The 5,6-dimethylbenzimidazole formed from riboflavin in cobalt-containing cells of Propionibacterium shermanii can only be isolated as a component of vitamin B_{12}. No free 5,6-dimethylbenzimidazole is detectable in these bacteria. In order to determine, if nevertheless the vitamin B_{12} biosynthesis proceeds via free 5,6-dimethylbenzimidazole, cells of P. shermanii were incubated with [5-^{15}N]riboflavin. One fraction of the vitamin B_{12} thus formed was degraded to 5,6-dimethylbenzimidazole α-0-ribofuranoside and acetylated to its 2',3',5'-tri-O-acetyl derivative. The other fraction of the vitamin B_{12} was methylated to 3,5,6-trimethylbenzimidazoleacetylamide and hydrolyzed to 1,5,6-trimethylbenzimidazole. The proton magnetic resonance spectra of both degradation products revealed that 60% of the \(^{15}N\) content of the 5,6-dimethylbenzimidazole moiety of vitamin B_{12} was localized in N-1 and 40% in N-3. This asymmetric distribution of \(^{15}N\) shows that 20% of the 5,6-dimethylbenzimidazole molecules formed are transformed regiospecifically into \([1-^{15}N]5,6\)-dimethylbenzimidazole α-0-ribofuranoside-5'-phosphate, the next intermediate in the biosynthesis of the nucleotide moiety of vitamin B_{12}.

From these results it can be concluded that the enzyme system transforming riboflavin into 5,6-dimethylbenzimidazole and the next enzyme in the biosynthetic pathway must be located very close together in the intact bacterial cell. A vitamin B_{12} reference compound in which the \(^{15}N\) was equally distributed between both nitrogens of its 5,6-dimethylbenzimidazole moiety was prepared by addition of synthetic \(^{15}N\),dimethylbenzimidazole to a cell constituent or migrates to the next intermediate in the biosynthesis of the nucleotide moiety of vitamin B_{12}.

EXPERIMENTAL PROCEDURES

Materials

\(\text{Na}^{15}\text{NO}_{3}\) (99.7 atom %) and \(\text{Na}^{15}\text{NO}_{2}\) (99.8 atom %) were obtained from B.O.C. Ltd, Prochem, London. Chromatographic solvents: I: \(\text{CHCl}_3/\text{EtOH}/\text{AcOH} = 85:15:1\). II: \(\text{Bu}2\text{OH}/\text{H}_{2}\text{O}/\text{AcOH} = 10\%\) aqueous KCN = 70:30:10:1. Thin layer chromatography was performed in all experiments with precoated preparative Silica Gel 60 F_{254} plates (20 x 20 cm, layer thickness 2 mm, Merck Nr. 5717).

\([5-^{15}N]\text{Ribo}f\text{lavin}\)

Nine hundred milligrams of N-ribityl-3,4-dimethylamine prepared according to Lambovy (5) was coupled with \([^{15}N]\text{Phenyldiazo}jium chloride (from 500 mg of } \text{Na}^{15}\text{NO}_{2} \text{and 455 mg of aniline) according to Shunk et al. (6). The 1 \text{d} \text{ribitylimino}2 \text{phenyldiazo}4,5 \text{dimethylbenzene was condensed with 670 mg of barbituric acid according to modification (7) of the method described by Tishler et al. (8) to yield 750 mg of [5-^{15}N]riboflavin.}\n
\([3-^{15}N]1,5,6\)-Trimethylbenzimidazole

Six hundred eighty milligrams of 1,5,6-trimethylamine, prepared as described for \(\text{Na}^1\text{methylamine} (9), \text{was dissolved in 5 ml of 1 N HCl.}

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* This investigation was supported by the Deutsche Forschungsgemeinschaft. (Grant Re 246/6). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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A solution of phenylazidochromium chloride (1.5 ml of concentrated HCl, 3.3 ml of H2O, 385 mg of Na2NO3, and 500 mg of aniline) was added, and the pH adjusted to 2.0 with 700 mg of sodium acetate. The mixture was stirred for 20 h at 0–5°C and the azo compound precipitated with 1.1 g of sodium acetate. The oily precipitate was dissolved in benzene and added to a column (1.7 x 7 cm) of basic alumina (activity level 1, Merck Nr. 1067, Darmstadt, Germany). A yellow impurity was eluted from the column with benzene and the azo compound with benzene/ethanol = 95:5 (v/v). After evaporation 30 mg (65%) of a red oil was obtained.

A solution of 3.1 g of Na2SO4 in 50 ml of EtOH/H2O = 1:1 was brought to 65°C. A solution of the azo compound in 10 ml of EtOH was added dropwise under stirring. The solution was stirred until it was light yellow, then brought to boiling, cooled, and evaporated to dryness in vacuo. The residue was dissolved in 1 ml of 1 N NaOH was added, and the N-1-methyleniminio-2-amino-4, 5-dimethybenzene extracted with 50 ml of CHCl3. The CHCl3 was evaporated, the residue dissolved in 3 ml of formic acid, and heated under reflux for 1.5 h. The solution was poured into 50 ml of 1 N NaOH, extracted three times with 50 ml of CHCl3, and the CHCl3 evaporated. The residue (540 mg) was dissolved in 30 ml of CHCl3, applied onto nine TLC plates, and chromatographed in Solvent I. The zone of trimethylbenzimidazole was eluted from CHCl3, concentrated NH4OH-100:5, the solvent evaporated, the residue dissolved in 2 ml of CH2Cl2 and applied onto a TLC plate. After TLC in Solvent I in addition to the band of trimethylbenzimidazolylcobamide isolated via the Amberlite XAD-2 procedure (12). Paper electrophoretic analysis in 0.5 M acetic acid revealed \( ^{14} \)N at 74.11.

**Organisms**

*P. shermanii* St33 was grown in the presence of CoSO4, as described previously (11). Packed cells (7.5 kg) were obtained from two 100 liter fermentors. *P. freudenreichii* (ATCC 6207) was grown similarly (11) in 5-liter carboys. The cells were centrifuged for 1 h at 5000 g. The clumps were washed with water and further purified by phenol extraction. This vitamin B12 was used for the \( ^{14} \)N NMR measurements.

**Aerobic Incubation Experiments**

P. *shermanii*. To a suspension of 600 g of wet cells in 4.5 liters of 66 mM phosphate buffer, pH 7.0 (5.5 g of KH2PO4 and 1.25 g of NaH2PO4, 2H2O per liter) were added 57 mg of \(^{15} \)N riboflavin, dissolved in 150 ml of H2O, and 50 ml of glucose (50% sterile solution). A stream of air (20 ml/min) was passed through the suspension for 48 h at 30°C. After 8 and 24 h the pH was adjusted to 7.0 with 20% acetic acid. The suspension was filtered through a column (2.5 x 17 cm) of Amberlite XAD-2 (50 to 100 μm). With MeOH/H2O = 3:7, a red band of vitamin B12 containing a small amount of riboflavin was eluted followed by a band of riboflavin containing a small amount of vitamin B13. The red band was rechromatographed on Amberlite XAD-2 with the same solvent. Pure vitamin B12 (368 mg from 7.5 kg of *P. shermanii*) was obtained. From the riboflavin fractions containing small amounts of vitamin B12, the two compounds were separated by descending paper chromatography on Whatman No. 3MM paper with Solvent II to yield an additional 76 mg of vitamin B13. From *P. freudenreichii* vitamin B12 was isolated in a similar manner with the exception that after the CM cellulose step vitamin B12 was separated from riboflavin by descending chromatography on Whatman No. 3MM paper with Solvent II. From the cells of a 6-liter fermentation, 7.3 to 18 mg of vitamin B12 was obtained (i.e. 78 mg from six experiments).

**Preparation of a Vitamin B12 Reference Compound Containing Equal Amounts of \(^{14} \)N in Both Nitrogens of Its 5,6-Dimethylbenzimidazole Moiety**

To a 16-liter culture of *P. shermanii* grown anaerobically in the presence of CoSO4, for 2 days as described previously (11), 100 mg of \(^{15} \)N trimethylbenzimidazole labeled in one nitrogen (see above), dissolved in 5 ml of 70% aqueous ethanol, was added. The culture was grown for another 4 days, and 900 g of wet cells were obtained. Vitamin B12 was isolated and purified on Dowex 2-X4 acetate and CM-cellulose, H’ form, as described above and 140 mg of vitamin B12 was obtained. Eighty milligrams were degraded to 5,6-dimethylbenzimidazole and acetylated to its tri-O-acetyl derivative. The solution was filtered through a TLC plate. After TLC in Solvent I in addition to the band of trimethylbenzimidazolylcobamide isolated via the Amberlite XAD-2 procedure (12). Paper electrophoretic analysis in 0.5 M acetic acid revealed that only traces of nonmethylated vitamin B12 were present.

**Hydrolysis of Trimethylbenzimidazolylcobamide to Trimethylbenzimidazole**

Trimethylbenzimidazolylcobamide (330 mg) was dissolved in 40 ml of 6 N HCl and maintained at 150–160°C for 5 h in a heavy wall sealed tube. Then 30 ml of a 50% solution of NaOH was added. The

The trivial names used are: **α-ribazole, 5,6-dimethylbenzimidazole**

**β-ribofuranoside; triacyetyl-α-ribazole, 2',3',5'-tri-O-acetyl-α-ribazole.**
trimethylbenzimidazole was extracted with three 50-ml portions of CHCl₃. The CHCl₃ was extracted three times with 50 ml of 1 N HCl. The aqueous phase was washed with 50 ml of CHCl₃, brought to pH 12 to 13 with NaOH, and extracted three times with CHCl₃. The CHCl₃ was washed with water, filtered through dry filter paper, and evaporated to dryness. The residue was dissolved in CHCl₃, the solution applied as a band onto two TLC plates and chromatographed in Solvent I. The band migrating like authentic trimethylbenzimidazole was eluted from the silica gel with EtOH/concentrated NH₃OHHCl = 100:5. The solution was evaporated, and the residue subjected twice to sublimation in vacuo at 150°C. The resulting material was dissolved in boiling 1 N HCl. The pH was brought to 10 with concentrated NH₃OH. After cooling 22 mg (58%) of white crystals were obtained, m.p. 143-145°C. The identity was further confirmed by UV spectroscopy. The presence of ¹⁵N in the molecule was checked by mass spectroscopy.

NMR Spectra—A Bruker 90 NMR spectrometer with Pulse-Fourier Transform unit was used in the ¹H lock mode for the ¹⁵N measurements at 9.12 MHz (with noise decoupling) and for the ¹H measurements at 90 MHz. One ¹H spectrum was recorded with a Bruker WP 60 Fourier NMR spectrometer at 60 MHz. The ¹⁵N signals were measured relative to ¹⁵NH₄Cl (95% enriched, saturated solution in 2 N HCl, external standard). However the ppm values given under “Results” are referred to CH₃'OH2N02,358 ppm downfield from ¹⁵NH₄Cl. All NMR measurements were performed at 300 K. Vitamin B₁₂ was dissolved in CDCl₃. Triacetyl-α-ribozole and trimethylbenzimidazole were dissolved in CDCl₃ (99.8% enriched; the signal of the CHCl₃ proton at 7.24 ppm was used as internal standard).

RESULTS

Vitamin B₁₂ was produced by P. shermanii and P. freudenreichii in a two-stage procedure. First the bacteria were grown anaerobically in the presence of a cobalt salt. Mainly cobinamide is formed during this phase. On aerobic incubation the cobinamide is transformed into vitamin B₁₂ (14). In this investigation [5-¹⁵N]riboflavin was added to the bacteria prior to the aerobic phase. Thus vitamin B₁₂ containing ¹⁵N-labeled dimethylbenzimidazole was obtained. The ¹⁵N NMR spectrum of this vitamin B₁₂ showed only one signal at 221 ppm, the signal of the glycosidic nitrogen of dimethylbenzimidazole. This could not be taken as evidence that only one of the two nitrogens of dimethylbenzimidazole was ¹⁵N-labeled because cobalt has a nuclear quadrupole moment (15), which may abolish the signal of the nitrogen bound to cobalt.

Therefore a vitamin B₁₂ reference compound was prepared by addition of synthetic dimethylbenzimidazole ¹⁵N-labeled in one nitrogen to an anaerobic P. shermanii fermentation. Due to the equivalence of the two nitrogens in free dimethylbenzimidazole the vitamin B₁₂ thus formed must consist of equivalent amounts of species in which the nitrogen bound to cobalt is ¹⁵N-labeled and of species in which the glycosidic nitrogen of dimethylbenzimidazole is ¹⁵N-labeled. As expected this vitamin B₁₂ showed the signal at 221 ppm in the ¹⁵N NMR spectrum. But because of the quadrupole moment of cobalt, the ¹⁵N bound to cobalt exhibits only a very broad and low peak at -130 ppm, which is hardly detectable (Fig. 1). Therefore the vitamin B₁₂ was degraded to α-ribozole and acetylated to triacetyl-α-ribozole in order to get a compound more soluble in CHCl₃. Part of the vitamin B₁₂ was methylated with (CH₃)₂SO₄ and degraded to trimethylbenzimidazole. The degradation procedures are illustrated in Scheme 1. The triacetyl-α-ribozole and the trimethylbenzimidazole were examined by ¹H NMR. The signals of the proton at C-2 of the dimethylbenzimidazole moiety of these compounds should reflect the ¹⁵N distribution between N-1 and N-3. Fig. 2A shows the signals of the proton at C-2 of the dimethylbenzimidazole moiety of triacetyl-α-ribozole originating from [5-¹⁵N]riboflavin. The singlet from molecules which contain no
**Biosynthesis of Vitamin B₁₂**

**Fig. 2.** 'H NMR spectra. Signals of the proton at C-2 of dimethylbenzimidazole. A, of triacetyl-α-ribazole from the experiment with *P. shermanii* and [5-15N]riboflavin; B, trimethylbenzimidazole from the experiment with *P. shermanii* and [5-15N]riboflavin; C, of triacetyl-α-ribazole from the experiment with *P. shermanii* and [15N]dimethylbenzimidazole; D, of synthetic [3-15N]trimethylbenzimidazole. Spectrum B was taken at 60 MHz, all other spectra at 90 MHz.

15N (from the reaction of endogenous riboflavin) is located at 8.1 ppm. Further two doublets are seen, one from the coupling of the C-2 proton with 15N-1 (J = 8.3 Hz) and one from the coupling with 15N-3 (J = 11 Hz). By comparison of the integrals of the singlet and the two doublets, it can be calculated that 27% of the dimethylbenzimidazole is unlabeled, and that 60% of the 15N-label is located at N-1 and 40% at N-3, i.e., N-1 contains a total amount of 44% 15N and N-3 of 29% 15N. The spectrum of trimethylbenzimidazole derived from [5-15N]riboflavin gives the same results (Fig. 2B). The heights of the doublets are reversed in this spectrum due to the substitution of that nitrogen with a methyl group, which was originally N-3 of the dimethylbenzimidazole moiety.

**Fig. 3.** The 90 MHz 'H NMR spectrum of triacetyl-α-ribazole from the experiment with *P. shermanii* and [5-15N]riboflavin (A), and a section of it showing the “triplet” (see “Results”) of the C-2' proton and its further splitting of 1.8 Hz due to the coupling with 15N-1 (B).

The vitamin B₁₂ obtained in the experiments with *P. freudenreichii* and [8-15N]riboflavin was also degraded to trimethylbenzimidazole by the method pointed out in Scheme 1. The 'H NMR spectrum revealed that also in this case the 15N is asymmetrically distributed in the dimethylbenzimidazole moiety. But the difference in the 15N content of N-1 and N-3 is less pronounced, i.e., 55% of the 15N is located at N-1 and 47% at N-3.

**DISCUSSION**

The unequal distribution of 15N label from [5-15N]riboflavin in the dimethylbenzimidazole moiety of vitamin B₁₂ found in the experiments with *P. shermanii* indicates that 20% of the riboflavin molecules are transformed so that N-5 of riboflavin becomes N-1 of the dimethylbenzimidazole moiety of vitamin B₁₂. Thus 80% of the dimethylbenzimidazole molecules formed get into a state where their two nitrogens became equivalent, but the rest reaches the trans-N-glycosidase in a sterically definite way. This is only possible if in the intact cell the two enzyme systems, i.e., the dimethylbenzimidazole synthase system and the trans N-glycosidase, are very closely associated. Then the distance between the active centers of the two enzymes is so small that a certain amount of molecules can reach the glycosidase without losing their asymmetry imposed by the 15N label. The asymmetric distribution of 15N label in dimethylbenzimidazole is greater in the experiments with *P. shermanii* than in those with *P. freudenreichii*. *P. freudenreichii*, grown in the absence of cobalt, produces dimethyl-
Biosynthesis of Vitamin $B_{12}$

Benzimidazole and $\alpha$-ribazole, when incubated aerobically with riboflavin (3). *P. shermanii* under the same conditions only produces minute amounts of these compounds, even in the presence of an additional substrate like glucose. The difference in asymmetric $^{15}$N labeling of dimethylbenzimidazole and the difference in the ability of the cobalt-free grown organisms to produce dimethylbenzimidazole might reflect a closer association of the dimethylbenzimidazole synthase and the trans-$N$-glycosidase in *P. shermanii* than in *P. freudenreichii*. Also it gives an indication that the dimethylbenzimidazole synthesis might be regulated in the intact cell by a more or less close association of the two enzymes. The data given in this paper are tempting to speculate that in vivo the enzyme system of the vitamin $B_{12}$ biosynthesis is arranged as a multienzyme complex, although the enzyme system for the corrin ring biosynthesis (16), the amidation of the carboxyl groups (17), the formation of the $\alpha$-glycosidic bond (4), and the enzymes transforming cobinamide to cobalamin (14) can be solubilized. If a gentle method were available for the hydrolysis of the cell wall of the lysozyme-insensitive propionic acid bacteria, one could perhaps isolate such a multienzyme system in an intact form.

REFERENCES