The 5,6-dimethylbenzimidazole formed from riboflavin in cobalt-containing cells of Propionibacterium shermanii can only be isolated as a component of vitamin B12. No free 5,6-dimethylbenzimidazole is detectable in these bacteria. In order to determine, if nevertheless the vitamin B12 biosynthesis proceeds via free 5,6-dimethylbenzimidazole, cells of P. shermanii were incubated with [5-15N]riboflavin. One fraction of the vitamin B12 thus formed was degraded to 5,6-dimethylbenzimidazole α-ribofuranoside and acetylated to its 2',3',5'-tri-O-acetyl derivative. The other fraction of the vitamin B12 was methylated to 3,5,6-trimethylbenzimidazole-α-n-ribofuranoside and hydrolyzed to 1,5,6-trimethylbenzimidazole. The proton magnetic resonance spectra of both degradation products revealed that 60% of the 15N content of the 5,6-dimethylbenzimidazole moiety of vitamin B12 was localized in N-1 and 40% in N-3. This asymmetric distribution of 15N shows that 20% of the 5,6-dimethylbenzimidazole molecules formed are transformed regiospecifically into [1-15N]5,6-dimethylbenzimidazole α-n-ribofuranoside 5'-phosphate, the next intermediate in the biosynthesis of the nucleotide moiety of vitamin B12.

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 B12 reference compound in which the 15N was equally distributed between both nitrogens of its 5,6-dimethylbenzimidazole moiety was prepared by addition of synthetic 5,6-dimethylbenzimidazole-15N-labeled in one nitrogen to a P. shermanii fermentation. The 15N-labeled vitamin B12 species were also examined by 15N nuclear magnetic resonance spectroscopy.

In experiments with whole cells of Propionibacterium shermanii, it was shown that the 5,6-dimethylbenzimidazole moiety of vitamin B12 is formed from riboflavin (1). Thereby the C-1' of riboflavin is transformed into C-2' of 5,6-dimethylbenzimidazole (2). Recently, it was demonstrated that Propionibacterium freudenreichii, grown in the absence of a cobalt salt, transforms riboflavin into free 5,6-dimethylbenzimidazole, which can be isolated from the incubation medium (3). On the other hand P. shermanii cells, also grown in the absence of a cobalt salt, do not produce appreciable amounts of free dimethylbenzimidazole. Both species, when grown in the presence of cobalt, form vitamin B12, but no free dimethylbenzimidazole.

From the fact that free dimethylbenzimidazole is formed under special conditions it might be concluded that free dimethylbenzimidazole is an intermediate in the biosynthesis of vitamin B12. This view is further supported by the findings of Friedmann (4), who purified a single displacement trans-N-glycosidase from P. shermanii. This enzyme forms α-ribazole 5'-phosphate, an intermediate in the biosynthesis of the nucleotide moiety of vitamin B12, from free, i.e. in Friedmann's experiments exogenous synthetic dimethylbenzimidazole: dimethylbenzimidazole + β-nicotinic acid mononucleotide → α-ribazole-5'-p + nicotinate + H'. If free dimethylbenzimidazole is formed during the normal course of the vitamin B12 biosynthesis, the glycosidic bond can be formed with either nitrogen, because in free dimethylbenzimidazole the two nitrogens are equivalent. But if dimethylbenzimidazole either remains bound to a cell constituent or migrates to the next enzyme in a sterically directed way, the subsequent glycosidation may only occur with one of the two nitrogens. The question, if free dimethylbenzimidazole is an intermediate in the biosynthesis of vitamin B12, can be answered by labeling one of the two nitrogens of dimethylbenzimidazole during its biosynthesis. This is achieved with [5-15N]riboflavin as precursor of dimethylbenzimidazole.

EXPERIMENTAL PROCEDURES

Materials

Na15NO3 (99.5 atom %) and Na15NO2 (99.8 atom %) were obtained from B.O.C. Ltd, Pochem, London. Chromatographic solvents: I: CHCl3/EtOH/ AcOH = 85:15:1. II: Bu-2-OH/H2O/ AcOH/10% aqueous KCN = 70:30:10:1. Thin layer chromatography was performed in all experiments with precoated preparative Silica Gel 60 F254 plates (20 × 20 cm, layer thickness 2 mm, Merck Nr. 5717).

[5-15N]Riboflavin

Nine hundred milligrams of N-α-ribityl-3,4-dimethylaniline prepared according to Lamborgo (5) was coupled with [15N]phenylazonium chloride (from 500 mg of Na15NO3 and 495 mg of aniline) according to Shunk et al. (6). The 1-α ribitylamino-2-phenylazo-4,5-dimethylenzene was condensed with 670 mg of barbituric acid acetylated to its 2',3',5'-tri-O-acetyl derivative. The other fraction of the vitamin B12 was methylated to 3,5,6-trimethylbenzimidazole.

[3-15N]1,5,6-Trimethylbenzimidazole

Six hundred eighty milligrams of N-α-ribityl-3,4-dimethylaniline, prepared as described for N-methylaniline (9), was dissolved in 5 ml of 1 N HCl.
A solution of phenylhydrazonium chloride (1.5 ml of concentrated HCl, 3.3 ml of H2O, 385 mg of NaNO2, 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 applied to a column (1.7 × 7 cm) of basic alumina (activity level I, 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 (50 mg) (95%) of a red oil was obtained.

A solution of 3.1 g of Na2S2O3 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 dissolved in 1 ml of 1 N NaOH was added, and the N1-methylaminol-2-amino-4,5-dimethylbenzene 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 with EtOH/concentrated NH4OH-1005, the solvent evaporated, the residue dissolved in 10 ml of 1 N HCl, heated, and the pH adjusted to 10 to 11 with concentrated NH4OH. After the crystals were collected, washed with H2O, and dried in vacuo to yield 35 mg of [15N]trimethylbenzimidazole, m.p. 141–143°C.

(15N)Dimethylbenzimidazole Labeled in One Nitrogen

3,4-Dimethylaniline (0.6 g) was dissolved in 10 ml of concentrated H2SO4 (95%) and cooled to −10°C. A solution of 0.425 g of Na2SO3 in 2.5 ml of concentrated H2SO4 was added dropwise. Four hundred sixty milligrams (65%) of [2-15N]-nitro-4,5-dimethylaniline isolated as described previously for the unlabeled compound (10). Two hundred four milligrams of [2-15N]-nitro-4,5-dimethylaniline was dissolved in 50 ml of 70% aqueous ethanol and hydrolyzed in the presence of 35 mg of PtO2. The solvent was evaporated, the residue dissolved in 5 ml of HCOOH (98 to 100%), and heated under reflux for 1 h. The HCOOH was evaporated in vacuo, the residue dissolved in 5 ml of 0.2 N HCl, and extracted three times with 2 ml of CHCl3 in order to remove impurities. The aqueous phase was brought to pH 10 with ammonia. The [15N]dimethylbenzimidazole was extracted with three 2-ml portions of chloroform. The chloroform was evaporated and the residue recrystallized from 30 ml of water to yield 100 mg (55%) of [15N]dimethylbenzimidazole; m.p. 203–205°C.

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 fermentations. P. freudenreichii (ATCC 6207) was grown similarly (11) in 5-liter carboys. The cells were centrifuged for 1 h at 5000 × g. The slimy sediment was directly used for the aerobic incubation experiments.

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 (3.5 g of KH2PO4 and 7.25 g of Na2HPO4, 2H2O per liter) were added 57 mg of [5-15N]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 a 20% solution of NaHCO3.

P. freudenreichii. The cells from a 5-liter fermentation were suspended in 1.5 liters of phosphate buffer, pH 7.0, and evenly distributed into three 1-liter Erlenmeyer flasks. To each flask 4.75 mg of [5-15N]-riboflavin, dissolved in 12.5 ml of water, was added. The flasks were shaken for 48 h at 30°C at 160 rpm.

Isolation of Vitamin B12

After the aerobic incubation KCN (1 g/liter) was added to the suspension of P. shermanii cells, and the pH was adjusted to 7.0 with glacial acetic acid. The suspension was heated for 20 min at 120°C in an autoclave. After cooling and centrifugation, a clear red supernatant was obtained. The corrinoids and unchanged riboflavin were absorbed on a column (4 × 20 cm) of Amberlite XAD-2 (100 to 200 μm, Serva, Heidelberg). The column was washed with 5 liters of water. The corrinoids and the riboflavin were eluted with CH3OH/H2O = 8:2 (12). The solvent was evaporated in vacuo and the residue dissolved in water. Acidic and basic components were removed by filtration through a column of Dowex 2-X4 acetate (2 × 10 cm) and CM-cellulose, H form (4 × 17 cm). In both cases, vitamin B12 and riboflavin were eluted with water. The aqueous solution was evaporated to dryness in vacuo. The material of 11 incubation mixtures was boiled for a few minutes with 100 ml of EtOH. On cooling, the bulk of riboflavin precipitated and was removed by filtration. The precipitate was washed with 100 ml of EtOH. The EtOH phases were combined and evaporated. The residue was dissolved in water and absorbed on a column (2.5 × 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 B12. The red band was chromatographed 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 B12. From P. freudenreichii vitamin B12 was isolated in a similar manner with the exception that 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 15N 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 [15N]dimethylbenzimidazole 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 α-ribazole1 and acetylated to its tri-O-acetyl derivative. Vitamin B12 (60 mg) was further purified by descending chromatography on Whatman No. 3MM paper with Solvent II. Vitamin B12 was eluted from the paper with water and further purified by phenol extraction. This vitamin B12 was used for the 15N NMR measurements.

2,3,5′-Tri-O-acetyl-α-ribazole from Vitamin B12

From 60 to 80 mg of vitamin B12 was degraded to α-ribazole and cobinamide as described before (11). The reaction products were dissolved in 5 ml of 0.02 N NaOH and the solution was evaporated for 48 h with CHCl3. The organic phase, containing 12 to 16 mg of α-ribazole, was evaporated, and the residue dried in vacuo. The residue was dissolved in 5 ml of 1 N NaOH, and the N1-riboside fraction containing small amounts of vitamin B12, the red band was chromatographed in Solvent I. The zone of trimethylbenzimidazole was eluted with three 2-ml portions of chloroform. The chloroform was evaporated and the residue recrystallized from 30 ml of water to yield 100 mg (55%) of [15N]dimethylbenzimidazole; m.p. 203–205°C.

Methylation of Vitamin B12

The methylation was carried out as described previously (13). The reaction mixture was neutralized with concentrated HCl and 3.5,6-trimethylbenzimidazolylcobamide isolated via the Amberlite XAD-2 procedure (12). Paper electrophoretic analysis in 0.5 M AcOH 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 walled tube. Then 30 ml of a 50% solution of NaOH was added. The

1 The trivial names used are: α-ribazole, 5,6-dimethylbenzimidazole α-ribofuranoside; triacetyl-α-ribazole, 2′,3′,5′-tri-O-acetyl-α-ribazole.
trimethylbenzimidazole was extracted with three 50-ml portions of CHCl.

Biosynthesis of Vitamin B12

trimethylbenzimidazole was extracted with three 50-ml portions of CHCl. The CHCl was extracted three times with 50 ml of 1 N HCl. The aqueous phase was washed with 50 ml of CHCl, brought to pH 12 to 13 with NaOH, and extracted three times with CHCl. The CHCl was washed with water, filtered through dry filter paper, and evaporated to dryness. The residue was dissolved in CHCl, 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 NH4OH = 100:5. The solution was evaporated, and the residue subjected twice to sublimation in vacuo at 150°C. The resulting material was dissolved in 1 N HCl. The pH was brought to 10 with concentrated NH4OH. 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 CH315NO2,358 ppm downfield from 15NH4Cl. All NMR measurements were performed at 300 K. Vitamin B12 was dissolved in (CD3)2SO. Triacetyl-α-ribazole and trimethylbenzimidazole were dissolved in CDCl3 (99.8% enriched; the signal of the CHCl 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 a-ribazole and acetylated to triacetyl-α-ribazole 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 triacyl-α-ribazole 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 triacyl-α-ribazole originating from [5-15N]riboflavin. The singlet from molecules which contain no 

![Scheme 1. Chemical degradation of vitamin B12 to triacyl-α-ribazole and to trimethylbenzimidazole (TBI), respectively. [Co] = corrin ring of vitamin B12.](image-url)
Biosynthesis of Vitamin B_{12}  

FIG. 2. $^1$H NMR spectra. Signals of the proton at C-2 of dimethylbenzimidazole. A, of triacetyl-$\alpha$-ribazole from the experiment with P. shermanii and [5-$^{15}$N]riboflavin; B, trimethylbenzimidazole from the experiment with P. shermanii and [5-$^{15}$N]riboflavin; C, of triacetyl-$\alpha$-ribazole from the experiment with P. shermanii and [15$^N$]dimethylbenzimidazole; D, of synthetic [3-$^{15}$N]trimethylbenzimidazole. Spectrum B was taken at 60 MHz, all other spectra at 90 MHz.

FIG. 3. The 90 MHz $^1$H NMR spectrum of triacetyl-$\alpha$-ribazole from the experiment with P. shermanii and [5-$^{15}$N]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 $^{15}$N-1 (B).

$^{15}$N (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 $^{15}$N-1 ($J = 8.3$ Hz) and one from the coupling with $^{15}$N-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 $^{15}$N-label is located at N-1 and 40% at N-3, i.e. N-1 contains a total amount of 44% $^{15}$N and N-3 of 29% $^{15}$N. The spectrum of trimethylbenzimidazole derived from [5-$^{15}$N]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.

As a reference for the interpretation of the aforementioned spectra, [3-$^{15}$N]trimethylbenzimidazole was prepared. The spectrum of this compound shows only the doublet from the coupling of C-2-H with $^{15}$N-3 with a $J$ ($^{15}$N-3,H-2) = 11.7 Hz (Fig. 2D). In addition to the symmetric distribution of $^{15}$N the $^1$H NMR spectrum of triacetyl-$\alpha$-ribazole (Fig. 3A) shows another interesting feature. The proton of C-2' of the ribose moiety but not the proton at C-1' couples with $^{15}$N 1 of dimethylbenzimidazole. Thus the “triplet” ($J$ (H-2', H-3') $\approx$ $J$ (H-2', H-1')) of the C-2'-proton is split as shown in Fig. 3B.

The vitamin B_{12} obtained in the experiments with P. freudenreichii and [5-$^{15}$N]riboflavin was also degraded to trimethylbenzimidazole by the method pointed out in Scheme 1. The $^1$H NMR spectrum revealed that also in this case the $^{15}$N is asymmetrically distributed in the dimethylbenzimidazole moiety. But the difference in the $^{15}$N content of N-1 and N-3 is less pronounced, i.e. 53% of the $^{15}$N is located at N-1 and 47% at N-3.

DISCUSSION

The unequal distribution of $^{15}$N label from [5-$^{15}$N]riboflavin in the dimethylbenzimidazole moiety of vitamin B_{12} 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 B_{12}. 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 $^{15}$N label. The asymmetric distribution of $^{15}$N 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-
benzimidazole and α-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 α-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.

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