THE INCORPORATION OF PIMELIC ACID AS A UNIT IN THE BIOSYNTHESIS OF BIOTIN

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Previous investigators have obtained indirect evidence that pimelic acid is a precursor of biotin (1,2,3,4,5). We have obtained direct evidence that pimelic acid serves as a precursor of biotin by isolating labeled biotin sulfone from hydrogen peroxide-treated eluates derived from culture filtrates of Aspergillus niger grown on a medium containing pimelic acid-1,7-C^{14} (6). The incorporation of pimelic acid into biotin was substantiated in a later report by Eisenberg in which labeled desthiobiotin was obtained after growth of Phycomyces blakesleeanus on a medium containing labeled pimelic acid-1,7-C^{14} (7). The experiments described in this paper provide evidence that pimelic acid is incorporated as a unit in the biosynthesis of biotin and offer no substantiation for the results of Eisenberg indicating a multiple entry of pimelic acid into the biotin molecule.

EXPERIMENTAL

Aspergillus niger was grown at 30°C with shaking on a Czapex-Dox medium which also contained 1 mg of pimelic acid-1,7-C^{14} (S.A. 1.147×10^7 cpm/mg) and 40 mg of lipoic acid per liter. After seven days of growth, the A. niger mycelia was removed by filtration through glass wool. The biotin vitamers were concentrated from the medium by absorption on Norit A and by elution with ethanol, water, and ammonia (10:10:1). The volume of eluate was reduced in vacuo and then extracted eight times with an equal volume of ether. This

extraction procedure removes over 99% of unconverted pimelic acid. The eluate was then further concentrated to 2-5 ml.

Although Wright et al. have shown that biotin-l-sulfoxide is the predominant form of biotin produced when A. niger is grown with aeration (8), the small amounts of compound produced, as well as the solubility characteristics of the compound, required for isolation purposes the use of carrier biotin and the conversion of biosynthetic biotin and biotin-l-sulfoxide together with carrier biotin to a common derivative. Experience has led to the choice of biotin sulfone. Thus 500 mg of unlabeled biotin and 100 ml of glacial acetic acid were added to the concentrated eluate. The mixture was treated with 25 ml of 30% hydrogen peroxide for 20 hours and then was evaporated to dryness in vacuo. The residue was recrystallized from water. Radioactivity of the biotin sulfone was determined with a Packard 'Tricarb' scintillation spectrometer on small portions of material dissolved in 10 ml of Bray's solution. Recrystallization of the biotin sulfone was continued until the crystals showed constant specific activity. The results of a typical experiment are shown in Table 1.

TABLE 1 Radioactivity of Recrystallized Biotin Sulfone

Recrystallization	M. p. (uncorrected)	Cpm/mg	Cpm/m mole
A	279 - 284°	571	157,000
B	278 - 283°	498	137,000
C	279 - 283°	432	119,000
D	280 - 285°	453	125,000

In order to determine whether or not the pimelic acid was incorporated as a unit in the biosynthesis of biotin, a decarboxylation of isolated biotin sulfone involving the Schmidt reaction was performed (9). If pimelic acid is incorporated into biotin as a unit, the Schmidt reaction should yield one half of the radioactivity of biotin sulfone in the form of carbon dioxide and one half of the radioactivity in the form of an amine.

Fifty mg of recrystallized biotin sulfone of constant specific activity was dissolved in 0.3 ml of fuming sulfuric acid. The mixture was chilled in an ice bath, and 90 mg of sodium azide was added. The temperature was raised to 100° over a twenty-minute period and kept at this temperature for 30 minutes. Throughout, a stream of nitrogen was passed through the system to carry over the evolved carbon dioxide. The gas stream was washed by passing it through two $KMnO_4$ traps after which it was passed into a $Ba(OH)_2$-$BaCl_2$ solution to precipitate evolved CO_2 as $BaCO_3$. The $BaCO_3$ was filtered, washed and dried. Radioactivity of the $BaCO_3$ was determined on the liquid scintillation counter after suspension of the material in Bray's solution according to the procedure of Hayes et al. (10).

The amine derivative of the decarboxylation reaction was isolated by ion exchange chromatography. The reaction mixture was applied to a Dowex-1 (OH^-) column (1.5 x 18.5 cm). The column was washed with water and eluted with 0.1 N HCl. Fifty ml fractions were collected and the radioactivity of each fraction determined by counting an aliquot in Bray's solution. The results of a typical experiment are summarized in Figure 1.

The combined fractions containing a neutral or basic radioactive compound that was not retained by a Dowex-1 (OH^-) column were applied to a Dowex-50 (H^+) column (1.5 x 15.3 cm). The column was washed with water and eluted with 0.1 N NaOH. Again 50 ml fractions were obtained, and an aliquot of each fraction was counted. The results of a typical experiment are summarized in Figure 2.

The fractions containing radioactive material that had been retained by the cation exchange column were combined and concentrated to a small volume. Crystals were obtained on cooling. The crystals were filtered, washed and dried. An infra-red spectrum on these crystals is compatible with reference spectra of biotin and biotin sulfone. All have major absorption peaks at 2.9, 5.9, and 6.7 microns. The first two are found in ureido-type structures whereas all three are characteristic of a monosubstituted amide, i.e. biotin.

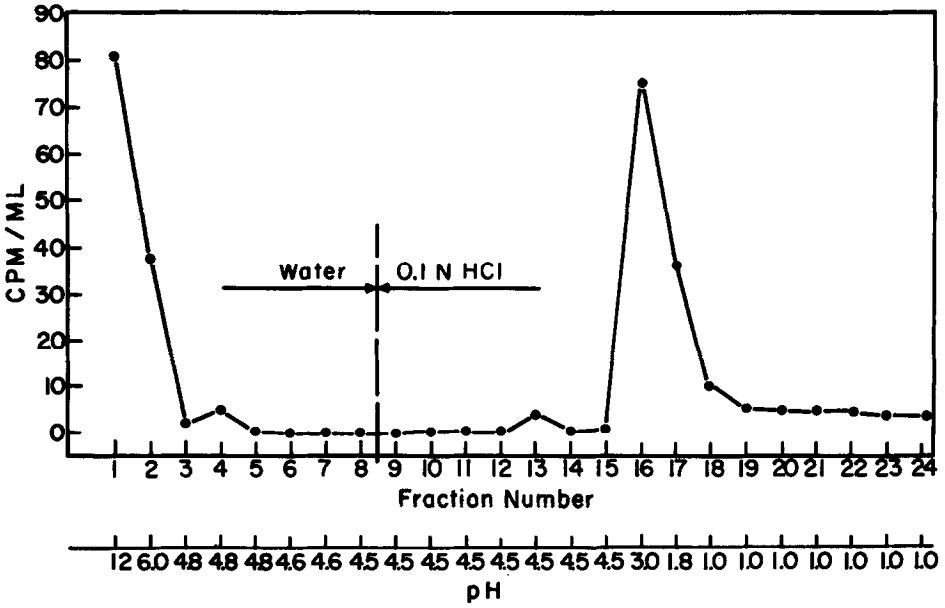

Figure 1. Anion exchange chromatography of the products of the Schmidt reaction on Dowex-1(OH^-).

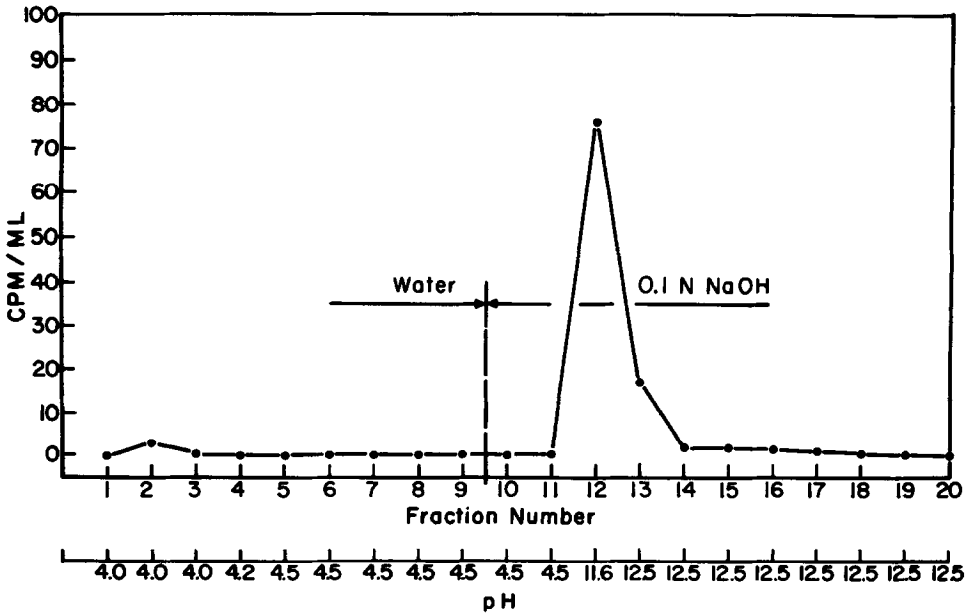

Figure 2. Cation exchange chromatography of the basic product of the Schmidt reaction on Dowex-50(H^+).

The amine derivative and biotin sulfone also possess major absorption peaks at 7.6 and 8.8 microns that are found in sulfone compounds. However, minor absorption peaks of 4.0 and 5.1 microns found in amido acid type compounds and seen in spectra of biotin and biotin sulfone are absent in the spectrum of the amine. In addition, a new small peak at 6.4 - 6.5 microns, characteristic of amine compounds, is seen in the amine spectrum but is absent in the spectra of biotin and biotin sulfone. The crystals were counted after being dissolved in Bray's solution.

The results of three experiments involving the counting of the products of the Schmidt reaction are summarized in Table 2.

TABLE 2 Radioactivity of Biotin Sulfone and Decarboxylation Products

Expt	Material counted	Cpm/mg	Cpm/m mole	% Radioactivity of Biotin sulfone
1	Biotin sulfone	650	180,000	--
	Barium carbonate	360	75,000	42
2	Biotin sulfone	721	199,000	--
	Barium carbonate	433	85,000	43
3	Biotin sulfone	721	199,000	--
	Barium carbonate	420	83,000	42
	Isolated amine	389	85,000	48

DISCUSSION AND CONCLUSION

The data indicate that the carbon dioxide produced from the decarboxylation of biotin sulfone derived from the culture filtrate of A. niger grown in the presence of labeled pimelic acid has, within experimental error, one half of the counts present in the original sulfone. Similarly, a basic compound presumed to be the amine, expected from the action of hydrazoic acid on biotin sulfone, has the other half of the counts present in the original sulfone. These results are interpreted as evidence that both carboxyl groups of pimelic acid participate in the biosynthesis of biotin. Similarly, pimelic acid as a unit must serve as a structural moiety rather than as a source of

smaller fractions which are biosynthetically active. Lastly, the results obtained indicate that only one mole of pimelic acid is involved in the biosynthesis of one mole of biotin.

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