Nonenzymatic Transformation of Riboflavin into 5,6-Dimethylbenzimidazole

Paul Renz, Rainer Wurm, and Joachim Hörig

Institut für Biologische Chemie der Universität Hohenheim

(Z. Naturforsch. **32 c**, 523–527 [1977]; received March 21, 1977)

5,6-Dimethylbenzimidazole, Riboflavin, Vitamin B_{12}, Nonenzymatic Formation, Cobalamin Analogues

Riboflavin, the biosynthetic precursor of the 5,6-dimethylbenzimidazole moiety of vitamin B_{12}, is transformed non-enzymatically into 5,6-dimethylbenzimidazole in small yield on treatment with 1 N or 5 N NaOH at 100 °C. Besides 5,6-dimethylbenzimidazole 1,2-diamino-4,5-dimethylbenzene, 1,2-dihydro-6,7-dimethyl-2-keto-1-D-ribityl-3-quinoxaline carboxylic acid and N-1-D-ribitylamino-2-amino-4,5-dimethylbenzene can be detected. When [1′-^{14}C]riboflavin is used the 5,6-dimethylbenzimidazole contains about 75 per cent of the specific radioactivity of riboflavin. N-1-D-ribitylamino-2-amino-4,5-dimethylbenzene is transformed into 5,6-dimethylbenzimidazole more efficiently than riboflavin. Oxygen enhances the yield of 5,6-dimethylbenzimidazole and 1,2-diamino-4,5-dimethylbenzene from riboflavin as well as from N-1-D-ribitylamino-2-amino-4,5-dimethylbenzene. 1,2-diamino-4,5-dimethylbenzene reacts together with formaldehyde but not with formate to form 5,6-dimethylbenzimidazole under alkaline conditions at 100 °C.

It is therefore suggested that the nonenzymatic reaction of riboflavin proceeds via N-1-D-ribitylamino-2-amino-4,5-dimethylbenzene and 1,2-diamino-4,5-dimethylbenzene, and that the latter reacts with formaldehyde preferably formed by oxidative degradation of C-1′ of the ribityl side chain to form 5,6-dimethylbenzimidazole via its unstable imidazoline derivative. The possible relevance of these results for the enzymatic process is discussed.

Vitamin B_{12} contains 5,6-dimethylbenzimidazole (DBI) as base part. DBI is formed biosynthetically from riboflavin [1]. Thereby the C-1′ of riboflavin is transformed into C-2 of DBI [2]. Since no further progress in the elucidation of the mechanism of the biosynthetic transformation could be made till now, we tried to find out, if this reaction also proceeds nonenzymatically. If this were the case we could perhaps get some information about the biosynthetic process.

In this paper experiments are described which demonstrate that riboflavin is indeed transformed nonenzymatically into DBI in small yield when treated with aqueous alkali at elevated temperature.

Material and Methods

Riboflavin was obtained from Merck (Darmstadt), N-1-D-ribitylamino-2-amino-4,5-dimethylbenzene from Merck-Schuchardt (München). 1,2-dihydro-6,7-dimethyl-2-keto-1-D-ribityl-3-quinoxaline carboxylic acid was prepared as described by Surrey and Nachod [3]. 7,8-dimethyl-10-(2′,3′-dihydroxy-1′-propyl)-isoalloxazine was synthesized according to Karrer *et al.* [4]. The 1-N-(2′,3′-dihydroxy-1′-propyl)-amino-2-nitro-4,5-dimethylbenzene used in this synthesis was prepared by a modification of the method of Karrer *et al.* [4] giving fourfold the yield reported by these authors. 1.11 g of 1-chloro-2-nitro-4,5-dimethylbenzene, 0.65 g of 1-aminopropan-2,3-diol and 0.97 g of anhydrous sodium acetate was heated to 125–130 °C under nitrogen for 12 h. Then 30 ml of ethanol was added. Insoluble material was removed by centrifugation, and the sediment washed with 5 ml of ethanol. The combined supernatants were poured onto a column (2 × 17 cm) of basic Al_2O_3 (Aktivitätsstufe I, Merck). Unreacted starting material was eluted with benzene. The product was eluted with pyridine/water/methanol = 1/1/2, and evaporated in vacuo. The residue was dissolved in ethanol and evaporated again in order to remove traces of pyridine, and this procedure was repeated three times. Then the product was dissolved in a small volume of ethanol, and 4 times this volume of water was added. On crystallization overnight 200 mg (11.3 per cent) of 1-N-(2′,3′-dihydroxy-1′-propyl)-amino-2-nitro-4,5-dimethylbenzene was obtained, m.p. 99–101 °C.

[1′-^{14}C]riboflavin was prepared using [1-^{14}C]-ribose as described by Kuhn and Stroebele [5]. The benzimidazole bases were identified and quantitatively determined spectrophotometrically. For the molar extinction coefficient of DBI see [6]. The molar extinction coefficient of 1,2-diamino-4,5-dimethylbenzene in ethanolic solution was determined: 2.4×10^3 cm²/mmol at 288 nm and 3.6×10^3 cm²/

Reprint requests should be sent to Prof. Dr. P. Renz, Institut für Biologische Chemie der Universität Hohenheim, Garbenstr. 30, *D-7000 Stuttgart 70*, W. Germany.

mmol at 300 nm. The UV-spectra were recorded with a Beckman Acta V-spectrophotometer, the mass spectra with a Varian MAT 311A-spectrometer, and radioactivity was measured with a Beckman LS 230 liquid scintillation counter. DBI was detected on chromatograms and electropherograms under UV-light of 254 nm.

Large scale experiment

In a 1 l three-necked round-bottomed flask equipped with a reflux condenser, gas inlet tube and thermometer 500 ml of 5 N NaOH was heated to 95 °C under magnetic stirring. 20 g of riboflavin was added in small portions within 5 min. Then the mixture was heated under reflux and a stream of air was passed through the solution in that way that excess foaming was prevented. After 25 min the solution was cooled and 65 ml of conc. sulfuric acid dissolved in 300 ml of water slowly added. The solution was extracted 3 times with 40 ml of chloroform, the chloroform phase washed 2 times with 40 ml of water, filtered through a dry filter paper and evaporated to dryness in vacuo. The residue was dissolved in 1.5 ml of chloroform, brought onto 2 precoated silica gel 60 F_{254}-plates (20 × 20 cm, layer thickness 0.25 mm, Merck) as 16 cm bands. The plates were chromatographed twice in chloroform/ethanol/acetic acid = 85/15/1. The band migrating like a DBI reference spot was scraped off, the DBI eluted from the silica gel with ethanol/conc. ammonia = 10/0.5 and the solvent evaporated. Usually 3 – 5 mg of crude DBI was obtained as measured by the UV-absorbance of the ethanolic solution at 288 nm [6]. The DBI of 3 experiments was combined, dissolved in 1 ml of 1 N HCl and extracted two times with 0.5 ml of chloroform which was discarded. The pH of the aqueous phase was adjusted to 10 – 12 with ammonia and the DBI extracted with three 1 ml-portions of chloroform. The chloroform was washed with water and evaporated.

The residue was further purified by sublimation *in vacuo* and by recrystallization from 1 ml of water in the presence of charcoal to remove a light yellow impurity. 5 – 6 mg of colourless needles were obtained. Mp and mixed mp with authentic DBI: 203 – 204 °C.

Small scale experiments of Table I

0.5 mmol (188 mg) of riboflavin or 0.5 mmol of the derivatives mentioned in Table I was dissolved in 50 ml of 1 N or 5 N NaOH and heated in a boiling water bath for 30 min. The color turned from orange to dark green and finally to yellow. In experiments with 1 N NaOH the solution was directly extracted 3 times with 5 ml of chloroform, in experiments with 5 N NaOH 200 ml of water was added and then the solution extracted 3 times with

Table I. Nonenzymatic transformation of riboflavin and some riboflavin derivatives into 5,6-dimethylbenzimidazole under varying conditions.

Substrate [a]	Conc. of NaOH [b]	Yields [μg] 5,6-dimethylbenzimidazole	1,2-diamino-4,5-dimethylbenzene [c]
I	1 N, N_2	9.2 [d]	24
I	1 N	10.9 [d]	184
I	1 N, air	12.6	
I	5 N, N_2	21.2	703
I	5 N	25.2	2 656
I	5 N, air	49.6	
[1′-^{14}C] **I** (4000 dpm/μmol)	5 N, air	37.0 [d] (3145 dpm/μmol) 23.0 [e] (3080 dpm/μmol)	
II	1 N	0	
II	1 N, air	0	
II	5 N	12.4	
III	1 N, N_2	45.9	1 217
III	1 N	68	
III	1 N, air	237 [f]	15 152
IV + formaldehyde [g]	1 N	2160	
IV + sodium formate [g]	1 N	0	
V	5 N, air	7 [d, h]	

[a] **I**, riboflavin; **II**, 1,2-dihydro-6,7-dimethyl-2-keto-1-D-ribityl-3-quinoxaline carboxylic acid; **III**, N-1-D-ribitylamino-2-amino-4,5-dimethylbenzene; **IV**, 1,2-diamino-4,5-dimethylbenzene; **V**, 7,8-dimethyl-10-(2′,3′-dihydroxy-1′-propyl)-isoalloxazine.

[b] For experimental details see under "Small Scale Experiments" in the experimental section. The numbers are mean values of 3 experiments. Where no special remarks are given, the samples were treated in the presence of the environmental air, "air" means that air was bubbled through the solution, "N_2" indicates that the experiments were carried out under nitrogen.

[c] Where no values are given in this column the amount of 1,2-diamino-4,5-dimethylbenzene was not determined.

[d] Additionally purified by paper electrophoresis.

[e] 5,6-dimethylbenzimidazole reisolated from the scintillation fluid (2,5-diphenyloxazole in toluene) and further purified by descending paper chromatography with butan-2-ol/water/acetic acid = 70/30/1.

[f] 3 thin-layer plates (5 × 20 cm) were used to separate the products instead of 1 plate.

[g] 1.5 mmol of formaldehyde or 1.5 mmol of sodium formate was added. A 2 per cent aliquot of the chloroform extract was used for thin-layer chromatography. A similar experiment in which the formaldehyde or the sodium formate was omitted, was used as a blank.

[h] On the electrophoresis besides 5,6-dimethylbenzimidazole a second band migrated to the cathode with about 80 per cent of the velocity of 5,6-dimethylbenzimidazole. This compound which showed the same fluorescence as 5,6-dimethylbenzimidazole, was not further characterized.

20 ml of chloroform. The chloroform was washed twice with 10 ml of water, filtered through a dry filter paper and evaporated to dryness *in vacuo*. The residue was dissolved in 0.3 ml of chloroform and applied to a precoated silica gel 60 F_{254}-plate (5 × 20 cm, layer thickness 0.25 mm, Merck) as a 2.5 cm-band. On chromatography for 2 h in chloroform/ethanol/acetic acid = 85/15/1 DBI (R_F 0.2) separates from 1,2-diamino-4,5-dimethylbenzene (R_F 0.6), the latter being present in a much larger amount than the former. The band migrating like a DBI reference spot or the band of 1,2-diamino-4,5-dimethylbenzene was scraped from the plate and the substance eluted from the silica gel with 3 ml of ethanol/conc. ammonia = 10/0.5. The concentration of DBI or 1,2-diamino-4,5-dimethylbenzene in this eluate was determined from its UV-absorbance at 288 nm. A blank was used for this measurement carried out as described above but without incubation in a boiling water bath. Although no DBI was formed in this experiment, this blank could account for impurities coming from the coating material of the thin layer plate.

In most experiments the DBI eluted from the silica gel showed the same UV-spectrum as an authentic sample of DBI. In some cases, especially in the experiments carried out with 1 N NaOH under nitrogen, the DBI was not completely pure. It was therefore further purified by paper electrophoresis on sheets (18 × 45 cm) of washed Schleicher a. Schuell-paper No. 2043 a or Whatman No. 1-paper in 0.5 M acetic acid at 10 V/cm for 2 h.

The band migrating like a DBI-reference spot was eluted from the dried paper with ethanol/water/conc. ammonia = 7/3/0.1 and the DBI-content measured as above.

In order to isolate the compounds remaining in the aqueous phase when the reaction mixture was extracted with chloroform the aqueous phase was neutralized with acetic acid and applied to a column (2 × 10 cm) of Amberlite XAD-2 (100 – 200 μ, Serva, Heidelberg). The column was washed with water and thus the bulk of 1,2-dihydro-6,7-dimethyl-2-keto-1-D-ribityl-3-quinoxaline carboxylic acid slowly removed. Several other compounds and especially N-1-D-ribitylamino-2-amino-4,5-dimethylbenzene were subsequently eluted with methanol. The methanol eluate was concentrated *in vacuo*, applied onto thin layer plates of silica gel 60 F_{254} as a 2 cm band together with the appropriate reference substances. The chromatography was carried out with chloroform/ethanol/acetic acid = 85/15/1 and with n-butanol/ethanol/water = 4/2/2 [7]. The band migrating like the N-1-D-ribitylamino-2-amino-4,5-dimethylbenzene reference substance was eluted with ethanol and further characterized by paper electrophoresis in 0.5 M acetic acid, where N-1-D-ribitylamino-2-amino-4,5-dimethylbenzene exhibits about 60 per cent of the mobility of DBI.

Results

Riboflavin forms DBI when heated in a sodium hydroxide solution at 100 °C. The identity of the DBI thus formed was confirmed by its melting point and mixed melting point, its UV- and mass spectrum and its chromatographic and electrophoretic behaviour. As seen from Table I the yield of DBI from riboflavin (**I**) is enhanced in the presence of oxygen and diminished, if the experiment is carried out under nitrogen. An equal effect of oxygen on the yield of 1,2-diamino-4,5-dimethylbenzene was found. 5 N NaOH raises the yield of DBI about 2 – 4 fold compared with 1 N NaOH, but the yield is rather sensitive to small changes in temperature and oxygen concentration. When [1′-^{14}C]riboflavin was used in these experiments approximately 75 per cent of the specific radioactivity was found in the DBI. Surrey and Nachod [3] found that from riboflavin, when heated at 80 °C in 1 N NaOH, 1,2-dihydro-6,7-dimethyl-2-keto-1-D-ribityl-3-quinoxaline carboxylic acid (**II**) is formed. This compound is not further transformed into DBI with 1 N NaOH in our system, but gives DBI with 5 N NaOH. N-1-D-ribitylamino-2-amino-4,5-dimethylbenzene (**III**) is transformed into DBI and 1,2-diamino-4,5-dimethylbenzene to a much greater extent than riboflavin. Also this reaction is greatly enhanced by oxygen. 1,2-diamino-4,5-dimethylbenzene (**IV**) gives DBI, when heated together with formaldehyde but not with sodium formate in 1 N NaOH. The riboflavin analogue 7,8-dimethyl-10-(2′,3′-dihydroxy-1′-propyl)-isoalloxazine (**V**) which has a glyceryl side chain instead of a ribityl side chain is also transformed into DBI, but a second compound with approximately 80 per cent of the mobility of DBI is seen under UV-light of 254 nm on the electrophoresis in 0.5 M acetic acid.

In experiments not mentioned in Table I we could show that 10-D-ribitylisoalloxazine is transformed into benzimidazole and that 7-methoxy-10-D-ribitylisoalloxazine yields 5-methoxybenzimidazole when heated with 1 N NaOH.

In our standard procedure to transform riboflavin or the other compounds mentioned into DBI we only isolate products which are soluble in chloroform. But in two cases we checked the aqueous phase for sub-

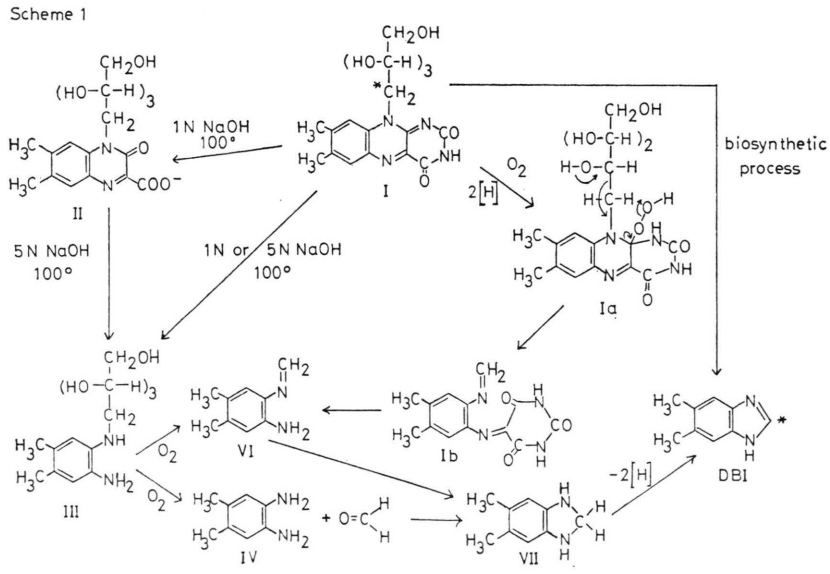
stances remaining there after chloroform extraction. In experiments with riboflavin and 1 N NaOH, 1,2-dihydro-6,7-dimethyl-2-keto-1-D-ribityl-3-quinoxaline carboxylic acid [3] is the main product besides small amounts of N-1-D-ribitylamino-2-amino-4,5-dimethylbenzene and several other compounds which where not further identified. In experiments with riboflavin and 5 N NaOH almost equal amounts of 1,2-dihydro-6,7-dimethyl-2-keto-1-D-ribityl-3-quinoxaline carboxylic acid and N-1-D-ribitylamino-2-amino-4,5-dimethylbenzene were found in the aqueous phase besides several minor constituents.

Discussion

From the data presented here and as pointed out in Scheme 1 we assume that the nonenzymatic transformation of riboflavin into DBI proceeds via N-1-D-ribitylamino-2-amino-4,5-dimethylbenzene which is cleaved to 1,2-diamino-4,5-dimethylbenzene under our reaction conditions. We suggest that the 1,2-diamino-4,5-dimethylbenzene thus produced reacts with formaldehyde formed from the ribityl side chain. It is remarkable that oxygen greatly enhances the formation of 1,2-diamino-4,5-dimethylbenzene and thus DBI from riboflavin and from N-1-D-ribitylamino-2-amino-4,5-dimethylbenzene probably due to an oxidative degradation of the ribityl side chain. This is in agreement with the well known effect that carbohydrates on treatment with alkali at elevated temperature especially in the presence of oxygen yield formaldehyde besides several other degradation products [8]. Although we were not able to isolate formaldehyde as an intermediate, our experiments with 1,2-diamino-4,5-dimethylbenzene and formaldehyde are a strong indication that formaldehyde may be the precursor of C-2 of DBI, forming DBI *via* the very unstable derivative **VII** (Scheme 1). It is rather surprising that C-1′ of riboflavin yields about 75 per cent of the precursor of C-2 of DBI as shown in the experiment with [1′-^{14}C]riboflavin. This is analogous to the biosynthetic process were C-1′ of riboflavin is exclusively the precursor of C-2 of DBI [2]. This result shows that formaldehyde is preferably formed from C-1′ of the ribityl side chain. But we can not exclude the possibility that DBI is also formed from the schiff base intermediate **VI**. This could either be generated from **III** or directly from riboflavin *via* the hydroperoxy derivative **Ia** [9] and compound **Ib** (Scheme 1). The hydroperoxy derivative **Ia** could be formed from riboflavin on reduction to dihydroflavin and reaction with oxygen. The green color emerging after a short heating period of riboflavin with alkali indicates that riboflavin is partly reduced despite the presence of oxygen. The riboflavin is probably reduced by degradation products of the ribityl side chain.

For the biosynthetic process we assume that a pathway like **I** → **Ia** → **Ib** → **VI** → **VII** → DBI leads to the 100 per cent yield of radioactivity from C-1′ of riboflavin in C-2 of DBI.

Scheme 1



In addition to vitamin B_{12} with DBI as base there are several natural vitamin B_{12} analogues containing a benzimidazole base (*i. e.* benzimidazole, 5-hydroxybenzimidazole, 5-methoxybenzimidazole, 5-methylbenzimidazole, naphthimidazole)[10]. The fact that the riboflavin analogues 10-D-ribitylisoalloxazine and 7-methoxy-10-D-ribitylisoalloxazine are transformed into benzimidazole and 7-methoxybenzimidazole respectively in our nonenzymatic system encourages the investigation of the biosynthesis of these benzimidazole bases.

We thank Dr. D. Spitzner and Mr. G. Schwinger, Institute of Organic Chemistry, University of Hohenheim, for recording the mass spectra mentioned in this publication. The financial support of this work by the Deutsche Forschungsgemeinschaft is greatfully acknowledged.

[1] P. Renz, FEBS Lett. **6,** 187–189 [1970].
[2] P. Renz and R. Weyhenmeyer, FEBS Lett. **22,** 124–126 [1972].
[3] A. S. Surrey and F. C. Nachod, J. Am. Chem. Soc. **73,** 2336–2338 [1951].
[4] P. Karrer, E. Schlittler, K. Pfaehler, and F. Benz, Helv. Chim. Acta **17,** 1516–1523 [1934].
[5] R. Kuhn and R. Stroebele, Ber. **70,** 773–787 [1937].
[6] R. Gompper, Biochemische Taschenbuch (H. M. Rauen, ed.), **Vol. 1,** p. 613, 2nd edn, Springer, Berlin 1964.
[7] M. Schuman Jorns, G. Schöllnhammer, and P. Hemmerich, Eur. J. Biochem. **57,** 35–48 [1975].
[8] R. L. Whistler and J. N. BeMiller, Advances in Carbohydrate Chemistry **13,** 289–329 [1958].
[9] H. I. X. Mager in Flavins and Flavoproteins, Proc. 5. Internat. Symp. (T. P. Singer, ed.), Elsevier, Amsterdam 1976, pp 23–37, and other papers on the flavin hydroperoxide problem published in this book.
[10] W. Friedrich, Fermente, Hormone, Vitamine (R. Ammon and W. Dirscherl, ed.), **Vol. III/2,** pp. 37–40, G. Thieme, Stuttgart 1975.