TABLE II: Reactivities of Tocopherols: Correlation between Singlet Oxygen Reactivity and Biological Activity.

Tocopherol Compound	$\beta \times 10^4$	k_T/k_α[b] × 100 (%)	Biological Activity[a]: Respiratory Decline in Rat Livers, *In Vitro* (%)	Respiratory Decline in Rat Livers, *In Vivo* (%)	Erythrocyte Hemolysis, *In Vitro* (%)	Erythrocyte Hemolysis, *In Vivo* (%)
α	1.4	100	100	100	100	100
β	2.8	50	46	55	40	23
γ	5.4	26	26	5	30	3–17
δ	13.5	10	18	4	20	2

[a] Century and Horwitt (1965). [b] Rel std dev = 30%.

oxygen reactivity of each tocopherol is correlated with respiratory decline in rat livers, the reactivity of each tocopherol falls between *in vivo* and *in vitro* biopotency. The correlation between singlet oxygen reactivity and erythrocyte hemolysis is similar except for β-tocopherol, for which the reactivity falls slightly above the range of reported biopotency.

Conclusion

We have demonstrated that oxidation of tocopherols with singlet oxygen in methanol is a good model reaction for certain functions of vitamin E. This correlation suggests that one function of vitamin E may be to protect membranes and lipids from the damaging effects of "active" oxygen.

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Biosynthesis of 5,6-Dimethylbenzimidazole from [1′-^{14}C,5-^{15}N]6,7-Dimethyl-8-ribityllumazine*

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ABSTRACT: [1′-^{14}C,5-^{15}N]6,7-Dimethyl-8-ribityllumazine was prepared and 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. This biosynthetic B_{12} was hydrolyzed and the resulting 5,6-dimethylbenzimidazole isolated. The ^{15}N and ^{14}C contents of the 5,6-dimethylbenzimidazole were determined and compared with the ^{15}N and ^{14}C contents of the added 6,7-dimethyl-8-ribityllumazine precursor. The location of the ^{14}C within the 5,6-dimethylbenzimidazole was also determined by degradation. The experimental results established that [1′-^{14}C,5-^{15}N]6,7-dimethyl-8-ribityllumazine is an efficient precursor of isotopically labeled 5,6-dimethylbenzimidazole and that the C-1′ carbon atom of 6,7-dimethyl-8-ribityllumazine is incorporated exclusively into the C-2 position of the 5,6-dimethylbenzimidazole. The results also indicated that the C-1′ and the N-5 atoms of 6,7-dimethyl-8-ribityllumazine are incorporated into 5,6-dimethylbenzimidazole as a unit. These results, in conjunction with previous observations, establish that all of the atoms of the 5,6-dimethylbenzimidazole moiety of vitamin B_{12} may be biosynthetically derived from 6,7-dimethyl-8-ribityllumazine.

Recently it was demonstrated that the methyl-^{14}C carbons of 6,7-[^{14}C]dimethyl-8-ribityllumazine are specific precursors of carbon atoms C-4(7) and C-8(9) of DBI[1] in *Propionibacterium shermanii* (Alworth *et al.*, 1971) (*cf.* Figure 1). These results established 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 that is involved in the biosynthesis of ring A of riboflavin (Harvey and Plaut, 1966) (*cf.* Figure 1). It had previously been found that the C-2 carbon atom of the DBI moiety of vitamin B_{12} may be derived from the C-1 position of ribose (Alworth *et al.*, 1969). It was proposed, therefore, that all of the carbon atoms of DBI were derived from 6,7-dimethyl-8-ribityllumazine. The

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[1] Abbreviation used is: DBI, 5,6-dimethylbenzimidazole.

TABLE I: Incorporation of [1′-^{14}C,5-^{15}N]6,7-Dimethyl-8-ribityllumazine into B_{12} by *P. shermanii*.

[1′-^{14}C,5-^{15}N]6,7-Dimethyl-8-ribityllumazine Added (mg)	Yield of Cell Paste (g)	Yield of B_{12} (mg)	Sp Act. of B_{12} (dpm/mmole)
77.9 (1.81 × 10^6 dpm/mmole)	610	12.87	7.01 × 10^5
74.2 (1.81 × 10^6 dpm/mmole)	459	11.72	1.32 × 10^6

C-6,7-dimethyl portion of the lumazine served as the precursor of the 4,5-dimethyl-1,2-phenylene unit, while, according to this proposal, the C-1′ carbon of the ribityl side chain of the lumazine was the immediate precursor of the C-2 carbon of the DBI. In order to test this proposed relationship between the C-1′ carbon of the lumazine precursor and the C-2 carbon of the DBI, and in an attempt to establish the biosynthetic origins of the nitrogen atoms of DBI, [1′-^{14}C,5-^{15}N]6,7-dimethyl-8-ribityllumazine was synthesized and supplied to *P. shermanii* cultures.

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 ^{15}N enrichments of the [1′-^{14}C,5-^{15}N]6,7-dimethyl-8-ribityllumazine and of the DBI were determined by gas chromatography–mass spectrometry at the Institute for Lipid Research, Baylor College of Medicine. An LKB 9000 instrument was used under the following conditions: ion source and carrier gas separator temperatures 250°; ionizing energy 70 eV; gas chromatographic inlet, 3 ft, 1% SE-30.

The 6,7-dimethyl-8-ribityllumazine precursor was synthesized according to the procedures described by Maley and Plaut (1959). To prepare the 6,7-dimethyl-8-ribityllumazine specifically labeled with ^{14}C in the C-1′ position, the published lumazine synthesis was carried out with [1-^{14}C]ribitylamine, obtained from the oxime of [1-^{14}C]ribose (New England Nuclear Corp.). To prepare the 6,7-dimethyl-8-ribityllumazine specifically enriched with ^{15}N in the N-5 position, the published lumazine synthesis was carried out with 4-ribitylamino-[^{15}N]5-nitroso-2,6-dihydroxypyrimidine, obtained by treating 4-ribitylamino-2,6-dihydroxypyrimidine with sodium [^{15}N]-nitrite (95% enriched, Stohler Isotope Chemicals). After the two isotopically labeled lumazine samples were separately chromatographed upon columns of acidic alumina and Lloyd Reagent (Hartman-Leddon Co.), suitable amounts of the two samples were combined and the [1′-^{14}C,5-^{15}N]6,7-dimethyl-8-ribityllumazine crystallized twice from 80% ethanol, mp 284–288° dec (Winestock and Plaut, 1961, mp 270–274° dec). *Anal.* Calcd for $C_{13}H_{18}N_4O_6$: C, 47.8; H, 5.5; N, 17.4.[2] Found: C, 47.9; H, 5.6; N, 17.2. The ultraviolet spectra of the crystallized lumazine in 0.1 N H_2SO_4 and in 0.1 N NaOH agreed with previously published spectral data (Alworth *et al.*, 1971).

[2] Calculated analysis based upon 70% ^{15}N in one nitrogen atom.

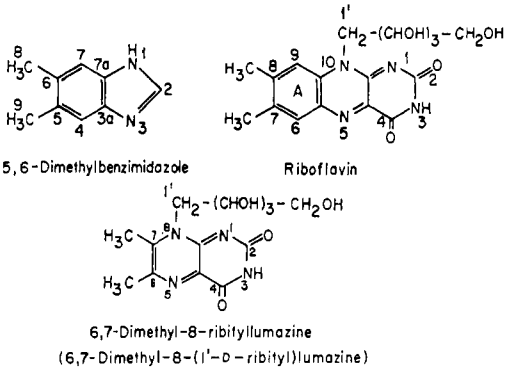

FIGURE 1: 5,6-Dimethylbenzimidazole, riboflavin, and 6,7-dimethyl-8-ribityllumazine (6,7-dimethyl-8-(1′-D-ribityl)lumazine) structures.

The ^{14}C content of the [1′-^{14}C,5-^{15}N]6,7-dimethyl-8-ribityllumazine was determined by liquid scintillation counting. The [1′-^{14}C,5-^{15}N]6,7-dimethyl-8-ribityllumazine sample pyrolyzed upon vaporization. Therefore the lumazine sample was dissolved in *N,O*-bis(trimethylsilyl)trifluoroacetamide and allowed to stand at 80–100° for 1 hr, and the ^{15}N content was established by glc–mass spectral analysis of the resulting trimethylsilyl derivative. Molecular ion peaks at *m/e* 758 and at 759, corresponding to (hexakis)trimethylsilyl derivatives of 6,7-dimethyl-8-ribityllumazine and of [^{15}N]6,7-dimethyl-8-ribityllumazine respectively, were used to establish a ^{15}N enrichment value of 71.2 ± 5%. This molecular weight is consistent with the trimethylsilyl derivative of the 7-methylene dilactim structure shown in Figure 2.

A related 7-methylene lactim tautomer of 6,7-dimethyl-8-ribityllumazine has been proposed as an intermediate in the observed hydrogen–deuterium exchange reaction of the 7-methyl group of lumazine (Beach and Plaut, 1970).

The [1′-^{14}C,5-^{15}N]6,7-dimethyl-8-ribityllumazine was dissolved in water and added to a culture (6 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 and the biosynthetic B_{12} was isolated and purified. The incubation media and growth conditions, and the procedures used to isolate and purify the B_{12} have been previously reported (Alworth and Baker, 1968; Alworth *et al.*, 1969). Table I summarizes the pertinent incorporation data from the two separate incubations which were carried out.

The B_{12} samples isolated from the two incubations described above were combined and hydrolyzed, and 0.837 mg of DBI was isolated by liquid–liquid extraction (Brink and Folkers, 1950). The DBI was then sublimed (120°, 10 μ) and analyzed for ^{14}C and ^{15}N by liquid scintillation counting and glc–mass

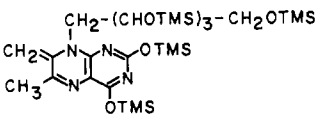

FIGURE 2: Proposed structure of the (hexakis)trimethylsilyl derivative of 6,7-dimethyl-8-ribityllumazine.

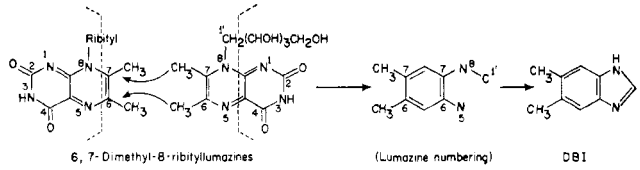

FIGURE 3: Schematic representation of DBI biosynthesis.

spectral analysis. The ^{15}N analysis was carried out with the trimethylsilyl derivative of the DBI sample. This derivative yielded predominately the mass spectrum of DBI since the trimethylsilyl group was lost during the introduction of the sample into the mass spectrometer. The molecular ion and M − 15 peaks of DBI (*m/e* 146 and 131) were therefore used to determine the ^{15}N content. The analysis established that the DBI was enriched 51.7 ± 2% with ^{15}N in one nitrogen.

The remaining biosynthetic DBI sample was then diluted with unlabeled DBI, recrystallized twice from water, and chemically degraded to establish the position of the ^{14}C label. The procedure used to convert DBI into 1,2-dibenzamido-4,5-dimethylbenzene and release the C-2 carbon as CO_2 has been previously described (Alworth and Baker, 1968).

Results

The experimental results pertaining to the biosynthesis of DBI from [1′-^{14}C,5-^{15}N]6,7-dimethyl-8-ribityllumazine by *P. shermanii* are summarized in Tables I, II, and III.

The data summarized in Tables I and II establish that [1′-^{14}C,5-^{15}N]6,7-dimethyl-8-ribityllumazine is an efficient precursor of the DBI moiety of vitamin B_{12} in aerated cultures of *P. shermanii*. The average specific activity of the isolated vitamin B_{12} can be calculated to be 1.0×10^6 dpm/mmole, while the most reliable value for the isolated DBI (based upon the specific activity of diluted, recrystallized DBI) is 0.92×10^6 dpm/mmole. Thus, the specific activity of the vitamin B_{12} formed within the cultures is about 55% of that of the added lumazine; 92% of this incorporated activity may be ascribed to the DBI moiety. The result of the partial chemical degradation summarized in Table III also establishes that ^{14}C label from the C-1′ atom of the ribityl side chain of lumazine was specifically incorporated into the C-2 position of this DBI moiety.

It was found that the ^{15}N enrichment in the biosynthetic DBI was 73% of the ^{15}N enrichment in the added lumazine samples, while the ^{14}C specific activity in the biosynthetic DBI was 51% of the specific activity in the added lumazine samples. Although the difference between these isotope incorporation values appears greater than experimental error, we feel that the data establish that the N-5 and C-1′ atoms of 6,7-dimethyl-8-ribityllumazine are incorporated into DBI as a unit. It seems possible that the lower ^{14}C incorporation value could arise from a small amount of ribityl–ribityl exchange during the biosynthesis. Alternatively, the original ribose may not have been exclusively labeled with ^{14}C at the C-1 carbon atom.

TABLE II: Incorporation of [1′-^{14}C,5-^{15}N]6,7-Dimethyl-8-ribityllumazine into DBI by *P. shermanii*.

	6,7-Dimethyl-8-ribityl-lumazine	DBI	DBI/Lumazine
^{15}N enrichment (%)	71	52	73%
^{14}C content (dpm/mmole)	1.81×10^6	1.0×10^6 [a]	
		0.92×10^6 [b]	51%

[a] Activity of isolated DBI by direct determination. [b] Activity of isolated DBI as calculated from activity of diluted, recrystallized DBI.

TABLE III: Distribution of the ^{14}C Label in the Isolated DBI.

	DBI[a]	Dibenzamidodimethylbenzene	CO_2	C-2
dpm/mmole	1.27×10^3	<20	1.16×10^3	
Percentage of DBI Label	100	<2	91	95[b]

[a] Diluted, recrystallized DBI. [b] Average C-2 value. [(DBI–dibenzamidodimethylbenzene) + CO_2]/2.

The above results, in conjunction with the results of incorporation experiments involving 6,7-[^{14}C]dimethyl-8-ribityl lumazine (Alworth *et al.*, 1971), establish that all of the atoms of the DBI moiety of vitamin B_{12} may be derived from 6,7-dimethyl-8-ribityllumazine. Figure 3 is a schematic representation of the indicated DBI biosynthetic pathway.

Renz (1970) has reported that [^{14}C]riboflavin is an efficient precursor of the DBI moiety of vitamin B_{12} in broken cell preparations of *P. shermanii*. It has also been established that *P. shermanii* cultures produce riboflavin (Janicki *et al.*, 1966). The labeling results reported here, as well as earlier labeling results from the 6,7-[^{14}C]dimethyl-8-ribityllumazine precursor, are consistent with two reasonable biosynthetic pathways. The DBI could be formed directly from a bimolecular lumazine condensation as implied by the scheme in Figure 3, or the DBI could be formed from a riboflavin precursor, derived in turn from a bimolecular lumazine condensation.

In these experiments the labeled lumazine precursors were supplied to the *P. shermanii* cultures after 4 days of vigorous growth in rich media. We feel that under these conditions the total flavin pool in the culture could not have become sufficiently labeled with ^{15}N to serve as the precursor of a DBI moiety which retained 73% of the original ^{15}N enrichment. Therefore, at present, we favor the view that related but branching pathways lead to the separate formation of riboflavin and DBI directly from the common 6,7-dimethyl-8-ribityllumazine precursor. It is possible, however, that a small, rapidly metabolized pool of riboflavin serves as a direct precursor to DBI in aerated *P. shermanii* cultures. Additional experiments, which we hope will resolve this ambiguity, are in progress.

Acknowledgment

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Expression of the *rel* Gene during R17 Phage Infection*

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ABSTRACT: Phage RNA synthesis has previously been reported to be under the influence of the *rel* gene of the host. It has also been suggested that guanosine 5′-diphosphate 3′- or 2′-diphosphate (ppGpp) is an inhibitor of host RNA synthesis, and may inhibit phage RNA synthesis as well. The effect of isoleucine starvation on the accumulation of ppGpp and the relative rate of phage RNA synthesis was compared in a stringent and a relaxed host infected with R17 RNA phage under conditions where host RNA and protein synthesis were inhibited by the addition of rifampicin. ppGpp was accumulated in the infected stringent but not in the infected relaxed cells upon isoleucine starvation. Since in the presence of rifampicin no ppGpp was formed in the uninfected host, the accumulation of ppGpp in the infected host must be phage specific. Phage RNA synthesis was not decreased in either a stringent or a relaxed host under these conditions. Therefore phage RNA synthesis is not influenced by the *rel* gene of the host in the same manner as is host RNA itself, nor is the accumulation of ppGpp dependent upon a decrease in RNA synthesis.

Amino acid starvation or restricted aminoacylation of tRNA causes a severe reduction of RNA accumulation (termed the stringent response) and the rapid accumulation of two guanosine nucleotides (MS I and MS II) in stringent (*rel*$^+$), but not relaxed (*rel*) strains of *Escherichia coli* (Cashel and Gallant, 1969; Cashel, 1969). Cashel and Kalbacher (1970) identified MS I as guanosine 5′-diphosphate 3′- or 2′-diphosphate (ppGpp). MS II, whose structure is not known, is produced in lesser amounts and is not characteristic of all stringent strains.

Travers *et al.* (1970b) have discovered a factor, ψ, which preferentially stimulates the synthesis of *E. coli* ribosomal RNA *in vitro*. This factor is found as a component of Qβ RNA phage replicase. This suggests the existence of similar regulatory mechanisms involving the factor for the synthesis of ribosomal and viral RNA. In fact, Travers *et al.* (1970a) demonstrated that ppGpp inhibits *in vitro* both ψ-stimulated RNA synthesis by bacterial RNA polymerase and poly(G) synthesis by Qβ replicase in a quantitatively similar manner.

Attempts to examine the effect of the *rel* gene on phage RNA synthesis *in vivo* have yielded conflicting results. Friesen (1969) and Khan and Yamazaki (1970) reported that the incorporation of radioactive uracil into phage RNA is influenced by the allelic state of the *rel* gene in a manner similar to that of the host. On the other hand, Siegel and Kjeldgaard (1971), using a spheroplast assay for infectious RNA, reported that the synthesis of Qβ phage RNA continues in a similar manner in both stringent and relaxed hosts deprived of a required amino acid. Therefore, there exists uncertainty concerning the relationships between the *rel* gene, ppGpp formation, and RNA synthesis during RNA phage infection.

In the present work we have examined the effect of amino acid starvation on ppGpp formation during R17 infection of a stringent and a relaxed host, and determined the rates of phage RNA synthesis under these conditions.

Materials and Methods

Bacteria, Phage, and Culture Conditions. Male strains, CP78 F$^+$ (*rel*$^+$) and CP79 F$^+$ (*rel*), were derived from CP78 and CP79 (Fiil and Friesen, 1968) by S. R. Khan in our laboratory. Both require arginine, histidine, leucine, threonine, and thiamine, and are isogenic except at the *rel* locus. F74 (F$^+$, *rel*$^+$, *met*, *his*) and F8 (F$^+$, *rel*, *met*, *his*, *thy*) were kindly provided by J. D. Friesen, York University, Toronto, Canada. A male specific RNA phage, R17, purified as described previously (Enger *et al.*, 1963), was used throughout.

For bacterial growth, Tris–maleate synthetic medium designated as TMM (Paranchych, 1966) was used throughout. Supplementation consisted of each required amino acid (a final concentration of 50 μg/ml) and thiamine (10 μg/ml). Bacteria were grown at 37° on a gyrotory water-bath shaker. Cell density was measured by absorbance at 500 nm (A_{500}) by means of a Bausch and Lomb spectrophotometer.

Chemicals. Rifampicin was purchased from Calbiochem;

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