The biosynthetic pathway of pyridoxal (vitamin B₆) in Rhizobium was clarified by studies on the incorporation of ¹⁴C- or ¹⁵N-labeled precursors into pyridoxal or its biosynthetic intermediates. Pyridoxal was formed by ring closure of two compounds, 1-deoxy- D-xylulose and 4-hydroxy- D-threonine, ⁴-aminopyridine, and ⁴-azobenzene-4'-sulfonamide. The former was formed from D-glyceraldehyde and pyruvate through decarboxylation of pyruvate, and the latter from glycine and glycolaldehyde.

Extensive studies have been carried out on the biosynthetic pathway of vitamin B₆ in Escherichia coli. Spenser and colleagues (1, 2) proposed that pyridoxal is synthesized from two compounds, 1-deoxy- D-xylulose and 4-hydroxy- D-threonine, which serve as the C₅ unit, C-2',3',3,4, and 4' and the C₃N unit, N-1, C-6,5, and 5', respectively, of pyridoxal (Structure 1). 1-Deoxy- D-xylulose (or its 5-phosphate) has been identified as a biosynthetic precursor of isopentenyl diphosphate in a nonmevalonate pathway (3), the thiazole moiety of thiamin diphosphate (4), and pyridoxal in the mevalonate pathway (3), the thiazole moiety of thiamin diphosphate (4), and pyridoxal 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 (5). For the formation of 4-hydroxy- D-threonine in E. coli, two biosynthetic routes have been proposed. One is from D-erythrose 4-phosphate by a four-step enzymatic reaction using the cell-free extract of E. coli IFO 13168 by the procedure reported by Yokota and Sasajima (12). 1-Deoxy- D-xylulose thus obtained had the following physicochemical properties: [a]D = +23.2° (c = 1.0, H₂O) (literature [a]D of 1-deoxy- D-xylulose, [a]D = +26.2° (c = 1, H₂O)). The ¹³C NMR spectrum of 1-deoxy- D-xylulose was observed as a mixture of isomers: an open form and two closed hemiketal α- and β-furanose forms. The NMR spectrum was: ¹³C NMR (100 MHz, D₂O). Open form δ values were 28.5 (d, J = 41.2 Hz, C-1) and 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- D-threonine was isolated from a culture broth of P. andropogonis ICMP 2809 according to the procedure of Mitchell et al. (14). ¹H NMR (400 Hz, D₂O) δ values were 3.57 (1H, dd, J = 5.5 Hz, 3.5 Hz), 3.58 (1H, dd, J = 4.5 Hz, 3.6 Hz), 3.64 (1H, d, J = 3.5 Hz), and 4.02 (1H, mult.). FAB-MS (negative) was 134 (M - H)⁻ and gave four low signals attributed to C-1 and 2. 4-Hydroxy- D-threonine was also prepared by culturing P. andropogonis ICMP 2809 in chemically defined medium in which ¹⁵N-labeled ammonium chloride was used. FAB-MS (negative) was 135 (M - H - ²H)⁻.

**Assay for Vitamin B₆ and Bio-autographic Detection of the Vitamin**

The amount of vitamin B₆ was quantified by the turbidity method with S. carlsbergensis ATCC 9080 (15). Pyridoxal formation was detectable by bioautogram of thin layer chromatography (TLC) plate (Silica gel 60, Merck, CHCl₃/MEOH = 3/1) using the microorganism as an indicator strain. Medium and Cultivation—R. meliloti IFO 14782 was cultivated in a medium consisting of 4% glucose, 2% peptone, 0.2% yeast extract, 0.05% MgSO₄·7H₂O, 0.05% MnSO₄·5H₂O, and 0.001% FeSO₄·7H₂O (pH 6.8) at 26°C.

**Formation of Vitamin B₆—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- D-threonine, 0.425% NaCl, and washed cells were shaken on a reciprocal shaker at 28°C for 25–30 min, then vitamin B₆ in the supernatant was assayed with S. carlsbergensis ATCC 9080.

To isolate vitamin B₆ synthesized from labeled substrates, 20 tubes (total volume: 200 ml) containing the reaction mixture with 2 mg each of [1, 2-³⁴C]1-deoxy- D-xylulose and [³¹⁵N]4-hydroxy- D-threonine, 0.425% NaCl, and washed cells were shaken on a reciprocal shaker at 28°C for 24 h. The reaction mixture containing vitamin B₆ of 10.2 µg/ml was centrifuged, and the vitamin produced was purified from the supernatant through column chromatography with Amberlite CG-120 (H⁺)–sodium chloride; FAB, fast atom bombardment; MS, mass spectrometry; HPLC, high pressure liquid chromatography.

**EXPERIMENTAL PROCEDURES**

**Microorganism**—The organism used in this study were R. meliloti IFO 14782, E. coli IFO 13168, Pseudomonas andropogonis ICMP 2809, and Saccharomyces 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), [1,2,3-¹⁵N₂,2,3,4]-glycine (99% ¹⁵N and ¹³C per N and C atoms), [1,2,3-¹⁵N]glycine (99% ¹⁵N per N atom), and [¹⁵N][NH₄]Cl (99% ¹⁵N per N atom). 4-(Dimethylamino)azobenzene-4'-sulfonamide (DABS) was used for determination of amino acids.

**The abbreviations used are:** DABS, 4-(dimethylamino)azobenzene-4'-sulfonamide; FAB, fast atom bombardment; MS, mass spectrometry; HPLC, high pressure liquid chromatography.
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, 52 mM glycine, and 1 mM 2-mercaptoethanol, were added to the reaction mixture (100 ml) was passed through a Dowex I×4 column by the same method as described in the previous paragraph and derivatized with DABS. The DABS derivative of 4-hydroxy-threonine was purified by reverse-phase TLC with C8 silica plates. A band having the same Rf value as 4-hydroxy-threonine was extracted by using a solvent mixture of CHCl3/MeOH (3:1) and then subjected to mass spectrometric analysis. FAB-MS (negative) was 423 (M−H−).

Formation of Vitamin B6 from 1-deoxy-d-xylulose and 4-hydroxy-l-threonine—To isolate the vitamin B6 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 following mixture: 40 mM glycolaldehyde, 52 mM glycine, 40 mM glycine, and 100 mM Tris-HCl buffer (pH 8.0), and the cells of R. meliloti IFO 14782 (final A600 = 20) were shaken on a reciprocal shaker at 28 °C for 24 h. The reaction mixture (100 ml) containing vitamin B6 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 B6 peak were collected, concentrated under reduced pressure, and subjected to 13C NMR structural analysis. 13C NMR (100 MHz, D2O) spectrum of the authentic pyridoxol showed only two signals, 146.6 and 18.0 ppm, which were assignable to C-2 and -2 of the skeleton, respectively (Fig. 1A). 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 assignable to C-2 and -2′ of the skeleton, respectively (Fig. 1B). The former signal appeared as a doublet (J1 = 18.0 Hz, J3 = 12.2 Hz), due to 13C15N enrichment in contiguous two carbon and one nitrogen atoms, C2′-C2-N1 of pyridoxol skeleton (Fig. 1B). The latter as a doublet (J1 = 48.0 Hz, J2 = 18.0 Hz, due to Fig. 1A) was present in the adjacent two carbon atoms, C2′-C2 of the skeleton (Fig. 1B). This result indicates that the double-labeled carbon bond, 13C15N 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 NC5 unit of 4-hydroxy-l-threonine enter the C0′ (C-2′, -2, -3, -4 and -4′) and NC5 (N-1, C-6, C-5, and C-5′) units, respectively, of the pyridoxol skeleton.
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 RF value as 1-deoxy-D-xylulose was observed in 1, 2, 3, or 4 h of incubation by staining with alkaline-tetrazolium chloride (Fig. 2). Further, incorporation of stable-labeled pyruvate into 1-deoxy-D-xylulose was elucidated by 13C NMR spectroscopy. The bulk production of 1-deoxy-D-xylulose was carried out by using [2,3-13C2]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 13C NMR structural analysis. The 13C NMR spectrum of the 13C-isotopically enriched 1-deoxy-D-xylulose showed only two signals of the open form, 215.8 and 28.5 ppm, which were attributable to C-2 and -1, respectively, of the skeleton (Fig. 3B). On the other hand, the 13C NMR spectrum of the 13C-isotopically enriched 1-deoxy-D-xylulose showed only two signals of the open form, 215.8 and 28.5 ppm, which were attributable to C-2 and -1, respectively, of the skeleton (Fig. 3B). In expanded spectra of 215.4–216.5 and 28–30 ppm spectral regions (Fig. 3B-1 and 2), two signals were observed as doublet peaks having the same coupling constant ($J = 41.2$ Hz) due to 13C enrichment in the contiguous carbon atoms, C1-C2, of the 1-deoxy-D-xylulose skeleton. This result indicates that the double-labeled carbon bond, 13C2-13C3, 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 [1,2,3-13C3]pyruvate showed a similar spectrum of 13C NMR to that isolated from the reaction mixture using [2,3-13C2]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-2 and -3 of D-glyceraldehyde, respectively. This result is consistent with the proposal of Yokota and Sasa-jima (17) that 1-deoxy-D-xylulose is formed from pyruvate and D-glyceraldehyde through decarboxylation of pyruvate.
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. 4. 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. 5B, lower spectrum), whereas the DABS derivative of 4-hydroxy-L-threonine had a 421 m/z corresponding to the molecular ion minus 1 (Fig. 5B, upper spectrum). These results indicate that a nitrogen and a carbon label of [1-15N, 2-13C]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 C2 unit of glycolaldehyde.

Recently, Lam and Winkler (6) 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. 6). Nevertheless, when we attempted to determine the presence of this enzyme activity in the cell-free extract of *R. meliloti* IFO 14782 according to the

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**Fig. 3.** 13C NMR spectra of unlabeled 1-deoxy-D-xylulose (A) and 1-deoxy-D-xylulose isolated from the enzyme reaction with [2,3-13C2]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.

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**Fig. 4.** 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.
method they reported, we were unable to detect this enzyme activity (data not shown). In other experiments, we have isolated a vitamin B6-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, we 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 was studied in an intact cell system of *R. meliloti* IFO 14782 with [1,2-13C2]1-deoxy-D-xylulose, [1-15N]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 13C NMR spectrometer. The spectrum was similar to that of pyridoxol formed from [1,2,13C2]1-deoxy-d-xylulose and [15N]4-hydroxy-L-threo-
nine. 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.

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