THE OXIDATIVE DISSIMILATION OF AMINO ACIDS AND RELATED COMPOUNDS BY BRUCELLA ABORTUS[1]

PHILIPP GERHARDT,[2] H. B. LEVINE,[3] AND J. B. WILSON

Department of Agricultural Bacteriology, University of Wisconsin, Madison, Wisconsin

Received for publication July 10, 1950

The enzyme systems of brucellae that are active in the dissimilation of amino acids have been investigated by Kien-Hun and Kan (1935), Steinbach (1940), and Mannozzi-Torini and Vendramini (1941*a*,*b*). The purpose of the present study, extending the findings of these workers, was to determine quantitatively which amino acids and related compounds are oxidatively metabolized by these organisms and to characterize certain of the reactions in terms of stereospecificity, substrate concentration effects, pH optima, and over-all stoichiometric ratios.

EXPERIMENTAL METHODS

Strain 19 of *Brucella abortus* was employed for the greater part of the experimental work because of its importance as a living vaccine for the immunization of cattle and its comparative safety for laboratory investigation. Unless otherwise stated, the data and discussion refer to this culture. Other strains of *B. abortus*, including some of relatively high virulence for the guinea pig and some requiring increased carbon dioxide for growth, were employed for comparisons.

Resting cell suspensions were prepared by growing the organism on glucose tryptose agar for 24 hours at 35 to 37 C. The cells were suspended in buffered saline (0.85 per cent NaCl in pH 6.8, M/15 phosphate buffer solution), centrifuged and resuspended twice, and finally adjusted to a turbidity such that 1 ml contained approximately 400 μg of cell nitrogen. Cells prepared in this manner may be stored in the refrigerator for a week without appreciable loss in activity.

The activity of resting cell suspensions was measured by conventional Warburg techniques (cf. Umbreit, Burris, and Stauffer, 1949) at 34 $\pm$ 0.1 C. One ml of cell suspension was used in each flask; total flask contents were 3.2 ml. Unless stated otherwise, reaction mixtures were at pH 6.8. The rate of oxygen consumption was expressed as $Q_{O_2}(N)$, i.e., the μl of oxygen consumed per mg cell nitrogen per hour in an air atmosphere. The evolution of carbon dioxide was determined by the "direct method," using a flask having three side arms with two in Siamese form. In the calculation of moles of gas exchange per mole of substrate

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This paper is based upon work sponsored in part by the Biological Division, Chemical Corps, Camp Detrick, Frederick, Maryland, under Contract No. W-18-064-CM-208 with the University of Wisconsin.

[2] National Institutes of Health Predoctorate Research Fellow. Present address: Department of Bacteriology, Oregon State College, Corvallis, Oregon.

[3] Present address: Office of Naval Research Task V, Department of Bacteriology, University of California, Berkeley, California.

utilized, correction was made for the endogenous activity; substrate utilization was assumed to be complete when the rate of oxygen uptake returned to approximately that of the endogenous. All determinations were in duplicate.

Cellular nitrogen analyses were made by a modification of the method of Johnson (1941). The production of ammonia from substrates by the organism was determined by preparing resting cell suspensions in the same manner as for the Warburg experiments, incubating 1.0 ml of the suspension with substrate and buffer at pH 6.8 and 34 C, stopping the reaction after 2 or 3 hours by the addition of trichloroacetic acid, and analyzing for ammonia by conventional aeration and Nesslerization procedures.

When highly virulent strains of *Brucella* were employed, a glass coupling assembly containing a cotton plug was used between the Warburg flask and manometer arm to control contamination of the manometer and to prevent dispersion of an aerosol. The coupling and flask could be removed together for sterilization before disassembly.

EXPERIMENTAL RESULTS

A number of amino acids and related nitrogen compounds first were surveyed to determine quantitatively the relative rates of oxidation and the amounts of ammonia produced by strain 19 of *B. abortus*. Representative data, which are given in table 1, show that the rate of oxygen uptake with glutamic acid was considerably greater than with any of the other compounds examined. Asparagine and alanine were oxidized at successively lesser rates. Oxidation rates for the remaining nitrogen compounds approximated that of the endogenous. The relative rates of oxidation given in table 1 were confirmed in replicate experiments. Thus, in the case of the three compounds metabolized at appreciable rates, $Q_{O_2}(N)$ values for M/300 L-glutamic acid over 18 comparable experiments varied from 508(42)[4] to 773(55) with a mean of 658(57); those for M/300 L-asparagine over 8 experiments varied from 196(54) to 313(68) with a mean of 258(60); those for M/150 DL-alanine over 4 experiments varied from 81(52) to 130(39) with a mean of 103(52). The negligible oxidation of aspartic acid as compared to the rapid oxidation of asparagine was paradoxical. Comparably slow rates of oxidation—$Q_{O_2}(N)$ of 85 to 98(83)—also were found when L-aspartic acid from different sources, DL-aspartic acid, and aspartic acid plus ammonium sulfate were compared.

The relative amounts of ammonia produced from these nitrogen compounds by strain 19 of *B. abortus* also are included in table 1. As in the case of oxygen consumption, markedly greater deamination or desamidization occurred with glutamic acid, asparagine, and alanine. With the possible exception of serine, ammonia production from the other compounds was relatively low or negligible. Although the data of table 1 suggested that ammonia was produced enzymatically from glutamine, controls proved that ammonia was released from glutamine in the absence of cells during the ammonia analysis. Neither asparagine nor

[4] The value in parenthesis indicates the endogenous $Q_{O_2}(N)$; this designation will be followed in the subsequent text and figures.

TABLE 1

Comparative rates of oxidative dissimilation of amino acids and related compounds by strain 19 of Brucella abortus

SUBSTRATE	$Q_{O_2}(N)$*	$\mu M\ NH_3$†
M/300 L-alanine	127(40)	
M/300 D-alanine	91(40)	
M/300 DL-alanine	115(40)	
M/150 DL-alanine	130(40)	2.88
M/300 *beta*-alanine	77(68)	0.16
M/150 DL-*alpha*-aminobutyric acid	76(82)	
M/300 L-asparagine	260(57)	7.07
M/300 D-asparagine	66(57)	
M/300 DL-asparagine	130(57)	
M/150 DL-asparagine	130(57)	
M/300 L-aspartic acid	59(54)	0.31
M/300 L-arginine	66(54)	0.36
M/300 L-cystine	59(54)	0.00
M/300 L-glutamic acid	646(49)	5.89
M/300 D-glutamic acid	100(49)	
M/300 DL-glutamic acid	498(49)	
M/150 DL-glutamic acid	510(49)	
M/300 L-glutamine	56(54)	7.02
M/300 glutathione	95(83)	0.26
M/300 D-glutamyl-polypeptide‡	56(51)	
M/300 glycine	47(54)	0.77
M/300 L-histidine	66(54)	0.36
M/300 hydroxy-L-proline	110(83)	0.36
M/150 DL-isoleucine	49(51)	0.11
M/300 L-leucine	49(51)	0.29
M/300 L-lysine	61(51)	0.11
M/300 L-methionine	49(51)	0.51
M/150 DL-norleucine	68(83)	0.60
M/150 DL-ornithine	74(83)	0.32
M/300 L-phenylalanine	51(54)	0.44
M/300 L-proline	110(83)	0.51
M/150 DL-serine	64(51)	1.27
M/150 DL-threonine	56(51)	0.12
M/300 L-tryptophan	54(54)	0.10
M/300 L-tyrosine	59(54)	0.00
M/150 DL-valine	56(51)	0.26
M/300 acetic acid	200(79)§	
M/300 *alpha*-ketoglutaric acid	167(82)	
M/300 citric acid	85(79)	
M/300 fumaric acid	96(82)	

* Control endogenous rates are given in parentheses.

† Ammonia determinations were made after 3 hours' incubation on shaker, all values being corrected for ammonia produced in the absence of substrate. Each tube contained 1.0 ml (0.48 mg N) of cell suspension and 3.0 ml total volume.

‡ Prepared from capsular material of *Bacillus subtilis* by R. J. Heckly of the University of Wisconsin. A hydrochloric acid hydrolyzate of the compound was oxidized slowly by the organism.

§ Rate is not constant. Figure given is average over initial 80-minute period after tipping in substrate.

TABLE 1—*Continued*

SUBSTRATE	$Q_{O_2}(N)$*	μM NH_3†
M/300 glutaric acid	82(82)	
M/300 isocitric acid	350(79)	
M/300 lactic acid	146(63)	
M/150 maleic acid	61(61)	
M/300 L-malic acid	406(82)§	
M/300 malonic acid	82(82)	
M/300 oxalacetic acid	119(82)	
M/150 oxalic acid	70(67)	
M/300 pyruvic acid	116(79)	
M/300 succinic acid	333(82)	

glutamic acid was so affected. Ammonia production by strain 19 from glutamic acid, asparagine, or alanine did not occur anaerobically to an appreciable extent, as shown by the data of table 2.

Because of their significance as possible intermediates in the dissimilation of glutamic acid, asparagine, or alanine, a number of related organic acids were compared for their relative rates of oxidation by strain 19 of *B. abortus*. Representative data for the study are included in table 1. L-Malic, isocitric, and succinic acids were oxidized most rapidly, whereas acetic, lactic, *alpha*-ketoglutaric, pyruvic, oxalacetic, and fumaric acids were oxidized at successively lower rates. The low rate of oxidation of *alpha*-ketoglutaric acid (i.e., a low rate in view of the rapid oxidation of its possible precursor, glutamic acid) could be increased by increasing the concentration of substrate. Several preparations of *alpha*-ketoglutaric acid were used and the presence of contaminating material in these preparations was considered minimal. Use of M/300 *alpha*-ketoglutaric acid and M/300 L-aspartic acid together resulted in only slightly greater oxygen uptake—Q_{O_2}(N) of 150(62)—than use of either compound alone—Q_{O_2}(N) of 122(62) for *alpha*-ketoglutaric acid and 85(62) for L-aspartic acid. This finding suggested that a transaminase system was not active under the experimental conditions used.

Certain of the results obtained with strain 19 were studied further using other strains of *B. abortus*, partially for confirmation of results and also for possible metabolic differences between virulent and avirulent strains. The highly virulent strain 2308 of *B. abortus* was found to have oxidative activity (or lack of activity) comparable to that of strain 19 with the following representative compounds: L-alanine and L-aspartic, formic, acetic, propionic, butyric, citric, lactic, and *alpha*-ketoglutaric acids. A similarity of activity between strains 19 and 2308 also was found to occur in the presence of five sugars (glucose, maltose, lactose, mannose, and fructose) and six alcohols (methanol, ethanol, propanol, isopropanol, butanol, and glycerol). A comparison of strain 19 with nine other strains (including strain 2308) of high and low virulence was made using L-glutamic acid as the substrate. Although the oxidative rates varied from a low Q_{O_2}(N) of 151(50) for strain 2308 upward to a mean value of 658(57) for strain 19, a negative corre-

TABLE 2

Production of ammonia from L*-glutamic acid,* L*-asparagine, and* DL*-alanine by strain 19 of Brucella abortus in the presence and absence of oxygen*

ATMOSPHERE	SUBSTRATE	μM NH_3*	
		Substrate equivalent	Recovery
Air	M/300 L-glutamic acid	10.0	5.48
	M/300 L-asparagine	20.0	6.93
	M/150 DL-alanine	20.0	1.45
Oxygen-free nitrogen	M/300 L-glutamic acid	10.0	0.13
	M/300 L-asparagine	20.0	0.49
	M/150 DL-alanine	20.0	0.20

* Ammonia determinations were made after 2 hours' static incubation, all values being corrected for ammonia produced in the absence of substrate. Each tube contained 1.0 ml (0.40 mg N) of cell suspension and 3.0 ml total volume.

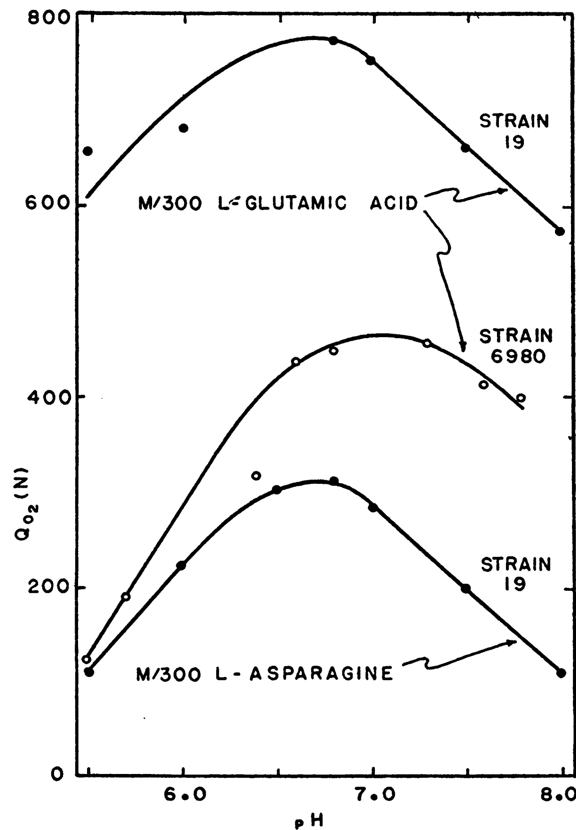

Figure 1. The effect of pH on the rate of oxidation of L-glutamic acid by strains 19 and 6980 and of L-asparagine by strain 19 of *Brucella abortus*.

lation was observed between metabolic activity and the relative degree of virulence among the various strains.

The stereospecificity of oxidation of amino acids by strain 19 of *B. abortus* was studied for those reactions in which an appreciable rate of activity occurred, i.e., the oxidation of glutamic acid, asparagine, and alanine. Representative data for the study are included in table 1. Whereas L-glutamic acid and L-asparagine were oxidized rapidly, relatively slight activity was obtained with the D-isomers. The intermediate rates of oxidation of the racemic mixtures indicated that the

TABLE 3

Stoichiometric ratios for the dissimilation of L-glutamic acid and L-asparagine by strains of Brucella abortus

DETERMINATION*	NON-CO_2-REQUIRING STRAINS		CO_2-REQUIRING STRAINS	
	19	6980	114277	24331
Moles O_2 consumed per mole L-glutamic acid	1.76	2.45	1.64	2.20
Moles CO_2 evolved per mole L-glutamic acid	2.60	3.31	2.36	3.03
Moles NH_3 evolved per mole L-glutamic acid	0.72	0.68		
Respiratory quotient	1.48	1.35	1.44	1.38
$Q_{O_2}(N)$†	608(64)	505(45)	183(86)	
Moles O_2 consumed per mole L-asparagine	0.79			
Moles CO_2 evolved per mole L-asparagine	1.88			
Moles NH_3 evolved per mole L-asparagine	1.73			
Respiratory quotient	2.39			
$Q_{O_2}(N)$†	184(75)			

* L-Glutamic acid was in M/600, L-asparagine in M/1,200 final concentration. Values given are for single representative experiments.

† Control endogenous *rates* are given in parentheses; all other quantitative ratios are corrected for endogenous values.

D-amino acids inhibited the oxidation of the natural isomers. When the ratio of D- to L-glutamic acid was increased to 4 to 1, the rate of oxidation was considerably lower than with the racemic mixture. Doubling the concentration of the racemic mixtures or replacing them with equal proportions of the separate enantiomorphs did not alter the comparisons appreciably. In the case of alanine, however, both the L- and the D-isomers apparently were oxidized by the organism. The rate of oxidation of M/300 DL-alanine was intermediate between the rates obtained with M/300 L- and M/300 D-alanine, although the comparison varied with substrate concentration.

The effect of concentration of substrate upon the rate of oxidation by strain 19 of *B. abortus* was studied with L-glutamic acid, L-asparagine, and DL-alanine.

Very little, if any, change in the rate of oxidation was found to occur throughout a concentration range of M/3,000 to M/150 L-glutamic acid or M/3,000 to M/300 L-asparagine. With DL-alanine, however, higher rates of oxidation occurred with an increase in the substrate concentration, and half-maximal activity for the system was reached at a concentration of M/1,200.

The effect of the pH of the system on the rate of oxidation of L-glutamic acid by strains 19 and 6980 and of L-asparagine by strain 19 of *B. abortus* is shown in figure 1. In each case, the optimum occurred at pH 6.7 to 7.1.

Over-all stoichiometric ratios for the gaseous exchange accompanying the dissimilation of L-glutamic acid and L-asparagine were determined for strain 19. Further comparisons of the ratios obtained with strain 19 and with certain other strains of *B. abortus* were made only with L-glutamic acid. A compilation of these data is given in table 3. In a representative experiment with strain 19, 1.8 moles of oxygen were consumed and 2.6 moles of carbon dioxide and 0.72 moles of ammonia were produced per mole of L-glutamic acid utilized. The addition of M/4,000 2,4-dinitrophenol increased the oxygen consumed to 3.1 moles per mole of L-glutamic acid utilized. For each mole of L-asparagine metabolized by strain 19, 0.79 moles of oxygen were consumed and 1.9 moles of carbon dioxide and 1.7 moles of ammonia were evolved. Similar values were obtained in replicate experiments. Comparison of the stoichiometric ratios that were obtained with four different strains revealed a variation in the oxygen and carbon dioxide ratios but a relative constancy in the corresponding respiratory quotients. The evolution of carbon dioxide by both carbon-dioxide-requiring and nonrequiring strains of *B. abortus* was comparable.

DISCUSSION

The ability of strain 19 of *B. abortus* to oxidize and deaminate only glutamic acid, asparagine, and alanine among the amino compounds surveyed was noteworthy, particularly insofar as glutamine and aspartic acid apparently were not metabolized. These results are in general agreement with those of Steinbach (1940) and Mannozzi-Torini and Vendramini (1941*a*,*b*). These and similar examples of selective oxidation possibly may be a manifestation of cell permeability rather than enzymatic specificity, as suggested by Rydon (1948). Rapid deterioration of the enzymes during the preparation of washed cell suspensions also may take place (cf. Gale, 1940), although the difference in activity with asparagine and glutamine, for example, is inconsistent with this possibility. Irrespective of cause, the selective oxidation of certain amino acids by nonproliferating cell suspensions of *B. abortus* is significant; a similar selection occurs when the organism is grown with single amino acids as the nitrogen source (Gerhardt, Tucker, and Wilson, 1950), and this nutritional specificity may be correlated with the respiratory specificity observed in the present experiments. Since the ammonium ion may serve as the sole source of nitrogen for growth (McCullough and Dick, 1943; Gerhardt and Wilson, 1948) and since ammonia is released in the oxidative dissimilation of these amino acids, a function of amino acids in the metabolism of the organism may be simply to serve as an organic source of ammonia, this in turn

serving as the primary nitrogen source for synthetic processes. The residual compound after deamination may (in the case of glutamic acid and alanine) function as a source of energy (Gerhardt, Tucker, and Wilson, 1950) or possibly may be or give rise to an essential metabolite.

The oxidative stereospecificity of the organism for L-asparagine and L-glutamic acid was of less interest than the apparent inhibition of these natural isomers by their optical antipodes. Thus, double- or equimolar concentrations of the racemic mixtures gave lower rates of oxygen uptake than the L-isomers alone. It was not determined whether the inhibition reaction was competitive. Oxidation of the D-isomer of alanine was shown to occur in the present case and similarly has been demonstrated with *Pseudomonas aeruginosa* and *Proteus vulgaris* (Bernheim, Bernheim, and Webster, 1935; Webster and Bernheim, 1936). Rydon (1948) has suggested that the utilization of D-amino acids by bacteria is not so unusual as is commonly supposed.

The comparison of over-all stoichiometric ratios for the dissimilation of glutamic acid by strain 19 and certain other strains of *B. abortus* did not support the probability of a fundamental difference in decarboxylase activity between carbon-dioxide-requiring and nonrequiring strains. The possibility had been postulated to explain the requirement of some strains for an increased atmosphere of carbon dioxide for growth. Among the four strains tested, there existed a considerable variation in the oxygen and carbon dioxide ratios. However, the corresponding respiratory quotients remained relatively constant (1.35 to 1.48), suggesting a basic constancy in the mode of dissimilation of glutamic acid by the four strains. The variation in carbon dioxide and oxygen ratios, the low recoveries of ammonia, the failure to obtain the whole or half-fractional gaseous ratios expected in a reaction of this nature, and the increased oxygen ratio for glutamic acid obtained with 2,4-dinitrophenol together suggested the occurrence of a variable amount of assimilation of the glutamic acid by the different strains.

SUMMARY

Of approximately 27 amino acids and related nitrogen compounds surveyed, only L-glutamic acid, L-asparagine, and D- or L-alanine were oxidized and deaminated at appreciable rates by strain 19 of *Brucella abortus*. Ammonia production from these compounds did not occur anaerobically to a significant extent. Among 14 related organic acids, L-malic, isocitric, and succinic acids were oxidized most rapidly, whereas acetic, lactic, *alpha*-ketoglutaric, pyruvic, oxalacetic, and fumaric acids were oxidized at successively lower rates.

The oxidative activity of the organism for glutamic acid and asparagine apparently was specific for the L-isomers; the D-forms not only were not metabolized but inhibited oxidation of the natural isomers. Both D- and L-alanine were oxidized. Very little change in the rate of oxidation occurred with varying concentrations of L-glutamic acid or L-asparagine, whereas half-maximal activity with DL-alanine was reached at a concentration of M/1,200. The optimal pH for the oxidation of L-glutamic acid or L-asparagine was approximately 6.8. Over-all stoichiometric ratios for the dissimilation of L-glutamic acid and L-asparagine

were determined for strain 19. A comparison was made of the ratios obtained with strain 19 and certain other strains with L-glutamic acid and the significance of the results was discussed.

REFERENCES

Bernheim, F., Bernheim, M. L. C., and Webster, M. D. 1935 Oxidation of certain amino acids by "resting" *Bacillus proteus*. J. Biol. Chem., **110**, 165–172.

Gale, E. F. 1940 Enzymes concerned in the primary utilization of amino acids by bacteria. Bact. Revs., **4**, 135–176.

Gerhardt, P., Tucker, L. A., and Wilson, J. B. 1950 The nutrition of brucellae: utilization of single amino acids for growth. J. Bact., **59**, 777–782.

Gerhardt, P., and Wilson, J. B. 1948 The nutrition of brucellae: growth in simple chemically defined media. J. Bact., **56**, 17–24.

Johnson, M. J. 1941 Isolation and properties of a pure yeast polypeptidase. J. Biol. Chem., **137**, 575–586.

Kien-Hun, B., and Kan, W. 1935 Über die Methylenblaureduktion durch *Bact. abort. infectiosi* Bang und *Bact. melitense*. Z. Hyg. Infektionskrankh., **117**, 399–402.

McCullough, N. B., and Dick, L. A. 1943 Growth of *Brucella* in a simple chemically defined medium. Proc. Soc. Exptl. Biol. Med., **52**, 310–311.

Mannozzi-Torini, M., and Vendramini, R. 1941*a* Studi sul metabolismo delle brucelle. 1. L'ossidazione degli aminoacidi da parte della *Brucella abortus*. Boll. ist. sieroterap. milan., **20**, 4–12.

Mannozzi-Torini, M., and Vendramini, R. 1941*b* Studi sul metabolismo delle brucelle. 2. Ulteriori ricerche sulla ossidazione degli aminoacidi da parte della *Brucella abortus*. Boll. ist. sieroterap. milan., **20**, 13–18.

Rydon, H. N. 1948 D-Amino acids in microbiological chemistry. Biochemical Society Symposia, **1**, 40–60.

Steinbach, K. F. 1940 Investigations into bacterial respiration with special reference to hydrogen donators. Thesis, University of Zurich. J. Hagmann, Dubstrasse 47, Zurich.

Umbreit, W. W., Burris, R. H., and Stauffer, J. P. 1949 Manometric techniques and tissue metabolism. Burgess Publishing Co., Minneapolis.

Webster, M. D., and Bernheim, F. 1936 Oxidation of amino acids by *Bacillus pyocyaneus* (*Pseudomonas aeruginosa*). J. Biol. Chem., **114**, 265–271.