

## BIOSYNTHESIS OF 5, 6-DIMETHYLBENZIMIDAZOLE FROM RIBOFLAVIN

### Transformation of C-1' of riboflavin into C-2 of 5, 6-dimethylbenzimidazole

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#### 1. Introduction

Recently it was found that riboflavin is transformed into the 5,6-DMBIA \*-moiety of vitamin B<sub>12</sub> by cell homogenates of *Propionibacterium shermanii* [1]. On the other hand Alworth et al. [2] showed in experiments with the same organism that with 1-<sup>14</sup>C-ribose as substrate, 40% of the radioactivity incorporated into 5,6-DMBIA is found in the C-2 position.

In this publication experiments are described showing that riboflavin, which is <sup>14</sup>C-labelled at C-1' of its ribityl side chain is transformed into 5,6-DMBIA exclusively labelled in its C-2 position.

#### 2. Materials and methods

1-<sup>14</sup>C-D-ribose (7.5 mCi/mmole) was purchased from Buchler a. Co., Braunschweig, Germany. Solvents for paper chromatography: I: butanol-2/water/acetic acid/10% aqueous KCN = 70/30/1/0.1. II: butanol-2/water/1% aqueous HCN = 70/29/0.5. Paper electrophoresis was performed in 0.5 M acetic acid (15 V/cm; 2–3 hr.). Riboflavin [3], vitamin B<sub>12</sub>, α-ribazole and 5,6-DMBIA were determined spectrophotometrically [4]. Radioactivity was measured in a liquid scintillation counter (Beckman LS 150) with a solution of 6 g

of PPO in 1 l of toluene and with internal standardization (<sup>14</sup>C-toluene, Beckman).

##### 2.1. Synthesis of 1'-<sup>14</sup>C-riboflavin [5]

3,4-Dimethylaniline-D-riboside was prepared according to Berger et al. [6] and hydrogenated with the catalyst described by Kuhn et al [5] to 1-D-ribitylamino-3,4-dimethylbenzene as described by Shunk et al. [7] with the exception that the hydrogenation was performed at 35° and atmospheric pressure for 44 hr. 1-D-Ribitylamino-2phenylazo-4, 5-dimethylbenzene was prepared according to [7] and reacted with anhydrous barbituric acid according to a modification [8] of the method described by Tishler et al. [9].

From 220 mg of 3,4-dimethylaniline (1.82 mmole) and 200 mg of 1-<sup>14</sup>C-D-ribose (1.33 mmole, 250 μCi) 91 mg (18% overall yield) of 1'-<sup>14</sup>C-riboflavin (264,000 dpm/μmole) were obtained. The purity was checked after chromatography in KCN-free solvent I by radioactivity-scanning (Dünnschicht-scanner II, Fa. Berthold, Wildbad, Germany) and by detection of fluorescence under UV-light of 254 nm. No impurity could be detected. The 260/450 nm-ratio of a solution of 1'-<sup>14</sup>C-riboflavin in 0.1 M phosphate buffer pH 7.0 was the same as that for pure riboflavin [3].

##### 2.2. Growth of *P. shermanii* and incubation with 1'-<sup>14</sup>C-riboflavin

*P. shermanii* St 33 was grown as described previously [10]. 35 g of wet cells from a 3 day-culture

\* Abbreviations: 5,6-DMBIA = 5,6-dimethylbenzimidazole; α-ribazole = 5,6-dimethylbenzimidazole-α-D-ribofuranoside.

were suspended in 400 ml of sterile 0.07 M phosphate buffer pH 7.0. After addition of 5 mg of  $1'-^{14}\text{C}$ -riboflavin the mixture was incubated with continuous shaking (100 rpm, 40 hr,  $25^\circ$ ). Vitamin  $\text{B}_{12}$  was isolated and purified on columns of Amberlite XAD-2, Dowex  $2 \times 8$  and CM-cellulose as described previously [11].

The vitamin  $\text{B}_{12}$  thus obtained from 4 of the above incubation mixtures was further purified by descending chromatography on Whatman 3 MM-paper with solvent I. After rechromatography with solvent II the vitamin  $\text{B}_{12}$  was finally chromatographed on a column ( $1 \times 10$  cm) of Amberlite XAD-2 [11].

The  $\alpha$ -ribazole obtained after cerous hydroxide degradation [10] of vitamin  $\text{B}_{12}$  was degraded to 5,6-DMBIA [12]. The 5,6-DMBIA was purified by paper electrophoresis in 0.5 M acetic acid and eluted from the paper with 70% aqueous ethanol. Degradation of 5,6-DMBIA to 1,2-dibenzamido-4,5-dimethylbenzene and formic acid [13], as well as the oxidation of the formic acid to  $\text{CO}_2$ , was performed as described by Alworth et al. [14].  $\text{CO}_2$  was absorbed in 2-phenylethylamine [15] in the absorption device of the decarboxylation apparatus described by Phares [16].

### 3. Results and discussion

In table 1 the data are given which demonstrate that radioactivity from  $1'-^{14}\text{C}$ -riboflavin is incorporated into the 5,6-DMBIA-moiety of vitamin  $\text{B}_{12}$  in *P. shermanii*. From these data it can be calculated that 10% of the  $1'-^{14}\text{C}$ -riboflavin added to the bacteria was transformed into 5,6-DMBIA. It is noteworthy that the specific radioactivity of riboflavin and of vitamin  $\text{B}_{12}$  differ only slightly. This means that the amount of endogenous riboflavin competing with the radioactive riboflavin does not exceed 10%. Table 2 shows that the total amount of radioactivity of 5,6-DMBIA is localized in the C-2 position. This proves that C-2 of 5,6-DMBIA is derived from C-1' of riboflavin.

Table 1  
Incorporation of radioactivity from  $1'-^{14}\text{C}$ -riboflavin into the 5,6-DMBIA-moiety of vitamin  $\text{B}_{12}$  \* by *P. shermanii*.

	Amount (mg)	Specific radioactivity (dpm/ $\mu$ mole)
$1'-^{14}\text{C}$ -riboflavin	20	264,000
Vitamin $\text{B}_{12}$		
1. After paper chromatography in solvent I	7.6	234,500
2. After paper chromatography in solvent II	6.15	227,500
3. After chromatography on Amberlite XAD-2	5.95	249,000
Products of cerous hydroxide degradation of 4.1 mg vitamin $\text{B}_{12}$		
Cobinamide	2.06	810
$\alpha$ -Ribazole	0.57	212,500
5,6-DMBIA		
1. From HCl-degradation of $\alpha$ -ribazole	0.26	205,500
2. After paper electrophoresis in 0.5 M acetic acid	0.23	225,000

\* 4 of the incubation mixtures described in Materials and methods were run. For the isolation of vitamin  $\text{B}_{12}$  the bacteria were pooled. led.

Table 2  
Evidence for the localization of the radioactivity of  $1'-^{14}\text{C}$ -riboflavin in C-2 of 5,6-DMBIA.\*

	Amount (mg)	Total dpm found	dpm/ $\mu$ mole
5,6-DMBIA**	29.23	354,000	1,770
1,2-dibenzamido-4,5-dimethylbenzene	38.4	2,520	23
$\text{CO}_2$ from C-2 of 5,6-DMBIA	—	201,400	1,675 <sup>†</sup>

\* For experimental details see Materials and methods.

\*\* For treatment with benzoyl chloride the 5,6-DMBIA mentioned in table 1 was diluted with 29 mg of non-radioactive 5,6-DMBIA.

<sup>†</sup> In several experiments with nonradioactive material it was found that the average yield of  $\text{CO}_2$  from 5,6-DMBIA was 60% determined as  $\text{BaCO}_3$ . This value was used to calculate the specific radioactivity of  $\text{CO}_2$ , since the amount of  $\text{CO}_2$  was not determined in the experiment with radioactive material.

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