OXIDATIVE DISSIMILATION OF AMINO ACIDS AND RELATED COMPOUNDS BY *SHIGELLA FLEXNERI*[1]

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The development of a synthetic medium for Shigella has been the subject of considerable investigation. Koser *et al.* (1938*a*, 1938*b*), Kligler and Grosowitz (1939), Weil and Black (1944), and Johnson (1954), among others, have reported the successful cultivation of Shigella in chemically-defined synthetic media of variable composition. All of these synthetic media consisted of inorganic salts, glucose, and various combinations of amino acids. It is surprising, therefore, that relatively little information is available concerning the enzyme systems of Shigella that are active in the dissimilation of amino acids, especially those utilized in the above media. This initial study was designed to furnish such information and to afford leads which might enable the formulation of a simpler, more effective synthetic medium. Specifically, the purpose of this study was to determine quantitatively which amino acids and related compounds are oxidatively metabolized by *Shigella flexneri* 3 and to characterize certain of the reactions in terms of stereospecificity, substrate concentration effects, and pH optima.

EXPERIMENTAL METHODS

Strain 1013 of *S. flexneri* 3 was employed throughout the experimental work. The species was selected on the basis of its implication in several widespread outbreaks of shigellosis on naval vessels in the Pacific Ocean and strain 1013 was selected because of its utilization as an immunizing vaccine (Barnes *et al.*, 1949). The strain was biochemically and serologically characteristic of the species and was maintained in a lyophilized state.

Resting cell suspensions were prepared by growing the organism on brain heart infusion agar (Difco) for 18 hr at 37 C. The cells were harvested with buffered saline (0.85 per cent sodium chloride in 0.067 M phosphate buffer, pH 6.8). After centrifuging and washing twice, the cells were resuspended in buffered saline and adjusted to a turbidity, such that 1 ml of cell suspension contained approximately 400 μg of cellular nitrogen. Cells prepared in this manner could be stored in a refrigerator for 5 days without appreciable loss in activity. As a safety factor, cells older than 4 days were not used.

Substrates were dissolved in buffer, adjusted to pH with either concentrated potassium hydroxide or phosphoric acid, and brought to volume. The quantity of substrate, unless otherwise stated, was 5 μmoles per cup.

The activity of resting cell suspensions was measured by conventional Warburg manometric techniques at 37 $\pm$ 0.1 C. One ml of cell suspension was used per flask and reaction mixtures were adjusted to pH 6.8. The rate of oxygen consumption was expressed as $Q_{O_2}(N)$, i. e., the μL of oxygen consumed per mg of cell nitrogen per hr in an air atmosphere. Control endogenous rates were subtracted. Carbon dioxide evolution was determined by the "indirect method" (Umbreit, Burris, and Stauffer, 1949) and results were expressed as $Q_{CO_2}(N)$. All determinations were made in duplicate. Unless specifically noted, rates were constant and linear for at least 80 min. Analyses of cellular nitrogen were made by the method of Johnson (1941). Ammonia production was determined by stopping the reaction at the desired time by addition of trichloroacetic acid to the reaction mixture and analyzing for ammonia by aeration and Nesslerization procedures.

RESULTS

A representative series of amino acids and related nitrogen compounds was surveyed to determine quantitatively the relative rates of oxidation and the amounts of ammonia produced by *S. flexneri* 3. The data presented in table 1 indi-

[1] The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

cate that oxygen uptake with asparagine, aspartate, and glutamate is considerably greater than with any of the other compounds tested. Serine, alanine, glutamine, glycyl-glycyl-glycine, glycylglycine, and threonine were oxidized at significant, but successively lesser, rates. Oxidation rates with the remaining nitrogen compounds were approximately equal to or slightly greater than the endogenous respiration. Also presented in table 1 are the amounts of carbon dioxide liberated during the oxidative dissimilation process; the calculated respiratory quotients usually were 1.0 or more. As in the case of oxygen consumption and carbon dioxide evolution, markedly greater ammonia formation occurred with asparagine, aspartic acid, threonine, serine, glutamine, glutamic acid and alanine. Significant but lesser amounts of ammonia were produced from glycine, glycyl-glycine, glycyl-glycyl-glycine, cystine and cysteine. Ammonia production from other compounds was negligible.

TABLE 1

Comparative rates of oxidative dissimilation of amino acids and related compounds by Shigella flexneri 3

Substrate	Q_{O_2} (N)	Q_{CO_2}(N)	R.Q.	μmoles NH_3*
D-α-Alanine	184	166	0.90	1.8
L-α-Alanine	208	214	1.03	2.2
DL-α-Alanine	222	201	0.91	2.2
β-Alanine	0	0	—	0
L-Arginine	0	0	—	0
D-Asparagine	26	12	0.46	1.4
L-Asparagine	926	1271	1.37	10.3
DL-Asparagine	732	1106	1.51	9.9
D-Aspartic acid	18	12	0.67	0
L-Aspartic acid	644	925	1.44	4.9
DL-Aspartic acid	446	605	1.36	2.6
L-Cystine	16	22	1.38	0.9
DL-Cystine	16	22	1.38	0.9
L-Cysteine	65	106	1.63	1.7
L-Glutamic acid	363	342	0.94	1.8
DL-Glutamic acid	311	301	0.97	1.5
DL-Glutamine	165	187	1.13	2.6
Glutaric acid	31	22	0.71	0
Glutathione	41	43	1.05	0
Glycine	49	62	1.27	2.5
Glycyl-glycine	166	223	1.34	5.4
Glycyl-glycyl-glycine	121	133	1.10	2.4
Histamine	0	0	—	0
L-Histidine	0	0	—	0
DL-Histidine	0	0	—	0
DL-Homocystine	0	0	—	0
Hydroxy-L-proline	0	0	—	0
DL-Isoleucine	0	0	—	0
D-Leucine	0	0	—	0
L-Leucine	40	53	1.29	0
DL-Leucine	0	0	—	0
L-Lysine	0	0	—	0
DL-Lysine	0	0	—	0
D-Methionine	19	22	1.16	0
L-Methionine	19	22	1.16	0
DL-Methionine	19	22	1.16	0
DL-Norleucine	0	0	—	0
DL-Ornithine	0	0	—	0
D-Phenylalanine	0	0	—	0
L-Phenylalanine	0	0	—	0
DL-Phenylalanine	0	0	—	0
L-Proline	0	0	—	0
DL-Proline	0	0	—	0
D-Serine	94	94	1.00	1.9
L-Serine	240	277	1.15	3.5
DL-Serine	256	277	1.08	3.6
L-Threonine	115	230	2.00	5.4
DL-Threonine	109	142	1.30	2.8
L-Tryptophan	33	41	1.24	0
DL-Tryptophan	20	30	1.50	0
L-Tyrosine	0	0	—	0
DL-Valine	14	22	1.57	0

Each Warburg flask contained: 1 ml (approximately 0.4 mg N) of cell suspension; 1.5 ml 0.067 M phosphate buffer pH 6.8; 0.5 ml substrate, 5 μmoles; 0.1 ml 10 per cent KOH (center well). Control endogenous rates subtracted (average 25 μL per hr).

* Terminal analysis after 2 hr incubation.

The rates of aerobic ammonia production from L-asparagine, DL-asparagine, L-aspartic acid, L-threonine, and glycyl-glycine are presented in figure 1. The rate of ammonia production increased with time with threonine, aspartic acid, and glycyl-glycine. With both L- and DL-asparagine, however, a relatively constant rate of ammonia production was maintained for about 60 min, followed by a 15 to 30 min period of negligible activity. Thereafter, a constant but decreased rate of ammonia production followed.

The anaerobic production of ammonia from seven of the most actively dissimilated amino acids was determined. These data are presented in table 2. Comparable levels of ammonia production occurred for asparagine, aspartic acid, serine,

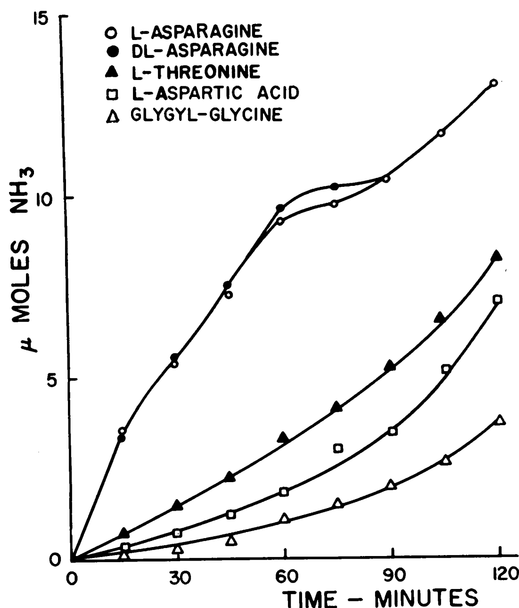

Figure 1. Rate of ammonia production from selected amino acids by strain 1013 of *Shigella flexneri* 3. Each flask contained: 1.0 ml (0.4 mg N) cell suspension; 1.0 ml 0.067 M phosphate buffer pH 6.8; 1.0 ml substrate (10 μmoles); temperature 37 C.

TABLE 2

Aerobic and anaerobic production of ammonia from selected amino acids by strain 1013 of Shigella flexneri 3

Substrate	Air	N_2
	μmoles NH_3*	μmoles NH_3*
L-Asparagine	10.34	9.72
DL-Asparagine	9.88	7.91
L-Aspartic acid	4.86	5.19
L-Serine	3.53	3.21
L-Threonine	5.40	5.19
DL-Glycine	2.54	0.14
Glycyl-glycine	5.40	0.66

* Ammonia determinations were made after 2-hr incubation on a shaker, all values being corrected for ammonia produced in the absence of substrate. Each flask contained 1.0 ml (0.40 mg N) of cell suspension and 10 μmoles substrate; total vol 3.1 ml.

and threonine, both in the presence and absence of oxygen. Ammonia production from glycine and glycyl-glycine, to the contrary, did not occur to any appreciable extent in the absence of oxygen.

Because of their significance as possible intermediates in the dissimilation of the above amino acids, a number of related organic acids were compared for their relative rates of oxidation. The data are presented in table 3. Pyruvate, succinate, lactate, malate, oxalacetate, fumarate, and isocitrate were found to be oxidized rapidly, whereas glutamate, α-ketoglutarate, acetate and *cis*-aconitate were oxidized at successively lower rates. Citrate was not oxidized by the intact cell.

The stereospecificity of oxidation of amino acids was studied for those reactions in which an appreciable rate of activity occurred, i. e., the oxidation of alanine, aspartic acid, asparagine and serine. The data included in table 1 indicate that both isomers of alanine, as well as the racemic mixture, are oxidized at the same rate. Although L-aspartic acid, L-asparagine, and L-serine were oxidized rapidly, relatively slight activity was obtained with the D-isomers. The intermediate rates of oxidation of the racemic mixtures of L-asparagine and L-aspartic acid suggested possible inhibition of the oxidation of the natural isomer by the D-form or a concentration dependency of the L-form. In contrast, the rate of oxidation of DL-serine suggested no such inhibition. Subsequent experiments in which the ratio of the D- to L-isomers was increased to 3 to 1 and 5 to 1 indicated that the D-isomers of serine, alanine, and asparagine produced no inhibitory effect on the oxidation of the enantiomorph. In

TABLE 3

Rate of oxidation of organic acids

Substrate	$Q_{O_2}(N)$	$Q_{CO_2}(N)$	R.Q.
Acetate	67	80	1.19
α-Ketoglutarate	131	173	1.32
cis-Aconitate	24	36	1.50
Citrate	0	0	—
Fumarate*	518	604	1.17
L-Glutamate	311	301	1.46
DL-Isocitrate*	399	382	0.96
L-Lactate*	454	438	1.04
L-Malate*	562	776	1.38
Oxalacetate*	360	496	1.35
Pyruvate*	360	496	1.38
Succinate*	532	609	1.15

Each Warburg flask contained: cellular suspension, 1 ml (0.4 mg N); 1.5 ml 0.067 M phosphate buffer pH 6.8; 0.5 ml substrate, 5 μmoles; 0.1 ml 10 per cent KOH (center well).

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

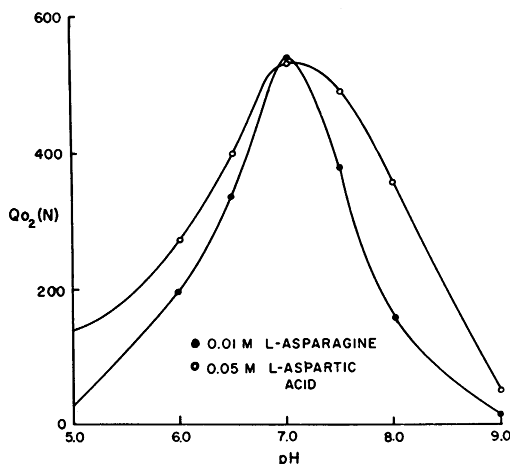

Figure 2. The effect of pH on the rate of oxidation of L-aspartic acid and L-asparagine by strain 1013 of *Shigella flexneri* 3. Each Warburg flask contained: 1.0 ml (0.4 mg N) cell suspension; 1.5 ml 0.067 M phosphate buffer; 0.5 ml substrate; 0.1 ml 10 per cent KOH (center well); temperature 37 C.

the case of alanine and serine, both the L- and D-isomers apparently are oxidized appreciably by the organism. No significant oxidation of D-asparagine was evident, nor was any inhibitory effect noted. In contrast, the oxidation of the natural isomer of aspartic acid was appreciably inhibited by increasing the proportion of the D- to L-isomers (10 per cent inhibition in a 1:1 ratio; 30 per cent inhibition in a 3:1 ratio; and 40 per cent inhibition in a 5:1 ratio).

The effect of substrate concentration upon the rate of oxidation was determined with aspartic acid and asparagine. Very little, if any, change in the rate of oxidation was found to occur within the concentration range of 0.005 M to 0.10 M with these substrates.

The effect of the pH of the system on the rate of oxidation of aspartic acid and asparagine is shown in figure 2. In each case the pH optimum occurred approximately at neutrality. A possible correlation between pH optima and substrate concentration was not found in subsequent investigation.

DISCUSSION

This investigation, designed as an empirical survey of the enzyme systems of Shigella that are active in the dissimilation of amino acids, has demonstrated that only 9 of 30 amino acids tested were oxidized and deaminated significantly. The data further indicate that the compounds tested were either, in general, rapidly dissimilated or not dissimilated. Of the 56 compounds tested, 17 had a Qo_2(N) of greater than 100; 5 between 25 and 100; and the remainder less than 25. Essentially comparable data were found with carbon dioxide evolution and ammonia production from the same substrates, i. e., very active dissimilation or none. These data indicate that the organism possesses very active enzyme systems for the degradation of certain amino acids and none for the others. These observations suggest the possibility of utilizing one of these actively degraded amino acids as a carbon, nitrogen, and energy source in nutritional studies.

Comparison of rates of oxidation and ammonia production from glycine and the glycine peptides suggests the organism may possess an active peptidase.

In contrast to the rate of ammonia production from threonine, aspartic acid, and glycyl-glycine which increased with time, a two-stepped rate was noted with L- and DL-asparagine. These data suggest a possible diauxie effect with an initial constitutive reaction (desamidation) and a secondary adaptive reaction (deamination). Total ammonia production from asparagine, threonine, and aspartic acid indicated complete deamination resulting after 3 hr incubation.

The stereospecificity of oxidation of amino acids by the organism appears to be inconsistent. Both optical antipodes of alanine were oxidized at significant rates. Oxidation of the D-isomer of alanine has also been shown in *Pseudomonas aeruginosa*, *Proteus vulgaris*, and *Brucella abortus* (Bernheim, Bernheim, and Webster, 1935; Webster and Bernheim, 1936; Gerhardt, Levine, and Wilson, 1950). In contrast D-serine was oxidized only slightly and D-asparagine and D-aspartic acid were not oxidized. However, the D-isomers of serine and asparagine were found to produce no inhibitory effect when present with the L-isomer in racemic mixtures, whereas D-aspartic acid was found to inhibit the oxidation of L-aspartic acid. The amount of inhibition proved to be dependent upon the proportion of the D-form present.

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SUMMARY

Of 30 amino acids and related nitrogen compounds tested, only 9 were oxidized and deaminated at appreciable rates by strain 1013 of *Shigella flexneri* 3. Of these 9 compounds, asparagine, aspartic acid and glutamic acid were most rapidly dissimilated. Except for glycine and glycyl-glycine, ammonia production from these compounds was found to be comparable under both aerobic and anaerobic conditions. Among 12 related organic acids, only citric acid was not oxidized.

The oxidative activity of the organism for asparagine, aspartic acid and serine apparently was specific for the L-isomer; the D-forms were not metabolized. D-aspartic acid inhibited the oxidation of the natural isomer. Both D- and L-alanine were oxidized.

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