The Biosynthesis of the 5,6-Dimethylbenzimidazole Moiety of Vitamin B_{12}[1]

Sir:

The established biosynthetic sequence[2-6] for the conversion of cobinamide to cobalamin (vitamin B_{12}) structures begins with the following reaction: 5,6-dimethylbenzimidazole + β-nicotinate mononucleotide → α-ribazole 5′-phosphate + nicotinate.[2,3] We wish to communicate results regarding the biosynthesis of the 5,6-dimethylbenzimidazole (DBI) involved in this reaction. We find that ribose-1-^{14}C is an effective precursor of both the C-2 carbon atom and the dimethylbenzene carbon unit of DBI in *Propionibacterium shermanii*.

The methods used to grow the bacteria, isolate the B_{12}, and obtain labeled DBI have been described.[7] Table I summarizes the efficiencies of incorporation of a series of precursors into the DBI moiety of B_{12}. The labeled precursors were added to the cultures after 3 days of anaerobic growth and the cultures then aerated for an additional 4–5 days before harvest. Glucose was added throughout the biosynthetic runs and therefore each of the labeled precursors was incorporated in competition with unlabeled glucose and glucose metabolites. To permit direct comparisons between the experiments, the total activity isolated as DBI has been corrected to the theoretical yield of DBI from the hydrolysis of B_{12} and then normalized to disintegrations per minute per microcurie of precursor added and per milligram of B_{12} isolated. The percentage of the total B_{12} activity found in the DBI moiety is also tabulated. The distribution of incorporated label between the dimethylbenzene carbons and the C-2 carbon of DBI was determined[7] and is summarized in Table I.

Table I. Relative Incorporation Efficiencies of Precursors into 5,6-Dimethylbenzimidazole (DBI)

Expt	Precursor[a]	Amount of ^{14}C supplied, mCi	Incorporation efficiency, dpm/(μCi mg of B_{12})	% of total B_{12} label in DBI	% of total DBI label at C-2[c]
1	Formate-^{14}C	1.0	0.059	0.87	18
2	Formate-^{14}C	1.0	0.032	0.87	4
3[b]	Formate-^{14}C	1.0	0.036	1.05	
4	Glycine-2-^{14}C	0.5	0.055	3.60	
5[b]	Glycine-2-^{14}C	0.5	0.024	0.66	
6	Bicarbonate-^{14}C	1.0	0.047	13.9	12
7	DL-Serine-3-^{14}C	0.2	0.084	1.50	14
8	Acetate-1-^{14}C	0.5	0.0014	0.11	
9	Acetate-2-^{14}C	0.5	0.030	1.10	51
10	DL-Lactate-1-^{14}C	0.5	0.23	30.0	5
11	Pyruvate-1-^{14}C	0.15	0.39	22.0	18
12	Pyruvate-2-^{14}C	0.15	0.56	31.0	9
13	D-Glucose-6-^{14}C	0.1	3.70	15.0	6
14	D-Glucose-1-^{14}C	0.1	4.58	32.0	24
15	D-Gluconate-1-^{14}C	0.1	0.00		
16	Erythritol-U-^{14}C	0.1	6.00	70.0	6
17	D-Ribose-1-^{14}C	0.15	18.0	45.0	40

[a] The labeled precursors were added to a total incubation medium of 12 l. Each experiment yielded 20–60 mg of purified biosynthetic B_{12}. [b] The labeled substrate was present in the culture media throughout the total incubation period (anaerobic and aerobic). [c] Determinations were performed as described in ref 7. Values listed represent the average of C-2 labeling as determined directly (as $BaCO_3$-^{14}C) and as determined by difference (DBI activity minus 1,2-dibenzamido-4,5-dimethylbenzene activity).

Renz and Reinhold have reported that lactate-2-^{14}C and lactate-2,3-^{14}C are incorporated into the dimethylbenzene portion of DBI by *P. schermanii*.[8] From a partial degradation of the labeled DBI, these workers concluded that an acetate condensation pathway was involved in the formation of the dimethylbenzene unit. In our opinion, the experimental data summarized in Table I argue against either acetate or lactate being directly involved in DBI formation by *P. shermanii*.

Acetate-1-^{14}C, acetate-2-^{14}C, and pyruvate-2-^{14}C are much less effective as precursors of the dimethylbenzene carbons of DBI than are ribose-1-^{14}C or erythritol-U-^{14}C. Although B_{12} contains a ribose unit in the α-ribazole structure, addition of ribose-1-^{14}C resulted in B_{12}-^{14}C with nearly half the activity in the DBI moiety. The total incorporation of ribose-1-^{14}C into DBI by *P. shermanii* was 0.07%. Since the incorporation of ribose-1-^{14}C into the dimethylbenzene carbons of DBI was of an order of magnitude greater than that of acetate-^{14}C, we conclude that ribose is a more immediate precursor of this portion of B_{12}.

Plaut established that the dimethylbenzene ring of riboflavin is formed by dimerization of 6,7-dimethyl-8-ribityllumazine.[9] This dimethylpteridine precursor is derived from a purine by loss of the C-8 carbon[10] and incorporation of a new four-carbon unit. The carbons of the dimethylbenzene ring of riboflavin are thus ultimately derived from a four-carbon unit. Several workers have proposed that this unit is derived from acetoin or 2,3-butanedione rather than acetate.[11,12]

The results of Renz and Reinhold[8] could potentially have resulted from condensations of pyruvate-^{14}C to form an acetoin-^{14}C or 2,3-butanedione-^{14}C precursor. However, all known pathways leading from pyruvate to acetoin or butanedione necessitate loss of the carboxyl group (C-1).[13-15] Since we find that pyruvate-2-^{14}C is only 1.4 times as effective (0.56/0.39) as pyruvate-1-^{14}C as a precursor of DBI, we conclude that the condensation–decarboxylation of a three-carbon unit to form an acetoin or butanedione precursor is not involved in DBI biosynthesis.

Ali and Al-Khalidi have reported that neither acetate nor acetoin is involved in the formation of the dimethylbenzene ring of riboflavin and have proposed that a pentose or tetrose intermediate is involved.[16] The observations that ribose-1-^{14}C and erythritol-U-^{14}C compete very efficiently with glucose for incorporation into the dimethylbenzene portion of DBI lead us to propose that a pentose–tetrose intermediate is also

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involved in dimethylbenzene ring formation in B_{12} biosynthesis.

Biochemical analogies would predict that the remaining carbon atom of DBI (C-2) is derived from a biological C-1 unit. If formation of the imidazole ring of DBI were analogous to the biosynthesis of histidine[17] or the purines,[18] the C-2 carbon atom would be derived from formate.

Table I illustrates that precursors of labeled C-1 units were found to be poor precursors of DBI, and that no preferential incorporation into the C-2 carbon from precursors of labeled C-1 units occurs. In *P. pentosaceum* erythritol is metabolized to formaldehyde and dihydroxyacetone phosphate.[19] The incorporation of erythritol-U-^{14}C was investigated in *P. shermanii* to determine if labeled formaldehyde produced by this route could serve as a C-1 unit for DBI biosynthesis. The data indicate that erythritol-U-^{14}C shows a marked specificity for incorporation into the dimethylbenzene portion of B_{12} and does not lead to significant labeling of the C-2 carbon of DBI. Since all tested precursors of labeled C-1 units result in approximately random C-2 labeling (11%), we conclude that the C-2 carbon of DBI is not derived from a biological C-1 unit.

The incorporation of ribose-1-^{14}C into DBI was significantly greater than that of any other precursor investigated, and since 40% of the label incorporated from ribose-1-^{14}C is localized in the C-2 position, we propose that ribose-1-^{14}C is a specific precursor of this carbon.[20]

Plaut and Broberg[21] observed that incorporation patterns of labeled glucose into the ribityl chain of riboflavin indicated incorporation of ribose formed by oxidative and nonoxidative pentose cycle reactions. Our observations of differential labeling of the C-2 atom of DBI by glucose-1-^{14}C and glucose-6-^{14}C, and the preferential incorporation of ribose-1-^{14}C into this position, are therefore analogous to observations concerning the labeling of the C-1′ carbon of the ribityl chain of riboflavin.

These observations regarding DBI biosynthesis and previous observations regarding riboflavin biosynthesis[16,21] serve to emphasize that the 1,2-diamino-4,5-dimethylbenzene unit is confined in biological systems to riboflavin and DBI, and that the 1-amino-4,5-dimethyl-2-ribitylaminobenzene unit in riboflavin may also be present in the biosynthetic precursor of DBI. We propose that these similarities cannot be ascribed to coincidence and that the biosyntheses of riboflavin and DBI are connected, as originally suggested by Woolley.[22] Experiments designed to test this proposal are in progress.

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Methoxymethyl Methanesulfonate. A Novel Active Oxyalkylating Agent

Sir:

Further research into the chemistry of mixed sulfonic–carboxylic anhydrides[1] has led us to investigate the reactions of such reagents with acetals and ketals. We wish, at this stage, to record the preparation and some of the properties of methoxymethyl methanesulfonate (**2**), the simplest member of a series of compounds of potential use as powerful oxyalkylating agents.

Equimolar admixture at 0° of acetyl methanesulfonate (**1**) and dimethoxymethane under anhydrous conditions led to rapid formation, in essentially quantitative yield, of the title compound **2**, plus methyl acetate. Room temperature evaporation left the sulfonate **2** which was distilled as a colorless oil, bp 73–75° (10^{-2} mm), characterized by microanalysis and by its nmr spectrum: three singlets at δ 3.10 (3 H, OSO_2CH_3), 3.60 (3 H, OCH_3), and 5.38 (2 H, OCH_2O).

$$\underset{\mathbf{1}}{CH_3COOSO_2CH_3} + CH_3OCH_2OCH_3 \longrightarrow \underset{\mathbf{2}}{CH_3OCH_2OSO_2CH_3} + CH_3COOCH_3$$

The sulfonate **2**, which was rapidly hydrolyzed in moist air, was stable at room temperature and, in the absence of acid, up to 150°, when rapid decomposition ensued, resulting in the formation of methyl methanesulfonate (**3**) and polymeric material derived from

$$\underset{\mathbf{2}}{CH_3OCH_2OSO_2CH_3} \xrightarrow{150^\circ} \underset{\mathbf{3}}{CH_3OSO_2CH_3} + [CH_2O]_n$$

formaldehyde. The efficacy of sulfonate as a leaving group led us to anticipate that the product **2** would have a greatly enhanced activity over the corresponding α-haloalkyl ethers[2] in introducing at nucleophilic sites the methoxymethyl group. An analogous consideration has been found to hold for mixed sulfonic–carboxylic anhydrides which are powerfully enhanced as acylating agents by the lability of the sulfonate group.[1]

Our work, summarized in Table I, has shown such anticipation to be justified. With primary and secondary alcohols reaction proceeded swiftly and cleanly, the only volatile product being the mixed acetal isolated in high yield by distillation. Tertiary amines gave the respective quaternary methanesulfonate salts in quantitative yield.[3]

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(3) C–N bond formation was shown to be reversible in the following manner: when N-methoxymethyl-N,N-dimethylbenzylamine methanesulfonate was refluxed for 16 hr in mesitylene a 70% yield of bis(2,4,6-trimethylphenyl)methane resulted. Alternate C–N bond cleavage to give phenyl(2,4,6-trimethylphenyl)methane by reaction of benzyl carbonium ion with mesitylene was not observed.