Chapter II

Biosynthetic study on vitamin B₆ of *Rhizobium*

II.1. Tracer studies on the biosynthesis of vitamin B₆

Introduction

During our screening search for vitamin B₆ overproducers, I found that *Rhizobium meliloti* IFO 14782 produced great amounts of vitamin B₆, 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 B₆ 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 C₅ unit, C-2',2,3,4, and 4' and the C₃N unit, N-1, C-6,5, and 5', respectively, of pyridoxol (Structure 1).¹⁸,²⁰
1-Deoxy-D-xylulose (or its 5-phosphate) has been identified as a biosynthetic precursor of isopentenyl diphosphate in a non-mevalonate pathway,\textsuperscript{34} the thiazole moiety of thiamin diphosphate,\textsuperscript{35} and pyridoxol in \textit{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.\textsuperscript{36} For the formation of 4-hydroxy-L-threonine in \textit{E. coli}, two biosynthetic routes have been proposed (Fig. 2). One is from D-erythrose 4-phosphate by a four-step reaction,\textsuperscript{14} and the other is from glycine and glycolaldehyde.\textsuperscript{37-40} When the former pathway was blocked in \textit{E. coli}, however, the microorganism became vitamin B\textsubscript{6} deficient. This finding suggested that only the former pathway is active in \textit{E. coli}. 

\section*{Structure 1. Pyridoxol}
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 B₆.

Chemicals. The following labeled compounds were purchased from Isotec Inc.: [2,3-¹³C₂]pyruvate (99% ¹³C per C atom), [1,2,3-¹³C₃]pyruvate (99% ¹³C per C atom), [1-¹⁵N,2-¹³C]glycine (99% ¹⁵N and ¹³C per N and C atoms), [1-¹⁵N]glycine (99% ¹⁵N per N atom), and [¹⁵N]NH₄Cl (99% ¹⁵N 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. ¹³C and ¹H NMR experiments were performed with a Jeol JNM GSX-400 spectrometer, and all compounds were dissolved in D₂O. Chemical shifts are shown with external reference to 3-(trimethylsilyl)-propionic acid·d₄ 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: \([\alpha]_D^\circ +23.2^\circ\) (c = 1.0, H\(_2\)O) (literature \([\alpha]_D^\circ\) of 1-deoxy-D-xylulose, \([\alpha]_D^\circ +26.2^\circ\) (c = 1, H\(_2\)O)). 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 \(\alpha^*\) and \(\beta^*\)-furanose forms. The NMR spectrum was: \(^{13}\)C NMR (100 MHz, D\(_2\)O). Open form \(\delta\) values were 28.5, 65.0, 74.2, 80.0, and 215.8. \(\alpha^*\) and \(\beta^*\)-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.\(^{42}\) \([1,2-^{13}\)C\(_2\)]1-Deoxy-D-xylulose was also prepared in the same way as mentioned above by using [2,3-\(^{13}\)C\(_2\)]pyruvate and D-glyceraldehyde as substrates. The NMR spectrum of the compound was: \(^{13}\)C NMR (100 MHz, D\(_2\)O). Open form \(\delta\) values were 28.5 (d, \(J=41.2\) Hz, C-1), 215.8 (d, \(J=41.2\) Hz, C-2). Two closed hemiketal \(\alpha^*\) and
\(\beta\)-furanose forms gave four low signals attributed to C-1 and C-2.

4-Hydroxy-L-threonine was isolated from a culture broth of \textit{P. andropogonis} ICMP 2809 according to the procedure of Mitchell \textit{et al.}\textsuperscript{43)} \(^1\)H NMR (400 Hz, D\textsubscript{2}O) \(\delta\) 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 \textit{P. andropogonis} ICMP 2809 in chemically defined medium in which \(^{15}\)N-labeled ammonium chloride was used. FAB-MS (Negative) was 135 (M·H)\(^-\).

\textit{Assay for vitamin B\textsubscript{6} and bio-autographic detection of the vitamin.}

The amount of vitamin B\textsubscript{6} was quantified by the turbidity method with \textit{S. carlsbergensis} ATCC 9080.\textsuperscript{31) Pyridoxol formation was detectable by bioautogram of thin layer chromatography (TLC) plate (Silica gel 60, Merck, CHCl\textsubscript{3}/MeOH = 3/1) using the microorganism as an indicator strain.}

\textit{Medium and cultivation.} \textit{R. meliloti} IFO 14782 was cultured in a medium consisting of 4\% glucose, 2\% Polypeptone, 0.2\% yeast extract, 0.05\% MgSO\textsubscript{4}·7H\textsubscript{2}O, 0.05\% MnSO\textsubscript{4}·5H\textsubscript{2}O, and 0.001\% FeSO\textsubscript{4}·7H\textsubscript{2}O (pH 6.8) at 28\textdegree 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 $\times$ 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 $\mu$g/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$_2$O) $\delta$: 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 phenylmethylsulfonyl 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 $\times$ 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 $\times$ g for 10 min. One microliter of the supernatant was loaded onto a TLC plate of Silica gel 60 (Merck, ethyl acetate/pyridine/H$_2$O =
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 [2,3-18C2]pyruvate, 100 mM D-glyceraldehyde, 80 mM Tris-HCl buffer, pH 8.0, 5 mM MgCl2, 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 x 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/H2O = 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, H2O; 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-xyllulose were as follows. $[\alpha]_D +26.5^\circ (c = 1, H_2O)$, $^{13}$C NMR (100 MHz, D$_2$O): open form $\delta$: 28.5 (d, $J=41.2$ Hz), 215.8 (d, $J=41.2$ Hz) and two closed hemiketal $\alpha$- and $\beta$-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 $\times g$, 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. The reaction mixture was analyzed by reversed-phase TLC on a silica C$_8$ plate (Tokyo Kasei Kogyo Co., Japan, acetonitrile/H$_2$O/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 \([1,2^{13}C_2]1\text{-deoxy-D-xy}lulose\), 32 mM 
\([1^{15}N]\)glycine, 40 mM glycolaldehyde, 100 mM Tris·HCl buffer, 
pH 8.0, and the cells of \(R. melli\)loti IPO 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 \(\mu\)g/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 \(^{13}\text{C}\) NMR structural 
analysis: \(^{13}\text{C}\) NMR (100 MHz, \(D_2O\)) \(\delta: 18.0 \text{ (d, } J_1=48.0 \text{ Hz)}, 
146.6 \text{ (dd, } J_1=48.0 \text{ Hz, } J_2=12.5 \text{ Hz}).\)
Results and Discussion

Pyridoxol formation from 1-deoxy-D-xylulose and 
4-hydroxy-L-threonine  Vitamin B₆ 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 B₆ was formed only when both 
of the substrates were present.

Table 7. Formation of Vitamin B₆ from 1-Deoxy-D-xylulose 
and 4-Hydroxy-L-threonine by Intact Cells of R meliloti 
IFO 14782

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Found vitamin B₆ (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>1-DX</td>
<td>0</td>
</tr>
<tr>
<td>HT</td>
<td>0</td>
</tr>
<tr>
<td>1-DX + HT</td>
<td>9.8</td>
</tr>
</tbody>
</table>

The abbreviations used are: 1-DX, 1-deoxy-D-xylulose; HT, 
4-hydroxy-L-threonine.
The vitamin B₆ 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, [1,2-¹³C₂]1-deoxy-D-xylulose and [¹⁵N]4-hydroxy-L-threonine, into a pyridoxol molecule was done and analyzed by ¹³C NMR spectrometry. The ¹³C 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. 3A). On the other hand, the ¹³C NMR spectrum of the ¹³C- and ¹⁵N-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. 3B). The former signal appeared as a double doublet (J₁=48.9 Hz, J₂=12.2 Hz), due to ¹³C,¹⁵N enrichment in contiguous two carbon and one nitrogen atoms, C2′-C2-N1 of pyridoxol skeleton (Fig. 3B·1) and the latter as a doublet (J₁=48.9 Hz), due to ¹³C enrichment in adjacent two carbon atoms, C2′-C2 of the skeleton (Fig. 3B·2). This result indicates that the double-labeled carbon bond, ¹³C1-¹³C2 of 1-deoxy-D-xylulose and ¹⁵N of 4-hydroxy-L-threonine were incorporated into the C2′-C2-N1 bond of the pyridoxol skeleton. This suggests that the C₅ unit of 1-deoxy-D-xylulose and the NC₃ unit of 4-hydroxy-L-threonine enter the C₅ (C·2′,·2,·3,·4, and ·4′)
and NC₃ (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  

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. 5A). 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. 5B). 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, $^{13}\text{C}2-^{13}\text{C}3$, 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 $[1,2,3·^{13}\text{C}_3]pyruvate$ showed a similar spectrum of $^{13}\text{C}$ NMR to that isolated from the reaction mixture using $[2,3·^{13}\text{C}_3]pyruvate$ (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\textsuperscript{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 C8 silica plate, and an orange spot having the same Rf 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 [1-15N, 2-13C]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 C8 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. 7B, 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. 7A, 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 [1-15N,2-13C]Glycine and Glycolaldehyde (B).
These results indicate that a nitrogen and a carbon label of \( [1^{\text{15}}N, 2^{\text{13}}C] \) 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).
Erythrose 4-phosphate → Erythronic acid 4-phosphate → 2-Oxo-erythronic acid 4-phosphate → 4-Phosphohydroxythreonine → 4-Hydroxythreonine → 1-Deoxy-D-xylulose → Pyridoxol

E4PD: Erythrose 4-phosphate dehydrogenase

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 \textit{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 \textit{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 \textit{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 \textit{R. meliloti} is different from that in \textit{E. coli}.

\textit{Incorporation of glycine into the pyridoxol molecule}

Incorporation of glycine into the pyridoxol molecule was studied in an intact cell system of \textit{R. meliloti} IFO 14782 with \([1,2^{13}C_2]1\text{-deoxy}\text{-}D\text{-xylulose}, [1^{15}N]\text{glycine}, \text{and glycolaldehyde}\) as substrates. Formed pyridoxol was purified from the supernatant of the reaction mixture by cation exchange column chromatography and then analyzed with the 13C NMR spectrometer. The spectrum was similar to that of pyridoxol formed from \([1,2^{13}C_2]1\text{-deoxy}\text{-}D\text{-xylulose and [}^{15}N\text{]4-hydroxy}\text{-}L\text{-threonine}\). 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 B₆ 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*.