JOURNAL OF BACTERIOLOGY, July, 1966
Vol. 92, No. 1

Alanine and Aspartate Formation During Growth on Valine-C^{14} by *Pseudomonas aeruginosa*

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Received for publication 4 March 1966

ABSTRACT

SOKATCH, J. R. (University of Oklahoma School of Medicine, Oklahoma City). Alanine and aspartate formation during growth on valine-C^{14} by *Pseudomonas aeruginosa*. J. Bacteriol. **92**:72–75. 1966.—*Pseudomonas aeruginosa* grown with DL-valine-*4,4'*-C^{14} synthesized alanine labeled mainly in carbons 1 and 3, indicating that the isopropyl carbons of valine were the precursors of pyruvate for alanine formation by a pathway which did not involve randomization of isotope. Alanine from cells grown on valine-*1*-C^{14} contained isotope only in the carboxyl carbon, suggesting another route to pyruvate from valine by carbon dioxide fixation. Oxidation of valine to propionyl-coenzyme A (CoA), as it occurs in animal tissues, followed by the oxidation of propionyl-CoA to acrylyl-CoA, lactyl-CoA, and pyruvate, would account for the isotope data. Cells grown on valine oxidized valine, isobutyrate, and propionate immediately, whereas cells grown on acetate did not oxidize valine or isobutyrate and required an induction period before propionate was oxidized. *P. aeruginosa* grown with propionate-*1*-C^{14} or propionate-*2*-C^{14} formed alanine-*1*-C^{14} and alanine-*2*-C^{14}, respectively, which agrees with the contention that at least part of the propionate is oxidized via the acrylate pathway. Aspartate formed from valine-*1*-C^{14} was labeled only in the carboxyl carbons, whereas that formed from valine-*4,4'*-C^{14} was labeled in all four carbons, but most heavily in carbons 1 and 3. These data suggest that the main route for the formation of the carbon skeleton of aspartate was by a C_3 plus C_1 condensation, with the C_3 unit derived from the isopropyl carbons of valine and the C_1 unit probably from carbon dioxide.

Pathways for the oxidation of the branched-chain amino acids are well established for animal tissues (5), but little is known about the bacterial metabolism of these compounds. *Pseudomonas aeruginosa* is capable of growth with DL-valine as the sole source of carbon and energy, and the approach used in these studies was to grow the organism with specifically labeled valine and to isolate and determine the isotope distribution of alanine and aspartate. The labeling patterns of these amino acids were considered to reflect the manner in which the three-carbon precursor of alanine was provided from valine. A preliminary account of these studies was presented earlier (J. Sokatch, Biochem. J. **92**:54P, 1964). The labeling patterns of alanine and other amino acids from several aerobic organisms grown on acetate have been reported (3), and the data indicate that the carbon skeletons of these amino acids are derived from tricarboxylic acid intermediates.

MATERIALS AND METHODS

Organisms and cultural conditions. P. aeruginosa was grown in synthetic medium, as previously described (13), with 0.5% DL-valine, isobutyrate, propionate, or acetate as the sole carbon and energy source. For the radioactive experiments, 10 μc of the appropriate substrate was added to 50 ml of medium, and the organism was grown at 37 C with aeration. The *Nocardia* species used for the decarboxylation of aspartate was obtained from the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland, accession number 8862. In the original literature (2), the organism was incorrectly identified as *N. globerula*, and the wrong accession number was given. The organism was grown in Roux bottles as described by Krebs and Bellamy (9), except that 25 mg of pyridoxine hydrochloride was added per 100 ml of medium.

Manometric procedures. Asparate was decarboxylated to carbon dioxide and alanine by the procedure of Krebs and Bellamy (9). The reaction mixture for the study of the oxidative patterns of whole cells of *P. aeruginosa* grown on DL-valine, isobutyrate,

or acetate contained cells equivalent to 5 mg (dry weight); 250 μmoles of phosphate buffer (*p*H 6.4); 10 μmoles of DL-valine, isobutyrate, or propionate in a volume of 2.9 ml; and 0.1 ml of 10% sodium hydroxide in the center well of the Warburg vessel.

Isolation and degradation of radioactive alanine and aspartate. When growth had ceased, 5 ml of 10% trichloroacetic acid was added to the medium, and the cells were harvested by centrifugation, and treated as described by Roberts et al. (14) for the preparation of the crude protein fraction. The material so obtained was hydrolyzed under vacuum for 16 hr in an autoclave at 121 C, and the hydrolysate was taken to dryness in a vacuum desiccator over sodium hydroxide. Alanine and aspartate were isolated according to the methods of Knight (8) and Hoare and Gibson (6). Two-dimensional paper chromatograms of isolated alanine and aspartate with *n*-butanol-acetic acid-water (4:1:5) and 90% phenol-0.6% ammonium hydroxide showed that these amino acids were the only ninhydrin-positive compounds. The yield of alanine and aspartate was determined by the quantitative ninhydrin reaction (12), and usually amounted to about 4 μmoles. Radioactive alanine was diluted approximately 125-fold with carrier alanine, and was degraded according to the procedures of Knight (8) and Hoare and Gibson (6). Carbon dioxide was absorbed in barium hydroxide-barium chloride bubblers (15), and radioactivity was measured on barium carbonate samples by use of a Nuclear D-47 detector as a proportional counter. Aspartate was decarboxylated to carbon dioxide and alanine with the *Nocardia* species (6, 8), and carrier alanine equal to approximately 100 times the carbon dioxide yield was added. Alanine was isolated from the decarboxylation reaction mixture by column chromatography over a Dowex 50 column (2 by 20 cm) with 0.5 N acetic acid as the eluant, and was degraded by the methods presented in the preceding portion of this paragraph.

RESULTS

Isotope distribution of alanine synthesized during growth on valine-C^{14}. Alanine isolated from *P. aeruginosa* grown on DL-valine-*4,4'-*C^{14} contained isotope in the highest concentration in carbons 1 and 3 (Table 1). This result suggests that a direct, i.e., nonrandomizing, conversion of carbons 3, 4, and 4' of valine is the main pathway to pyruvate for alanine biosynthesis. On the other hand, alanine isolated from *P. aeruginosa* after growth on DL-valine-*1-*C^{14} contained isotope only in carbon 1 and at a low specific activity. The results with valine-*1-*C^{14} suggest that isotope from this position was incorporated as a result of carbon dioxide fixation.

Sequential induction patterns of P. aeruginosa after growth on valine, isobutyrate, and acetate. Oxidation of valine through isobutyryl-Coenzyme A (CoA) and propionyl-CoA as in animal tissues (5), followed by oxidation of propionyl-CoA via the acrylate pathway outlined by Stadtman and Vagelos (16, 17), would account for the data obtained in the experiment with valine-*4,4'-*C^{14}. Evidence to support the contention that *P. aeruginosa* oxidized valine by way of isobutyrate and propionate was obtained by the finding that cells grown on valine oxidized valine, isobutyrate, and propionate immediately (Table 2). Isobutyrate cells oxidized isobutyrate and propionate, but not valine, whereas acetate cells oxidized none of these compounds immediately, but did oxidize propionate after induction period.

Isotope distribution of alanine synthesized during growth on propionate-C^{14}. If the nonrandomizing pathway functions in the oxidation of propionyl coenzyme A by *P. aeruginosa*, then alanine from cells grown on labeled propionate should be labeled as is propionate. This result was obtained in growth experiments with propionate-*1-*C^{14} and propionate-*2-*C^{14}, which resulted in alanine-*1-*C^{14} and alanine-*2-*C^{14}, respectively (Table 3).

Isotope distribution of aspartate synthesized during growth on valine-C^{14}. A nonrandomizing pathway such as the acrylate pathway from propionate to pyruvate involves only C_3 intermediates, which raises the problem of the biosynthetic route to C_4 dicarboxylic acids during growth on

TABLE 1. *Distribution of isotope in carbons of alanine from Pseudomonas aeruginosa grown on valine-1-*C^{14} *and valine-4,4'-*C^{14}

Alanine carbon	Percentage of total radioactivity in alanine carbons after growth on	
	Valine-*1-*C^{14}	Valine-4,4'-C^{14}
COOH	100	50
$CHNH_2$	0	11
CH_3	0	39

TABLE 2. *Oxidative patterns of whole cells of Pseudomonas aeruginosa after growth on valine, isobutyrate, or acetate*[a]

Substrate	Ability to oxidize test substrate after growth on		
	DL-Valine	Isobutyrate	Acetate
DL-Valine	+	−	−
Isobutyrate	+	+	−
Propionate	+	+	*lag*

[a] Symbols: + = immediate oxidation; − = no oxidation in the time of the experiment; *lag* means that the substrate was oxidized after an induction period.

valine. Aspartate formed during growth on valine-*1*-C^{14} was labeled only in the carboxyl carbons (Table 4), which is similar to aspartate formed during growth of aerobic organisms on acetate-*1*-C^{14} or with added $C^{14}O_2$ (3). Aspartate formed from valine-*4,4'*-C^{14} was labeled in every carbon, but most heavily carbons 1 and 3. These results could be explained by assuming that two species of labeled oxaloacetate contribute to aspartate formation, one labeled in carbons 1 and 3, and the other labeled in all four carbons. The former would be obtained as a result of the carboxylation of pyruvate-*1,3*-C^{14}, formed from propionate-*1,3*-C^{14} by the acrylate pathway, and the latter by the carboxylation of propionyl-CoA to methylmalonyl-CoA and succinyl-CoA (Fig. 1). Oxidation of pyruvate-*1,3*-C^{14} to acetate-*2*-C^{14} with metabolism of acetate via the tricarboxylic acid cycle would also result in aspartate labeled in all four carbons, but with a higher isotope concentration in carbons 2 and 3.

TABLE 3. *Distribution of isotope in carbons of alanine from Pseudomonas aeruginosa grown on propionate-1-C^{14} and propionate-2-C^{14}*

Alanine carbon	Percentage of total radioactivity in alanine carbons after growth on	
	Propionate-*1*-C^{14}	Propionate-*2*-C^{14}
COOH	86	11
$CHNH_2$	0	79
CH_3	14	10

TABLE 4. *Distribution of isotope in carbons of aspartate from Pseudomonas aeruginosa grown on valine-1-C^{14} and valine-4,4'-C^{14}*

Aspartate carbon	Percentage of total radioactivity in aspartate carbons after growth on	
	Valine-*1*-C^{14}	Valine-*4,4'*-C^{14}
COOH	66	41
$CHNH_2$	0	16
CH_2	0	29
COOH	34	14

DISCUSSION

The labeling patterns of alanine produced from valine and propionate by *P. aeruginosa* contrast with the results obtained in animal experiments with labeled valine and propionate. Fones et al. (4) fed DL-valine-*4,4'*-C^{13} to rats and found that liver glycogen was uniformly labeled. In a similar series of experiments, Lorber et al. (11) found that carbons 2 and 3 of propionate equilibrated before being converted to glycogen. These results are now explainable on the basis of the formation of propionyl-CoA from the isopropyl carbons of valine, and of the known route of propionate metabolism in animal tissues by carboxylation of propionyl-CoA to methylmalonyl-CoA, succinyl-CoA, and oxidation via the tricarboxylic acid cycle (7).

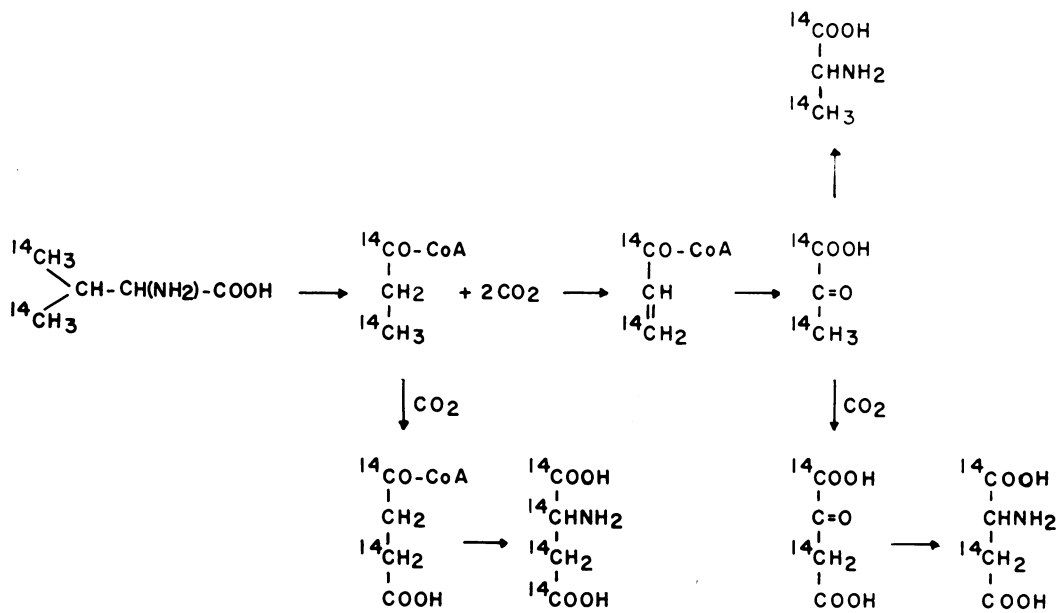

FIG. 1. *Catabolic pathways which would account for the observed labeling pattern of alanine and aspartate after growth of Pseudomonas aeruginosa on valine-4,4'-C^{14}.*

On the other hand, *Clostridium propionicum* (10) and *Peptostreptococcus elsdenii* (J. N. Ladd, Biochem. J. **67**:4P, 1957) both ferment lactate to propionate without randomization of carbons 2 and 3. Stadtman and Vagelos (16) studied propionate oxidation by *C. propionicum* and obtained evidence for the oxidation of propionyl-CoA to acrylyl-CoA. They postulated the hydration of acrylyl-CoA to lactyl-CoA, but were unable to obtain evidence for this reaction in extracts of *C. propionicum*. They later succeeded in demonstrating the hydration of acrylyl-CoA by an enzyme from a propionate-oxidizing pseudomonad (17). Recently, Baldwin et al. (1) confirmed the occurrence of the acrylate pathway in *P. elsdenii* by purifying the lactyl-CoA dehydrase of this organism and demonstrating the formation of lactyl-CoA from acrylyl-CoA.

The evidence presented here shows that the main pathway for the metabolism of propionate to pyruvate for alanine biosynthesis by *P. aeruginosa* does not involve randomization of carbons 2 and 3. This, in turn, suggests that the acrylate pathway of propionate metabolism functions in this organism (Fig. 1). Formation of aspartate and, by inference, C_4 dicarboxylic acids related to aspartate, is not quite so clear-cut. Condensation of pyruvate, or a derivative thereof, with a C_1 compound to yield oxaloacetate-*1,3-*C^{14} plus a randomizing pathway resulting in uniformly labeled oxaloacetate would account for the observed isotope distribution of aspartate. Carboxylation of propionyl-CoA-*1,3-*C^{14} eventually yielding uniformly labeled succinate is one pathway which would provide uniformly labeled aspartate (Fig. 1). Van der Linden and Thijsse (18) proposed that propionate, which is produced in the oxidation of odd-numbered hydrocarbons by *P. aeruginosa*, is oxidized mainly by the methylmalonate pathway rather than the acrylate pathway. However, this suggestion is based on evidence from whole-cell experiments, and is considered tentative until the enzymes of propionate metabolism by *P. aeruginosa* have been studied.

ACKNOWLEDGMENTS

I would like to express my appreciation to S. R. Elsden for the courtesies extended to me during the time spent in his laboratory at the University of Sheffield where this work was started, and to M. M. Knight for his valuable advice on the isolation and degradation of alanine and aspartate.

This investigation was supported by grants G-9868 and G-554 from the National Science Foundation, and by National Institutes of Health Research Career Award No. 5-K3-GM-18343.

LITERATURE CITED

1. BALDWIN, R. L., W. A. WOOD, AND R. S. EMERY. 1965. Lactate metabolism by *Peptostreptococcus elsdenii*: evidence for lactyl coenzyme A dehydrase. Biochim. Biophys. Acta **97**:202–213.
2. CRAWFORD, L. V. 1957. Studies on the aspartic decarboxylase of *Nocardia globerula*. Biochem. J. **68**:221–225.
3. EHRENSVARD, G. 1955. Metabolism of amino acids and proteins. Ann. Rev. Biochem. **24**:275–310.
4. FONES, W. S., T. P. WAALKES, AND J. WHITE. 1951. The conversion of L-valine to glucose and glycogen in the rat. Arch. Biochem. Biophys. **32**:89–95.
5. GREENBERG, D. M. 1961. Carbon catabolism of amino acids, p. 79–172. *In* D. M. Greenberg [ed.], Metabolic pathways, vol. II. Academic Press, Inc., New York.
6. HOARE, D. S., AND J. GIBSON. 1964. Photoassimilation of acetate and the biosynthesis of amino acids by *Chlorobium thiosulphatophilum*. Biochem. J. **91**:546–559.
7. KAZIRO, Y., AND S. OCHOA. 1964. The metabolism of propionic acid. Advan. Enzymol. **26**:283–378.
8. KNIGHT, M. 1962. The photometabolism of propionate by *Rhodospirillum rubrum*. Biochem. J. **84**:170–185.
9. KREBS, H. A., AND D. BELLAMY. 1960. The interconversion of glutamic and aspartic acid in respiring tissues. Biochem. J. **75**:523–529.
10. LEAVER, F. W., H. G. WOOD, AND R. STJERNHOLM. 1955. The fermentation of three carbon substrates by *Clostridium propionicum* and *Propionibacterium*. J. Bacteriol. **70**:521–530.
11. LORBER, V., N. LIFSON, W. SAKAMI, AND H. G. WOOD. 1950. Conversion of propionate to liver glycogen in the intact rat, studied with isotopic propionate. J. Biol. Chem. **183**:531–538.
12. MOORE, S., AND W. H. STEIN. 1948. Photometric ninhydrin method for use in the chromatography of amino acids. J. Biol. Chem. **176**:367–388.
13. NORTON, J. E., G. S. BULMER, AND J. R. SOKATCH. 1963. The oxidation of D-alanine by cell membranes of *Pseudomonas aeruginosa*. Biochim. Biophys. Acta **78**:136–147.
14. ROBERTS, R. B., D. B. COWIE, P. H. ABELSON, AND E. T. BOLTON. 1955. Biosynthesis in *Escherichia coli*. Carnegie Inst. Wash. Publ. 607.
15. SOKATCH, J. R., AND I. C. GUNSALUS. 1957. Aldonic acid metabolism. I. Pathway of carbon in an inducible gluconate fermentation by *Streptococcus faecalis*. J. Bacteriol. **73**:452–560.
16. STADTMAN, E. R., AND P. R. VAGELOS. 1958. Propionic acid metabolism. Proc. Intern. Symp. Enzyme Chem. Tokyo and Kyoto, 1957 **2**:86–92.
17. VAGELOS, P. R., J. M. EARL, AND E. R. STADTMAN. 1959. Propionic acid metabolism. II. Enzymatic synthesis of lactyl pantetheine. J. Biol. Chem. **234**:765–769.
18. VAN DER LINDEN, A. C., AND G. J. E. THIJSSE. 1965. The mechanism of microbial oxidation of petroleum hydrocarbons. Advan. Enzymol. **26**:469–546.