of great importance for use in further studies of this new pathway. These compounds should also prove very useful for studies on the enzymic conversion of alkylglyceryl ethers to plasmalogens.

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Biosynthesis of 5,6-Dimethylbenzimidazole from 6,7-Dimethyl-^{14}C-8-ribityllumazine*

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ABSTRACT: 6,7-Dimethyl-^{14}C-8-ribityllumazine was added to anaerobically grown cultures of *Propionibacterium shermanii*. After 5 additional days of aerobic growth, the cells were harvested and the vitamin B_{12} was isolated and purified. The 5,6-dimethylbenzimidazole-^{14}C obtained from hydrolysis of this biosynthetic B_{12} represented about 7% of the ^{14}C originally added to the culture. A carbon-by-carbon degradation of the 5,6-dimethylbenzimidazole-^{14}C established that the ^{14}C was entirely confined to carbon atoms C-4(7) and C-8(9).

These results indicate that the 4,5-dimethyl-1,2-phenylene unit of the 5,6-dimethylbenzimidazole was formed by biosynthetic condensation of two molecules of the added 6,7-dimethyl-^{14}C-8-ribityllumazine precursor.

The 1,2-diamino-4,5-dimethylbenzene structural unit is confined in biological materials to riboflavin and its derivatives and to the DBI[1] moiety of vitamin B_{12} (Figure 1). Twenty years ago, Woolley (1951) proposed that this structural relationship between riboflavin and DBI resulted from a common biosynthetic pathway. Woolley found that 1,2-diamino-4,5-dichlorobenzene was toxic to those organisms which did not have a nutritional need for riboflavin and vitamin B_{12}, but that 1,2-diamino-4,5-dichlorobenzene did not retard the growth of those organisms which required exogenous sources of the two vitamins. Woolley postulated that 1,2-diamino-4,5-dimethylbenzene was a biosynthetic precursor of both riboflavin and the DBI moiety of vitamin B_{12}, and that the 1,2-diamino-4,5-dichlorobenzene was acting as an antimetabolite of this common precursor.

Subsequent experiments indicated that 1,2-diamino-4,5-dimethylbenzene was efficiently incorporated into the DBI moiety of B_{12} by *Propionibacterium arabinosum* (Perlman and Barrett, 1958). In the case of riboflavin however, nutritional studies and studies employing labeled precursors both established that the biosynthetic pathway originated within purine metabolism, rather than from 1,2-diamino-4,5-dimethylbenzene derivatives. (For reviews see Plaut (1961) and Goodwin (1970)). The investigations of Plaut (1960, 1963; Harvey and Plaut, 1966) then established that 6,7-dimethyl-8-ribityllumazine was the sole direct precursor of the riboflavin molecule. (For the recent identification of a biosynthetic intermediate between purines and 6,7-dimethyl-8-ribityllumazine see Bacher and Lingens, 1970.) The accumulated experimental evidence, therefore, tended to indicate that the biosyntheses of DBI and riboflavin were unrelated. It was clearly established that riboflavin biosynthesis began with the pyrimidine portion of the molecule and proceeded through 6,7-dimethyl-8-ribityllumazine; while, in contrast, it was interpreted that DBI biosynthesis began with the dimethylphenylene portion of the molecule and proceeded through 1,2-diamino-4,5-dimethylbenzene intermediates (*cf.* Plaut, 1961).

Nevertheless, the results of recent investigations have indicated that the biosyntheses of riboflavin and of the DBI moiety of vitamin B_{12} are connected. It was found that various labeled compounds tested as potential precursors of DBI (Renz and Reinhold, 1967; Alworth *et al.*, 1969, 1970) led to incorporation efficiencies and patterns that closely paralleled the results of earlier investigations into the biosynthetic

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[1] Abbreviations used are: DBI, 5,6-dimethylbenzimidazole; DBAB, 1,2-dibenzamido-4,5-dimethylbenzene; IDC, imidazole-4,5-dicarboxylic acid.

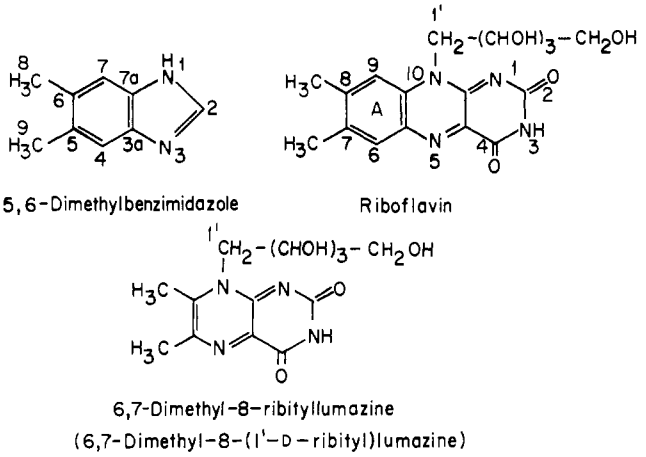

FIGURE 1: 5,6-Dimethylbenzimidazole, riboflavin, and 6,7-dimethyl-8-ribityllumazine (6,7-dimethyl-8-(1′-ribityl)lumazine) structures.

origin of ring A of riboflavin (Plaut, 1954; Ali and Al-Khalidi, 1966). It was also established that the C-2 carbon of DBI was derived from the C-1 carbon of ribose, rather than from the usual C-1 metabolic unit precursors (Alworth and Baker, 1968; Alworth *et al.*, 1969). Since the 6,7-dimethyl-8-ribityllumazine molecule contains a *N*-1-ribityl linkage (Figure 1), this biosynthetic origin for C-2 of DBI was also indicative of a close relationship to the established 6,7-dimethyl-8-ribityllumazine → riboflavin pathway. Most recently, Renz (1970) reported that randomly labeled riboflavin-^{14}C was efficiently converted into DBI-^{14}C in broken cell preparations of *P. shermanii*.[2] We now wish to report that 6,7-dimethyl-^{14}C-8-ribityllumazine is an effective precursor of the DBI moiety of vitamin B_{12}. Furthermore, the labeling pattern within the resulting DBI-^{14}C is totally consistent with biosynthesis of the 4,5-dimethyl-1,2-phenylene unit of DBI *via* the same type of bimolecular 6,7-dimethyl-^{14}C-8-ribityllumazine condensation previously established for the biosynthesis of ring A of riboflavin (Plaut, 1963).

Materials and Methods

Melting points were determined on a calibrated Fisher-Johns apparatus. Elemental analyses were performed by Spang Microanalytical Laboratory, Ann Arbor, Mich. Ultraviolet spectra were obtained with a Cary 14 recording spectrophotometer. The specific activities of all samples were determined by liquid scintillation counting in a Beckman series 200 instrument using external standardization.

The 6,7-dimethyl-8-ribityllumazine substrate specifically labeled with ^{14}C in the methyl carbons was synthesized according to the procedure described by Plaut (1963). The synthetic 6,7-dimethyl-^{14}C-8-ribityllumazine was purified by column chromatography upon acidic alumina and Lloyd Reagent (Hartman-Leddon Co.), and finally crystallized from 80% ethanol, mp 280–283° dec (Winestock and Plaut, 1961, mp 270–274° dec). *Anal.* Calcd for $C_{13}H_{18}N_4O_6$: C, 47.9; H, 5.6; N, 17.2. Found: C, 47.7; H, 5.6; N, 17.1.

TABLE I: Distribution of ^{14}C in DBI Labeled Biosynthetically from 6,7-Dimethyl-^{14}C-8-ribityllumazine.

Compound Analyzed	Carbon Atoms Represented	Specific Activity (dpm/mM)	Per Cent of Total DBI Activity
DBI[a]	All	9145	100
$BaCO_3$	C-2	<3	0
DBAB[a]	All but C-2	9371	102
IDC[a]	C-2,3a,4,7,7a	4742	52
$BaCO_3$	C-4(7)	2026[b]	22[b]
Imidazole perchlorate	C-2,3a,7a	<10	0
2-Methylbenzimidazole[a]	C-5,9(6,8)	2345[b]	26[b]
$BaCO_3$	C-8(9)	1692[b]	19[b]
Benzimidazole perchlorate	C-5(6)	<30[b]	0[b]

[a] Satisfactory elemental analyses (C, H, N) were obtained for these degradation products. [b] Values determined and reported in the table represent the activity in one of the equivalent atoms, or groups, of the total DBI molecule.

The ultraviolet spectrum of the crystallized lumazine in 0.1 N H_2SO_4 closely matched the published spectral data (Maley and Plaut, 1959; Winestock and Plaut, 1961). The spectrum in 0.1 N NaOH, however, differed from the published spectral data in that a third strong maximum at 228 nm (log ϵ 4.387) was observed.[3] The purity of the synthetic 6,7-dimethyl-^{14}C-8-ribityllumazine was also checked by paper chromatography with three different developing solvent systems (Bu-OH–EtOH–H_2O, 50:15:35; 5% Na_2HPO_4, and H_2O-saturated *tert*-amyl alcohol (Maley and Plaut, 1959)). In each case only a single yellow-green fluorescent spot was detected on the developed chromatograph.

The 6,7-dimethyl-^{14}C-8-ribityllumazine (100.2 mg, 2.07×10^6 dpm) was dissolved in water and added to a culture (12 l.) of *P. shermanii* that had been incubated for 4 days under anaerobic conditions. Aeration of the culture was then begun. After 5 additional days of aerobic growth, the cells were harvested (670 g of cell paste) and the biosynthetic B_{12} was isolated and purified (36 mg, 1.9×10^5 dpm). The incubation media and growth conditions and the procedures used to isolate and purify B_{12} have been previously described (Alworth and Baker, 1968; Alworth *et al.*, 1969).

The isolated B_{12} was diluted with 64 mg of nonradioactive B_{12} (Sigma Chemical Co.) and then hydrolyzed and the DBI was isolated (Brink and Folkers, 1950). The DBI sample obtained by hydrolysis of the B_{12} was diluted with 2 g of nonradioactive DBI (Aldrich Chemical Co.), crystallized twice from water, and then sublimed, mp 205–206°. The specific activity of the diluted, purified DBI-^{14}C was determined and

[2] In this communication Renz also cited unpublished data from his laboratory (P. Renz and H. Kühnle) which indicated that "^{14}C-6,7-dimethyl-8-ribityllumazine" served as a specific DBI precursor. Such an experiment is clearly related to the results described here. However, to the best of our knowledge, these data of Renz and Kühnle have not been published.

[3] Judging from their published spectrum, it seems quite possible that Maley and Plaut failed to record the absorption below about 230 nm and thus overlooked this 228-nm maximum.

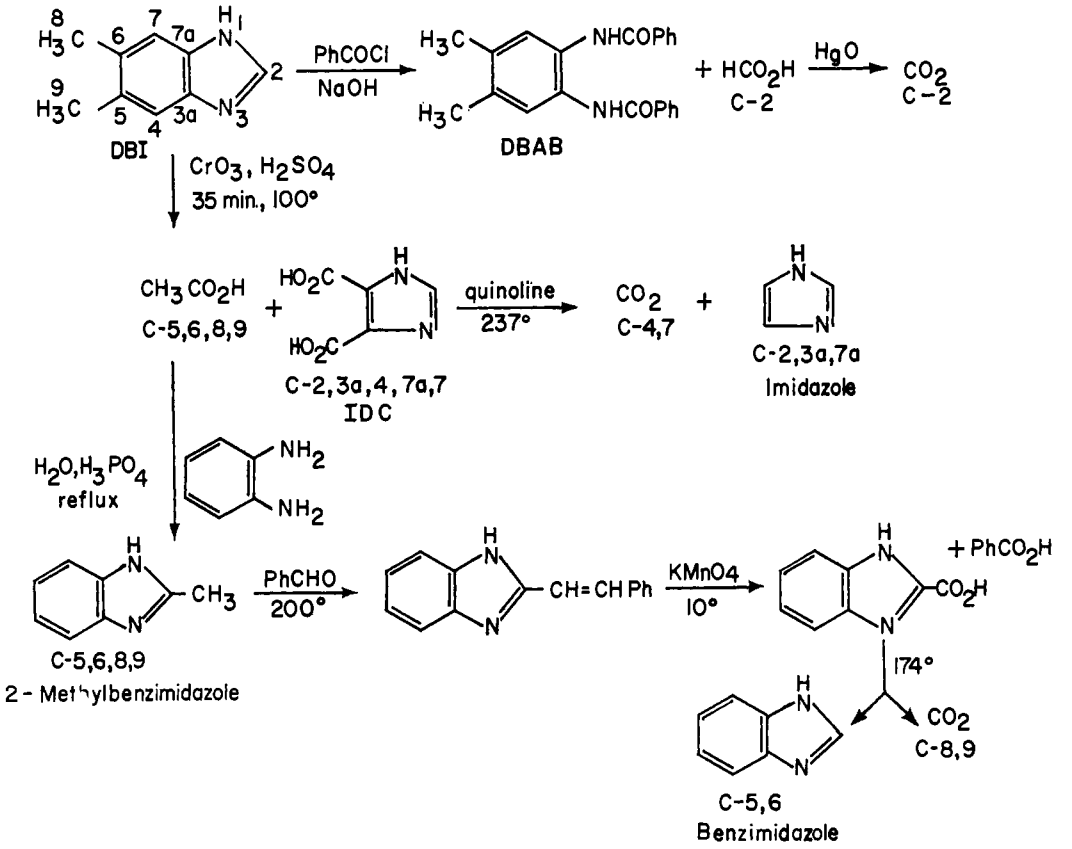

FIGURE 2: Chemical degradation scheme for DBI-^{14}C.

the incorporation data were corrected to take into account the observed yield of DBI (59%) from hydrolysis of the B_{12}.

The labeling pattern within the diluted, purified DBI-^{14}C was established by the chemical degradation scheme outlined in Figure 2. The procedure used to convert DBI into DBAB and release the C-2 carbon as CO_2 has been previously outlined (Alworth and Baker, 1968). Determination of the labeling pattern within the remainder of the DBI molecule (the 4,5-dimethyl-1,2-phenylene structural unit) was based upon a Kuhn–Roth oxidative cleavage (*cf.* Reinhold and Renz, 1969). After the acetic acid product of the Kuhn–Roth oxidation was removed by steam distillation (yield 51%), the reaction mixture was cooled, and the crude IDC product was collected by centrifugation (yield 47%). The IDC was precipitated several times from dilute NH_4OH solutions by the addition of HCl, and then purified by ion-exchange column chromatography with AG-1 anion-exchange resin (Bio-Rad Lab.).

The distribution of label within the acetic acid product was established *via* the Roseman procedure (Roseman, 1953). The Roseman degradation yielded benzimidazole containing the C-1 of the acetic acid (C-5 and C-6 of the original DBI) and CO_2 containing the C-2 of the acetic acid (C-8 and C-9 of the original DBI). The distribution of label within the IDC product was determined by decarboxylating the purified IDC to yield CO_2 (C-4 and C-7 of the DBI) and imidazole (C-2,3a,7a of the DBI). The benzimidazole and the imidazole samples produced in the degradation sequence were sublimed, and then purified as the perchlorate salts. The CO_2 samples yielded in the degradation sequence were trapped in carbonate-free NaOH solutions, precipitated as $BaCO_3$, and the specific activities determined according to the procedure of Woeller (1961).

Results and Discussion

Slightly over 9% of the ^{14}C originally added to the *P. shermanii* culture as 6,7-dimethyl-^{14}C-8-ribityllumazine was recovered in the form of purified radioactive vitamin B_{12}. When adjusted to the theoretical yield of DBI from the hydrolysis of B_{12}, the specific activity of the diluted, purified DBI indicated that about 75% of the total B_{12} activity was present in the DBI moiety. The data in Table I summarize the results of the carbon-by-carbon degradation of this DBI-^{14}C. The distribution of radioactivity within the degradation products indicated that the ^{14}C label within the biosynthetic DBI-^{14}C was completely confined to C-4(7) and C-8(9). Furthermore, within experimental error, the activity of C-4(7) equaled that of C-8(9).[4]

These results demonstrate that the methyl-^{14}C carbons of 6,7-dimethyl-^{14}C-8-ribityllumazine are specific biosynthetic precursors of carbon atoms of C-4(7) and C-8(9) of DBI. Plaut (1963) established that a 6,7-dimethyl-^{14}C-8-ribityllumazine precursor is converted into a riboflavin-^{14}C molecule specifically labeled in the methyl carbons and in carbons C-6 and C-9. The results reported here therefore indicate that the 4,5-dimethyl-1,2-phenylene structural unit of DBI is derived by the same type of bimolecular 6,7-dimethyl-8-ribityllumazine condensation as is involved in the biosynthesis of ring A of riboflavin (Harvey and Plaut, 1966). This observation

[4] For unknown reasons, the specific activities of the $BaCO_3$ samples determined in this experiment were apparently several per cent low. Since, however, the data summarized in Table I established that the degradation products representing all the carbons other than C-4(7) and C-8(9) contained less than 1% of the total DBI activity, the low $BaCO_3$ determinations cannot significantly affect the conclusions.

clearly establishes that Woolley (1951) was correct when he proposed that the biosyntheses of riboflavin and of the DBI moiety of vitamin B_{12} were intimately connected. The common precursor to the two structures, however, is 6,7-dimethyl-8-ribityllumazine rather than the 1,2-diamino-4,5-dimethylbenzene originally proposed.

Previously it has been found that the C-2 carbon atom of the DBI moiety of B_{12} is biosynthetically derived from the C-1 position of ribose (Alworth *et al.*, 1969). The present results indicate that all of the carbon atoms of DBI may be derived from 6,7-dimethyl-8-ribityllumazine. The C-6,7-dimethyl portion of the lumazine serves as the precursor of the 4,5-dimethyl-1,2-phenylene unit of DBI, while C-1′ of the ribityl chain of the lumazine is probably the immediate precursor of the C-2 carbon of the DBI.

Since 6,7-dimethyl-^{14}C-8-ribityllumazine is an established precursor of specifically labeled riboflavin-^{14}C (Plaut, 1963), the results reported here are consistent with Renz's observation (1970) that randomly labeled riboflavin-^{14}C is converted into the DBI moiety of vitamin B_{12} by broken *P. shermanii* cell preparations. It remains to be definitively established, however, that riboflavin is an obligatory intermediate in the biosynthesis of DBI in *P. shermanii* as proposed by Renz. It seems possible that related but branching pathways could lead to the formation of both riboflavin and DBI directly from the common 6,7-dimethyl-8-ribityllumazine precursor.

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Specificity for Phosphorylcholine of Six Murine Myeloma Proteins Reactive with *Pneumococcus* C Polysaccharide and β-Lipoprotein*

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ABSTRACT: Six murine IgA myeloma proteins, precipitating with the C-polysaccharide of *Pneumococcus*, recognize the same chemical determinant, phosphorylcholine. The myeloma proteins may be divided into three classes on the basis of inhibition of precipitation by the compounds choline, phosphorylcholine, glycerophosphorylcholine, and phosphonocholine.

The characteristic inhibition patterns are: class I, phosphorylcholine = glycerophosphorylcholine > choline > phosphonocholine; class II, phosphorylcholine > glycerophosphorylcholine = phosphonocholine ≫ choline; and class III, phosphorylcholine > glycerophosphorylcholine > phosphonocholine ≫ choline. Although choline is a poor inhibitor of the proteins in classes II and III, the quaternary nitrogen group appears to be a prerequisite for binding of the phosphate ester, since phosphorylethanolamine and glycerophosphate are not inhibitors of precipitation. All six myeloma proteins agglutinate erythrocytes coated with human β-lipoprotein. Representative proteins from each of the three classes precipitated human β-lipoprotein.

Investigations of the structure of antibody binding sites have been limited by the heterogeneity of the antibody population present in a normal antiserum. General properties of the binding site regions of normal heterogeneous antibodies, such as the presence of basic amino acids in or near sites directed toward negatively charged antigens (Freedman *et al.*, 1968),

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