The Biogenetic Anatomy of Vitamin B₆

A \(^{13}\)C NMR INVESTIGATION OF THE BIOSYNTHESIS OF PYRIDOXOL IN ESCHERICHIA COLI

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It is shown by incorporation experiments with \(^{13}\)C bond-labeled substrates, followed by analysis by means of \(^{13}\)C NMR spectroscopy, that two compounds, 1-deoxy-D-xylulose (12) and 4-hydroxy-L-threonine (13), serve as precursors of pyridoxol (vitamin B₆) (1) in Escherichia coli. Together, these two compounds account for the entire C₈N skeleton of the vitamin. 1-Deoxy-D-xylulose supplies the intact C₅ unit, C-2,2,3,4,4 of pyridoxol. 4-Hydroxy-L-threonine undergoes decarboxylation in supplying the intact C₃N unit, N-1,C-6,5,5. Both precursors are ultimately derived from glucose. The C₅ unit of pyridoxol that is derived from 1-deoxy-D-xylulose originates by union of a triose phosphate (yielding C-3,4,4) with pyruvic acid (which decarboxylates to yield C-2'). \(\Delta\)-Erythroate (11) enters the C₃ unit, C-6,5,5', and is therefore an intermediate on the route from glucose into 4-hydroxy-L-threonine.

Our early work on the biosynthesis of vitamin B₆ in Escherichia coli mutants WG2 and WG3, based on studies originally using substrates labeled with \(^{14}\)C and \(^{3}\)H (1–4), and later with substrates singly labeled with \(^{13}\)C (5), established the pattern of incorporation into the skeleton of pyridoxol (1; see Structure 1) of the carbon atoms of glycerol, glucose, and pyruvic acid. From these results it was inferred that pyridoxol is constructed from three glucose-derived triose phosphates, two of which enter intact, supplying the C₃ fragments, C-3,4,4' and C-6,5,5' of pyridoxol, with the carbon atom holding the phosphate ester group yielding C-4' and C-5', while the third triose phosphate unit yields a C₂ unit, by decarboxylation of pyruvate, whose CH₂-CO moiety then supplies the C₂ unit, C-2',2', i.e. the CH₃ group and the adjacent ring carbon of pyridoxol.

We now furnish definitive proof that these inferences were indeed correct. This direct evidence comes from the results of incorporation studies with substrates labeled with \(^{13}\)C at contiguous sites, so called "bond-labeled" compounds, whose mode of incorporation into pyridoxol was determined by \(^{13}\)C nuclear magnetic resonance spectroscopy (\(^{13}\)C NMR).

The power of this method lies in the circumstance that, when contiguous carbon atoms within a compound are 100% enriched in \(^{13}\)C, this fact is indicated by the presence, within the \(^{13}\)C NMR spectrum of the compound, of \(^{13}\)C-\(^{13}\)C coupling, which is indicated by the appearance of peaks that are not present in the spectrum of a natural abundance sample or of a singly \(^{13}\)C-enriched sample. These new peaks are detectable even if the fully \(^{13}\)C enriched sample is a minor component of a mixture that consists mainly of the unenriched compound.

The appearance of peaks due to \(^{13}\)C-\(^{13}\)C coupling, in the signals of the \(^{13}\)C NMR spectrum of a biosynthetic product, as a result of incorporation of contiguously 100% \(^{13}\)C-\(^{13}\)C-enriched (i.e. bond-labeled) substrates, provides direct evidence for the transfer from substrate into the biosynthetic product of an intact C-C unit. The application of bond-labeled samples thus constitutes a powerful tool for the demonstration of the transfer of intact multi-carbon fragments in biosynthetic investigations; provided adequate incorporation can be achieved in a tracer experiment with a \(^{13}\)C bond-labeled substrate, these are the tracers of choice. Neither radioactive tracer methods nor the use of substrates that are enriched with stable isotopes at single sites can show incorporation of intact multi-carbon units. Furthermore, \(^{13}\)C NMR does not only detect the site of labeling, but at the same time confirms the identity of the labeled sample and determines its degree of chemical purity. A precondition of the application of NMR methods for the analysis of biosynthetic incorporation patterns is the reliable assignment of each spectral signal to the individual atom that gives rise to it.

Support for the inferences drawn from our tracer studies with \(^{14}\)C-labeled substrates, that the pyridoxine skeleton was constructed from three glucose-derived subunits, came from an experiment with [1,2,3,4,5,6-\(^{13}\)C₆]D-glucose (referred to as Experiment 1 in Table I) (6, 7), which demonstrated that, as predicted, only two carbon-carbon bonds, those between C-2 and C-3 and between C-4 and C-5, are newly formed in the course of the biosynthetic derivation of pyridoxol from glucose, and that glucose does indeed supply three intact multicarbon units, as the building blocks of the three fragments, C-2',2', C-3,4,4', and C-6,5,5', of pyridoxol (Fig. 1, A).

The results of the tracer studies with \(^{14}\)C bond-labeled substrates that are here presented define more precisely the mode of incorporation of glucose carbon atoms into the glucose-derived subunits, C-2',2', C-3,4,4' and C-6,5,5', of pyridoxol. They show, further, that an intact C₅ unit, derived from C-3,2 of pyruvic acid, serves as the precursor of the C₂-unit, C-2',2', and that the C₅ chain, C-2',2,3,4,4' of pyridoxol, originates by linear combination of the pyruvate-derived C₂ unit and one of the glucose-derived C₅ units. Furthermore, the results establish the identities of the two multicarbon precursors whose union accounts for the formation of the complete skeleton of pyridoxine. It is shown that 1-deoxy-D-xylulose (12) supplies the intact C₅ chain, C-2',2,3,4,4', while the C₅ unit, C-1,C-6,5,5', is derived intact from 4-hydroxy-L-threonine (13), which is thereby proven to be an intermediate of the biosynthetic route. \(\Delta\)-Erythroate (11) is shown to serve as an intermediate between glucose and 4-hydroxy-L-threonine. Preliminary reports of part of this work have appeared elsewhere (8–10).
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Organism

The organism used in these investigations was E. coli B strain WG2. This is a pyridoxine auxotroph (pdxH⁻) that lacks pyridoxol-phosphate oxidase (EC 1.1.1.65 or EC 1.4.3.65).

Media

**Nutrient Medium**—This was a nutrient broth medium (Oxoid Ltd., London, United Kingdom) prepared according to the supplier’s instructions.

**Minimal Salts Medium**—The minimal salts medium contained the following salts: 7 g/liter KH₂PO₄, 3 g/liter KH₂PO₄, 1 g/liter (NH₄)₂SO₄, 0.1 g/liter MgSO₄, and 0.01 g/liter CaCl₂. (Experiments 1–3, 5, 7, and 8) or n-xylene (Experiments 4, 6, and 9) served as the general carbon source. Pyridoxal hydrochloride was added to a concentration of 6 × 10⁻⁵ M when the minimal medium was used to grow the pdxH⁻ mutant.

All media were prepared in distilled water and were sterilized by autoclaving. The pyridoxal hydrochloride supplement solution was sterilized by filtration.

**Stock Cultures**

Stock cultures of E. coli B WG2 were maintained on monthly slants of the nutrient broth medium. After subculturing from the previous month’s stock, fresh slants were incubated 24 h at 37 °C and were then stored at 4 °C. Every time fresh stock slants were prepared, slants of minimal salts medium, with and without pyridoxal supplementation, were inoculated and incubated 24 h at 37 °C in order to monitor for the presence of wild-type revertants.

**Labeled Compounds**

The following labeled compounds were acquired from a commercial source (Cambridge Isotope Laboratories, Inc. (CIL)): [1,2,3,4,5,6-¹³C₆]D-glucose (99% ¹³C), [1,2-¹³C₂]D-glucose (99% ¹³C), and sodium [2,3-¹³C₂]4-Hydroxy-L-threonine was synthesized (11) from [1,2-¹³C₂]pyruvate (99% ¹³C).

**EXPERIMENTAL PROCEDURES**

**Organism**

Two-month-old cultures of E. coli strain B WG2 were maintained on monthly slants of the nutrient broth medium. After subculturing from the previous month’s stock, fresh slants were incubated 24 h at 37 °C and were then stored at 4 °C. Every time fresh stock slants were prepared, slants of minimal salts medium, with and without pyridoxal supplementation, were inoculated and incubated 24 h at 37 °C in order to monitor for the presence of wild-type revertants.

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The following labeled compounds were prepared from commercial starting materials by multi-step syntheses devised for the purpose.

**Scheme 1. The synthesis of potassium [2,3-¹³C₂]D-erythroate (11) from [1,2-¹³C₂]lacteylene (2).**

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**Scheme 1. The synthesis of potassium [2,3-¹³C₂]D-erythroate (11) from [1,2-¹³C₂]lacteylene (2).**
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4.45–4.75 (m, 1H), 3.62–4.00 (m, 5H), 1.36 (s, 3H), 1.32 (s, 3H), 0.96 (s, 3H).

1<sup>3</sup>C NMR (75.5 MHz, CD<sub>2</sub>Cl<sub>2</sub>): δ 135.6, 135.5, 133.5, 133.4, 129.6, 127.7, 108.4, 77.4 (enriched), 63.2 (X part of a deceptively simple ABX system, δ<sub>AB</sub> = 37 Hz, δ<sub>AX</sub> = 40 Hz, δ<sub>BX</sub> = 5 Hz), 27.8, 26.7, 25.3, 19.1.

IR (film): 1112 (v<sub>COO</sub>) cm<sup>-1</sup>.

ms (CI): m/z 658 (25%, m + NH<sub>4</sub>), 659 (95%), 363 (100%).

(2,3,4-<sup>2</sup>3<sup>13</sup>C<sub>3</sub>)-2,3-O-Isopropylidenederythritol [4,5-<sup>2</sup>13C<sub>2</sub>]meso-4-Dihydroxy-2,2-dimethyl-1,3-dioxolane (7) (5.05 g, 7.95 mmol) was dissolved in tetrahydrofuran (40 ml). Water (0.5 ml, 28 mmol) and a solution of triethylamine (10 ml) were added and the mixture stirred vigorously at room temperature. Florisil<sup>®</sup> (8.5 g) was then added with stirring, which was continued for 10 min, and the mixture was then filtered through a pad of silica gel and washed with ethyl acetate/methanol (1:1 v/v) to give 7 (1.11 g, 95%).

The residue, obtained after evaporation of the solvent, was chromatographed on silica gel (ethyl acetate/hexane, 1:2 v/v), followed by ethyl acetate/methanol/1:1 v/v). The oily product was further purified by vacuum distillation (Kugelrohr, 1 mm Torr, 145°C). Diol (8) was obtained as an oil (1.2 g, 93%), which crystallized (m.p. 47°C; literature data 46°C).

Each tracer experiment was started by subculturing from the current month's nutrient broth stock slant onto a pyridoxine-supplemented minimal medium slant, which had been prepared with either 0.5% w/v n-glucose (Experiments 1–3, 5, 7, and 8) or 0.5% w/v d-xylene (Experiments 4, 6, and 9) as the carbon source. The slant was incubated 24 h at 37°C. Cells from this slant were then used to inoculate 2 × 500 ml samples of the same medium, one of which was supplemented with pyridoxal hydrochloride, the other without pyridoxal supplementation. These cultures were incubated on a rotary shaker (New Brunswick Scientific) at 37°C, until an optical density measurement at 600 nm indicated that growth in the supplemented culture was well into the exponential phase (approximately 12 h). Any growth in the un-supplemented culture served to indicate the presence of wild-type revertants or possible contaminants. If this were observed, the experiment could have been aborted at this stage without any wastage of labeled substrate. Fortunately, in our hands the pds<sup>H</sup> mutation in strain W2G is very stable, and so far such an occasion has not arisen. Nevertheless, the control un-supplemented culture was included in every experiment as a precautionary measure.

The cells from the pyridoxine-supplemented culture were harvested by centrifugation (10 min at 7000 rpm) and washed with sterile 0.9% saline (3 × 10 ml). The washed cells were divided into two equal portions, each one of which was resuspended in minimal salts medium (500 ml) without pyridoxal but containing the appropriate carbon source, the labeled substrate and other additives. These cultures were incubated 6 h on the rotary shaker (400 rpm, 37°C). In order to obtain sufficient material for NMR investigation, each incubation was repeated five times, except in Experiment 9, where labeled substrate for only three repeat incubations was available. Details of the components used in each of the experiments are presented in Table I.

Isolation of Pyridoxol from the Culture Medium

Work-up of Bacterial Cultures—The contents of each 500-ml culture flask from each of the two 500-ml incubation experiments with 1<sup>3</sup>C-labeled substrate were centrifuged 10 min at 7000 rpm, and the supernatant solutions decanted and combined. The solution was concentrated to 200 ml in vacuo on a rotary evaporator. The concentrated solution was treated with acid to hydrolyze phosphate esters; sulfuric acid (1 ml, 80 ml) was added until the final pH of the solution was 1.5, and the mixture was autoclaved (121°C, 3 h). The solution was then evaporated to dryness in vacuo on a rotary evaporator. The residue was dissolved in potassium acetate/acetic acid buffer (0.2 ml, pH 4.5, 200 ml) and the solution was filtered through a fine sintered glass filter. Pyri-adoxol hydrochloride (2.5 mg) was added to the filtrate as a carrier. This solution was then subjected to ion exchange chromatography, as follows.

Chromatography—A cation exchange column was prepared as follows. Dowex 50-X8 (200–400 mesh) was washed, in succession, with water, hydrochloric acid (3 M), water, potassium hydroxide solution (6 M), and water, until free of dye particles. The concentrated solution was treated with acid to hydrolyze phosphate esters; sulfuric acid (1 ml, 80 ml) was added until the final pH of the solution was 1.5, and the mixture was autoclaved (121°C, 3 h). The solution was then evaporated to dryness in vacuo on a rotary evaporator. The concentrated solution was treated with acid to hydrolyze phosphate esters; sulfuric acid (1 ml, 80 ml) was added until the final pH of the solution was 1.5, and the mixture was autoclaved (121°C, 3 h). The solution was then evaporated to dryness in vacuo on a rotary evaporator. The residue was dissolved in potassium acetate/acetic acid buffer (0.2 ml, pH 4.5, 200 ml) and the solution was filtered through a fine sintered glass filter. Pyridoxol hydrochloride (2.5 mg) was added to the filtrate as a carrier. This solution was then subjected to ion exchange chromatography, as follows.

The solution containing the concentration of the incubate (pH 4.5, 200 ml, see above) was applied to this column. Elution of the column was carried out by stepwise increase in pH, with the following buffer sequence: potassium acetate (0.2 M)/acetic acid (0.2 M), pH 5.0, 100 ml; potassium acetate (0.2 M)/acetic acid (0.2 M), pH 5.5, 100 ml; potassium acetate (0.2 M)/acetic acid (0.2 M), pH 6.0, 50 ml; boric acid (0.2 M)/ potassium chloride (0.2 M)/acetic acid (0.2 M), 200 ml, adjusted to pH 6.6 by addition of sodium hydroxide (0.2 M). Fractions (10 ml) were collected and assayed by UV spectroscopy in order to determine the position of

as a yellow powder.

The crude product (from several runs) (855 mg) was dissolved in water (1.7 ml), methanol (5.1 ml) was added, and the mixture was stored 2 d in the refrigerator. Pure potassium erythrate (11) (650 mg, 76%) was obtained as colorless leaflets (a<sub>D</sub> = +110° (c = 1.12, H<sub>2</sub>O)).

1<sup>3</sup>C NMR (200 MHz, D<sub>2</sub>O): δ 4.14–4.43 (m, 1H), 3.24–3.62 (m, 3H).

1<sup>3</sup>C NMR (50.3 MHz, D<sub>2</sub>O): δ 178.5 (X part of a deceptively simple ABX system, δ<sub>AB</sub> = 28 Hz, δ<sub>AX</sub> = 42 Hz, δ<sub>BX</sub> = 70 Hz, δ<sub>AX</sub> = 6 Hz, 74.0 (enriched, δ<sub>AX</sub> = 40 Hz), 73.1 (enriched, δ<sub>AX</sub> = 40 Hz), 61.9 (X part of a deceptively simple ABX system (δ<sub>AB</sub> = 13 Hz, δ<sub>AX</sub> = 40 Hz, δ<sub>BX</sub> = 45 Hz), δ<sub>AX</sub> = −3 Hz).

IR (KBr): 3405, 3194, 1711, 1605 (v<sub>COO</sub>) cm<sup>−1</sup>.

ms (ES): m/z 137 (100% m<sup>−1</sup>).
Each experiment (except Experiment 9) consisted of five 1-liter incubations. Pyridoxol hydrochloride was isolated from the culture fluid of each of the five incubations, after removal of the cells by centrifugation and addition of unlabeled pyridoxol hydrochloride (2.5 mg) as carrier.

### TABLE I

**Experimental details**

| Exp. no. | Substrates | Weight (mg/liter (mmol)) | 13C NMR spectrum of isolated pyridoxol HCl |
|----------|------------|--------------------------|-------------------------------------------|
| 1        | [1,2,3,4,5,6-^13C^6]-D-glucose | 200 (5.6) | A (Fig. 1; Refs. 6 and 7) |
| 2        | [1,2-^13C]-D-glucose | 300 (5.6) | B (Fig. 2) |
| 3        | Sodium [2,3-^13C]-Pyruvate | 150 (1.3) | C (Fig. 2) |
| 4        | Sodium [2,3-^13C]-Pyruvate | 200 (1.8) | Not shown |
| 5        | [1,2,3,4,5,6-^13C^6]-D-glucose | 200 (5.6) | D (Fig. 3) |
| 6        | D-Xylose | 750 (5.6) | (3.3) |
| 7        | [2,3-^13C]-1-Deoxy-D-xylulose | 200 (1.5) | E (Fig. 3) |
| 8        | 4-Hydroxy-L-threonine | 160 (0.74) | (Fig. 4) |
| 9        | L-Threonine | 20 (0.17) | (Fig. 4) |
| 10       | [1,2,3,4,5,6-^13C^6]-D-glucose | 200 (5.6) | G (Fig. 5) |
| 11       | D-Glucose | 800 (2.8) | (Fig. 5) |
| 12       | 4-Hydroxy-L-threonine | 20 (0.17) | (Fig. 5) |
| 13       | L-Threonine | 20 (0.17) | (Fig. 5) |
| 14       | Potassium [2,3-^13C]-d-erythrose | 200 (1.1) | H (Fig. 5) |
| 15       | D-Xylose | 500 (3.3) | (Fig. 5) |

a In Experiments 4, 6, and 9, D-glucose was used as the general carbon source in order to avert the possibility that the presence of glucose might limit the uptake and incorporation of the labeled carbohydrate substrates, [2,3-^13C]-1-deoxy-D-xylulose (Experiment 6) and [2,3-^13C]-d-erythrose (Experiment 9). Experiment 4 served as a test of D-glucose as the general carbon source. This change in the general carbon source was made after consideration of the results of the [1,2,3,4,5,6-^13C^6]-D-glucose displacement experiments, Experiments 5 and 7, and after failure to observe ^13C incorporation in an early experiment with [2,3-^13C]-1-deoxy-D-xylulose. Whereas unlabeled 4-hydroxy-L-threonine completely suppressed the incorporation, into C-6,5,5′ of pyridoxal, from [1,2,3,4,5,6-^13C^6]-D-glucose (Experiment 7), unlabeled 1-deoxy-D-xylulose only partially suppressed the incorporation, into C-2′,2,3,4,4′ of pyridoxal, from [2,3-^13C]-D-glucose (Experiment 5). Furthermore, in an experiment with [2,3-^13C]-1-deoxy-D-xylulose in which D-glucose served as the general carbon source, no ^13C enrichment was detectable in the pyridoxal that was isolated. We reasoned that these results may have been the consequence of an “inducer exclusion effect,” a phenomenon that occurs in bacterial systems, whereby certain carbohydrates (so-called “PTS-carbohydrates”), e.g. glucose, inhibit the transport and metabolism of other carbohydrates (so-called “class I non-PTS carbohydrates”; PTS = phosphoenolpyruvate:carbohydrate phosphotransferase system) (16) of which 1-deoxy-D-xylulose might be one. We surmised that in our short term (6 h) incubations, D-glucose might have inhibited the uptake of 1-deoxy-D-xylulose, whereas entry of the amino acid, 4-hydroxy-L-threonine, had not been affected. If this reasoning were correct, the problem might be overcome by using as the general carbon source in this experiment a non-PTS-carbohydrate such as D-xylene, in place of D-glucose, a PTS-carbohydrate. Cultures of E. coli mutant WG2 were established on the minimal medium, with D-xylene as the general carbon source, and in a test experiment (Experiment 4) it was found that under these conditions incorporation of label from sodium [2,3-^13C]-pyruvates matched the result obtained when D-glucose served as the general carbon source (Experiment 3). Having thereby established that D-xylene could replace D-glucose as the general carbon source, we proceeded with Experiments 6 and 9 (Table I).

b When this experiment (Experiment 6) was performed, it was already known, from Experiments 7 and 8, that 4-hydroxy-L-threonine serves as a direct precursor of pyridoxol. A sample of the amino acid was added since it was observed that this stimulates pyridoxol biosynthesis.

c The spectrum does not provide information concerning the identity of the fragments of glucose from which these fragments of pyridoxol, of ^13C from [1,2,3,4,5,6-^13C^6]-D-glucose (Experiment 5). Furthermore, in an experiment with [2,3-^13C]-1-deoxy-D-xylulose in which D-glucose served as the general carbon source, no ^13C enrichment was detectable in the pyridoxal that was isolated. We reasoned that these results may have been the consequence of an “inducer exclusion effect,” a phenomenon that occurs in bacterial systems, whereby certain carbohydrates (so-called “PTS-carbohydrates”), e.g. glucose, inhibit the transport and metabolism of other carbohydrates (so-called “class I non-PTS carbohydrates”; PTS = phosphoenolpyruvate:carbohydrate phosphotransferase system) (16) of which 1-deoxy-D-xylulose might be one. We surmised that in our short term (6 h) incubations, D-glucose might have inhibited the uptake of 1-deoxy-D-xylulose, whereas entry of the amino acid, 4-hydroxy-L-threonine, had not been affected. If this reasoning were correct, the problem might be overcome by using as the general carbon source in this experiment a non-PTS-carbohydrate such as D-xylene, in place of D-glucose, a PTS-carbohydrate. Cultures of E. coli mutant WG2 were established on the minimal medium, with D-xylene as the general carbon source, and in a test experiment (Experiment 4) it was found that under these conditions incorporation of label from sodium [2,3-^13C]-pyruvate matched the result obtained when D-glucose served as the general carbon source (Experiment 3). Having thereby established that D-xylene could replace D-glucose as the general carbon source, we proceeded with Experiments 6 and 9 (Table I).

d Only three 1 liter cultures (rather than five) were employed in this experiment.

**RESULTS AND DISCUSSION**

The ^13C NMR spectrum (Fig. 1, A) of the sample of pyridoxol hydrochloride isolated from a culture of E. coli B mutant WG2 after incubation with [1,2,3,4,5,6-^13C^6]-D-glucose (6, 7) shows that the three fragments C-2′, 2′, C-3,4′, and C-6,5,5′ are derived from glucose as intact multi-carbon units (Scheme 2, A). However, the spectrum does not provide information concerning the identity of the fragments of glucose from which these fragments of pyridoxol are derived.
units are derived.

The answer to this question can now be conclusively deduced from three sets of labeling data. First, the $^{13}$C NMR spectrum (Fig. 2, B)$^2$ of the sample of pyridoxol from $\Delta$-[1,2,3,4,5,6-$^{13}$C$_6$]glucose (Experiment 2) shows that each of the signals due to the carbon atoms of the three carbon pairs C-2',2, C-4',4, and C-5',5 of pyridoxol intact. Second, it had been found earlier (3) that label from [1-$^{14}$C]-glucose (or from [6-$^{14}$C]-glucose) entered the three carbon atoms, C-2', C-4', and C-5' of pyridoxol, and only these three carbon atoms. This defines the orientation of entry into pyridoxol of the intact multicarbon units of glucose. The glucose carbon atoms yielding the three intact fragments derived from [1,2,3,4,5,6-$^{13}$C$_6$]glucose enter as follows: C-2 (or C-6,5)$^3$ of glucose yield carbon atoms C-2',3, of pyridoxol; and carbon atoms C-1,2,3 (or C-6,5,4)$^3$ of glucose enter C-4' and C-5', C-4 and C-5, and C-3 and C-6, respectively, of the two C$_2$ units C-4',4, and C-5',5,6 of pyridoxol. The early inference that the two C$_2$ units C-4',4, and C-5',5,6 of pyridoxol were derivable intact from C-1,2,3 (or C-6,5,4)$^3$ of glucose, with C-1 (or C-6)$^3$ of the sugar giving rise to C-2',3, C-4', and C-5', is thus confirmed, lending strong support to the early conclusion that the two C$_2$ units C-4',4, and C-5',5,6 of pyridoxol are derivable intact from triose phosphates.$^4$

Third, it is now confirmed (Fig. 2, C) by the experiments with sodium [2,3-$^{13}$C$_2$]pyruvate (Experiments 3 and 4) that the C$_2$ unit, C-2',2, of pyridoxol, inferred (3) to be derived intact from the CH$_3$CO- fragment of pyruvic acid, is indeed so derived. Satellites appear only at the signals due to C-2' and C-2 (δ 16.5, 144.7, $^{13}$C-13C coupling 46.4 Hz). The stage is thus set for an investigation of the identity of compounds that might serve as more advanced precursors of the B$_6$ skeleton. Since only two new C-C bonds, C-2,3 and C-4,5, are generated in the course of pyridoxol biosynthesis from $\Delta$-glucose (Scheme 2, A) and since it may reasonably be assumed that these two bonds are not generated simultaneously, only two sets of advanced precursors need to be considered. One alternative set of advanced precursors consists of a C$_5$ compound and a C$_4$N unit. The C$_5$ compound would be generated by closure of the bond destined to become the C-2,3 bond.

$^2$ Figs. 2–5, A–H: 125.776-MHz proton decoupled $^{13}$C NMR spectra of pyridoxol hydrochloride (in 100 μl of D$_2$O), isolated from E. coli B mutant WG 2 after incubation with the $^{13}$C bond-labeled substrate that is listed below each figure. The spectra were acquired on a Bruker DRX 500 spectrometer, operating at 11.74 T, using a Bruker 2.5 mm microprobe, with a 90° pulse width (8 μs), spectral width 28985.5 Hz, and a recycle time of 10.6 s. Digital resolution was 0.88 Hz per data point.

$^3$ Since the two C$_2$ fragments of glucose, C-1,2,3 and C-6,5,4, are interconvertible via triose-phosphate isomerase (EC 5.3.1.1)-catalyzed equilibration of dihydroxyacetone 1-phosphate (from C-1,2,3 of glucose) and glyceraldehyde 3-phosphate (from C-4,5,6 of glucose), the experiment does not distinguish between the ultimate derivation of these three C$_2$ units from C-1,2,3 or from C-6,5,4 of glucose.

$^4$ It must be stated at this point that the inferences that we drew (19, 20) concerning the identity of the triose phosphate that entered each the two C$_2$ units, on the basis of the quantitative distribution within pyridoxol of radioactivity from [1-$^{13}$C]- and [6-$^{14}$C]-glucose (4), were incorrect. This will be referred to later.
of pyridoxol, as the precursor of the C\text{5}_\text{a} unit, C-2'-2-3-4-4' of pyridoxol, originating by union of the CH\text{3}-CO fragment of pyruvic acid (C-2'-2) with a triose phosphate (C-3-4-4'). The remaining portion of the pyridoxol skeleton would then require a C\text{3}_\text{a} precursor of the fragment C-6-5-5', or possibly a C\text{3}_\text{N} precursor of the fragment N-1-C-6-5-5'.

The other alternative assumes closure of the bond destined to become the C-4,5 bond of pyridoxol. The advanced precursors would then be a branched chain C\text{6}_\text{b} compound, serving as the source of the C\text{6}_\text{b} unit, C-3-4-(-4')-5-5'-6, while the remaining portion of the pyridoxol skeleton, C-2'-2,N-1, would require a C\text{2}_\text{N} unit derived from the CH\text{3}-CO fragment of pyruvic acid, together with a nitrogen source.

In the first instance we decided to focus on the former of the two alternatives, a possible origin of the pyridoxol skeleton by union of a C\text{5}_\text{a} plus C\text{3}_\text{N} unit. This turned out to be the right
This alternative demands that the C₅ unit must be derived from the CH₃-CO fragment of pyruvate plus a triose phosphate. The reaction of a triose phosphate, D-glyceraldehyde 3-phosphate (or of D-glyceraldehyde itself), with pyruvic acid, catalyzed by pyruvate dehydrogenase (EC 1.2.4.1), occurs in many micro-organisms, including *E. coli* (21, 22). The reaction is accompanied by loss of the pyruvic acid carboxyl group, to yield a C₅ compound, 1-deoxy-D-xylulose 5-phosphate (or 1-deoxy-D-xylulose (12), Scheme 2), respectively.

Preliminary evidence that 1-deoxy-D-xylulose serves as a precursor, supplying the C₅ unit, C-2’,2,3,4,4’ came from an experiment with [1,1,1-(RS)-5-²H₄]1-deoxy-D-xylulose. Deuterium from this substrate entered pyridoxol in the predicted manner (23). It remained to generate conclusive evidence that 1-deoxy-D-xylulose is indeed an intermediate on the route from glucose into pyridoxol and that it is incorporated intact. To prove intermediacy, it had to be demonstrated that the presence of excess 1-deoxy-D-xylulose in the system spared the incorporation of glucose carbon into the C₅ unit C-2’,2,3,4,4’ of pyridoxol. To prove intact incorporation, it had to be demonstrated that the bond, generated in the formation of the compound, i.e. the bond destined to become C-2,3 of pyridoxol, remains intact in the course of entry of 1-deoxy-D-xylulose into pyridoxol.

Evidence that each of the two conditions is fulfilled is provided by Experiments 5 and 6, respectively. The ¹³C NMR spectrum (Fig. 3, D) of the sample of pyridoxol (Scheme 2, D), isolated after administration of [1,2,3,4,5,6-¹³C₆]D-glucose in the presence of excess 1-deoxy-D-xylulose (12, Scheme 2), shows that the signals due to ¹³C enrichment at C-2’ (δ 16.5), C-2 (δ 144.7), C-3 (δ 154.7), C-4 (δ 142.5) and C-4’ (δ 59.0) are less intense than the corresponding signals in the spectrum of pyridoxol derived from [1,2,3,4,5,6-¹³C₆]D-glucose alone (Fig. 1, A), whereas the signals due to C-6 (δ 131.8), C-5 (δ 138.8), and C-5’ (δ 60.1) remained unchanged (8). The extent of decrease in the incorporation of ¹³C into the carbon atoms C-2, C-2’, C-3, C-4, and C-4’, relative to that into carbon atoms C-6, C-5, and C-5’, is illustrated in the expanded spectrum (Fig. 3, D’), which shows that the satellites at the signal due to C-4’ (δ 59.0) are approximately 2.5 × less intense than those at the signal due to C-5’ (δ 60.1). Thus, the presence of excess 1-deoxy-D-xylulose had reduced the level of incorporation of glucose-derived ¹³C into the C₅ unit, C-2’,2,3,4,4’ of pyridoxol. It follows that 1-deoxy-D-xylulose lies on the route from glucose into pyridoxol.

The evidence is thus complete that the intact C₅ chain of 1-deoxy-D-xylulose serves as source of the C₅ unit C-2’,2,3,4,4’.
of pyridoxol. It remains for the future to determine whether it is the free deoxypentulose or its 5-phosphate ester that serves as the actual precursor.\(^5\)

We now turn our attention to the identity of the C\(_3\)N precursor of N-1,C-6,5,5\(^9\) of pyridoxol.

Since the C\(_3\) unit C-6,5,5\(^9\) is generated from C-3,2,1 (or from C-4,5,6) of glucose, we postulated (2) in our original working hypothesis that a triose phosphate is implicated in the derivation of this unit. Difficulties arose with this notion when it was discovered that the C2 unit, C-5\(^9\), was derivable from glycolaldehyde (25, 26) and that the CN unit, C-6,N-1, originated intact from the fragment -CH\(_2\)-NH\(_2\) of glycine (27). On the basis of these results, we inferred (20) that the C\(_3\)N fragment, N-1,C-6,5,5\(^9\), of pyridoxol might be derived from 4-hydroxy-L-threonine, which in turn might be formed by an aldolase type condensation of glycolaldehyde plus glycine, in the manner of the formation of L-serine from a formaldehyde equivalent plus glycine, catalyzed by serine hydroxymethylase (EC 2.1.2.1). In an attempt to achieve a reconciliation of the incorporation results with glucose on the one hand and with glycolaldehyde and glycine on the other, we postulated (20) intermediacy of 1-aminopropane-2,3-diol, which might arise either from 4-hydroxy-L-threonine by decarboxylation, or from D-glyceraldehyde by transamination. An attempt to support this notion experimentally failed. Label from a \(^2\)H-labeled sample of 1-aminopropane-2,3-diol was not incorporated into pyridoxol (28).

A solution to the problem was conceived by Lam and Winkler (29), who suggested, on the basis of genetic studies, that the glucose-derived C\(_3\) unit, C-6,5,5\(^9\), of pyridoxol was generated not by way of a glycolytic triose phosphate intermediate, but via intermediates originating from the pentose phosphate pathway. The major impetus for this idea was the parallelism, genetic, enzymic and structural, between the conversion of D-glyceraldehyde 3-phosphate to L-serine (via glyceric acid 3-phosphate, 3-hydroxypropionic acid 3-phosphate, and 3-phosphoserine) and the conversion, in the homologous series, of D-erythrose 4-phosphate into 4-hydroxy-L-threonine (via erythrose 4-phosphate, 3,4-dihydroxy-2-oxobutanoic acid 4-phosphate, and 4-hydroxy-L-threonine 4-phosphate). This new hypothesis is consistent with the results of the tracer experiments with \(^14\)C-labeled substrates on which the earlier hypothesis had been based (30). The glycolaldehyde/glycine route to 4-hydroxy-L-threonine was regarded as an alternative minor pathway.

We now provide the first direct experimental evidence in support of the ideas of Lam and Winkler.

To prove that 4-hydroxy-L-threonine (13, Scheme 2) lies on the route from glucose into pyridoxol, it had to be demonstrated that its presence in the system in excess spared the incorporation of glucose carbon into the C\(_3\) unit C-6,5,5\(^9\) of pyridoxol. To prove intact incorporation of 4-hydroxy-L-threonine, it had to be demonstrated that the bond, generated in the formation of the compound from glycolaldehyde and glycine, i.e. the bond destined to become C-5,6 of pyridoxol, remains intact in the course of entry of 4-hydroxy-L-threonine into pyridoxol. Finally, to substantiate the proposal of Lam and Winkler, that the compound originated from glucose via the pentose phosphate route, direct evidence of the intermediacy of a compound related to the proposed route was required.

Evidence that each of these three conditions is fulfilled is provided by Experiments 7, 8, and 9, respectively. The \(^13\)C NMR spectrum (E, Fig. 3) of the sample of pyridoxol, isolated after administration of \([1,2,3,4,5,6-\text{\(^{13}\)C}_6]\)D-glucose in the presence of excess 4-hydroxy-L-threonine (Experiment 7) shows that the signals due to C-6, (\(\delta 131.8\)), C-5 (\(\delta 138.8\)), and C-5\(^9\) (\(\delta 60.1\)) appear as singlets, whereas those due to the other five carbon atom all maintain their multiplicity (8). The presence of 4-hydroxy-L-threonine had completely suppressed incorporation of glucose carbon into the C\(_3\) unit C-6,5,5\(^9\) of pyridoxol, while incorporation of glucose-derived \(^13\)C into the rest of the molecule was unaffected (Scheme 2, F; Fig. 3). Thus, 4-hydroxy-L-threonine lies on the route from glucose into pyridoxol. The \(^13\)C NMR spectrum (F, G) of the sample of pyridoxol, isolated after administration of \([2,3,\text{\(^{13}\)C}_2]\)4-hydroxy-L-threonine (11) (Experiment 8) shows satellites at the signals

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\(^5\) A suggestion (24), conceived to explain recent microbiological observations, that under anaerobic conditions 1-deoxy-D-xylulose is replaced as the precursor of the C\(_3\) unit by the corresponding aldehyde, \((2S,3R)-2,3\text{-dihydroxy-4-oxopentalen}\), remains entirely hypothetical.
due to C-5 and C-6 ($\delta$ 138.8, 131.8, $^{1}J_{5,6} = 64.9$ Hz), and nowhere else, proving incorporation of the substrate without cleavage of the C-2,3 bond (9).

Finally, the $^{13}$C NMR spectrum (Fig. 5, $H$) of the sample of pyridoxol, isolated from an experiment with [2,3-$^{13}$C$_{2}$]D-erythroic acid (Experiment 9), showed that bond-label had been transferred from the substrate into C-6,5 of pyridoxol. This result represents the first direct evidence in support of the proposal of Lam and Winkler (29). Satellites, which are clearly visible in the expanded spectrum (Fig. 5, $H'$), appear at the signals due to C-5 and C-6 ($\delta$ 138.8, 131.8, $^{1}J_{5,6} = 64.9$ Hz).

The evidence is thus complete that the C$_{6}$N unit that serves as the source of N-1,C-6,5,5 of pyridoxol is derived from 4-hydroxy-L-threonine, and that the latter is derived from glucose via erythroic acid. Recent results indicate that the first vitamin B$_{6}$ compound to be formed in E. coli is pyridoxol 5'-phosphate, rather than pyridoxin (31, 32). Even though the nonphosphorylated compounds, 4-hydroxy-L-threonine and D-erythroic acid, were incorporated intact into the C$_{6}$N unit, N-1,C-6,5,5 of pyridoxol, it would appear more likely that 4-hydroxythreonine 4-phosphate (33) and erythroic acid 4-phosphate, rather than the non-phosphorylated compounds, serve as the precursors. The presence in E. coli of a nonspecific kinase would account for the utilization, in our experiments, of the nonphosphorylated compounds.

The status of the alternative source of the C$_{6}$N unit N-1,C-6,5,5 of pyridoxol and, presumably, also of its precursor, 4-hydroxy-L-threonine, i.e. its derivation from glycolaldehyde plus glycine, must now be evaluated.

Glycolaldehyde satisfies the nutritional requirement for pyridoxol in the pdxB$^{-}$ E. coli B mutant, WG3 (34). The evidence is unequivocal that glycolaldehyde is incorporated into pyridoxal. In this mutant (25), as well as in the pdxH$^{-}$ mutant WG2 (26), label from specifically $^{14}$C-labeled glycolaldehyde enters C-5,5. The aldehyde carbon of glycolaldehyde supplies C-5 of pyridoxal and the carbinol carbon supplies C-5 (25, 26). Furthermore, in mutant WG3, the N-C bond of the NH$_{2}$-CH$_{2}$- group of glycine yields the N-1,C-6 bond of pyridoxal (27). This incorporation pattern is entirely consistent with the notion that 4-hydroxy-L-threonine, the precursor of the C$_{6}$N unit N-1,C-6,5,5, is synthesized not only from D-glucose via D-erythroic acid, but also from glycine plus glycolaldehyde (20), by a
reaction analogous to that normally catalyzed by serine hydroxymethylase.

Whether or not this alternative derivation of 4-hydroxy-L-threonine is a normal, if minor, source of this compound or whether it is induced only if glycolaldehyde is supplied to the culture medium. Whether or not this alternative derivation of 4-hydroxy-L-threonine is a normal, if minor, source of this compound or whether it is induced only if glycolaldehyde is supplied to the culture medium.

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