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Oxidation of D- and L-Valine by Enzymes of *Pseudomonas aeruginosa*

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ABSTRACT

NORTON, J. E. (University of Oklahoma School of Medicine, Oklahoma City), AND J. R. SOKATCH. Oxidation of D- and L-valine by enzymes of *Pseudomonas aeruginosa*. J. Bacteriol. **92**:116–120. 1966.—Cell-free extracts prepared from *Pseudomonas aeruginosa* grown on DL-valine catalyzed the consumption of oxygen with several D-amino acids, but not with the corresponding L-amino acids. The product of D-valine oxidation was identified as 2-oxoisovalerate by the preparation and characterization of 2-oxoisovalerate 2,4-dinitrophenylhydrazone. The enzyme catalyzing D-amino acid oxidation was present in extracts of cells grown on valine, but not on glucose, had a *p*H optimum of approximately 9.0, consumed 1 atom of oxygen per mole of keto acid produced, and was not stimulated by any of the usual electron transport cofactors. It was not possible to demonstrate either the direct oxidation of L-valine or the conversion of L- to D-valine by these enzyme preparations. However, a possible route of L-valine metabolism by transamination with 2-oxoglutarate with regeneration of the amino group acceptor by glutamate oxidation was established by identification of the transaminase and L-glutamate dehydrogenase in these enzyme preparations.

Very little is known of the details of the catabolism of the branched-chain amino acids by bacteria. Valine, leucine, and isoleucine all serve as hydrogen donors in the Stickland reaction and are oxidized to the next lower fatty acids (13). Nisman (7) reported that extracts of *Clostridium sporogenes* contained a nicotinamide adenine dinucleotide (NAD)-linked alanine dehydrogenase and catalyzed the uptake of oxygen with L-valine when supplemented with NAD. Sanwal and Zink (11) purified an NAD-linked L-leucine dehydrogenase from *Bacillus cereus* which was active with all three branched-chain amino acids. Sanwal and Zink favored the belief that their enzyme served a catabolic rather than a biosynthetic function.

In the preceding paper (12) of this series, we presented evidence which indicated that *Pseudomonas aeruginosa* catabolized DL-valine by oxidation to isobutyrate and propionate. Alanine formed during growth on DL-valine-*4,4'*-C^{14} was labeled in carbons 1 and 3, which suggested the direct oxidation of propionate derived from the isopropyl carbons of valine to pyruvate for alanine biosynthesis, possibly by the acrylate pathway. The derivation of aspartate from labeled valine was less certain, but apparently a C_3 plus C_1 condensation was involved in the conversion of propionate to oxaloacetate for aspartate biosynthesis. This publication reports our studies of the metabolism of D- and L-valine by enzyme extracts of *P. aeruginosa*.

MATERIALS AND METHODS

Organism and cultural conditions. P. aeruginosa was maintained in stock culture and grown in 15-liter batches with 0.5% DL-valine or 0.5% glucose as the sole carbon source, as previously described (8). Fresh whole cells oxidized both D- and L-valine at equal rates, with Q_{O_2} values between 150 and 300 μliters per hr per mg (dry weight). The enzyme system responsible for D-amino acid oxidation slowly deteriorated in frozen cells, and fresh cells were prepared every 2 to 3 months.

Enzymatic methods. With the exception of the data presented in *Requirements for D-valine and D-amino acid oxidation* in Results, all enzyme studies were made with extracts prepared from cells grown in DL-valine medium. (For this aspect of the investigation, extracts prepared from cells grown on glucose were also used.) To reduce the viscosity of the cell suspensions prior to sonic disintegration, 5 g of frozen cells in 15 ml of distilled water was treated with 3 mg of deoxyribonuclease and stirred at room temperature for 10 min. All other operations were performed at between 1 and 5 C. The organisms were treated with sonic vibration

for 15 min with a Raytheon model DF101 10-kc sonic oscillator. Cellular debris was removed by centrifugation at 12,000 × *g*, and the protein extracts were freed from small molecules either by passage through a Sephadex G-25 column or by dialysis.

D-Amino acid oxidation was assayed manometrically by the measurement of oxygen consumption. The complete reaction mixture contained 450 μmoles of glycine-sodium hydroxide buffer (*p*H 9.2), 150 μmoles of neutralized semicarbazide, 75 μmoles of D-valine or other D-amino acid, and approximately 10 mg of enzyme protein, in a final volume of 3.2 ml. Other buffers, such as phosphate and tris(hydroxymethyl)-aminomethane (Tris), could also be used. Sodium hydroxide was not required in the center well of the Warburg vessel when semicarbazide was present, because carbon dioxide was not evolved in the course of valine oxidation. With this assay, the rate of oxygen uptake with fresh enzyme preparations was 5 to 10 mμatoms per min per mg of protein. L-Glutamate oxidation was also assayed manometrically, and, in this case, the complete reaction mixture contained 100 μmoles of phosphate buffer (*p*H 7.0), 0.4 mg of NAD or nicotinamide adenine dinucleotide phosphate (NADP), and 10 μmoles of L-glutamate or D-glutamate, in a final volume of 3.0 ml. Sodium hydroxide, 0.2 ml of a 10% solution, was added to the center well. 2-Oxoglutarate was also oxidized rapidly by unfractionated extracts under these conditions.

Alanine racemase was assayed, as described by Wood and Gunsalus (16), by use of pig kidney D-amino acid oxidase. This procedure was also used to detect valine racemase activity, since the kidney enzyme utilizes D-valine.

Reaction mixtures for the assay of transamination with 2-oxoglutarate contained 50 μmoles of phosphate buffer (*p*H 8.5), 20 μmoles of amino acid previously adjusted to *p*H 8.5, 20 μg of pyridoxal phosphate and enzyme, equivalent to 2 mg of protein, in a total volume of 0.5 ml. The reaction mixtures were incubated in a water bath at 37 C in stoppered tubes under an atmosphere of nitrogen. Reactions were started by injection of enzyme through the rubber serum stopper with a hypodermic syringe and stopped by injection of 0.5 ml of 0.5 M perchloric acid. Precipitated protein was removed by centrifugation, and the supernatant solution was neutralized with 0.5 ml of 0.5 M potassium hydroxide. The precipitate of potassium perchlorate was removed by centrifugation, and a sample of the reaction mixture was taken for glutamate determination by quantitative paper chromatography. The initial velocity of transamination with fresh enzyme extracts was about 0.09 μmoles of glutamate formed per min per mg of protein with L-valine plus 2-oxoglutarate. The rate dropped off rapidly owing to substrate depletion (Fig. 2).

Analytical and chemical methods. Protein was determined by the Bücher (2) method with the volume reduced to 1 ml. 2-Oxoisovalerate was measured colorimetrically by use of the toluene extraction procedure devised by Friedemann and Haugen (3) for the determination of pyruvate as the 2,4-dinitrophenylhydrazone. 2-Oxoisovalerate was used to prepare the standard curve, and was obtained from Sigma Chemical Co. (St. Louis, Mo.) and from Calbiochem. A modification of the method of Heilmann et al. (5) was used for the quantitative determination of glutamate and other amino acids by paper chromatography. The solvent used routinely was *n*-butanol-acetic acid-water (75:13:12), except for the separation of glutamate from basic amino acids and asparate, in which case *t*-butanol-formic acid-water (75:13:12) was used. The color was developed as described by Heilmann et al. (5) and eluted by extracting the colored zones with methanol at 60 C for 10 min, and the color was measured at 510 mμ.

2-Oxoisovalerate 2,4-dinitrophenylhydrazone was isolated from large-scale reaction mixtures which contained 4 mmoles of glycine-sodium hydroxide buffer (*p*H 9.2), 1 mmole of neutralized semicarbazide, 0.5 mmole of D-valine, and 200 mg of enzyme protein, in a total volume of 30 ml. After incubation for 3 hr at 37 C with shaking, the protein was precipitated by adding 2 ml of 10 N sulfuric acid and removed by centrifugation. The deproteinized reaction mixtures were extracted continuously with ether for 16 hr, and the extracts from six such mixtures were pooled, titrated to the phenol red end point, and brought to dryness in a rotary evaporator. The sodium salt of 2-oxoisovalerate was dissolved in 30 ml of 0.67 N sulfuric acid, and a twofold excess of 2,4-dinitrophenylhydrazine in 9 ml of ethyl alcohol, plus 1 ml of concentrated sulfuric acid, was added. After 30 min, the precipitate was removed by centrifugation, and the derivative was recrystallized three times with a minimal amount of ethyl alcohol.

RESULTS

Stoichiometry between oxygen consumption and oxoacid production. In the initial experiments, it was found that extracts of *P. aeruginosa* catalyzed the uptake of oxygen with D-valine, but not L-valine. The stoichiometry between oxygen consumed and oxoacid produced, assumed to be 2-oxoisovalerate, was variable with these preparations, but usually more oxygen was consumed than could be accounted for by oxoacid production (i.e., when the net oxygen uptake was 11.4 μmoles, the net 2-oxoisovalerate produced was 4.9 μmoles). However, when semicarbazide was added to the reaction mixture to trap the oxoacid as it was formed, the ratio of oxygen consumed to oxoacid produced was consistently 1:2 (net oxygen uptake, 5.3 μmoles; net 2-oxoisovalerate produced, 10.3 μmoles).

Product of D-valine oxidation. The product of D-valine oxidation was identified as 2-oxoisovalerate by the preparation and characterization of its crystalline 2,4-dinitrophenylhydrazone. The melting point of the derivative obtained from the reaction mixture was 195 to 196 C, the melting point of the 2,4-dinitrophenylhydrazone prepared from authentic 2-oxoisovalerate was 194 to 196 C, and the reported melting point is 194 to

195 C (9). The mixed melting point of the two specimens was 195 to 196 C.

Requirements for D-valine and D-amino acid oxidation. The enzyme which catalyzed D-valine oxidation was inducible, since extracts prepared from cells grown on glucose were unable to oxidize D-valine as compared with those prepared from cells grown on DL-valine (extracts from cells grown on glucose consumed 0.25 mμatom of oxygen per min per mg of protein in comparison with 9.0 consumed by extracts of cells grown on DL-valine). Glucose oxidation by these preparations is included for comparison: with extracts of cells grown on glucose, 14.0 mμatoms of oxygen per min per mg of protein was utilized, whereas, with extracts from cells grown on DL-valine, the rate was only 2.5 mμatoms of oxygen per min per mg of protein. (Reactions were assayed manometrically with cell-free extracts prepared from cells grown on glucose or on DL-valine, as indicated. Valine oxidation was measured in phosphate-semicarbazide buffer, *p*H 8.5, and glucose was measured in the same buffer without semicarbazide.) We did not test extracts prepared from cells grown on the pure isomers, and it is unknown whether the enzyme is induced by growth on one or both valine isomers. Neither NAD, NADP, flavin adenine dinucleotide (FAD), nor any of the several ions tested stimulated the rate of oxygen uptake. The *p*H optimum for valine oxidation in the manometric assay with semicarbazide was approximately 9.0 (Fig. 1). Several other D-amino acids were oxidized when tested in the usual manometric assay, but the corresponding L-amino acids, with the exception of L-alanine and L-aspartic acid, were not oxidized (Table 1).

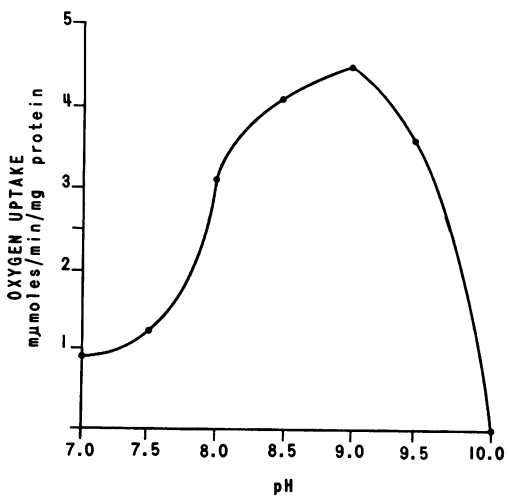

FIG. 1. *Specific activity of D-valine oxidase as a function of pH. Tris buffer was used from pH 7.0 to 8.5, and glycine-sodium hydroxide buffer from 9.0 to 10.0. All vessels contained semicarbazide; 12 mg of enzyme protein was used per vessel.*

Pathway of L-valine oxidation. Enzyme extracts of *P. aeruginosa* grown on DL-valine did not oxidize L-valine when supplemented with NAD, NADP, or FAD, nor did these extracts catalyze an L-valine dependent reduction of a large number of dyes tested. A possible alternate pathway to the direct oxidation of L-valine was the conversion of L- to D-valine by a racemase and oxidation of D-valine by the enzyme described in the preceding section. The enzyme preparations contained a high level of alanine racemase activity (112 mμmoles of oxygen taken up per min per mg of protein when pig kidney D-amino acid oxidase was included in the assay system), but no detectable valine racemase activity. Deamination of L-valine by transamination and oxidation of the amino-group acceptor was another possible route of L-valine oxidation. Of a number of oxoacids tested, 2-oxoglutarate was the best amino-group acceptor with L-valine as the amino donor (Fig. 2). Other L-amino acids which transaminated with 2-oxoglutarate are shown in Table 2. The

TABLE 1. *Relative rates of amino acid oxidation by cell-free extracts of Pseudomonas aeruginosa*

Amino acid	Rate[a]
D-Valine	1.0
D-Serine	1.1
D-Phenylalanine	1.9
D-Norvaline	1.4
D-Tryptophan	0.0
D-Threonine	1.0
D-Alanine	2.3
D-Methionine	2.8
D-Alloisoleucine	0.45
D-Leucine	1.3
D-Glutamic acid	0.0
L-Alanine	0.79
L-Aspartic acid	0.56
α-Aminoisobutyric acid	0.13

[a] The numbers in this table were obtained by dividing the rate of oxygen uptake obtained with the indicated amino acid by the rate obtained with D-valine which was included in each experiment. Glycine-semicarbazide buffer was used with all amino acids except glycine, in which case phosphate-semicarbazide buffer was used. No detectable rate was found with: L-valine, L-serine, L-proline, L-phenylalanine, L-norvaline, L-tryptophan, L-threonine, L-methionine, L-lysine, L-histidine, L-isoleucine, L-leucine, L-α-amino-*n*-butyric acid, L-norleucine, L-glutamic acid, glycine.

observed activities are generally similar to the combined action of transaminases A and B of *Escherichia coli* (10), with the exception of the low rate observed with tryptophan.

L-Glutamate oxidation. L-Glutamate was rapidly oxidized when extracts of *P. aeruginosa* were supplemented with either NAD or NADP, when the manometric assay was used (Fig. 3). 2-Oxoglutarate was also rapidly oxidized under the conditions of this assay, and it is apparent from the data of Fig. 3 that L-glutamate was oxidized beyond the stage of 2-oxoglutarate, since 10 μ-moles of substrate was used. It was possible to demonstrate a net oxidation of reduced NADP ($NADPH_2$) spectrophotometrically which depended on 2-oxoglutarate and ammonium ion, but this was not possible with reduced NAD ($NADH_2$), probably because of the high rate of $NADH_2$ oxidation without other additions. The reason for the oxidation of L-glutamate with unsupplemented extracts is not known, but may be due to traces of pyridine nucleotide not removed by dialysis.

DISCUSSION

The reactions in the metabolism of D- and L-valine which have been demonstrated to occur in enzyme extracts of *P. aeruginosa* may be summarized as follows. D-Valine was oxidized directly to 2-oxoisovalerate, whereas L-valine was deaminated by transmination with 2-oxoglutarate. Glutamate resulting from transamination was itself deaminated, regenerating 2-oxoglutarate. L-Glutamate deamination occurred by the action of L-glutamate dehydrogenase, and probably also

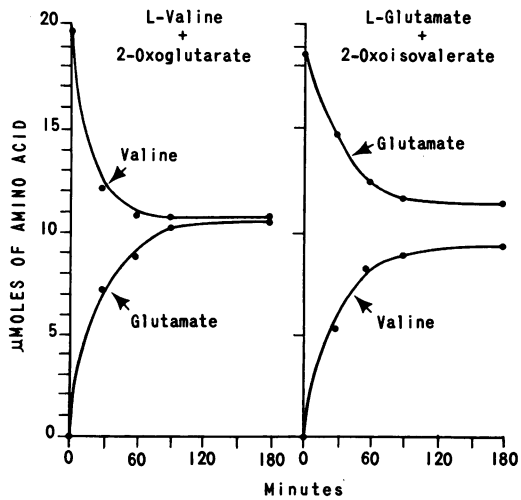

FIG. 2. *Transamination between L-valine plus 2-oxoglutarate and L-glutamate plus 2-oxoisovalerate.*

TABLE 2. *Relative rates of transamination of L-amino acids with 2-oxoglutarate*

Amino acid	Rate[a]
L-Alanine	0.65
L-α-Amino-*n*-butyric acid	0.24
L-Aspartic acid	1.22
L-Cysteic acid	0.69
L-Cysteine	1.15
L-Histidine	0.28
L-Isoleucine	1.22
L-Leucine	1.04
L-Lysine	0.28
L-Methionine	0.87
L-Norleucine	1.15
L-Norvaline	1.61
L-Phenylalanine	1.00
L-Serine	0.00
L-Threonine	0.00
L-Tryptophan	0.07
L-Valine	1.00

[a] The numbers in this table were obtained by dividing the rate of glutamate formation with the indicated amino acid plus 2-oxoglutarate by the rate obtained with L-valine and 2-oxoglutarate. Each experiment contained a tube with L-valine and 2-oxoglutarate as a standard.

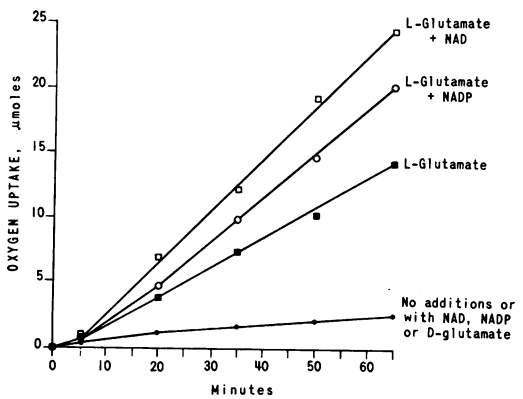

FIG. 3. *Oxidation of L-glutamate by an enzyme extract prepared from Pseudomonas aeruginosa.*

by secondary transamination reactions with other 2-oxoacids, resulting in amino acids for protein synthesis. This latter pathway could represent an appreciable route of glutamate deamination when *P. aeruginosa* is grown with valine as the sole source of nitrogen, as well as carbon and energy.

The oxidation of D-amino acids by enzymes of *P. aeruginosa* again raises the question of the natural function of D-amino acid oxidases. Yoneya and Adams (1, 17) found that L-hydroxyproline was metabolized by *P. striata* by conversion to D-allohydroxyproline. D-Allohydroxy-

proline was oxidized by a particulate enzyme which was specific for D-allohydroxyproline. Stumpf and Green described the oxidation of L-amino acids by similar preparations from *Proteus vulgaris* (14) and D-amino acids by *Proteus morganii* (Federation Proc. **5**:157, 1946). In none of these cases was a soluble cofactor required, and the nature of the active site of the enzymes is unknown. D-Alanine (8) and D-valine oxidation by *P. aeruginosa*, D-allohydroxyproline oxidation by *P. striata* (17), and D-amino acid oxidation by *P. morganii* all were found to occur with the uptake of 1 atom of oxygen per mole of amino acid oxidized, which favors the contention that water is a product of the bacterial oxidation of D-amino acids, possibly because of the participation of the cytochrome system. Such a result would also be obtained by the action of catalase, but the cell membrane preparations of *P. aeruginosa* which were used for the study of D-alanine oxidation (8) were found to be free from catalase, and neither Yoneya and Adams (17) nor Stumpf and Green (Federation Proc. **5**:157, 1946) were able to demonstrate peroxide formation in their respective studies. These findings are in contrast with the stoichiometry obtained with flavoprotein enzymes such as kidney D-amino acid oxidase, where 1 mole of oxygen is consumed per mole of amino acid oxidized and peroxide is a product.

The pathway of L-valine utilization by way of transamination with 2-oxoglutarate has been suggested several times as a general method of L-amino acid deamination (6). Vender et al. (15) and Halpern and Lupo (4) obtained mutants of *Escherichia coli* capable of using glutamate as the sole carbon source from parent strains unable to use glutamate. Mutants obtained by both groups contained only NADP glutamate dehydrogenase, which was repressed by growth on L-glutamate. However, the mutant obtained by Vender et al. (15) had lost the ability to decarboxylate L-glutamate, and growth on glutamate was accompanied by an increase in aspartase. These authors visualized the pathway of glutamate oxidation as occurring by way of transamination of L-glutamate with oxaloacetate to produce aspartate which was deaminated by aspartase. They attribute the ability of their mutant to use glutamate for growth to the loss in glutamate decarboxylase, whereas Halpern and Lupo (4) concluded that the ability of their mutant to use glutamate was due to a change in the permeability of the cell to glutamate. In contrast, our strain of *P. aeruginosa* can grow with L-glutamate as the sole carbon source, lacks glutamic decarboxylase, can oxidize L-glutamate when either NAD or NADP is present, and contains aspartase or a similar enzyme which causes the disappearance of aspartate without other additions (Norton and Sokatch, *unpublished data*).

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