PHENYLALANINE BIOSYNTHESIS FROM PHENYLACETIC ACID BY ANAEROBIC BACTERIA FROM THE RUMEN

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Received October 15, 1964

Amino acid biosynthesis has not been studied in many anaerobic bacteria but in several instances it has been shown that the biosynthetic mechanisms are different from those that have been demonstrated in aerobic bacteria. Evidence for this has been presented by Cutinelli et al. (1951) with Rhodospirillum rubrum, Tomlinson (1954 a,b) with Clostridium kluyveri, and by Hoare and Gibson (1964) with Chlorobium thiosulphatophilum.

Further evidence for distinct biosynthetic pathways has been obtained with anaerobic bacteria from the rumen which biosynthesize leucine, valine and probably isoleucine using the carbon skeletons of branched-chain fatty acids containing one carbon less than the amino acid product. With Ruminococcus flavefaciens, carbon dioxide, but not formate, contributes to the carboxyl carbon of these amino acids, and a significant amount of C^{14} from $C^{14}O_2$ was noted in other amino acids including phenylalanine (Allison and Bryant, 1963).

Scott et al. (1964) found that rumen liquor from sheep contained significant amounts of phenyl substituted fatty acids, including phenylacetic acid, and determined that these were produced from phenylalanine, tyrosine and tryptophan by rumen microorganisms. It seemed possible that the phenylalanine carbon skeleton could be synthesized by a carboxylation of phenylacetic acid using a mechanism similar to that used to synthesize the branched-chain amino acid carbon skeletons from branched-

chain fatty acids. The experiments reported here were conducted to determine if this occurred.

Materials and Methods

Ruminal ingesta was obtained from an adult sheep maintained on an alfalfa hay ration. Microscopic observation of the ruminal ingesta indicated the presence of an apparently normal cilliate protozoan population, mainly Entodinia and Isotricha species. Ruminococcus flavefaciens strain C94 and Bacteroides succinogenes strain S85 were obtained from Dr. M. P. Bryant. Both of these cellulolytic anaerobes from the rumen were cultured using the anaerobic technique of Hungate (1950) in the medium of Bryant and Robinson (1961) except casein hydrolysate was deleted. Escherichia coli, strain B, obtained from Joseph Songer of this Laboratory, was cultured in medium C of Roberts et al. (1957).

Phenylacetic acid-1-C^{14} (Cal. Biochem. Corp.) SA 3.0 mc/mM was sterilized using ethylene oxide after which it was dissolved in anaerobic dilution solution (Bryant and Burkey, 1953) and added to both the anaerobic culture medium and medium C at similar levels. Paper chromatograms of the phenylacetic acid-1-C^{14} showed that it was not contaminated with labelled phenylalanine. Growth was estimated as optical density (OD) at 600 mμ in 18 x 150 mm Pyrex tubes. Cells were fractionated and protein was hydrolyzed under N_2 with HCl in sealed tubes using the methods of Roberts et al. (1957). Protein hydrolysates were chromatographed by descending development in one dimension using several solvent systems. The benzyl alcohol solvent system at pH 6.2 described by McFarren (1951) gave good separation of phenylalanine from other amino acids. Radioactive areas were positioned using a windowless gas flow detector and a chromatogram strip scanner and also by preparation of radioautographs.

Results

A significant quantity (4.4%) of the radioactivity from phenylacetic

acid-1-C^{14} was incorporated into microbial cells during in vitro fermentation of the mixed ruminal population (Table 1). No attempt was made to distinguish between the C^{14} in bacterial and protozoal cells. Most of the cellular radioactivity was in protein and phenylalanine was the only amino acid with radioactivity that was detected when paper chromatograms of protein hydrolysates were examined. No radioactivity was noted in a NaOH trap through which gases from the fermentation mixture were bubbled at the end of the fermentation.

Both pure cultures of rumen bacteria incorporated carbon from phenylacetic acid-1-C^{14}. In both organisms, most of the cellular radioactivity was in the protein fraction of the cells, but the distribution of C^{14} between other cellular fractions was quite different (Table 2). R. flavefaciens incorporated approximately 55% of the C^{14} in the medium during growth to an OD of 0.46 in 29 hrs. The culture of B. succinogenes

TABLE 1

Distribution of Radioactivity After In Vitro Fermentation of Rumen Fluid in the Presence of Phenylacetic Acid-1-C^{14}.[a]

	Radioactivity (dpm)[b]
Whole Culture	5,760,000
Particulate Fraction[c]	
Ethanol-Ether Extract	47,600
Hot TCA Extract	7,400
Wash of Protein	1,600
Protein	196,000

[a] Rumen contents from a sheep on an alfalfa hay ration strained through two layers of cheese cloth and 3.0 ml of the filtrate added to 3.0 ml anaerobic dilution solution containing phenylacetic acid-1-C^{14}. Fermentation was under CO_2 atmosphere for 2 hrs. at 39°C.

[b] Disintegrations per minute measured using a liquid scintillation counter and counting efficiency determined by addition of internal standard.

[c] Centrifuged 15 min. at 7,000 x g at 2°C; washed in anaerobic dilution solution and fractionated by methods of Abelson, Bolton and Aldous (1952).

TABLE 2

Distribution of Radioactivity After Growth of Two Species of Cellulolytic Bacteria From the Rumen in a Medium Containing Phenylacetic Acid-1-C^{14}.

	R. flavefaciens dpm[a]	B. succinogenes dpm
Whole Culture	2,160,000	2,092,000
Cells[b]		
Cold TCA Extract	234,000	5,600
Ethanol-Ether Extract	18,600	44,500
Hot TCA Extract	31,200	11,500
Wash of Protein	13,900	2,900
Protein	884,000	196,000

[a] Disintegrations per minute measured using a liquid scintillation counter with internal standard added to determine counting efficiency.

[b] Cells fractionated using the methods of Roberts et al. (1957).

grew to an OD of 0.75 in 29 hrs. but incorporated only 12.5% of the C^{14} into cells. The E. coli culture was harvested at 17 hrs. (OD 0.45) and less than 0.1% of the radioactivity from phenylacetic acid-1-C^{14} was incorporated into the cells.

The only amino acid with appreciable radioactivity in hydrolysates of the protein from either mixed or pure cultures of rumen bacteria migrated with the phenylalanine area on paper chromatograms (Rf 0.37 in the benzyl alcohol system). The data thus show that phenylalanine was biosynthesized using the carbon skeleton of phenylacetic acid, and also that phenylacetic acid-1-C^{14} was not decarboxylated and tyrosine was not formed from phenylalanine or its immediate precursors. Preliminary data obtained by degradation of phenylalanine biosynthesized from phenylacetic acid-1-C^{14} indicated that carbon 2 was labeled.

Discussion

Phenylalanine biosynthesis was essential for growth of the pure cultures studied here. Cysteine was the only amino acid present in the

anaerobic medium used to grow the rumen bacteria and medium C used to culture E. coli did not contain amino acids.

In yeast the side chains of phenylalanine and tyrosine arise from a 3-carbon unit of glycolysis which branches off somewhere above pyruvate (Gilvarg and Block, 1952). The data given here do not preclude the function in the rumen of the pathway described in aerobic organisms (Davis, 1955), but do indicate the operation of a different pathway, the relative importance of which is not yet known.

The present results indicating that E. coli B does not use phenylacetic acid carbon for phenylalanine biosynthesis agree with results of Davis (1951). He studied a mutant of E. coli that could utilize shikimate or phenylpyruvate, but not phenylacetate as a substitute for phenylalanine.

Phenylacetic acid may be carboxylated, presumably to produce phenylpyruvate which is transaminated to produce phenylalanine. The mechanism is perhaps similar to that by which the branched-chain amino acids are synthesized from branched-chain fatty acids, but details of the reactions are not yet known. It is suggested that para-hydroxy-phenylacetic acid and 3-indoleacetic acid may be similarly carboxylated to produce the carbon skeletons of tyrosine and tryptophan, and experiments to test this are in progress.

Since phenylacetic acid is present in the rumen, it may be more economical for microorganisms in the rumen to use phenylacetic acid in phenylalanine biosynthesis than to synthesize the carbon skeleton from carbohydrate or other substances. Furthermore, since there is a tendency for microorganisms to lose unnecessary or unused biosynthetic capabilities, it is suggested that bacteria might be found in the rumen that have become nutritionally dependent upon an exogenous supply of phenyl-substituted precursors of amino acids.

Acknowledgment

The technical assistance of Jerry A. Bucklin is gratefully acknowledged.

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