Biosynthesis of Phenylalanine from Phenylacetate by *Chromatium* and *Rhodospirillum rubrum*

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Cultures of *Chromatium* strain D and *Rhodospirillum rubrum* incorporated ^{14}C from phenylacetate-1-^{14}C during anaerobic growth. The radioactivity in the protein fraction of cells was mainly in phenylalanine. Phenylalanine from *Chromatium* cells grown in phenylacetate-1-^{14}C was labeled at carbon 2. Incorporation of phenylacetate by *Chromatium* was decreased in the presence of exogenous phenylalanine, and de novo synthesis of phenylalanine from bicarbonate was less in medium containing either phenylalanine or phenylacetate. These organisms, and also certain anaerobic rumen bacteria, apparently carboxylate phenylacetate to synthesize the phenylalanine carbon skeleton. The mechanism of the carboxylation is unknown; however, it appears to be dependent upon anaerobic conditions, since *R. rubrum* did not synthesize phenylalanine from phenylacetate during aerobic growth in the dark.

In yeast and in certain other aerobic organisms, the side chains of phenylalanine and tyrosine arise as 3-carbon units from glycolysis (17, 25, 27). Certain anaerobic bacteria from the rumen, however, appear to use a different biosynthetic pathway, since, during growth in the presence of phenylacetate-1-^{14}C, ^{14}C was incorporated into phenylalanine but not into other amino acids (1). It was suggested that synthesis involves carboxylation of phenylacetate to form phenylpyruvate which is then aminated to produce phenylalanine.

The present study demonstrates that anaerobic bacteria other than ruminal bacteria also synthesize phenylalanine by use of the carbon skeleton of phenylacetate.

MATERIALS AND METHODS

Cultures of *Chromatium* strain D and *Chlorobium thiosulfatophilum* 8327 were obtained from D. S. Hoare, University of Texas. *Rhodospirillum rubrum* strain 13A5 was obtained from P. A. Hartman, Iowa State University. The inorganic medium described by Hendley (19) and the "malate" medium of Arnon et al. (6) were used to culture *Chromatium*. The chemically defined liquid medium of Ormerod et al. (28) was used for culture of *R. rubrum*, and *C. thiosulfatophilum* was grown in the medium of Larsen (24). Media for anaerobic culture were prepared and maintained under oxygen-free N_2 or CO_2 by use of the anaerobic technique described by Hungate (21). The culture vessels were rubber-stoppered tubes (18 by 150 mm). The photosynthetic bacteria were cultured at 28 to 32 C, and light was supplied by two 15-w fluorescent bulbs and one 100-w incandescent lamp. The same medium used for anaerobic growth

of *R. rubrum* was used for aerobic culture. In the latter case, however, cotton-stoppered 500-ml Erlenmeyer flasks, containing 100 ml of medium, incubated in the dark on a shaker were used. The Erlenmeyer flasks had attached side-arm tubes to permit measurement of growth in the culture vessels. Growth was measured as optical density at 600 mμ in 18-mm tubes.

Phenylacetate-1-^{14}C was obtained from Nuclear-Chicago Corp., Des Plaines, Ill. (specific activity, 3.98 mc/mmole), and from New England Nuclear Corp., Boston, Mass. (specific activity, 4.4 mc/mmole). Aqueous solutions of NaH $^{14}CO_3$ (specific activity, 1.84 mc/mmole; Volk Radiochemical Co., Skokie, Ill.) and of the labeled phenylacetate were filtered through sterile membrane filters (type HA, 0.45 μ; Millipore Filter Corp., Bedford, Mass.) before use.

Cells were washed with an anaerobic mineral dilution solution (9) and were fractionated by use of the methods of Roberts et al. (29). Protein hydrolysates were chromatographed on paper by use of a butanol-acetic acid solvent system proposed by Smith (34), a benzyl alcohol solvent system buffered at *p*H 6.2, and the benzyl alcohol-butanol solvent system buffered at *p*H 8.4 described by McFarren (26). The latter systems separated phenylalanine from other amino acids. Radioactive areas on paper chromatograms were located by preparation of radioautographs and with a chromatogram strip scanner. To measure the quantity of phenylalanine, the specific activity of phenylalanine was determined from eluates from paper chromatograms by the method of Rosen (30).

Radioactivity was usually measured with a liquid scintillation counter and the xylene, dioxane, Cellosolve solvent of Bruno and Christian (8). Counting efficiency and quenching were measured with toluene-

^{14}C as an internal standard. The rate of incorporation of phenylacetate-1-^{14}C during growth of *Chromatium* cells was determined by filtering and washing cells on membrane filters (0.45 μ, type HA; Millipore Filter Corp.). The filters were cemented to planchets with rubber cement and dried, and radioactivity was measured with a windowless gas-flow counter.

Unlabeled carrier phenylalanine (300 μmoles) was added to labeled phenylalanine (0.07 μmole) isolated by chromatography from hydrolyzed protein of *Chromatium* cells grown in phenylacetate-1-^{14}C. One such sample was decarboxylated with ninhydrin (31), and another sample was partially degraded to benzoic acid with dichromate by use of the methods of Gilvarg and Block (17) as modified by Hoare and Gibson (20). Carbon dioxide was trapped in 2.5 N NaOH; the carbonate was precipitated as $BaCO_3$, washed, dried, and was counted as a suspension with the liquid scintillation counter (12). The counting efficiency (42% with 50-mg samples) was determined by use of $Ba^{14}CO_3$ of known specific activity. A sample of uniformly labeled ^{14}C-phenylalanine was degraded as a control to test the effectiveness of the methods.

RESULTS

Chromatium cells incorporated most of the ^{14}C from phenylacetate-1-^{14}C during illuminated anaerobic growth on a medium with carbonate as the main carbon source and on a medium containing malate as the major carbon source (Table 1). The radioactivity was mainly in the hot-trichloroacetic acid precipitate and the ethyl alcohol-ether extract of the cells. Acid hydrolysates of the hot-trichloroacetic acid precipitate fraction were chromatographed on paper by use of several different solvent systems, and, in all cases, the ^{14}C migrated with phenylalanine. Other fractions of the cells were not studied. When *Chromatium* cells were grown in the carbonate medium (19) plus isobutyrate-1-^{14}C, isovalerate-1-^{14}C, or indoleacetate-1-^{14}C, less than 1% of the ^{14}C from the medium was incorporated by the cells.

When *R. rubrum* was grown with phenylacetate-1-^{14}C anaerobically in the light, 51% of the ^{14}C from the growth medium was incorporated into the cells (Table 2). The distribution of ^{14}C in fractions of *R. rubrum* cells was similar to that found with *Chromatium*. The ^{14}C in hydrolysates of the hot-trichloroacetic acid precipitate of these cells also migrated with phenylalanine during paper chromatography (Fig. 1). *R. rubrum* cells grown aerobically in the dark, in the same medium used for anaerobic growth, incorporated only 0.16% of the ^{14}C from phenylacetate-1-^{14}C.

The data given in Table 2 also indicate the extent of incorporation of ^{14}C from isobutyrate-1-^{14}C and indoleacetate-1-^{14}C by *R. rubrum* during anaerobic illuminated growth. Radioautographs of paper chromatograms of hydrolysates of the

TABLE 1. *Incorporation of phenylacetate-1-^{14}C by Chromatium and distribution of ^{14}C in fractions of the cells*

Determination	Carbonate medium[a]	Malate medium[b]
^{14}C from medium incorporated into cells (percentage of ^{14}C in culture)	82	91
Distribution of ^{14}C in fractions of cells (percentage of total ^{14}C in cells)		
Cold-trichloroacetic acid extract	0 5	1.2
Ethyl alcohol-ether extract	38	38
Hot-trichloroacetic acid extract	3.1	4.1
Wash of hot-trichloroacetic acid precipitate	2.8	7.7
Hot-trichloroacetic acid precipitate	55	49

[a] Medium of Hendley (19) plus phenylacetate-1-^{14}C (2.3 $\times$ 10^{-5} M, 1.75 $\times$ 10^6 dpm). Optical density increased from 0.12 to 1.05 in 39 hr.

[b] Medium of Arnon et al. (6) plus phenylacetate-1-^{14}C (10^{-4} M, 2.14 $\times$ 10^6 dpm). Optical density increased from 0.06 to 1.2 in 46 hr.

hot-trichloroacetic acid precipitates show that the ^{14}C from these two labeled materials was present in several amino acids (Fig. 1).

The rate of incorporation of ^{14}C from phenylacetate-1-^{14}C by growing cells of *Chromatium* is given in Fig. 2. The optical density of the cell suspension in the growth medium (19) at 0 hr was 0.8, and the phenylacetate-1-^{14}C concentration was 4 $\times$ 10^{-6} M (specific activity, 3.98 mc/mmole). The culture was subdivided, and additions as indicated in Fig. 2 were made at 30 min when 6% of the ^{14}C in the culture had been incorporated into the cells.

Addition of phenylalanine (final concentration, 10^{-3} M) greatly reduced incorporation of ^{14}C from phenylacetate-1-^{14}C as did 250-fold dilution of the specific activity with unlabeled phenylacetate. Addition of lower levels of phenylacetate and phenylalanine reduced incorporation of ^{14}C through 7 hr, but after 24 hr the uptake of ^{14}C in these cultures approached that of cultures to which only water was added (94% of the ^{14}C incorporated into cells). The data indicate that, with excess phenylalanine in the medium, synthesis of phenylalanine by use of phenylacetate carbon is repressed. With 10^{-5} M phenylalanine, the repression of phenylacetate incorporation was less and temporary, perhaps because of utilization of most of the exogenous phenylalanine by the cells. Phenylpyruvate (10^{-4} M) had little effect on the

TABLE 2. *Incorporation of ^{14}C from labeled acids by Rhodospirillum rubrum and distribution of ^{14}C in fractions of the cells*

Determination	Phenylacetate-1-^{14}C		Isobutyrate-1-$^{14}C^a$	Indoleacetate-1-$^{14}C^b$
	Illuminated anaerobic growth[c]	Dark aerobic growth[d]		
^{14}C from medium incorporated into cells (percentage of total ^{14}C in culture)...........................	51	0.16	16	7.7
Distribution of ^{14}C in fractions of cells (percentage of total ^{14}C in cells)				
Cold-trichloroacetic acid extract....................	1.8	13	9.8	7.9
Ethyl alcohol-ether extract.........................	43	28	30	32
Hot-trichloroacetic acid extract.....................	6.1	3.3	41	39
Wash of hot-trichloroacetic acid precipitate.........	1.9	0	1.4	0.6
Hot-trichloroacetic acid precipitate.................	47	56	18	20

a Isobutyrate-1-^{14}C (1.6×10^{-4} M, 9.6×10^6 dpm); optical density increased from 0.05 to 0.70 in 366 hr.

b Indoleacetate-1-^{14}C (5.6×10^{-5} M, 9.9×10^6 dpm); optical density increased from 0.05 to 0.85 in 336 hr.

c Phenylacetate-1-^{14}C (8.3×10^{-5} M, 7.3×10^6 dpm) added to medium of Ormerod (28); optical density increased from 0.05 to 0.74 in 228 hr.

d Phenylacetate-1-^{14}C (8.5×10^{-5} M, 7.5×10^6 dpm); optical density increased from 0.04 to 0.75 in 47 hr.

incorporation of ^{14}C from phenylacetate. The culture incubated in the dark did not grow, and there was no measurable incorporation of phenyl acetate.

The results obtained by degradation of phenylalanine from *Chromatium* cells grown in phenylacetate-1-^{14}C are given in Table 3. The recoveries of carbonate were greater than theoretical from both the ninhydrin and the dichromate oxidation reactions. It is evident, however, that the radioactivity in the phenylalanine was in carbon number 2, since a negligible amount of ^{14}C was recovered from the carboxyl carbon obtained by reaction with ninhydrin or in carbons 3 to 9 isolated as benzoic acid.

When *Chromatium* cells were grown in the inorganic medium (19) containing NaH $^{14}CO_3$ (0.086 $\mu c/\mu$mole), the amount of ^{14}C incorporated into phenylalanine and the specific activity of cellular phenylalanine were depressed by the presence of either phenylalanine or phenylacetate in the culture medium (Table 4). Phenylpyruvate had less effect upon the incorporation of ^{14}C into phenylalanine. The effect of exogenous phenylalanine and phenylacetate on the utilization of carbonate for phenylalanine biosynthesis was also seen with radioautographs prepared from paper chromatograms of the protein hydrolysates (Fig. 3).

C. thiosulphatophilum differed from *R. rubrum* and *Chromatium* in that less than 0.1% of the ^{14}C was incorporated into cells when it was grown in a

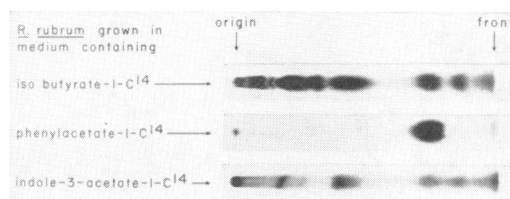
FIG. 1. *Radioautograph of paper chromatograph (with butanol-acetic acid) of protein hydrolysates from Rhodospirillum rubrum cells grown in media containing labeled compounds as indicated.*

medium containing phenylacetate-1-^{14}C. This organism incorporated 2% of the ^{14}C from indoleacetate-1-^{14}C and 21% of the ^{14}C from isobutyrate-1-^{14}C into cells. Most of the radioactivity from the latter two materials was in the ethyl alcohol-ether extract of the cells. No evidence was obtained for the carboxylation of these acids by *C. thiosulphatophilum* to synthesize amino acid carbon skeletons.

Several attempts were made to obtain phenylalanine biosynthesis from phenylacetate-1-^{14}C with cell-free preparations from *Chromatium*. The methods used were similar to those used by Buchanan et al. (10). Cell extracts containing an active hydrogenase were incubated in a H_2 atmosphere with and without added ferredoxin. These cell-free extracts fixed $^{14}CO_2$, but synthesis of phenylalanine or phenylpyruvate was not detected.

DISCUSSION

Much of current information concerning pathways of amino acid biosynthesis was obtained with aerobic and facultative microorganisms. Evidence is accumulating, however, that biosynthetic pathways in obligate anaerobes are not necessarily the same, as in, for instance, *Escherichia coli, Neurospora,* and yeast (3, 14, 16, 20, 22, 35, 36).

In *E. coli,* the side chains of both phenylalanine and tyrosine arise as 3-carbon units from phosphoenolpyruvate via prephenate (32, 39). The distributions of ^{14}C from acetate and bicarbonate in the side chains of phenylalanine synthesized by *C. thiosulphatophilum* (20) and tyrosine synthesized by *R. rubrum* (15) were also as expected if the side chains were derived directly from phosphoenolpyruvate. Evidence for or against the involvement of prephenate in biosynthesis of phenylalanine by *R. rubrum* and *Chromatium* is lacking; however, if the prephenate pathway is

functional, then at least two biosynthetic mechanisms are possible since phenylacetate is not an intermediate in that pathway.

The failure of cells of *R. rubrum* to utilize phenylacetate in phenylalanine biosynthesis during aerobic growth in the absence of light indicates that this biosynthesis is dependent upon anaerobic metabolic systems.

If the pathway for de novo biosynthesis of phenylalanine in these photosynthetic anaerobes does not involve phenylacetate as an intermediate, the importance of phenylacetate carboxylation as a biosynthetic process may depend upon the presence and concentration of exogenous phenylacetate. Information concerning the occurrence of phenylacetate in the natural environments of photosynthetic anaerobes is lacking. The purple bacteria occur in nature as a secondary flora, depending, for their growth, on the production of simple breakdown products by a primary and varied microflora (37). *Chromatium* and other members of the *Thiorhodaceae* have been found in high numbers in anaerobic waste digestion lagoons (13), and catabolism of phenylalanine to phenylacetate in these environments, as has been found in the rumen (23, 33), seems likely. Phenylacetate has also been detected as an end product of metabolism of phenyldecane, phenyldodecane, and phenyloctadecane by a *Nocardia* species from the soil (38).

Synthesis of phenylalanine from phenylacetate probably occurs in many anaerobic environments. Anaerobic photosynthetic bacteria are widely distributed in nature, and it is probable that organisms in addition to *Chromatium* and *R. rubrum* have this capacity. Bacteria similar to or identical with the ruminal organisms that use phenylacetate in phenylalanine biosynthesis (1, 2) have been found in several other anaerobic environments (7, 18). The ability to use phenylacetate as a phenylalanine precursor in these environments would reduce energy requirements as compared with synthesis of phenylalanine from less complex substances, and may indicate a fitting of organisms to an ecological niche.
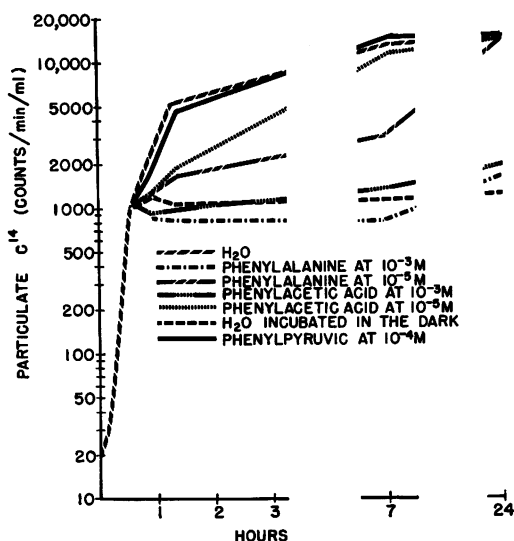

FIG. 2. *Effect of indicated additions on incorporation of ^{14}C from phenylacetate-1-^{14}C by Chromatium.*

TABLE 3. *Degradation of phenylalanine from Chromatium cells grown in phenylacetate-1-^{14}C*

Carbon	Reaction	Material counted	Quantity recovered	Radioactivity
			mmoles	
1	Ninhydrin[a]	BaCO₃	0.55	5 dpm/50 mg[b]
1 and 2	Dichromate[a] oxidation	BaCO₃	1.17	1,540 dpm/50 mg[b]
3–9	Dichromate[a] oxidation	Benzoic acid	0.11	0 dpm/13.3 mg[c]

[a] Degraded 0.3 mmoles of phenylalanine (19,500 dpm).
[b] Duplicate BaCO₃ suspensions (50 mg each) counted.
[c] Hexane solution counted in XDC solvent (8).

TABLE 4. *Isotopic competition studies with Chromatium cells grown in medium containing $^{14}CO_2$*

Competitor added	Relative amount of ^{14}C in phenylalanine[a]	Specific activity of phenylalanine[b]
None.....................	5.3	26,900
Phenylalanine, 10^{-3} M......	2.4	12,700
Phenylacetate, 10^{-3} M......	3.1	17,300
Phenylpyruvate, 10^{-3} M.....	4.8	21,571

[a] Radioactivity in the phenylalanine area of the paper chromatogram expressed as percentage of total ^{14}C present in the protein hydrolysate.

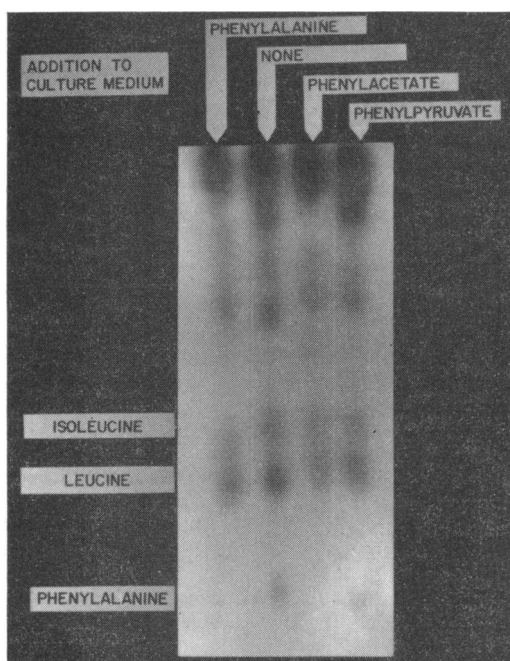[b] Disintegrations per minute per micromole of phenylalanine.

FIG. 3. *Radioautograph of a paper chromatogram (with benzyl-butyl alcohol buffered at pH 8.4) of protein hydrolysates from Chromatium cells grown in media containing $^{14}CO_2$ with additions as given at the top of the figure.*

Anaerobic rumen bacteria are capable of synthesizing amino acid carbon skeletons other than that of phenylalanine by carboxylating acids with one less carbon. Thus, isovalerate and isobutyrate are used in synthesis of leucine and valine, respectively (3), and tryptophan is synthesized from indole-3-acetate (Allison and Robinson, *unpublished data*). Experiments were conducted with some of these acids to determine whether the

photosynthetic anaerobes also had these capabilities. Radioactivity from isobutyrate-1-^{14}C and indole-3-acetate-1-^{14}C was well distributed in fractions of cells of *R. rubrum* and was present in most or all of the amino acids. This indicates that these acids were degraded and that the carboxyl carbon was used for biosynthesis (Fig. 1). Use of isobutyrate as a carbon source for *R. rubrum* had been demonstrated by van Niel (37). Evidence for carboxylation of indoleacetate and isobutyrate to produce tryptophan and valine carbon skeletons, as found with rumen bacteria, was not obtained. With *C. thiosulphatophilum*, radioactivity from isobutyrate-1-^{14}C was not well distributed in cell fractions but was incorporated mainly into the fraction extracted with ethyl alcohol-ether. This suggests that the isobutyrate was not significantly catabolized. From previous experience with anaerobic rumen bacteria which incorporate isobutyrate into cell lipids (4), we think it likely that ^{14}C from isobutyrate was incorporated into higher even-numbered branched-chain fatty acids.

The data in Fig. 2 demonstrate that synthesis of phenylalanine using phenylacetate carbon was decreased when excess phenylalanine was present in the medium. Synthesis of phenylalanine using labeled carbonate was also less when either phenylacetate or phenylalanine was present in the medium (Table 4). Inhibition of de novo biosynthesis of phenylalanine by exogenous phenylacetate supports the concept that significant quantities of phenylalanine carbon could arise from phenylacetate in the natural environment of these bacteria. Phenylpyruvate did not greatly affect the rate of biosynthesis of phenylalanine from either phenylacetate or bicarbonate, but failure to compete with these carbon sources may merely indicate an impermeability of the cells toward phenylpyruvate.

Synthesis of phenylalanine-2-^{14}C from phenylacetate-1-^{14}C probably involves carboxylation of phenylacetate, or a derivative, to produce phenylpyruvate, which is then transaminated to produce phenylalanine. The mechanism of the carboxylation reaction is unknown, but the most probable model for the reaction is the reduced ferredoxin-dependent carboxylation of acetyl-coenzyme A (CoA) to produce pyruvate, which has recently been demonstrated in several anaerobic microorganisms including *Chromatium* (5, 10). A similar reductive carboxylation of succinyl-CoA to produce α-ketoglutarate has recently been demonstrated in *C. thiosulphatophilum* (11). Reductive carboxylations of carboxyl groups must also be involved in synthesis of leucine from isovalerate, valine from isobutyrate, and tryptophan from indole-3-acetate by anaerobic bacteria from the

rumen. Studies to determine the nature of the carboxylation reactions are in progress.

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