BENZIMIDAZOLE RIBOSIDE, A POSSIBLE PRECURSOR OF VITAMIN B_{12} IN *PROPIONIBACTERIUM SHERMANII*

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Basing their argument on the comparative utilization by resting *E. coli* cells of 5,6-dimethylbenzimidazole, the corresponding 1-α-ribofuranoside and 1-α-ribofuranoside phosphate, Dellweg *et al.* (1956) concluded that the free base rather than the nucleoside or nucleotide is incorporated directly into vitamin B_{12}. Later studies from the same laboratory with *Propionibacterium shermanii* led to a similar conclusion (Bernhauer *et al.*, 1959). On the other hand Barchielli *et al.* (1960) and Barbieri *et al.* (1962) working with *Nocardia rugosa* have presented convincing evidence for the incorporation of the riboside into B_{12}, perhaps by condensation with guanosine diphosphate cobinamide. They infer that the terminal reactions may be different in *Nocardia* and *Propionibacterium*. We wish to report evidence which strongly suggests that the ribofuranosides of benzimidazole and of 5,6-dimethylbenzimidazole are intermediates in the biosynthesis of B_{12} by *P. shermanii*.

P. shermanii [ATCC 9614] was grown in a medium based on that of Bernhauer *et al.* (1959) containing 10.8 mg of $CoCl_2 \cdot 6H_2O$ and of $ZnCl_2$ per liter. Five grams of fresh cells were transferred to 25 ml. of fresh medium containing a solution of 3.23 μmoles benzimidazole-2-C^{14} (1.04×10^4 c.p.m. per μmole). The labelled benzimidazole had been synthesized from o-phenylenediamine, formic acid and sodium formate-C^{14} (cf. Wagner and Millett, 1939). The medium was adjusted to pH 7 with

30% Na_2CO_3 daily, a small volume of 50% glucose being added when no pH change occurred overnight. Samples of the cultures were taken on 5 successive days, the supernatant medium separated and a lyophilized pyridine extract of the washed bacteria prepared. The media and extracts were subjected to paper electrophoresis in 0.5 M acetic acid [12.9 volts/cm for 2 hours] and counted in a Nuclear Actigraph II strip counter. Extracts of the washed bacteria contained, in addition to benzimidazole, three new peaks [A, B, C] migrating towards the cathode. Approximate mobilities relative to benzimidazole were A) 0.18, B) 0.45 and C) 0.69. The medium on the other hand exhibited, in addition to benzimidazole, one new peak corresponding to (C), the fastest of the above three peaks.

The extract of the cells was shown to contain benzimidazolyl-cobamide (BC) coenzyme by measurement of its activity in the enzymatic conversion of glutamate to mesaconate by crude extracts of Clostridium tetanomorphum H1 (Barker et al., 1960, Weissbach et al., 1961). On the basis of this fact, the radioactivity of the spots, and their relative electrophoretic mobility, peaks A and B were tentatively identified as BC hydroxide and coenzyme respectively. This identification was strengthened by the observation that photolysis of the extract (100 watt bulb, 15 cm, 60 minutes) led to an increase in the magnitude of peak A at the expense of peak B as expected from the properties of the BC coenzyme (Barker et al., 1960). Moreover, in extracts prepared on successive days and carefully shielded from light, peak A decreased relative to peak B. This is in agreement with the observations of Brady and Barker (1961) and of Weissbach et al. (1961) that the hydroxo compound is a precursor of the light-labile BC coenzyme which is the final product of biosynthesis.

Our primary attention was drawn to peak C which appeared to represent a compound not hitherto reported in extracts of B_{12} synthesizing organisms. Accordingly, we undertook to isolate the compound in larger

quantities, using its characteristic absorption spectrum (see below) to monitor the isolation. The material was purified as follows from a 5-day culture containing initially 440 g P. shermanii and 440 mg unlabelled benzimidazole in 3.5 l. The medium was concentrated in vacuo to about one-thirtieth of the original volume (pH about 6.5) and vigorously extracted four times with equal volumes of isobutanol. The clear, successively less yellow isobutanol extracts were recovered by centrifugation. Removal of the isobutanol in vacuo at 35-45° in the presence of extra water was followed by adjustment of the pH to about 9 with concentrated NH_4OH. Most of the free benzimidazole and some other impurities were removed by addition of a slight excess of concentrated ammoniacal $AgNO_3$. Excess silver was removed with concentrated HCl. Most of the inorganic ions were removed by a new extraction with four equal portions of isobutanol after adjustment of the pH to about 9 with NaOH. The alcohol was removed as before. The clear aqueous liquid was applied to a washed Dowex AG 50W-X2 (Bio-Rad Laboratories) column (H^+ form) and eluted by a gradient of HCl from 0 to 2 N in a total volume of 2 l. The desired compound was eluted when the HCl concentration reached 0.7 N (13 to 18 bed volumes). It was rechromatographed on a similar column using a weaker gradient of HCl [0 to 1 N].

The absorption spectrum of the material in peak C and of the purified compound was similar to but not identical with that of free benzimidazole. Absorption bands at 262, 268.5, and 275 mμ were homologous to bands occurring at slightly lower wavelengths in the spectrum of benzimidazole. The relative absorbancies of the three bands were however different. In more striking contrast was the absence of a band at 240 mμ and the presence of a broad shoulder with a minor peak at 254 mμ.

These data suggested that the unknown substance was a derivative of benzimidazole, an inference strengthened by the observation that the compound was not precipitated by ammoniacal silver as is benzimidazole

which is unsubstituted at N-1. The presence of a carbohydrate was indicated by a positive furfural spot test (cf. Feigl, 1954). In 0.25 M tris-phosphate buffer (pH 8.6) the compound migrated to the cathode as it does in acetic acid. However, in 0.25 M tris-borate buffer (pH 8.6) the compound migrated to the anode as would be expected for a borate complex of a glycoside with cis vicinal hydroxyl groups. Tests for phosphate were negative. It was tentatively inferred that the substance in question was 1-α-D ribofuranosyl benzimidazole.

Detailed comparison of the absorption spectrum with that of a sample of 1-β-D-ribofuranosyl benzimidazole synthesized by Davoll and Brown (1951) revealed no significant differences. The ratios of absorbance at 262, 268.5 and 275 mμ to that at 254 mμ were 1.055, 1.24, and 1.04, in good agreement with the values calculated from Davoll and Brown's data (1.07, 1.27, 1.10 respectively). It should be noted that the absorption spectrum of 1-β-D-glucopyranosyl benzimidazole differs significantly from that of the riboside (Davoll and Brown, 1951). A similar preparation starting with 5,6-dimethylbenzimidazole yielded a compound with a spectrum identical to that described by Davoll and Brown for 1-β-D-ribofuranosyl-5,6-dimethylbenzimidazole, which again differs from those of the free base and the β-D-glucopyranoside (Davoll and Brown, 1951). The electrophoretic mobilities of Davoll and Brown's compounds in acetic acid were identical to those of the compounds isolated here.

These data strongly suggest that the compounds isolated from media and demonstrated in extracts of the bacteria are the ribofuranosides of benzimidazole and of 5,6-dimethylbenzimidazole. In view of the structure of vitamin B_{12} and the findings of Barbieri et al. (1962) it seems probable that these compounds are the α-D-ribosides, but sufficient material has not yet been obtained to permit determination of the optical rotation.

The accumulation by P. shermanii of derivatives of benzimidazole and 5,6-dimethylbenzimidazole, which appear from their chemical reactions

and absorption spectra to be the α-D-ribosides, suggests that these compounds may be intermediates in the biosynthesis of vitamin B_{12} in P. shermanii as they seem to be in Nocardia rugosa (Barbieri et al., 1962). It seems most unlikely that the compounds described here are breakdown products of vitamin B_{12} since P. shermanii repeatedly subcultured in media to which no Co^{++} was added was still able to form the labelled riboside, although it formed little if any B_{12}.

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