RIBOFLAVIN AS PRECURSOR IN THE BIOSYNTHESIS OF THE 5,6-DIMETHYLBENZIMIDAZOLE-MOIETY OF VITAMIN B_{12}

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1. Introduction

In previous experiments with *Propionibacterium shermanii* it has been shown [1] that ^{14}C-lactic acid is incorporated into the 5,6-DMBIA*-moiety of vitamin B_{12} with the same isotope distribution pattern as that found by Plaut [2] for the dimethylbenzene moiety of riboflavin.

These results suggested the possibility that the dimethylbenzene moiety of riboflavin and of 5,6-DMBIA are formed by similar pathways, or that riboflavin could be the precursor of the dimethylbenzene moiety of 5,6-DMBIA, a hypothesis already put foreward by Woolley [3].

In this publication experiments are described which show that the radioactivity from uniformly labeled riboflavin is incorporated into the 5,6-DMBIA-moiety of vitamin B_{12}.

2. Materials and methods

Uniformly ^{14}C-labeled riboflavin was prepared by growing 40 ml cultures of *Ashbya gossypii* NRRL Y-1056 according to [4] in the presence of 125 μCi U-^{14}C-D-glucose (Boehringer, Mannheim, specific activity 4.56 μmoles/mCi). Riboflavin was isolated and purified by known procedures [5] to yield a compound with the 260/450 nm-ratio of pure riboflavin [6]. *P. shermanii* was first grown anaerobically in the presence of cobalt(II)-nitrate [7]. Since riboflavin is poorly taken up by intact cells, *P. shermanii* cells from 2 days old cultures were broken at −30°C in the X-press (AB Biox, Nacka, Sweden). 20 g of broken cells were then suspended in 250 ml of sterile 0.067 M phosphate buffer, pH 7.0, in a 1 l-shake culture flask. The uniformly ^{14}C-labeled riboflavin was added and the mixture incubated with shaking (100 rpm, 40 hr, 28°C. During this aerobic incubation, cobalamin is formed from the incomplete corrinoids synthesized during the anaerobic growth phase [8]. The corrinoids were isolated in the presence of KCN and purified by phenol extraction [9]. Acidic and basic corrinoids were removed from B_{12} by chromatography on Dowex-2-acetate and on CM-Sephadex, respectively. Vitamin B_{12} was further purified by paper chromatography on Schleicher a. Schüll-paper No. 2043a ausgew. (butan-2-ol/acetic acid/water/HCN = 70: 1:30:0.01) and by thin-layer chromatography on silica gel (ethanol/water = 8 : 2). Vitamin B_{12} was degraded to 5,6-DMBIA, which was isolated by chloroform extraction [10]. The final purification of 5,6-DMBIA was achieved by descending paper chromatography (butan-2-ol/acetic acid/water = 70 : 1 : 30). Radioactivity was determined in a liquid scintillation counter (Beckman LS 150) with an internal standard (^{14}C-toluene, Beckman).

* Abbreviations: 5,6-DMBIA, 5,6-dimethylbenzimidazole; α-ribazole, 5,6-dimethylbenzimidazole-α-D-ribofuransoide, B_{12}, vitamin B_{12} (Cyanocobalamin).

Scheme 1.

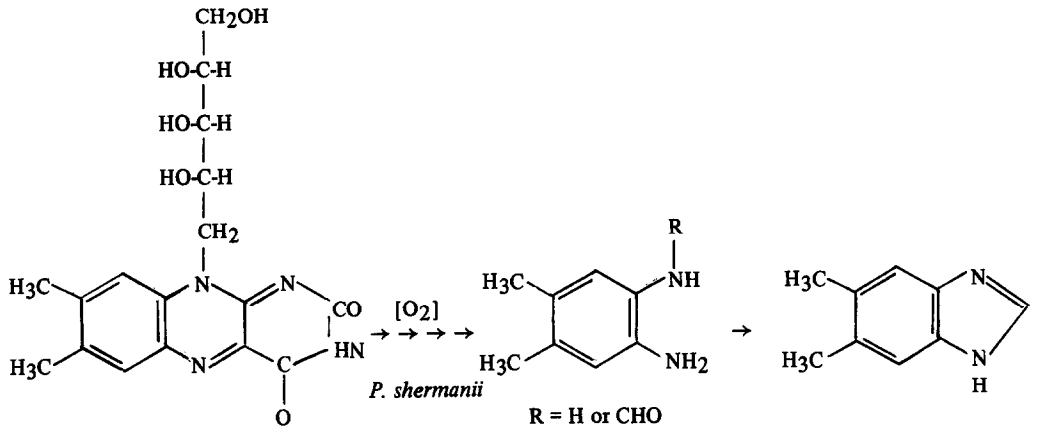

Table 1.
Incorporation of radioactivity from uniformly ^{14}C-labeled riboflavin into vitamin B_{12} and into its 5,6-dimethylbenzimidazole moiety [a].

	Amount (μg)	Specific radioactivity (dpm/μmole)
Riboflavin	713	308 000
Vitamin B_{12} [b]		
1. After paper chromatography	356	19 400
2. Rechromatographed on paper (same solvent)	281	17 500
3. After thin-layer chromatography	194	17 900
5,6-Dimethylbenzimidazole [c]	4.6	19 800

[a] For experimental details see: Materials and methods. [b] The purity of the B_{12} was measured by the 278/361 nm-ratio of its solution in water. This value changed from 0.64 after the first paper chromatography to 0.60 after thin-layer chromatography. Values within the same range are obtained when pure vitamin B_{12} (278/361 nm-ratio 0.55 [12]) is subjected to paper chromatography and eluted from the paper. [c] For the degradation, B_{12} was diluted with nonradioactive B_{12}. For comparison, the value here was calculated referring to undiluted B_{12}.

3. Results and discussion

Table 1 shows the incorporation of radioactivity of riboflavin into vitamin B_{12} and into its 5,6-DMBIA-moiety. Since the B_{12} and the 5,6-DMBIA have the same specific acitivity, the ribityl-side chain does not seem to be involved in the formation of the ribose moiety of B_{12}. This was confirmed in experiments in which the B_{12} was degraded to cobinamide and α-ribazole [11]. The cobinamide was not radioactive. On degradation of α-ribazole to 5,6-DMBIA, the specific activity also remained constant. Thus, under aerobic conditions, which are necessary for the formation of B_{12} from incomplete corrinoids in *P. shermanii*, riboflavin is broken down to yield free 5,6-DMBIA (scheme 1). These findings are consistent with the results of Friedmann [13] that the nucleoside of vitamin B_{12} is formed from free 5,6-DMBIA and nicotinic acid mononucleotide.

The results in this paper are corroborated by experiments in which riboflavin was substituted by 6,7-dimethyl-8-ribityl-lumazin, its direct precursor [14]. In these experiments, using ^{14}C-6,7-dimethyl-8-ribityil-lumazin, the B_{12} formed was also exclusively labeled in the 5,6-DMBIA-unit [15]. Further experiments are necessary to show whether only the 1,2-diamino-4,5-dimethylbenzene unit of riboflavin is used to form the 5,6-DMBIA, or if in addition the CH_2-group of the ribityl-side-chain is transformed into C-2 of 5,6-

DMBIA. The latter possibility was suggested by Alworth et al. [16] from experiments with 1-^{14}C-ribose, in which they showed that the label from 1-^{14}C-ribose is very efficiently incorporated into C-2 of 5,6-DMBIA.

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References

[1] P.Renz and K.Reinhold, Angew. Chem. 79 (1967) 1073.
[2] G.W.E.Plaut, J. Biol. Chem. 211 (1954) 111.
[3] D.W.Woolley, J. Exptl. Med. 93 (1951) 13.
[4] F.W.Tanner, C.Vojnovich and J.M.Van Lanen, J. Bacteriol. 58 (1949) 737.
[5] F.M.Heunnekens and S.P.Felton, Methods in enzymology, Vol. 3 (1957) p. 950.
[6] L.G.Withby, Biochem. J. 54 (1953) 440.
[7] K.Bernhauer, E.Becher and G.Willharm, Arch. Biochem. Biophys. 83 (1959) 248.
[8] J.D.Speedie and G.W.Hull, Brit. Pat. 829 232 (1960). P.Renz, Z. Physiol. Chem. 349 (1968) 979.
[9] W.Friedrich and K.Bernhauer, in: Medizinische Grundlagenforschung, Vol. 12, ed. K.F.Bauer (Thieme-Verlag, Stuttgart, 1959) p. 663.
[10] N.G.Brink and K.Folkers, J. Am. Chem. Soc. 72 (1950) 4442.
[11] W.Friedrich and K.Bernhauer, Chem. Ber. 89 (1956) 2507.
[12] W.Friedrich and K.Bernhauer, in: Biochemisches Taschenbuch, ed. H.M.Rauen (Springer, Berlin, Göttingen, Heidelberg, 1956) p. 478.
[13] H.C.Friedmann and H.L.Harris, J. Biol. Chem. 240 (1965) 406.
[14] G.W.E.Plaut, J. Biol. Chem. 238 (1963) 2225.
[15] P.Renz and H.Kühnle, unpublished data.
[16] W.C.Alworth, H.N.Baker, D.A.Lee and B.A.Martin, J. Am. Chem. Soc. 91 (1969) 5662.