Chapter II

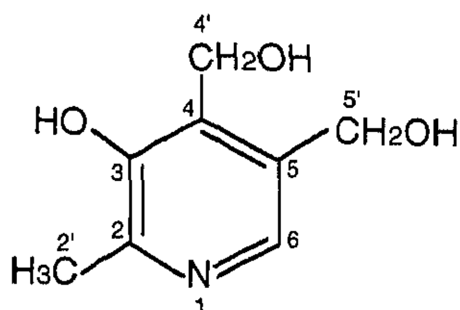
Biosynthetic study on vitamin B_6 of *Rhizobium*

II.1. Tracer studies on the biosynthesis of vitamin B_6

Introduction

During our screening search for vitamin B_6 overproducers, I found that *Rhizobium meliloti* IFO 14782 produced great amounts of vitamin B_6, 78 mg of pyridoxol per liter, as described in Chapter I. But its productivity is still too low to compete with the current chemical process, used industrially. Thus, I need to understand both its biosynthetic pathway and its regulation system to construct an economically competitive pyridoxol producer.

Extensive studies have been carried out on the biosynthetic pathway of vitamin B_6 in *Escherichia coli*. Spenser and his colleagues proposed that pyridoxol is synthesized from two compounds, 1-deoxy-D-xylulose and 4-hydroxy-L-threonine, which serve as the C_5 unit, C-2′,2,3,4, and 4′ and the C_3N unit, N-1, C-6,5, and 5′, respectively, of pyridoxol (Structure 1).[18,20]



Structure 1. Pyridoxol

1-Deoxy-D-xylulose (or its 5-phosphate) has been identified as a biosynthetic precursor of isopentenyl diphosphate in a non-mevalonate pathway,[34] the thiazole moiety of thiamin diphosphate,[35] and pyridoxol in *E. coli*. Its formation from pyruvate and D-glyceraldehyde (or its 3-phosphate) has recently been demonstrated by an enzyme system using 1-deoxy-D-xylulose-5-phosphate synthase.[36] For the formation of 4-hydroxy-L-threonine in *E. coli*, two biosynthetic routes have been proposed (Fig. 2). One is from D-erythrose 4-phosphate by a four-step reaction,[14] and the other is from glycine and glycolaldehyde.[37-40] When the former pathway was blocked in *E. coli*, however, the microorganism became vitamin B_6 deficient. This finding suggested that only the former pathway is active in *E. coli*.

In this Chapter, I elucidated the biosynthetic pathway of pyridoxol in *R. meliloti* by tracer studies with regard to the following points:

1) Formation of pyridoxol from 1-deoxy-D-xylulose and 4-hydroxy-L-threonine;
2) Formation of 1-deoxy-D-xylulose from pyruvate and D-glyceraldehyde;
3) Formation of 4-hydroxy-L-threonine from glycine and glycolaldehyde.

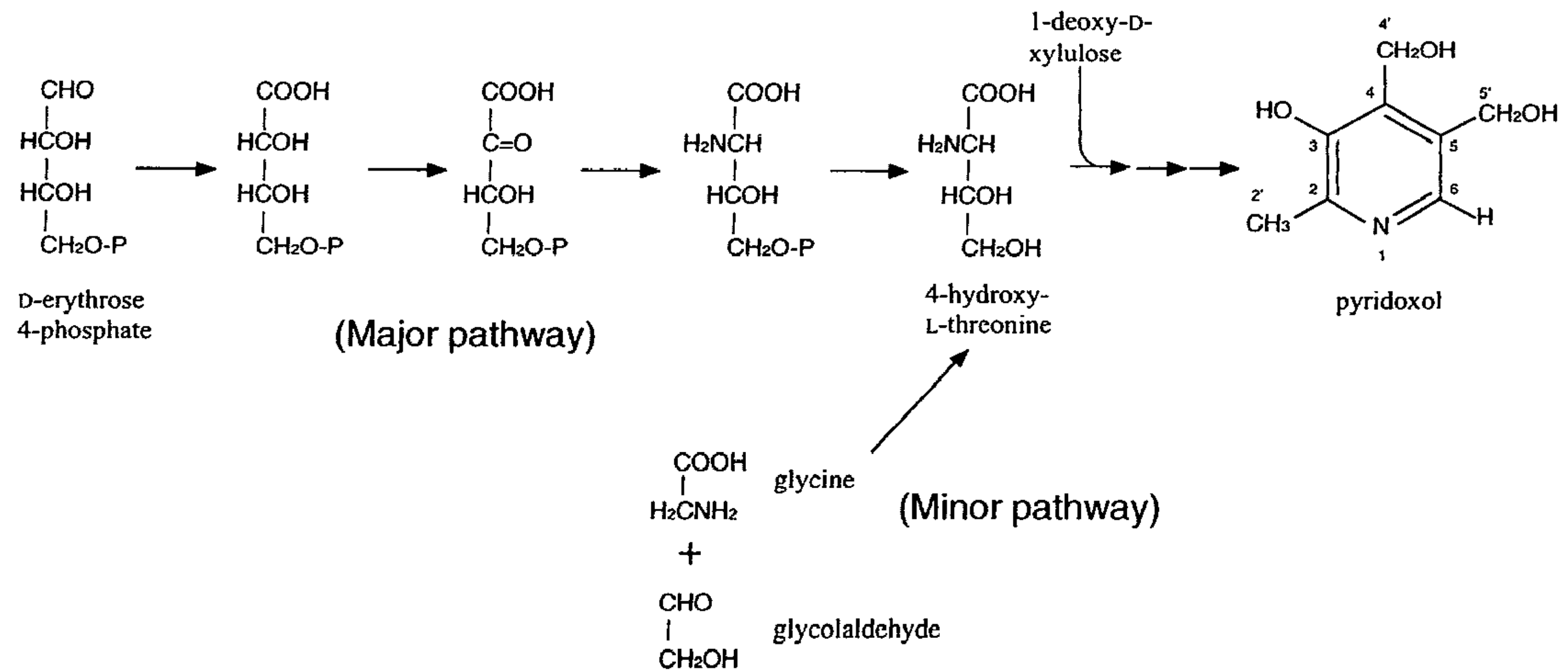


Fig. 2. Two Synthetic Sequences of 4-Hydroxy-L-threonine in *Escherichia coli*.

Materials and Methods

Microorganisms. The organism used in this study were *R. meliloti* IFO 14782, *E. coli* IFO 13168, *Pseudomonas andropogonis* ICMP 2809, and *S. carlsbergensis* ATCC 9080 for quantitative determination of vitamin B_6.

Chemicals. The following labeled compounds were purchased from Isotec Inc.: [2,3-$^{13}C_2$]pyruvate (99% ^{13}C per C atom), [1,2,3-$^{13}C_3$]pyruvate (99% ^{13}C per C atom), [1-^{15}N,2-^{13}C]glycine (99% ^{15}N and ^{13}C per N and C atoms), [1-^{15}N]glycine (99% ^{15}N per N atom), and [^{15}N]NH_4Cl (99% ^{15}N per N atom). 4-(Dimethylamino)azobenzene-4′-sulfonyl chloride (DABS) was used for determination of amino acids.

NMR and negative fast atom bombardment (FAB)-MS experiments. ^{13}C and ^{1}H NMR experiments were performed with a Jeol JNM GSX-400 spectrometer, and all compounds were dissolved in D_2O. Chemical shifts are shown with external reference to 3-(trimethylsilyl)-propionic acid-d_4 sodium salt (0 ppm). FAB-MS experiments were carried out with a Jeol SX-102/102 mass spectrometer equipped with a Hewlett Packard Apollo Series 400 data system. *m*-Nitrobenzyl

alcohol was used as the liquid matrix for the FAB-MS experiments.

Preparation of 1-deoxy-D-xylulose and 4-hydroxy-L-threonine, and their labeled compounds.

1-Deoxy-D-xylulose was prepared by an enzymatic reaction using the cell-free extract of *E. coli* IFO 13168 by the procedure reported by Yokota and Sasajima.[41] 1-Deoxy-D-xylulose thus obtained had the following physicochemical properties: $[\alpha]_D$ +23.2° (c = 1.0, H_2O) (literature $[\alpha]_D$ of 1-deoxy-D-xylulose, $[\alpha]_D$ +26.2° (c = 1, H_2O)). The ^{13}C NMR spectrum of 1-deoxy-D-xylulose was observed as a mixture of three isomers: an open form and two closed hemiketal α- and β-furanose forms. The NMR spectrum was: ^{13}C NMR (100 MHz, D_2O). Open form δ values were 28.5, 65.0, 74.2, 80.0, and 215.8. α- and β-Furanose forms gave many low signals. 1-Deoxy-D-xylulose has been reported to give signals derived from three forms in the ^{13}C NMR spectrum.[42] $[1,2\text{-}^{13}C_2]$1-Deoxy-D-xylulose was also prepared in the same way as mentioned above by using $[2,3\text{-}^{13}C_2]$pyruvate and D-glyceraldehyde as substrates. The NMR spectrum of the compound was: ^{13}C NMR (100 MHz, D_2O). Open form δ values were 28.5 (d, J=41.2 Hz, C-1), 215.8 (d, J=41.2 Hz, C-2). Two closed hemiketal α- and

β-furanose forms gave four low signals attributed to C-1 and -2.

4-Hydroxy-L-threonine was isolated from a culture broth of *P. andropogonis* ICMP 2809 according to the procedure of Mitchell *et al.*[43] ^1H NMR (400 Hz, D_2O) δ values were 3.57 (1H, dd, *J*=5.5 Hz), 3.58 (1H, dd, *J*=4.5 Hz), 3.64 (1H, d, *J*=3.5 Hz), 4.02 (1H, mult.). FAB-MS (Negative) was 134 $(M-H)^-$. [^{15}N]4-Hydroxy-L-threonine was also prepared by culturing *P. andropogonis* ICMP 2809 in chemically defined medium in which ^{15}N-labeled ammonium chloride was used. FAB-MS (Negative) was 135 $(M-H)^-$.

Assay for vitamin B_6 and bio-autographic detection of the vitamin. The amount of vitamin B_6 was quantified by the turbidity method with *S. carlsbergensis* ATCC 9080.[31] Pyridoxol formation was detectable by bioautogram of thin layer chromatography (TLC) plate (Silica gel 60, Merck, $CHCl_3$/MeOH = 3/1) using the microorganism as an indicator strain.

Medium and cultivation. *R. meliloti* IFO 14782 was cultured in a medium consisting of 4% glucose, 2% Polypepton, 0.2% yeast extract, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% $MnSO_4 \cdot 5H_2O$, and 0.001% $FeSO_4 \cdot 7H_2O$ (pH 6.8) at 28°C.

Formation of vitamin B_6. Cells of *R. meliloti* IFO 14782 were harvested from 3-day culture broth by centrifugation, washed twice with sterile 0.85% saline, and suspended in a small amount of sterile water. In a tube, 10 ml of the following mixture [2 mg each of 1-deoxy-D-xylulose and 4-hydroxy-L-threonine, 0.425% NaCl, and washed cells (final OD_{600}=20)] was prepared and incubated on a reciprocal shaker (285 rpm) at 28°C. After shaking for 24 h, the reaction mixture was centrifuged at 10,000 × *g* for 10 min, and then vitamin B_6 in the supernatant was assayed with *S. carlsbergensis* ATCC 9080.

To isolate vitamin B_6 synthesized from labeled substrates, 20 tubes (total volume: 200 ml) containing the reaction mixture with 2 mg each of [1,2-$^{13}C_2$]1-deoxy-D-xylulose and [^{15}N]4-hydroxy-L-threonine, 0.425% NaCl, and washed cells were shaken on a reciprocal shaker at 28°C for 24 h. The reaction mixture containing vitamin B_6 of 10.2 μg/ml was centrifuged, and the vitamin produced was purified from the supernatant through column chromatography with Amberlite CG-120 (H^+) (Rohm and Haas Company, Philadelphia, PA., USA) cation exchange resin (1.2 cm in diameter and 15 cm in length) developed by 5% ammonium solution. Fractions containing a vitamin B_6 peak were pooled, concentrated under reduced

pressure, and subjected to ^{13}C NMR structural analysis. ^{13}C NMR (100 MHz, D_2O) δ: 18.0 (d, J_1=48.9 Hz), 146.6 (dd, J_1=48.9 Hz, J_2=12.2 Hz).

Formation of 1-deoxy-D-xylulose. Cells of *R. meliloti* IFO 14782 were harvested from 200 ml of 3-day culture broth by centrifugation. The pellet was washed first with 100 ml of saline and subsequently with 100 ml of 10 mM Tris-HCl buffer, pH 7.5, containing 5 mM $MgCl_2$, 1 mM thiamin pyrophosphate, 2 mM EDTA, 0.1 mM phenylmethane sulfonyl fluoride, and 5 mM 2-mercaptoethanol, and finally suspended in 20 ml of the same buffer (final OD_{600}=150). The cells were disrupted with a French press homogenizer. After centrifugation at 13,000 × *g* for 60 min, the supernatant was used as the cell-free extract for the following enzyme reaction. The reaction mixture containing 100 mM sodium pyruvate, 100 mM D-glyceraldehyde, 80 mM Tris-HCl buffer, pH 8.0, 5 mM $MgCl_2$, 0.5 mM thiamin pyrophosphate, 2 mM EDTA, and the cell-free extract (5.6 mg protein) in a total volume of 1ml was incubated at 37°C. After incubation for 1, 2, 3, or 4 h, the reaction mixture was heated in a boiling bath for 3 min and then centrifuged at 10,000 × *g* for 10 min. One microliter of the supernatant was loaded onto a TLC plate of Silica gel 60 (Merck, ethyl acetate/pyridine/H_2O =

90/5/3) and developed. The 1-deoxy-D-xylulose formed was detected by staining the plate with alkaline-tetrazolium chloride.

To isolate the 1-deoxy-D-xylulose synthesized from labeled substrate, the enzyme reaction was carried out by incubating a tube containing the following reaction mixture (7.5 ml) at 37°C: 100 mM [2,3-$^{13}C_2$]pyruvate, 100 mM D-glyceraldehyde, 80 mM Tris-HCl buffer, pH 8.0, 5 mM $MgCl_2$, 0.5 mM thiamin pyrophosphate, 2 mM EDTA, and the cell-free extract of *R. meliloti* IFO 14782 (42 mg protein). After incubation for 4 h, a five-times volume of methanol was added to the mixture, and then it was centrifuged at 10,000 × *g* for 10 min. The supernatant was concentrated under reduced pressure, dissolved in a small amount of methanol, and then chromatographed on a column (2.3 cm in diameter and 25 cm in length) of Silica gel 60 with ethyl acetate/pyridine/H_2O = 90/5/3. 1-Deoxy-D-xylulose was followed by TLC on silica gel 60 plates, and the fractions containing 1-deoxy-D-xylulose were collected and concentrated under reduced pressure. The residue was dissolved in a small amount of water and further purified by HPLC under analytical conditions as follows: column, Daisopack SP-120-5-ODS-BP (Daiso Co., Osaka, Japan); mobile phase, H_2O; flow rate, 0.5 ml/min; detector, RI. Fractions containing 1-deoxy-D-xylulose were

collected and concentrated under reduced pressure. Physicochemical properties of the obtained 1-deoxy-D-xylulose were as follows. $[\alpha]_D$ +26.5° (c = 1, H_2O), ^{13}C NMR (100 MHz, D_2O): open form δ; 28.5 (d, J=41.2 Hz), 215.8 (d, J=41.2 Hz) and two closed hemiketal α- and β-furanose forms; four low signals.

Formation of 4-hydroxy-L-threonine. Cells of *R. meliloti* IFO 14782 were prepared by the same procedure as described under "*Formation of vitamin B_6.*" Formation of 4-hydroxy-L-threonine was carried out by shaking a tube containing 10 ml of the following mixture: 40 mM glycolaldehyde, 32 mM glycine, 100 mM Tris-HCl buffer, pH 7.6, and washed cells (final OD_{600}=20) on a reciprocal shaker (285 rpm) at 28°C for 24 h. The reaction mixture was centrifuged at 10,000 × *g*, adjusted to pH 8.5 with 5% ammonium solution, and passed through an anion exchange column (Dowex 1X4) to remove Tris in the buffer. 4-Hydroxy-L-threonine was eluted with 0.2 N HCl solution, and the effluent was concentrated under reduced pressure and derivatized with DABS.[44] The reaction mixture was analyzed by reversed-phase TLC on a silica C_8 plate (Tokyo Kasei Kogyo Co., Japan, acetonitrile/H_2O/acetic acid = 5/5/0.4). The DABS derivative of the formed

4-hydroxy-L-threonine was observed as an orange spot having an R_F value of 0.42. Alternatively, the spot was extracted from the TLC plate by using $CHCl_3$/MeOH (3:1) and analyzed by mass spectrometry. FAB-MS (Negative) was 421$(M-H)^-$. Isolation of 4-hydroxy-L-threonine from labeled substrate was done as follows. Ten tubes each containing 10 ml of the following mixture: 40 mM glycolaldehyde, 32 mM [1-^{15}N, 2-^{13}C]glycine, 100 mM Tris-HCl buffer, pH 7.6, and the cells of *R. meliloti* IFO 14782, were shaken on a reciprocal shaker at 28°C for 24 h. The total reaction mixture (100 ml) was passed through a Dowex 1X4 column by the same method as described in the previous paragraph and derivatized with DABS. The DABS derivative of 4-hydroxy-L-threonine was purified by reversed-phase TLC with C_8 silica plates. A band having the same R_F value as 4-hydroxy-L-threonine was extracted by using a solvent mixture of $CHCl_3$/MeOH (3:1) and then subjected to mass spectrometric analysis. FAB-MS (Negative) was 423 $(M-H)^-$.

Formation of vitamin B_6 from labeled [1,2-$^{13}C_2$]1-deoxy-D-xylulose, [1-^{15}N]glycine, and glycolaldehyde.

To isolate the vitamin B_6 formed from 1-deoxy-D-xylulose, glycine, and glycolaldehyde, an intact cell reaction was done as described below. Ten tubes each containing 10 ml of the

mixture of 1.5 mM [1,2-$^{13}C_2$]1-deoxy-D-xylulose, 32 mM [1-^{15}N]glycine, 40 mM glycolaldehyde, 100 mM Tris-HCl buffer, pH 8.0, and the cells of *R. meliloti* IFO 14782 (final OD_{600}=20) were shaken on a reciprocal shaker at 28°C for 24 h. The reaction mixture (100 ml) containing vitamin B_6 of 8.6 μg/ml was centrifuged, and the produced vitamin was purified from the supernatant by chromatography through an Amberlite CG-120 (H^+) column (1.2 cm in diameter and 15 cm in length) developed by 5% ammonium solution. Fractions containing a vitamin B_6 peak were collected, concentrated under reduced pressure, and subjected to ^{13}C NMR structural analysis: ^{13}C NMR (100 MHz, D_2O) δ: 18.0 (d, J_1=48.0 Hz), 146.6 (dd, J_1=48.0 Hz, J_2=12.5 Hz).

Results and Discussion

Pyridoxol formation from 1-deoxy-D-xylulose and 4-hydroxy-L-threonine Vitamin B_6 formation from 1-deoxy-D-xylulose and 4-hydroxy-L-threonine as substrates was examined by an intact cell system of *R. meliloti* IFO 14782. As shown in Table 7, vitamin B_6 was formed only when both of the substrates were present.

Table 7. Formation of Vitamin B_6 from 1-Deoxy-D-xylulose and 4-Hydroxy-L-threonine by Intact Cells of *R meliloti* IFO 14782

Substrate	Found vitamin B_6 (μg/ml)
None	0
1-DX	0
HT	0
1-DX + HT	9.8

The abbreviations used are: 1-DX, 1-deoxy-D-xylulose; HT, 4-hydroxy-L-threonine.

The vitamin B_6 formed in the reaction mixture was analyzed by TLC of silica gel 60, and identified as pyridoxol. Further, an incorporation test of the stable-labeled substrates, [1,2-$^{13}C_2$]1-deoxy-D-xylulose and [^{15}N]4-hydroxy-L-threonine, into a pyridoxol molecule was done and analyzed by ^{13}C NMR spectrometry. The ^{13}C NMR spectrum of authentic pyridoxol showed eight signals, 155.5, 145.5, 143.3, 139.5, 132.5, 60.9, 59.7, and 17.2 ppm which were assignable to C-3,-2,-4,-5,-6,-4′,-5′, and -2′ of the skeleton, respectively (Fig. 3*A*). On the other hand, the ^{13}C NMR spectrum of the ^{13}C- and ^{15}N-isotopically enriched pyridoxol showed only two signals, 146.6 and 18.0 ppm, which were assigned to be C-2 and -2′ of the skeleton, respectively (Fig. 3*B*). The former signal appeared as a double doublet (J_1=48.9 Hz, J_2=12.2 Hz), due to ^{13}C,^{15}N enrichment in contiguous two carbon and one nitrogen atoms, C2′–C2–N1 of pyridoxol skeleton (Fig. 3*B-1*) and the latter as a doublet (J_1=48.9 Hz), due to ^{13}C enrichment in adjacent two carbon atoms, C2′–C2 of the skeleton (Fig. 3*B-2*). This result indicates that the double-labeled carbon bond, $^{13}C1$–$^{13}C2$ of 1-deoxy-D-xylulose and ^{15}N of 4-hydroxy-L-threonine were incorporated into the C2′–C2–N1 bond of the pyridoxol skeleton. This suggests that the C_5 unit of 1-deoxy-D-xylulose and the NC_3 unit of 4-hydroxy-L-threonine enter the C_5 (C-2′,-2,-3,-4, and -4′)

and NC_3 (N-1, C-6, C-5, and C-5′) units, respectively, of the pyridoxol skeleton.

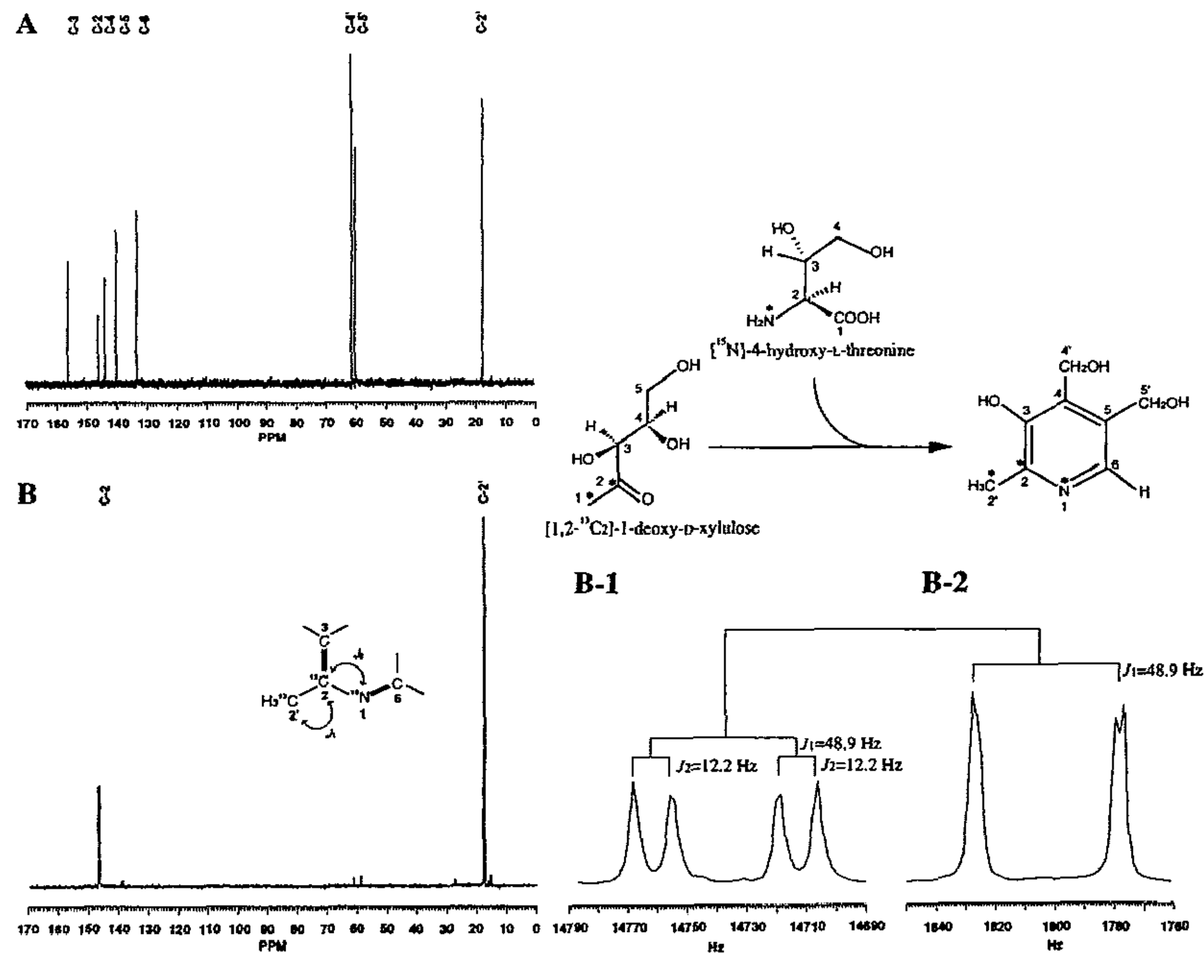


Fig. 3. ^{13}C NMR Spectra of Unlabeled Pyridoxol Hydrochloride (*A*), and Pyridoxol Hydrochloride Isolated from the Intact Cell Reaction with [1,2-$^{13}C_2$]1-Deoxy-D-xylulose and [^{15}N]4-Hydroxy-L-threonine (*B*), and the Expanded Spectra of 14690–14790 Hz (146.1–147.1 ppm) (*B-1*), and 1760–1850 Hz (17.5–18.4 ppm) (*B-2*), Spectral Regions.

Formation of 1-deoxy-D-xylulose Formation of 1-deoxy-D-xylulose was examined for the enzyme system of *R. meliloti* IFO 14782 by using pyruvate and D-glyceraldehyde as substrates. The time course of the reaction was analyzed by TLC on a silica gel 60 plate, and a purple spot having the same R_F value as 1-deoxy-D-xylulose was observed in 1, 2, 3, or 4 h of incubation by staining with alkaline-tetrazolium chloride (Fig. 4).

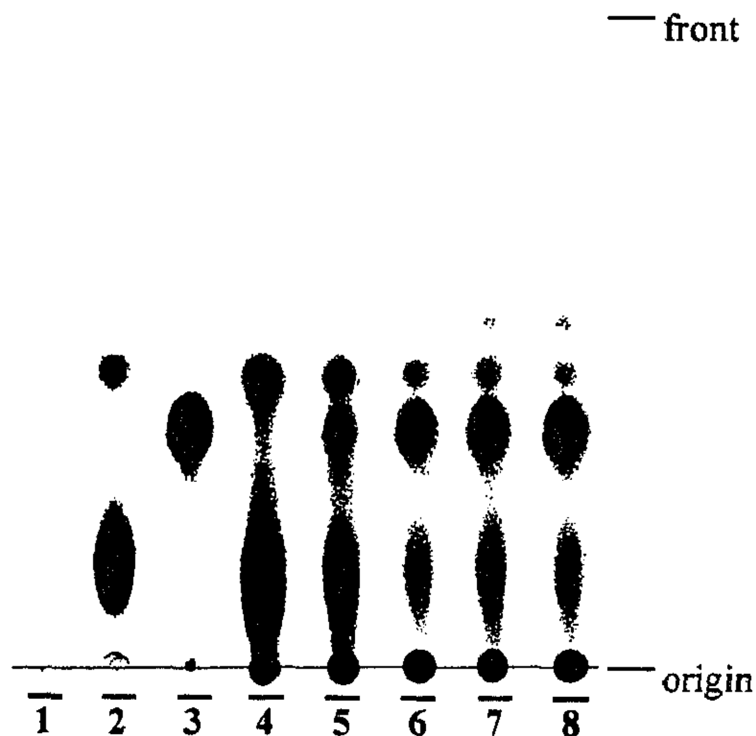


Fig. 4. Thin Layer Chromatograms of the Reaction Mixture Obtained from Pyruvate and D-Glyceraldehyde.

Lanes 1 and 2, pyruvate and D-glyceraldehyde; lane 3, authentic 1-deoxy-D-xylulose; *lanes* 4–8, reaction mixtures after incubation for 0,1,2,3, and 4 h, respectively. Commercial D-glyceraldehyde contained an unidentified impurity (the upper position of lane 2), but the material was used without further purification.

Further, incorporation of stable-labeled pyruvate into 1-deoxy-D-xylulose was elucidated by ^{13}C NMR spectroscopy. The bulk production of 1-deoxy-D-xylulose was carried out by using [2,3-$^{13}C_2$]pyruvate and D-glyceraldehyde as substrates. After the reaction, 1-deoxy-D-xylulose was purified by column chromatography in Silica gel 60, followed by HPLC, and then it was subjected to ^{13}C NMR structural analysis. The ^{13}C NMR spectrum of authentic 1-deoxy-D-xylulose showed five signals of the open form, 215.8, 80.0, 74.2, 65.0, and 28.5 ppm, which were assignable to C-2,-3,-4,-5, and -1, respectively, of the skeleton (Fig. 5*A*). On the other hand, the ^{13}C NMR spectrum of the ^{13}C-isotopically enriched 1-deoxy-D-xylulose showed only two signals of the open form, 215.8 and 28.5 ppm, which were attributed to C-2 and -1, respectively, of the 1-deoxy-D-xylulose skeleton (Fig. 5*B*). In expanded spectra of 215.4–216.5 and 28–30 ppm spectral regions (Fig. 5, *B-1* and *2*), two signals were observed as doublet peaks having the same coupling constant (J=41.2 Hz) due to ^{13}C-enrichment in the contiguous carbon atoms, C1–C2, of the 1-deoxy-D-xylulose skeleton.

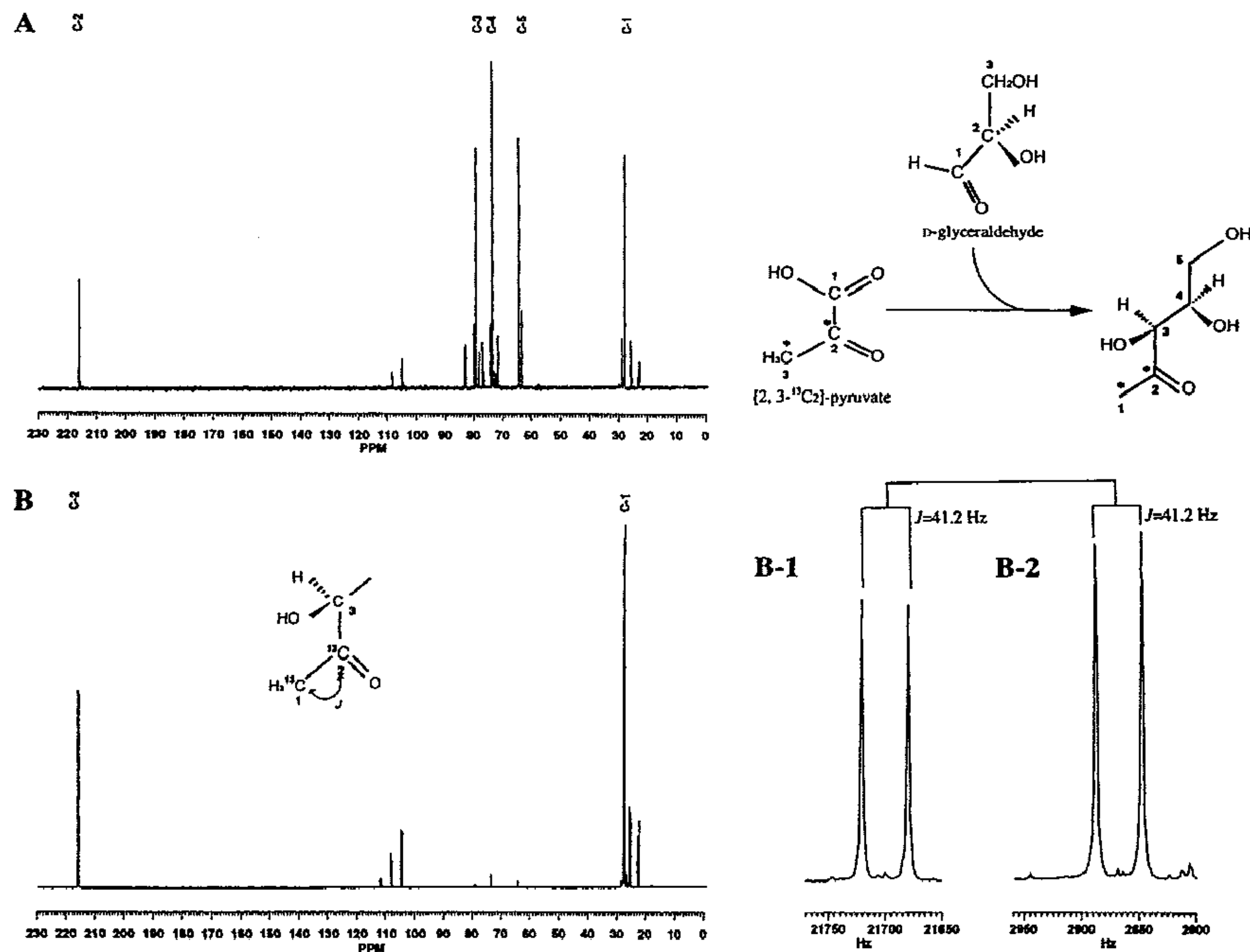


Fig. 5. ^{13}C NMR Spectra of Unlabeled 1-Deoxy-D-xylulose (*A*) and 1-Deoxy-D-xylulose Isolated from the Enzyme Reaction with [2,3-$^{13}C_2$]Pyruvate and D-Glyceraldehyde (*B*), and the Expanded Spectra of 21650–21760 Hz (215.4–216.5 ppm) (*B-1*) and 2810–3015 Hz (28.0–29.5 ppm) (*B-2*) Spectral Regions.

This result indicates that the double-labeled carbon bond, $^{13}C2-^{13}C3$, of pyruvate was incorporated into the C-1 and -2 carbons of the 1-deoxy-D-xylulose skeleton. Furthermore, 1-deoxy-D-xylulose isolated from the reaction mixture using [1,2,3-$^{13}C_3$]pyruvate showed a similar spectrum of ^{13}C NMR to that isolated from the reaction mixture using [2,3-$^{13}C_3$]pyruvate (the spectrum not shown). This indicates that C-2 and -3 of pyruvate were incorporated into C-1 and -2 of the 1-deoxy-D-xylulose skeleton without the incorporation of C-1 of pyruvate. These results suggest that the C_2 (C-1 and -2) and C_3 (C-3,-4, and -5) units of the 1-deoxy-D-xylulose skeleton are derived from C-3 and -2 of pyruvate and C-1,-2, and -3 of D-glyceraldehyde, respectively. This result is consistent with the proposal of Yokota and Sasajima[45] that 1-deoxy-D-xylulose is formed from pyruvate and D-glyceraldehyde through decarboxylation of pyruvate.

Formation of 4-hydroxy-L-threonine Formation of 4-hydroxy-L-threonine was studied in the intact cell system of *R. meliloti* IFO 14782 with glycolaldehyde and glycine as substrates in Tris-HCl buffer. The reaction product was identified by comparing it with a DABS derivative of authentic 4-hydroxy-L-threonine. With this derivatization, 4-hydroxy-L-threonine can be separated from the glycine used

as one of the substrates. The reaction mixture was analyzed by reversed-phase TLC on a C_8 silica plate, and an orange spot having the same R_F value as 4-hydroxy-L-threonine was observed on the plate as shown in Fig. 6.

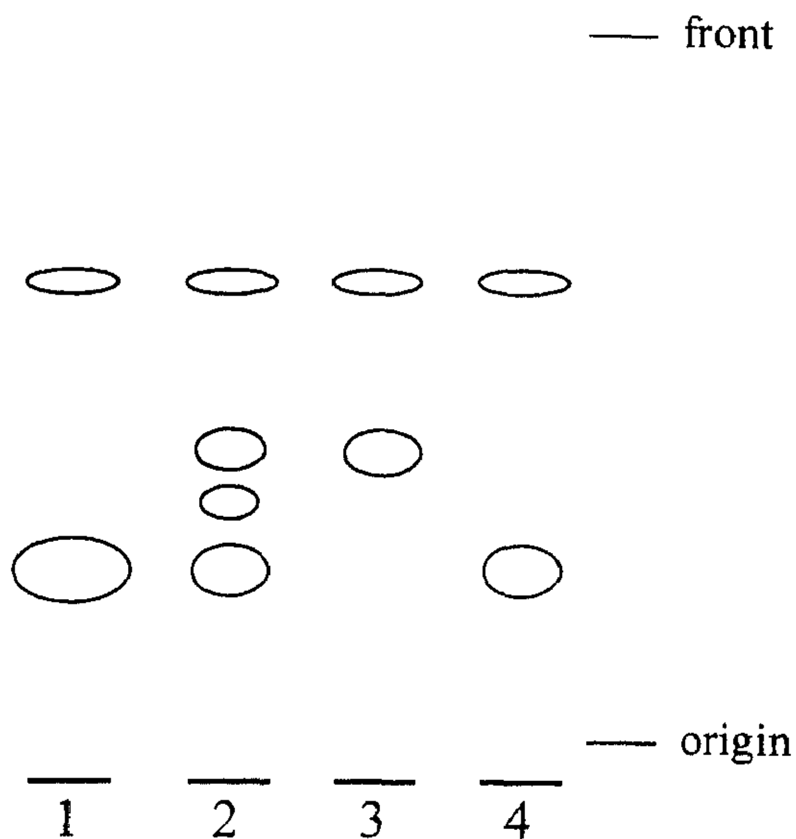


Fig. 6. Thin Layer Chromatograms of the Reaction Mixture Obtained from Glycolaldehyde and Glycine.

Lane 1, before reaction; *lane* 2, reaction mixtures after incubation for 24 h; *lane* 3, authentic 4-hydroxy-L-threonine; lane 4, authentic glycine.

Further, incorporation of labeled glycine into 4-hydroxy-L-threonine was elucidated by mass spectrometry. Then, the formation of 4-hydroxy-L-threonine in the intact cell system was carried out by using [1-^{15}N, 2-^{13}C]glycine instead of glycine as the substrate. After incubation for 24 h, the reaction mixture was passed through a Dowex 1X4 column, derivatized with DABS, and purified by reversed-phase TLC on C_8 silica plates. The mass spectrum of the DABS derivative of 4-hydroxy-L-threonine enriched with labeled glycine and glycolaldehyde had a 423 *m/z* corresponding to the molecular ion minus 1 (Fig. 7*B*, the lower spectrum), whereas the DABS derivative of 4-hydroxy-L-threonine had a 421 *m/z* corresponding to the molecular ion minus 1 (Fig. 7*A*, the upper spectrum).

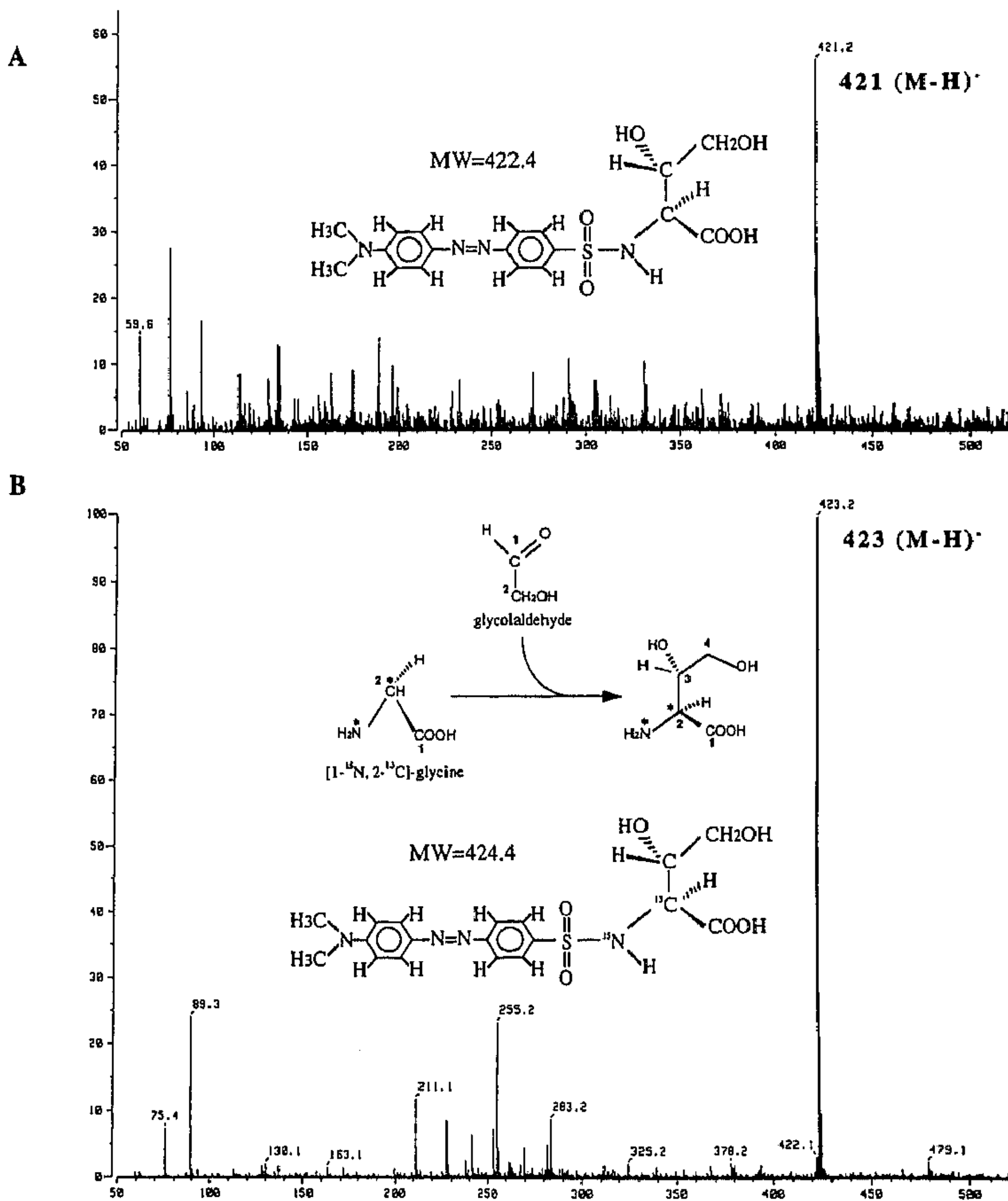


Fig. 7. FAB-MS (negative) Spectra of Unlabeled 4-Hydroxy-L-threonine (*A*), and 4-Hydroxy-L-threonine Isolated from the Intact Cell Reaction with [1-^{15}N,2-^{13}C]Glycine and Glycolaldehyde (*B*).

These results indicate that a nitrogen and a carbon label of [1-^{15}N, 2-^{13}C]glycine were incorporated into the 4-hydroxy-L-threonine molecule, and that the 4-hydroxy-L-threonine might be constructed from the NC unit of glycine and the C_2 unit of glycolaldehyde.

Recently, Lam and Winkler[14] have proposed that 4-hydroxy-L-threonine would be formed from D-erythrose 4-phosphate by four stepwise reactions as the major pathway in *E. coli*, and that activity of the first enzyme, D-erythrose-4-phosphate dehydrogenase, on the pathway was found in the cell-free extract of an *E. coli* strain (Fig. 8).

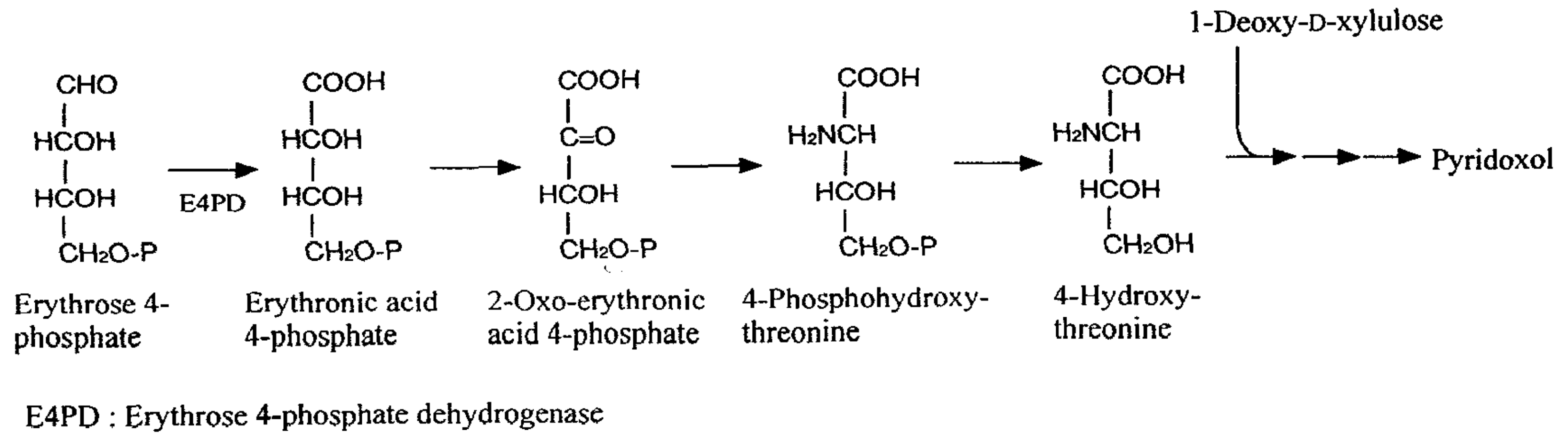


Fig. 8. Hypothetical Synthetic Sequence of 4-Hydroxy-L-threonine in *E. coli* by Lam and Winkler.[14)]

Nevertheless, when I attempted to determine the presence of this enzyme activity in the cell-free extract of *R. meliloti* IFO 14782 according to the method they reported, I was unable to detect this enzyme activity (data not shown). In other experiments, I have isolated a vitamin B_6-requiring mutant derived from *R. meliloti* IFO 14782 that is defective in formation of 4-hydroxy-L-threonine from glycine and glycolaldehyde (data not shown). These results support that *R. meliloti* IFO 14782 synthesizes 4-hydroxy-L-threonine from glycine and glycolaldehyde, but not from D-erythrose 4-phosphate. Accordingly, I conclude that the biosynthetic pathway of 4-hydroxy-L-threonine in *R. meliloti* is different from that in *E. coli*.

Incorporation of glycine into the pyridoxol molecule

Incorporation of glycine into the pyridoxol molecule was studied in an intact cell system of *R. meliloti* IFO 14782 with [1,2-$^{13}C_2$]1-deoxy-D-xylulose, [1-^{15}N]glycine, and glycolaldehyde as substrates. Formed pyridoxol was purified from the supernatant of the reaction mixture by cation exchange column chromatography and then analyzed with the ^{13}C NMR spectrometer. The spectrum was similar to that of pyridoxol formed from [1,2-$^{13}C_2$]1-deoxy-D-xylulose and [^{15}N]4-hydroxy-L-threonine. The result indicates that the

labeled nitrogen of glycine enters the N-1 position of pyridoxol, and that the NC (N-1 and C-6) unit of pyridoxol would be derived from the NC unit of glycine.

In conclusion, the biosynthetic pathway of vitamin B_6 in *R. meliloti* is summarized as follows. Pyridoxol is synthesized from 1-deoxy-D-xylulose and 4-hydroxy-L-threonine; the former is from pyruvate and D-glyceraldehyde through decarboxylation of pyruvate, and the latter is from glycolaldehyde and glycine (Fig. 9).

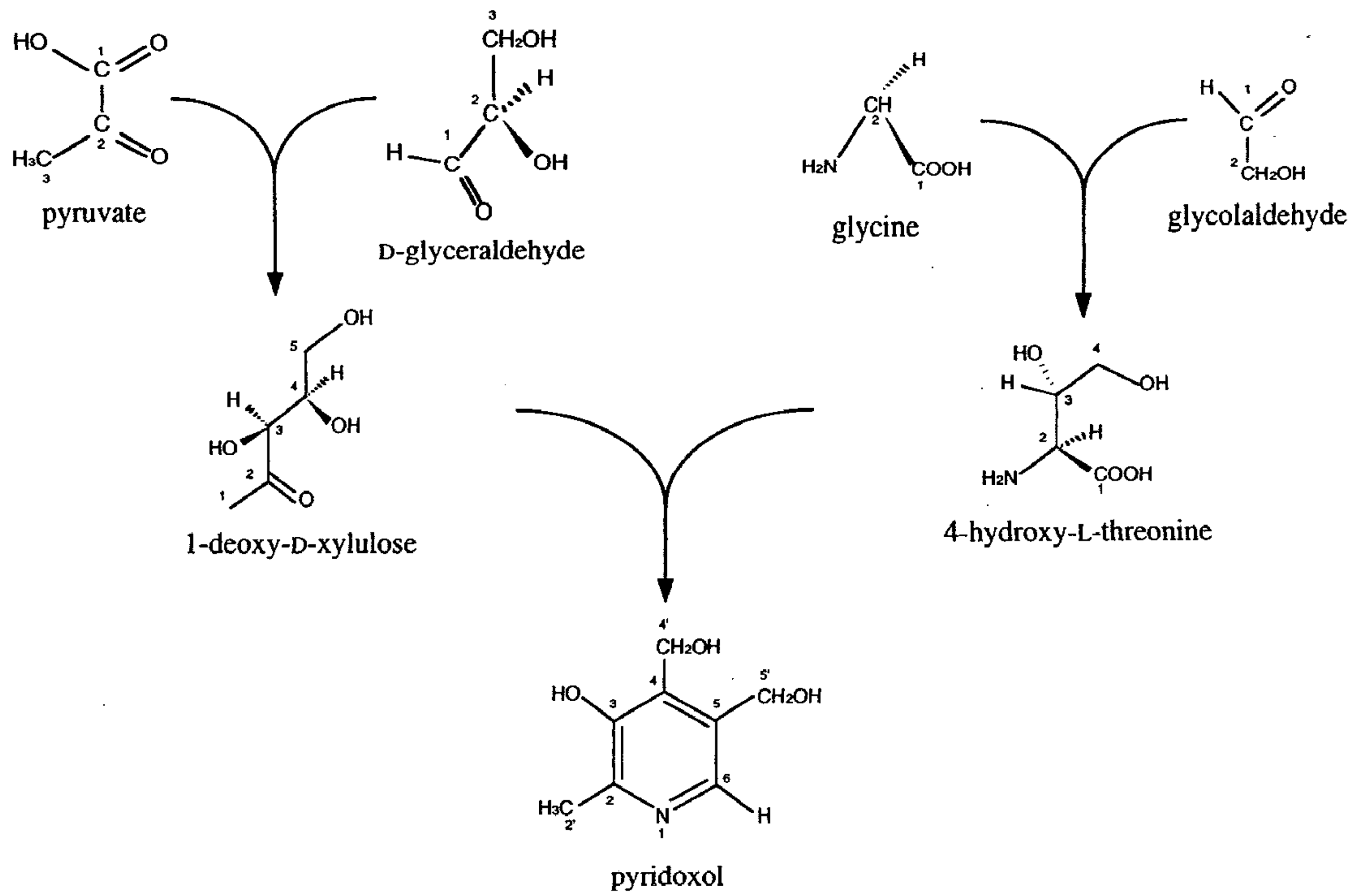


Fig. 9. Biosynthesis of Pyridoxol in *Rhizobium meliloti*.