BIOSYNTHESIS OF THE 5,6-DIMETHYLBENZIMIDAZOLE MOIETY OF VITAMIN B_{12}

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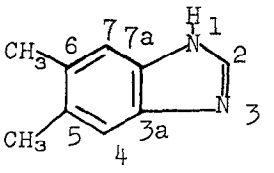
Received January 29, 1968

Among the several novel chemical groupings present in the cyanocobalamin molecule is the unique nucleotide 1-α-D-ribofuranosyl-5,6-dimethylbenzimidazole-3' -phosphate (α-ribazole phosphate). Biosynthetic studies have been carried out which show that the corrin ring structure is derived by modification of the established pathway leading to the porphyrin ring structure (Corcoran and Shemin, 1957; Bray and Shemin, 1963). The sequence of reactions involved in the formation of the complete cobalamin structure from the combination of cobinamide and α-ribazole derivatives has also been investigated (Friedmann and Harris, 1962). However, little is known regarding the mode of formation of the unique 5,6-dimethylbenzimidazole moiety.

Perlman and Barrett (1958) observed that 1,2-diamino-4,5-dimethylbenzene was incorporated into complete cobalamin structures by P. shermanii. More recently, Renz and Reinhold (1967) have published evidence for the formation of the 3a, 4, 5, 6, 7 and 7a ring carbon atoms and the two C-methyl groups of 5,6-dimethylbenzimidazole from four molecules of acetate. These results prompt us to communicate our evidence regarding the origin of the remaining carbon atom of 5,6-dimethylbenzimidazole, i.e., the C-2 carbon.



5,6-Dimethylbenzimidazole

5,6-Dimethylbenzimidazole (5,6-DMBI) isolated from cyanocobalamin is subjected to the Schotten-Baumann procedure with benzoyl chloride to form 1,2-dibenzamido-4,5-dimethylbenzene (1,2-DBAB) and formic acid (Bamberger and Berle, 1893). The formic acid solution is then oxidized with red mercuric oxide (Osburn, Wood and Werkman, 1933), to produce CO_2 which is isolated as $BaCO_3$. This procedure permits the determination of the activity present at C-2 directly ($BaCO_3$) or by difference (5,6-DMBI - 1,2-DBAB). We have applied this degradative scheme to 5,6-DMBI produced by P. shermanii supplied with formate-^{14}C, sodium bicarbonate-^{14}C and glycine-2-^{14}C. All three labeled compounds were incorporated to a slight extent into the 5,6-DMBI molecule, but, contrary to expectations, we find that formate-^{14}C is not preferentially incorporated into the C-2 carbon atom of 5,6-DMBI. The results also indicate that neither glycine-2-^{14}C nor bicarbonate-^{14}C are precursors of the C-2 carbon atom.

Methods

Radioactive determinations were carried out in toluene solution with PPO as the scintillator using a Beckman Liquid Scintillation System. Samples were counted for about 200 minutes to minimize the counting error. External standardization was used to determine the counting efficiency. Analyses were performed

by G. I. Robertson, Jr., Florham Park, New Jersey. Melting points were taken with a calibrated Fisher-Johns apparatus.

P. shermanii (ATCC 13763) were grown in Erlenmeyer flasks at 30°C. in the media described by Bernhauer et al.,(1960). After three days of standing culture, the media was neutralized to pH 6.8 with NH_4OH, additional glucose was added, the radioactive compound introduced, and shaking of the flasks begun to aerate the cultures. After two days of aerobic growth the pH of the media was again adjusted to 6.8 and more glucose added. After an additional two days of aerobic growth, the cells were harvested. The cobalamin coenzymes were extracted from the cell paste with ethanol, purified by ion exchange chromatography, and converted to cyanocobalamin which was then purified by liquid-liquid extraction and ion exchange chromatography according to published procedures (Barker et al., 1960; Toohey et al., 1961; Bray and Shemin, 1963).

The radioactive vitamin B_{12} was diluted with cold B_{12} (Sigma) to yield a total of 100 mg of material. The diluted sample was hydrolyzed with 6 N HCl and the 5,6-DMBI isolated according to the method of Brink and Folkers (1950). This 5,6-DMBI was diluted with enough cold 5,6-DMBI (Aldrich Chemical Co., recrystallized before use) to give a total sample of 500 mg, which was recrystallized twice from ethanol-water and then sublimed. Mp. 205-206°C. Calc. for $C_9H_{10}N_2$: C, 73.94; H, 6.90; N, 19.16. Found: C, 74.35; H, 6.75; N, 19.31.

5,6-DMBI (50-100mg) was suspended in 12 ml of 1 N NaOH at 0°C. and 2 ml of benzoyl chloride was added to the mechanically stirred mixture. After 2 hours an additional 12 ml of base was added and the mixture warmed to 50°C. The mixture was filtered to remove the 1,2-DBAB which was recrystallized several times

from dimethylformamide-water and then sublimed. Mp. 267-270°C. Calc. for $C_{22}H_{20}O_2N_2$: C, 76.72; H, 5.85; N, 8.13. Found: C, 76.44; H, 6.01; N, 8.24.

The aqueous filtrate was extracted with CH_2Cl_2, acidified with H_3PO_4, and extracted again with CH_2Cl_2. The acidic aqueous solution was then heated to 90°C. and N_2 bubbled through to remove any CO_2 present. The resulting formic acid solution was reacted with solid HgO at 90°C. and the CO_2 produced was trapped in a NaOH solution, and precipitated as $BaCO_3$. The $BaCO_3$ was converted to CO_2 and counted according to the method of Woeller (1961).

Results

Table 1

Radioactive Compound Added	Determined Activities (dpm/mM)		
	5,6-DMBI	1,2-DBAB	$BaCO_3$
Formate -^{14}C (1 mc)	319	282 (88%)	80 (25%)
Bicarbonate-^{14}C (1 mc)	58[a]	46[a] (79%)	5 (9%)
Glycine-2-^{14}C (0.5 mc)	61[a]	46[a] (75%)	7[a] (11%)

[a]Average of two separate determinations.

The results of the partial degradation of 5,6-DMBI are summarized in Table 1. The determination of the partial labeling pattern in material of such low specific activity was possible because Woeller's method (1961) allows millimolar quantities of CO_2 to be counted at high efficiency (86%); and because use of 1 ml of dimethylformamide to dissolve 5,6-DMBI and 1,2-DBAB allows 25-30 mg samples of these compounds to be counted at high efficiency (84%). Despite the low activity of the samples involved, the fact that the C-2 activities, as determined directly

($BaCO_3$) or as determined by difference (5,6-DMBI minus 1,2-DBAB), are in reasonably good agreement, indicates that the data in Table 1 are accurate enough to justify the conclusions reached below.

Discussion

The observation of Perlman and Barrett (1958) that 1,2-diamino-4,5-dimethylbenzene is incorporated into the cobalamin molecule and the recent experiments of Renz and Reinhold (1967) which indicate all of the carbon atoms of 5,6-DMBI except the C-2 carbon can be derived from acetate imply that the C-2 carbon is derived from a biological C-1 unit. Of the likely precursors to the C-2 atom, formate seemed the most probable. The condensation of o-phenylenediamines with carboxylic acid groups to form benzimidazoles is a standard heterocyclic synthesis. Furthermore, if formation of the imidazole ring in 5,6-DMBI were analogous to the formation of the imidazole ring in histidine or the purines (Mahler and Cordes, 1966) the formate carbon would be incorporated at C-2.

The data in Table 1 indicate, however, that only 12-25% of the label incorporated into 5,6-DMBI from formate-^{14}C is located at the C-2 position. Since random incorporation of formate into any of the nine carbon atoms of 5,6-DMBI would be 11%, we conclude that formate is not the biological precursor of the C-2 atom in this molecule. Similarly, the low level of incorporation of bicarbonate-^{14}C into the C-2 atom leads to the conclusion that this potential one carbon unit is not the biological precursor for C-2. Finally, the lack of preferential incorporation of ^{14}C from glycine-2-^{14}C into the C-2 position of 5,6-DMBI indicates that this molecule is not derived by a

condensation with glycine followed by cyclization and decarboxylation.

Acknowledgements

This work was supported by Research Grant AM 10067 from the National Institute of Arthritis and Metabolic Diseases of the U. S. Department of Health, Education, and Welfare.

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