74.2	0	458
<i>Iso</i> -citric acid—500 μ M Na ₂ HAsO ₄ —M/30	68.0	trace	440
<i>Iso</i> -citric acid—500 μ M	trace	trace	495
Citric acid—500 μ M Na ₂ HAsO ₄ —M/30	114	81.0	398
Citric acid—500 μ M	trace	78.7	433
Na ₂ HAsO ₄ —M/30	43.9	0	480
None	0	0	480

1.29 mg nitrogen per ml in the initial inoculum. Total volume 10 ml. Shaken for 18 hours at 37 C. The secondary substrate, 500 μ M L-asparagine, was incubated with fresh cells in evacuated Thunberg tubes 48 hours at 37 C.

the factors of concentration by evaporation, substrate permeability, etc., complicate interpretation.

Since transamination mechanisms were demonstrated, it was of interest to determine the oxidation of proven intermediates of the tricarboxylic acid cycle to α -ketoglutaric acid, and the oxidation of C_4 compounds to pyruvate. A two step procedure was adopted for an investigation of this point. As a first step, the cell suspension was incubated with the particular compound involved (initial substrate) for 18 to 24 hours under conditions of vigorous aeration (shaking machine). Arsenite ion was added to prevent further oxidation of any α -keto compounds formed. After initial incubation the suspension was filtered through sintered glass to remove the bacteria. L-Asparagine (secondary substrate) was added to the filtrate and dissolved. This solution was then added to freshly grown *B. abortus* cells and diluted to 10 ml with $M/10$ phosphate buffer, pH 7.4. This suspension was incubated anaerobically for 24 to 48 hours. Therefore, any α -ketoglutaric acid or pyruvic acid that might have been formed from oxidation of primary substrates would participate in transamination or direct amination and would appear as glutamic acid or alanine, respectively. Control experiments showed that arsenite produces no inhibition of glutamic acid synthesis and only 30 per cent inhibition of alanine formation from L-asparagine and α -ketoglutaric acid. The results of several experiments are presented in table 8. It is readily apparent that the tricarboxylic acids are oxidized to α -ketoglutarate. In addition, the greater yield of alanine found in arsenite-treated filtrates demonstrates an accumulation of pyruvate (from residual cell materials?), as previously recorded for *Escherichia coli* by Ajl (1950). The initial incubation under vigorous aeration was insufficient to oxidize all the initial substrate past the C_5 state in the absence of arsenite; but the pyruvate available was much less. The results obtained with C_4 compounds demonstrated their conversion to pyruvate yet gave no indication that $C_2 + C_1$ condensations were occurring to a measurable degree. However, it is certain that this organism will oxidize tricarboxylic acid intermediates if they are provided.

DISCUSSION

The presence of highly efficient transamination systems suggests that they comprise an important synthetic mechanism in *Brucella abortus*, strain 19. It appears that *one* route of alanine synthesis proceeds via two transamination steps. An over-all picture of the process of alanine synthesis from L-asparagine and α -ketoglutarate seems to be as follows: (1) hydrolysis of L-asparagine to aspartic acid, (2) transamination between aspartate and α -ketoglutarate to produce glutamate and oxalacetate, (3) formation of pyruvate from oxalacetate, presumably by the loss of CO_2 , and (4) transamination between glutamate and pyruvate to produce alanine. The relative importance of transamination and of reductive amination in synthesis of alanine under normal growth conditions remains undetermined.

The anaerobic conditions of growth and of the resting cell suspension experiments severely limit both the oxidative activity of the cells and the generation of α -ketoglutarate. The study of Still *et al.* (1950) of the oxidation of L-alanine by the cyclophorase system showed that α -ketoglutarate or glutamate

accelerated alanine oxidation. If α -ketoglutarate was regenerated rapidly by *B. abortus*, a small amount of this compound should act as a "sparker" and initiate rapid synthesis of alanine. This did not occur and constitutes evidence that the mechanisms producing α -ketoglutarate are poorly operative under the conditions of resting cell experiments. It is therefore probable that, in *B. abortus*, strain 19, alanine synthesis from asparagine proceeds as stated, but is limited in these experiments by the available α -ketoglutarate.

Recent investigations (Dulberg *et al.*, 1950) have indicated that anaerobic glycolysis by *B. abortus*, strain 19, parallels the degradation sequence discovered in animal tissues. The data presented in this paper are evidence that the oxidative phase of carbohydrate utilization likewise conforms to classic pathways. The extensive occurrence of the tricarboxylic acid cycle of carbohydrate oxidation in bacterial cells has not been demonstrated. In fact, evidence has appeared that indicates that Krebs' cycle may not operate at all in *Azotobacter agilis* (Karlsson and Barker, 1948) or may constitute an adaptive phenomenon in the coli-aerogenes group (Ajl, 1950). If oxidation of Krebs' intermediates by *B. abortus* is adaptive, the complex medium usually employed for growth would ensure adaptation. Oxidation of citric acid, *cis*-aconitic acid, and isocitric acid to α -ketoglutarate does occur and indicates that cells of this organism are capable of metabolizing carbohydrates via the Krebs' cycle.

An effort was made to correlate the effect of pyrophosphate and manganous ions upon variation with the synthesis of alanine. No stimulatory or inhibitory effect upon alanine production by these ions was observed under the specific experimental conditions described.

SUMMARY

Smooth *Brucella abortus*, strain 19, possesses both aspartic-glutamic and glutamic-alanine transaminases. In resting cell suspensions alanine is synthesized from simple substrates both by transamination mechanisms and by direct or reductive amination. The relationship of the degree of aeration to alanine synthesis has been described. The effectiveness of various substrates for production of alanine is recorded and discussed, and a mechanism for synthesis of this amino acid from simple substrates is proposed.

By means of an enzymatic inhibitor, arsenite ion, this organism has been shown to possess the necessary enzymes for oxidation of tricarboxylic acid intermediates of the Krebs' cycle. Other evidence has been presented to illustrate that carbohydrate utilization proceeds via pathways established in other cells.

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