Biosynthesis of 5,6-Dimethylbenzimidazole from 6,7-Dimethyl-10-[1′-^{14}C]formylmethylisoalloxazine

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Summary 6,7-Dimethyl-10-[1′-^{14}C]formylmethylisoalloxazine was added to growing cultures of *Propionibacterium shermanii;* the vitamin B_{12} subsequently isolated was found to be ^{14}C-labelled exclusively in the C-2 carbon of the 5,6-dimethylbenzimidazole unit.

EXPERIMENTS carried out in the laboratories of Renz and of Alworth have established that riboflavin and its immediate precursor, 6,7-dimethyl-8-ribityl-lumazine, serve as biosynthetic precursors of the 5,6-dimethylbenzimidazole (DBI) unit of vitamin B_{12}.[1] The observation by Renz and Weyhenmeyer that [1′-^{14}C]riboflavin is converted into the DBI unit of B_{12} with the incorporated label located exclusively in the C-2 position of DBI[2] is especially relevant to the new results described here.

We are interested in establishing the nature of the biosynthetic intermediates in the indicated pathway from riboflavin to DBI. Labelled lumiflavin (6,7-dimethyl-10-methylisoalloxazine), 1-amino-2-D-ribitylamino-4,5-dimethylbenzene, and 2-(*ribo*-1,2,3,4-tetrahydroxybutyl)-5,6-dimethylbenzimidazole were prepared and added to cultures of *P. shermanii*. No incorporation of label from any of these potential precursors of DBI into B_{12} could be detected.[3] These negative results indicate that none of these compounds are intermediates of the biosynthetic pathway leading from riboflavin to DBI.

Alternatively, cell-free extracts of *P. shermanii* were analysed for enzymatic activities capable of metabolizing riboflavin or known riboflavin catabolites. This approach led to the detection of an aldehyde reductase activity that converts 6,7-dimethyl-10-formylmethylisoalloxazine (DFI) into 6,7-dimethyl-10-(2-hydroxyethyl)isoalloxazine and an aldehyde oxidase activity that converts DFI into 6,7-dimethyl-10-carboxymethylisoalloxazine.[4] DFI, 6,7-dimethyl-10-(2′-hydroxyethyl)isoalloxazine, and 6,7-dimethyl-10-carboxymethylisoalloxazine have been established as products of riboflavin catabolism in animals by West and Owen, who also described enzymatic activities that convert DFI into 6,7-dimethyl-10-(2-hydroxyethyl)isoalloxazine and 6,7-dimethyl-10-carboxymethylisoalloxazine.[5] These activities, however, have not been previously detected in *Propionibacteria*. Since our experiments established that DFI, a known riboflavin catabolic product, is enzymatically converted by *P. shermanii* extracts, [1′-^{14}C]DFI was prepared and tested as a precursor of the DBI unit of B_{12} in *P. shermanii* cultures. The results, summarized in the Table, establish that DFI is converted into the DBI unit of vitamin B_{12} by *P. shermanii* and that the label from [1′-^{14}C]DFI is incorporated exclusively into the C-2 position of the DBI.

Sodium cyanoborohydride was used for the reductive amination of D-[1-^{14}C]ribose (ICN) with 3,4-dimethylani-

TABLE. Summarized incorporation data.

Sample	Specific activity (mCi mol^{-1})	Total activity (μCi $\times$ 10^3)
B_{12} isolated ..	$3{\cdot}6 \times 10^{-1}$ [a]	6·1
B_{12} hydrolysed	$3{\cdot}7 \times 10^{-2}$	3·1
DBI isolated	$5{\cdot}5 \times 10^{-2}$ [b]	0·97[c]
DBI degraded	$1{\cdot}4 \times 10^{-3}$	0·68
DBA isolated	0 ($<1{\cdot}2 \times 10^{-8}$)	0

[a] Based upon spectroscopic determination of amount of B_{12} isolated and the total activity in the diluted B_{12} sample recrystallized to constant specific activity. [b] Based upon spectroscopic determination of amount of DBI isolated from the hydrolysis and the total activity in the diluted DBI sample recrystallized to constant specific activity. [c] The chemical yield of DBI, based upon spectroscopic determination was 20·8%. This total activity therefore represents 150% of the activity in the B_{12} hydrolysed.

line.[6] The resulting *N*-(D-[1′-^{14}C]-1′-ribityl)-3,4-dimethylaniline was converted into [1′-^{14}C]riboflavin by the method described by Tishler *et al.*[7] and the labelled riboflavin was then oxidized to [1′-^{14}C]DFI with periodic acid according to the procedure of Fall and Petering.[8] Since the conversion of labelled riboflavin into DBI by *P. shermanii* had been previously observed,[2,9] the labelled DFI was carefully purified by preparative t.l.c. (1 mm silica gel, $CHCl_3$–MeOH, 9:1), before being used in a biosynthetic experiment. The purified [1′-^{14}C]DFI displayed a single yellow fluorescent spot when examined by both analytical silica gel t.l.c. ($CHCl_3$–MeOH, 9:1 and BuOH–EtOH–H_2O 7:2:1) and paper chromotography (BuOH–HOAc–H_2O, 7:2:1 on $NaHSO_3$ impregnated paper).

The purified [1′-^{14}C]DFI (55·1 mg, 80·2 mCi mol^{-1}) was dissolved in aqueous acid, sterilized by ultrafiltration, and added to a *P. shermanii* culture (7 l) that had been growing under anaerobic conditions for 4 days. Aeration of the culture was begun after the [1′-^{14}C]DFI was added; after 4 days of aerobic growth the cells were harvested and the B_{12} isolated.[10,11]

To establish the position of the incorporated label, the isolated B_{12} was diluted with unlabelled B_{12}, crystallized to constant specific activity, and then hydrolysed to release the DBI. The isolated DBI was diluted, crystallized to constant specific activity, and treated with benzoyl chloride and NaOH to yield 1,2-dibenzamido-4,5-dimethylbenzene (DBA) and formic acid. Finally, DBA was recrystallized three times from dimethylformamide–water and its specific activity determined.[10,12] All measurements of radioactivity were made with a Beckman series 200 liquid scintillation spectrometer using external standardization.

Since the DBA degradation product contains all of the carbon atoms in DBI except C-2, the determined specific activity of the DBA establishes the extent of the labelling at C-2 by difference. No activity could be detected in the final DBA degradation product; thus, the ^{14}C-label within the DBI unit is exclusively in the C-2 position. Furthermore, comparison of the activity of the DBI isolated with that of the B_{12} hydrolysed established that *all* of the incorporated label in B_{12} is accounted for by the DBI unit. [1′-^{14}C]DFI is therefore converted into labelled B_{12} by *P. shermanii* with the incorporated label appearing exclusively in the C-2 position of DBI (see Figure).

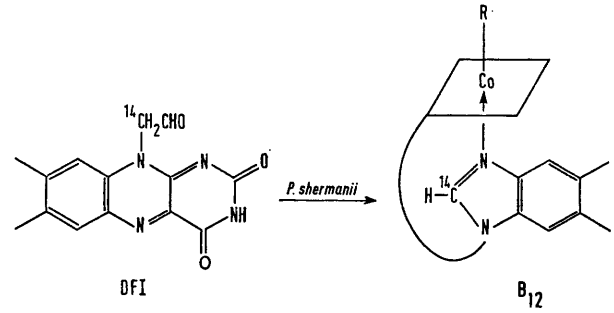

FIGURE. Detected biosynthetic conversion.

The incorporation of label from [1′-^{14}C]DFI into the DBI unit of B_{12} and the specificity of the observed incorporation both establish that DFI can serve an intermediate in the biosynthesis of DBI in intact cells of *P. shermanii*. The observed incorporation of activity, however, is only 0·39% and the observed dilution factor is 223. These values demonstrate that under the experimental conditions, added DFI is a relatively inefficient precursor of DBI. Perhaps the DFI added to the culture medium is not effectively transported into the intact cells. Even within the cell the added DFI may not compete effectively with a DFI intermediate generated as part of a riboflavin-to-DBI biosynthetic pathway. Finally, since DFI can be converted into 6,7-dimethyl-10-(2-hydroxyethyl)isoalloxazine and 6,7-dimethyl-10-carboxymethylisoalloxazine by enzymatic activities present in *P. shermanii*, it is possible that the actual biosynthetic intermediate is not DBI but one of these related riboflavin catabolites.

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