The Regiochemistry and Stereochemistry of the Biosynthesis of Vitamin B₆ from Triose Units*

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3¹C and ²H NMR spectroscopy has been employed to probe the biosynthesis of vitamin B₆ in Escherichia coli. The ³¹C NMR spectrum of a sample of pyridoxol derived biosynthetically from d-[1,2,3,4,5,6-³¹C₆]glucose shows that the bonds, C(2)-C(3) and C(4)-C(5), of the pyridine nucleus are the only two carbon-carbon bonds of pyridoxol which are generated de novo in the course of its biosynthesis from glucose. It follows that the pyridoxol skeleton is generated from two intact triose units and a triose-derived two-carbon unit, all of which are supplied by glucose.

From the ²H NMR spectra of samples of pyridoxol derived from (R)-[1,1-²H₂]glycerol and (S)-[1,1-²H₂]glycerol, respectively, it can be deduced that the reduced monomethyl group of glycerol enters C-2', C-4', and C-5' of the pyridoxol skeleton. It follows that each of the three fragments is derived from glycerol in stereospecific fashion.

These results answer questions concerning the regiochemistry and the stereochemistry of pyridoxol biosynthesis.

In studies of the biosynthesis of vitamin B₆ by tracer experiments with putative precursors labeled with ¹⁴C and ³¹C (2-6), we have employed Escherichia coli B mutant WG2 (7). This mutant, one of the Group IV (pdxH) mutants of E. coli B (8), lacks the enzyme pyridoxol-phosphate oxidase (EC 1.1.1.65 or EC 1.4.3.5) (9). The mutant elaborates the complete ring skeleton of vitamin B₆, as exemplified by pyridoxol, and differs from the wild-type strain of E. coli B merely in that there is a block in the final step of vitamin B₆ biosynthesis, oxidation of pyridoxol (and its 5'-phosphate) into pyridoxal (and its 5'-phosphate). A study of the biosynthesis of pyridoxol in this mutant thus represents an investigation of the normal biosynthetic process leading from primary precursors into the C₆N ring skeleton of the vitamin.

The incorporation pattern within pyridoxol of label from ¹⁴C-labeled samples of glyceraldehyde and glucose permitted us to put forward a chemically rational hypothesis of the biosynthetic route from triose units into pyridoxol (2, 10). In advancing this hypothesis, we made assumptions concerning the location of newly formed bonds and concerning the identity of the triose intermediates involved in the biosynthetic process.

We now present evidence which locates the bonds that are newly formed in the process of biosynthesis of pyridoxol from glucose. Furthermore, we report results which establish the stereochemistry of the incorporation of glycerol.

These results answer questions relating to the regiochemistry and stereochemistry of the incorporation of triose precursors into pyridoxol and add support to the model of pyridoxol biosynthesis which we had advanced on the basis of earlier tracer evidence (2, 10).

MATERIALS AND METHODS

Labeled Compounds

D-[1,2,3,4,5,6-³¹C₆]Glucose (enrichment 99% ¹³C per carbon atom) (Experiment 1 (see Table I)) was obtained from the Los Alamos National Laboratory, National Institutes of Health Stable Isotope Resource. d-[¹³C]Glucose (specific activity, 270 mCi/mmol (Experiment 1) and [2-¹³C]Glycerol (specific activity, 24 mCi/mmol) (Experiments 2 and 3) were commercial products (Amersham Corp.).

Synthesis of Chirally Deuterated Glycerol (Experiments 2 and 3) (Scheme 1)

(1)-[1,1-²H₂]Glycerol (6) from L-serine (11) (cf. Refs. 11 and 12)

(R)-2,2-Dimethyl-4-hydroxy[²H₂]methyl-1,3-dioxolane (5) (R)-2,2-Dimethyl-4-carboxymethyl-1,3-dioxolane (6) (3), obtained (11) from L-serine in three steps, was dissolved in dry ether (30 ml), and the solution was added dropwise to a suspension of lithium aluminium deuteride (3 g, MSD Isotopes) in dry ether (25 ml). The mixture was refluxed 0.5 h, ethyl acetate was added, and the precipitate was filtered off and washed with ether. The ether solution was dried (MgSO₄) and evaporated, and the residue fractionated.

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(S)-[1,1-²H₂]Glycerol (6) from L-serine (11) (cf. Refs. 11 and 12)

(R)-2,2-Dimethyl-4-hydroxy[²H₂]methyl-1,3-dioxolane (5) (1 g) in water (2 ml) was hydrolyzed by addition of a drop of concentrated hydrochloric acid. The solution was concentrated in vacuo at 30 °C to remove acetone. The concentrate, containing (S)-[1,1-²H₂]glycerol, was used without further purification in a tracer experiment with E. coli B W20 (Experiment 3). Deuterium content at C-1 (by 500-MHz ¹H NMR, relative signal area at 3.77 (1H, dd, H-2) compared to those at 3.56 (1H, dd, H-3) and 3.65 (1H, dd, H-3), 1:1; cf. Fig. 2A) was >95%.

(S)-[1,1-²H₂]Glycerol (6) from L-serine (11) (cf. Refs. 11 and 12)

(R)-2,2-Dimethyl-4-hydroxy[²H₂]methyl-1,3-dioxolane (5) (1 g) in water (2 ml) was hydrolyzed by addition of a drop of concentrated hydrochloric acid. The solution was concentrated in vacuo at 30 °C to remove acetone. The concentrate, containing (S)-[1,1-²H₂]glycerol, was used without further purification in a tracer experiment with E. coli B W20 (Experiment 3). Deuterium content at C-1 (by 500-MHz ¹H NMR, relative signal area at 3.77 (1H, dd, H-2) compared to those at 3.56 (1H, dd, H-3) and 3.65 (1H, dd, H-3), 1:1; cf. Fig. 2A) was >95%.

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2,2-dimethyl-4-carbomethoxy-1,3-dioxolane (9) (5.5 g) was hydrolyzed in water (2 ml) and the solution was evaporated. The residue was dissolved in benzene, and the solution was applied to a silica column (4 g). The column was eluted in sequence with benzene (10 ml, fractions 1 and 2), benzene/diethyl ether (9:1, 15 fractions 3–5), benzene/diethyl ether (8:2, 50 ml, fractions 6–10), benzene/diethyl ether (7:5:2.5, 40 ml, fractions 11–20), and diethyl ether/methanol (9:8:0.2, 10 ml, fractions 21 and 22).

Tri-O-acetylpyridoxol (15), a viscous oil, was obtained from fractions 6–16 (Rf 0.4, Kieselgel 60 F254 diethyl ether). Yield was 158 mg (91%).

Mass spectrum (70 eV), m/z (relative abundance): 296 (32) M+ + 1, no M+, 253 (35) [M+ + 1]-CH3CO, 235 (15) [253-18], 210 (11) [253-CH2CO], 193 (65) [M+ + 1-2 CH2CO-CH2], 179 (100), 123 (53), 122 (18), 106 (42), 94 (18), 65 (10), 51 (15). 'H NMR (90 MHz, CDCl3): 2.03, 2.09, 2.40, 2.43 (4 × 3H, 4 × 3H, 4 × 3H), 5.04, 5.16 (2 × 1H, 2 × 2H, C-4', C-5' CH3), ppm. IR (CHCl3): 1750 cm⁻¹ carbon=oxygenc.

A small sample was converted into tri-O-acetylpyridoxol N-oxide, m.p. 146–148 °C (from methanol/diethyl ether). m.p. (literature) 157 °C (17).

3-Acetoxy-4,5-di(acetoxyethyl)-2-methylpyridine (Tri-O-acetylpyridoxol N-Oxide) (18) (cf. Ref. 18)

3-Acetoxy-4,5-di(acetoxyethyl)-2-methylpyridine (15) (158 mg) in chloroform (10 ml) was mixed with 80% m-chloroperbenzoic acid (260 mg) in chloroform (5 ml) at room temperature. The mixture was allowed to stand overnight and was then shaken at 0–10 °C with a
SATURATED SOLUTION OF AQUEOUS SODIUM BICARBONATE (3 X 5 ml) AND THE CHLOROFORM LAYER WAS DRIED (MgSO4, Na2SO4, ANHYDROUS) AND EVAPORATED. THE CRYSSTALLINE RESIDUE WAS DISSOLVED IN BENZENE, AND THE SOLUTION WAS APPLIED TO A SILICA COLUMN (3.5 g). THE COLUMN WAS ELUTED, IN SEQUENCE, WITH BENZENE (6 ml, FRACTION 1), DIETHYL ETHER (20 ML, FRACTIONS 2-4), CHLOROFORM/METHANOL (9.5:0.5, 30 ML, FRACTIONS 5-8), AND CHLOROFORM/METHANOL (9:1:0.1, 20 ML, FRACTIONS 9-12). THE FRACTIONS WERE MONITORED BY TLC (KIELSELM 60 F254/DIETHYL ETHER). FRACTIONS 2-5 CONTAINED THE TRICHLOROMETHYLATED RESIDUE (16) WHICH CRYSTALLIZED IN NEEDLES FROM BENZENE/HAXANE (3:2). YIELD WAS 150 MG (72%). M.P. 110-112°C. M.P. (LITERATURE) 114-115°C (18).

Mass spectrum (70 eV), m/z (relative abundance): 311 (15) M*, 269 (8), 251 (5), 226 (17), 209 (62), 192 (19), 168 (55), 150 (100), 122 (10), 69 (35). 22N MR (90 MHz, CDCl3) δ: 2.10, 2.32, 2.64 (4 X s, 4 X 3H, 4 X CH3), 5.06, 5.23 (2 X s, 2 X 2H, C-4', C-5' CH3), 8.33 (1H, C-6 H) ppm. IR (CDCl3): 1750, 1760 cm-1 CARBON=OXYGEN. 1120 cm-1 NITROGEN=OXYGEN.

3-Acetoxy-4,5-di(acetoxymethyl)-6-chlorine (Tri-O-acetylpyridoxol-1-oxide (17) (cf. Ref. 18))

Freshly distilled phosphorus oxychloride (0.45 ml) was added to 3-acetoxy-4,5-di(acetoxymethyl)-2-methylpyridine (16) (110 mg). The mixture was heated at 100°C for 15 min and at 120°C for 15 min. The resulting solution was cooled, added to ice water (2 ml), neutralized with potassium bicarbonate, and extracted with diethyl ether (3 x 30 ml). The ether layer was washed with water and dried (anhydrous MgSO4). The resulting solution was evaporated in sequence, with benzene (5 ml), diethyl ether (20 ml), and chloroform/methanol (9:5:1, 20 ml, fractions 2-4), chloroform/methanol (9.5:0.5, 30 ml, fractions 5-8), saturated solution of aqueous sodium bicarbonate (3 g), and glycerol (Experiments 2 and 3). The details of the tracer experiments are summarized in Table I.

The 13C NMR spectrum of 13C-enriched pyridoxol (Experiment 1) was determined on a Bruker AM 500 spectrometer. 1H and 13C NMR spectra were determined on a Bruker AM 500 spectrometer. Spectra are presented in Figs. 1-4 where spectral details are also reported.

RESULTS

(R)- and (S)-[1,2,3,4,5,6-13C6]Glycerol—Samples of the enantiomers of [1,1-13H2]glycerol were obtained by adaptation of published methods (11, 12). (S)-L-Serine (1) was converted, via (S)-L-glyceraldehyde (2), into methyl (S)-2,3-O-isopropylenediglycerate ([S]-2,3-dimethyl-4-carboxymethoxy-1,3-dioxolane) (3). The latter yielded (R)-L-2,3-O-isopropylenediglycerol ([R]-2,2-dimethyl-4-hydroxymethyl-1,3-dioxolane) (4) on reduction with lithium aluminium hydride (11) and (R)-L-[1,1-2H2]-2,3-O-isopropylenediglycerol ([R]-2,2-dimethyl-4-hydroxy[1-13C6]methyl-1,3-dioxolane) (5) with lithium aluminium deuteride (12). Acid hydrolysis of the latter yielded (S)-[1,1-2H2]glycerol (6) (Scheme 1). In analogous steps (R)-d-serine (7) gave (S)-d-[1,1-2H2] 2,3-O-isopropylenediglycerol ((S)-2,2-dimethyl-4-hydroxy[1-13C6]methyl-1,3-dioxolane) (11) and (R)-L-[1,1-2H2]glycerol (12).

The 1H NMR spectra of methyl and ethyl esters of 2,3-O-isopropylenediglyceric acid (methyl ester: RS (12), S (11, 13); ethyl ester: RS (19), isopropyleneglycol (RS, Solketal (20-22), R (13), 2,3, 24, S (13, 26)). (S)-[1,2,3,4,5,6-13C6]-2,3-O-isopropylenediglycerol (14), and RS-[1,1-2H2]-2,3-O-isopropylenediglycerol (12) have been reported but have been incorrectly (22) or incompletely assigned. A full assignment of signals including those due to each of the two diastereotopic geminal methyl groups and each of the two diastereotopic protons of the CH3 group of the dioxolane ring is now possible (Fig. 1).

These assignments are based on nuclear Overhauser effect measurements. It had previously been assumed (20), on the basis of empirical considerations but without direct evidence, that the signal due to the methyl group in the cis position relative to the R group, in a 2,2-dimethyl-4-ethyl-substituted-1,3-dioxolane such as 9 or 10, would appear at higher frequency (i.e. downfield) than that due to the trans-methyl group. This assumption turns out to have been correct.

NMR Spectra of 13C- and 1H-Labeled Samples of Pyridoxol—The samples of pyridoxol hydrochloride which were isolated from E. coli B W2 which had been incubated with D-[1,2,3,4,5,6-13C6]glucose (Experiment 1) and (R)- and (S)-[1,1-2H2]glycerol plus [2-13C6]glycerol (Experiments 2 and 3) were analyzed by NMR spectroscopy. The 13C NMR spectrum of pyridoxol hydrochloride from Experiment 1 is shown in Fig. 3. The 1H NMR spectra of the samples from Experiments 2 and 3 are shown in Fig. 4 (B and C), which also shows a 1H NMR spectrum of pyridoxol hydrochloride (A). An interpretation of the spectra is offered in the discussion which follows.

Degradation of 13C,1H-Labeled Pyridoxol Derived from 2-13C, 1-1H2Glycerol—The sample of pyridoxol was degraded by the reaction sequence shown in Scheme 2. The 1H/13C ratio of the final product, tri-O-acetyl-6-chloropyridoxol (17), was identical with that of the initial pyridoxol sample. The results of the degradation are summarized in Table II.

Bacterial Strains, Conditions of Culture, and Isolation of Pyridoxol Hydrochloride

Mutant strain W202 of E. coli B was obtained from W. B. Dempsey (Veterans Administration Hospital, Dallas, TX). Culture conditions and general methods of administering labeled compounds have been described (2).

Labeled pyridoxol hydrochloride was isolated and purified by established methods (2) after dilution with unlabeled carrier, from the cultures of E. coli B W2 which had been incubated with D-[1,2,3,4,5,6-13C6]glucose (Experiment 1) and with (R)- and (S)-[1,1-2H2]glycerol (Experiments 2 and 3). The details of the tracer experiments are summarized in Table I.
Biosynthesis of Vitamin B<sub>6</sub> from Triose Units

### TABLE I

Incorporation of labeled substrates into pyridoxol

| Experiment no. | Substrate | Enrichment or specific activity | Total weight or total activity | Culture medium volume | Pyridoxol HCl carrier added |
|---------------|-----------|---------------------------------|--------------------------------|-----------------------|----------------------------|
| 1             | d-[1,2,3,4,5,6-<sup>14</sup>C]Glucose | 99% <sup>13</sup>C | 0.2 g | 0.8 g | 270 μCi/mmol | 12.5 μCi | 1 liter | 250 μCi |
| 2             | (R)-[1,1,<sup>2</sup>H<sub>2</sub>]Glycerol + [2-<sup>14</sup>C]glycerol | | | | | | | 1 liter | 50 μCi |
| 3             | (S)-[1,1,<sup>2</sup>H<sub>2</sub>]Glycerol + [2-<sup>14</sup>C]glycerol | | | | | | | 24 mCi/mmol | 250 μCi |

**FIG. 1.** 500.13-MHz <sup>1</sup>H NMR spectra of chiral isopropylidene derivatives. A, isopropylidene derivative of methyl glycerate ([(R)-2,2-dimethyl-4-carbomethoxy-1,3-dioxolane (9)]. Chemical shifts: δ 3.19 (3H, CH<sub>3</sub>-si), 1.49 (3H, CH<sub>3</sub>-re), 3.75 (3H, CO<sub>2</sub>CH<sub>3</sub>), 4.09 (1H, dd, H-5re), 4.22 (1H, dd, H-5si), 4.59 (1H, dd, H-4) ppm. Coupling constants (±0.1 Hz): J<sub>H-5-si,H-4</sub> = -8.5, J<sub>H-5-re,H-4</sub> = 5.2, J<sub>H-5-re,H-5si</sub> = 5.2 Hz (cf. Refs. 11 and 13). B, isopropylidene derivative of [1,1-<sup>2</sup>H<sub>2</sub>]glycerol ([(S)-2,2-dimethyl-4-hydroxy[<sup>2</sup>H<sub>2</sub>]methy1-1,3-dioxolane (11)]. Chemical shifts: δ 3.19 (3H, CH<sub>3</sub>-si), 1.43 (3H, CH<sub>3</sub>-re), 3.76 (1H, dd, H-5re), 4.03 (1H, dd, H-5si), 4.20 (1H, dd, H-4) ppm. Coupling constants (±0.1 Hz): J<sub>H-5-si,H-4</sub> = -8.0, J<sub>H-5-re,H-4</sub> = 6.7, J<sub>H-5-re,H-5si</sub> = 6.7 Hz. C, isopropylidene derivative of glycerol (Soketal) ([(S)-2,2-dimethyl-4-hydroxymethyl-1,3-dioxolane (10)]. Chemical shifts: δ 3.15 (3H, CH<sub>3</sub>-si), 1.43 (3H, CH<sub>3</sub>-re), 3.76 (1H, dd, H-5re), 4.03 (1H, dd, H-5si), 4.20 (1H, dd, H-4) ppm. Coupling constants (±0.1 Hz): J<sub>H-5-si,H-4</sub> = -8.2, J<sub>H-5-re,H-4</sub> = -11.6, J<sub>H-5-re,H-5si</sub> = 6.5, J<sub>H-5-si,H-5re</sub> = 6.6, J<sub>H-4,H-5si</sub> = 3.8, J<sub>H-4,H-5re</sub> = 5.3 Hz (cf. ref. 13).

**DISCUSSION**

In our earlier tracer work (2-6), we demonstrated that in E. coli B mutant WG2, the entire carbon skeleton of pyridoxol is derived from glycerol in a very specific manner: five of the eight carbon atoms of pyridoxol (C-2', C-3, C-4', C-5', and C-6) are derived from the primary carbon atoms of glycerol, and the other three (C-2, C-4, and C-5) from its secondary carbon atom. On the basis of these results we concluded that the CR skeleton of pyridoxol was derived from three glycerol units. We surmised that two glycerol units were incorporated intact, supplying the two C<sub>3</sub> fragments, C-3/C-4/C-4', and C-5'/C-5/C-6 of the vitamin; whereas the third glycerol unit loses one of its primary carbon atoms on route to supplying the C<sub>2</sub> unit, C-2'/C-2, of the vitamin.

Experiments with specifically <sup>14</sup>C-labeled samples of pyruvate and D-glucose supported this view. Label from [3-<sup>14</sup>C]pyruvate entered C-2' and no other site, and all activity from [2-<sup>14</sup>C]pyruvate was confined to C-2 of pyridoxol (2). Furthermore, addition of unlabeled pyruvate to a culture containing [2-<sup>14</sup>C]glycerol spared the incorporation of label into C-2 of pyridoxol, but did not affect its entry into C-4 or C-5 (6). Thus, the C<sub>2</sub> unit, C-2'/C-2, of pyridoxol originates from a C<sub>2</sub> unit derived from pyruvate by decarboxylation.

Since glycerol itself is not sufficiently reactive, chemically or biochemically, to undergo either the carbon-carbon bond-breaking reaction that leads to the C<sub>2</sub> unit serving as the precursor of C-2'/C-2 or the two carbon-carbon bond-making reactions that are required to produce the vitamin skeleton from two C<sub>3</sub> fragments and one C<sub>2</sub> fragment, we surmised...
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Further that it was much more likely that the reactive species were the triose intermediates of glycolysis, 3-phosphoglyceraldehyde and dihydroxyacetone 1-phosphate, which are generated from glycerol (2).

The normal source of these two triose phosphates is glucose. Glycolytic breakdown of D-[1-14C]- and D-[6-14C]glucose yields triose phosphates labeled at one site only, namely at the terminal carbon atom carrying the phosphate ester. Incorporation of these triose phosphates into pyridoxol would be expected to deliver label into only three sites: C-2', the carbon atom derivable from the methyl group of pyruvate; and two other sites, one in each of the C3 segments, either C-3 or C-4', but not both as was the case with [1-14C]glycerol, and either C-5' or C-6, but not both. Indeed, activity of pyridoxol derived from D-[1-14C]glucose (2, 3) or from D-[6-14C]glucose (3) was located at C-2', C-4', and C-5' and at no other site.

This, in summary, is the evidence on the basis of which we proposed that the C₃ units of pyridoxol, C-3/C-4/C-4' and C-5'/C-5/C-6, are derived from intact triose phosphate generated from glucose by the normal glycolytic sequence and that the C₂ unit, C-2'/C-2, of pyridoxol is generated from one such triose phosphate by loss of a terminal carbon (2, 4).

If this interpretation is correct, then only two carbon-carbon bonds within pyridoxol, C(2)-C(3) and C(4)-C(5), are generated de novo in the course of biosynthesis. All other carbon-carbon bonds of pyridoxol, C(2')-C(2), C(3)-C(4), C(4')-C(4'), C(5')-C(5), and C(5)-C(6), represent bonds already preformed within glucose serving as the precursor. The first objective of the present work was to submit this proposition to critical experimental examination.

**Fig. 3.** 75.47-MHz proton-decoupled 13C NMR spectrum of pyridoxol hydrochloride (6.5 mg in 60 μl of D₂O), derived biosynthetically from D-[1,2,3,4,5,6-13C₆]glucose (Experiment 1). The spectrum was determined on a Bruker AM 300 spectrometer. Spectral parameters were: 180,234 transients; spectral width, 18,500 Hz; pulse flip angle, 25°; acquisition time, 0.88 s; line broadening, 0.3 Hz; memory size, 32K, zero-filled to 128 K; final digital resolution, 0.28 Hz/data point. For chemical shifts and coupling constants, see Table III.

Furthermore, the sequence from glucose to pyridoxol via triose phosphates does not assign a function to glycerol, a compound which has been shown to serve as a source of all eight carbon atoms of pyridoxol (2, 5). In our biogenetic model we had assumed that glycerol acts as a precursor of pyridoxol because it serves as an alternative source of triose phosphate. The second objective of the present investigation was to test this assumption experimentally.

13C NMR Spectroscopy as a Probe in the Detection of Intact Bonds Transferred from Precursors into Product. Incorporation of [1,2,3,4,5,6-13C₆]Glucose into Pyridoxol—The use, in biosynthetic studies, of intramolecularly multiply 13C-enriched substrates which are 13C-enriched at contiguous carbon atoms and the detection, by means of 13C NMR spectroscopy, of incorporation into biosynthetic products of intact fragments of such "bond-labeled" precursors was pioneered by Seto and co-workers (27-29) who were the first to employ intramolecularly doubly 13C-enriched acetate, i.e. sodium [1,2-13C₂]acetate, in the study of polyketide biosynthesis. The 13C NMR spectrum of the product into which an intact precursor-derived 13C-13C unit is incorporated exhibits characteristic signals due to the enriched carbon sites. These signals appear as doublets (or higher multiplets if a 13C-13C-13C unit had entered the product) as a consequence of coupling of contiguous 13C atoms (spin ½).

We have examined the 13C NMR spectrum of a sample of pyridoxol isolated from cultures of E. coli B mutant WG9 which had been incubated with a mixture consisting of 20%...
formed during the biosynthetic process. Furthermore, the $^{13}$C-$^{13}$C spin-coupling pattern will indicate the size of each intact multicarbon unit and therefore the number of intact units that had been used to form the pyridoxol skeleton from glucose. Thus, the signals corresponding to carbon atoms that entered as part of an intact two-carbon unit should appear as triplets, composed of a central line (due to natural abundance $^{13}$C and, if present, due to enriched $^{13}$C attached to $^{12}$C) straddled by a doublet caused by $^{13}$C-$^{12}$C coupling between the enriched carbon atoms of the $^{12}$C$_3$ unit. In the same way, carbon atoms that entered pyridoxol as the primary carbons of an intact $^{13}$C$_3$ unit are bonded to a single neighbor, i.e. to the central carbon of the unit, and their signals will also appear as triplets. The central carbon of such a $^{13}$C$_3$ unit, being bonded to two primary carbons, will yield a quintet, composed of a doublet on each side of a central line. In turn, more complex but equally predictable signals would be associated with carbon atoms that are derived from interior carbons of larger intact $^{13}$C units.

The sample of pyridoxol, whose $^{13}$C NMR spectrum was determined (Fig. 3), consisted of a mixture of newly biosynthesized pyridoxol (~80 μg/l-litter culture), derived from E. coli B WG2 which had been incubated in the presence of a 1:4 mixture of $[1,2,3,4,5,6,^{13}$C$_6]$glucose (99% $^{13}$C per carbon atom) and unenriched glucose (1.1% $^{13}$C per carbon atom), and natural abundance pyridoxol (2.5 mg) which had to be added as carrier in order to facilitate isolation and purification of the enriched sample.

From the composition of this mixture, the relative signal areas of the central line, due to the natural abundance component of each signal, and of the multiplet straddling the central line, due to the coupled $^{13}$C-enriched component, can be calculated. The calculated value$^1$ for the ratio, area of the central line/total area of the outer doublet(s), is 64:36. Thus, the central line of each carbon signal contributes 64%, whereas the outer lines together contribute 36% of the total signal area. For example, each line of the outer doublet associated with the signal from a carbon atom, which is attached to a single neighbor, will contribute 18% to the total signal area; whereas each line of the two outer doublets, which are associated with the signal from a carbon that enters pyridoxol as the central carbon of an intact C$_3$ unit, will constitute 9% of the total signal area.

The $^{14}$NMR spectrum of the enriched sample of pyridoxol (Fig. 3) shows that each of the signals, due to C-2', C-2, C-3, C-4', C-5', and C-6 of pyridoxol, appears as a central line, whereas the outer lines together contribute 36% of the total signal area.
relative area of 64, within experimental error, straddled by a doublet, total relative area 96, within experimental error (see Table III). Thus, each of these six carbon atoms shows direct coupling to only one other carbon. The two carbon atoms, C-4 and C-5, on the other hand, must each be the central carbon atom of a C3 unit: the signals due to these two carbon atoms (Fig. 3) consist of a central line, relative area 82, straddled by a doublet, total relative area 18 (Table III). This indicates that the signal is in fact degenerate, consisting of a central natural abundance line, relative area 64, straddled by a pair of doublets, total relative area 36. The inner lines of the pair of doublets are not resolved from the central line.

Comparison of the measured separation of the outer lines with calculated values confirms this interpretation. The separation of the outer lines, 109.6 Hz for C-4 and 112.6 Hz for C-5 (Table III), corresponds to that calculated from the measured values of the coupling constants $J_{c,4,c,5} = 64.5$ Hz, $J_{c,4,c,4} = 45.1$ Hz and $J_{c,4,c,5} = 48.8$ Hz, $J_{c,4,c,5} = 64.9$ Hz, respectively (Table III). The calculated separation of the outer lines is 64.5 + 45.1 + 64.9 = 174.5 Hz for C-4 and 64.9 + 48.8 + 64.9 = 178.6 Hz for C-5, in good agreement with the observed values.

The calculated values for the separation of the inner lines of the doublets is 64.5 = 45.1 = 19.4 Hz for C-4 and 64.9 - 48.8 = 16.1 Hz for C-5. These lines are not resolved from the central line due to the signal width at the base of the central lines (22 Hz). The relatively simple appearance of the $^{13}$C NMR spectrum indicates that no carbon fragment larger than C3 is implicated in the biosynthesis of pyridoxol from glucose. From the coupling constants and relative areas it can be inferred that there is biosynthetic connectivity between C-2' and C-2, but not between C-2 and C-3. Thus, the bond C(2)-C(3) is newly formed in the course of biosynthesis. Similarly, each of C-3 and C-4' and each of C-6 and C-5' is shown to have biosynthetic connectivity to a neighbor, C-4 and C-5, respectively. However, there is no measurable coupling between C-4 and C-5 (predicted ~65 Hz, cf. $J_{c,4,c,4}$ and $J_{c,5,c,5}$; Table III) or between C-3 and C-6 (predicted ~14 Hz, cf. pyridine $J_{c,3,c,5} = 14$ Hz (30)). Thus, there is no connectivity between C-4 and C-5, and the bond C(4)-C(5) is also newly formed in the course of biosynthesis.

These results establish that, in E. coli B, pyridoxol is derived by the combination of a C3 unit, which supplies C-2'/C-2 of the vitamin, and two C3 units, which supply C-3/C-4/C-4' and C-5'/C-5/C-6. These three units are derived intact from glucose. Thus, the inferences that were drawn from earlier tracer experiments (2-6) are confirmed. On the basis of this tracer work with $^{14}$C-labeled substrates, we had inferred that two bonds of pyridoxol, C(2)-C(3) and C(4)-C(5), were newly formed in the course of the biosynthesis of pyridoxol from glucose. The present experiment provides direct experimental support for the earlier inference and shows that C(4)-C(3) and C(4)-C(5) are, indeed, the only carbon-carbon bonds of the pyridoxol skeleton which are formed de novo in the course of its biosynthesis from glucose.

$^1$H NMR Spectroscopy as a Probe of Biosynthetic Prochirality: Incorporation of (R)- and (S)-[1,1-$^3$H$_2$]Glycerol into Pyridoxol—It has been shown by means of tracer experiments with [1-$^{14}$C]-, [1,3-$^{13}$C]$, - and [2-$^{14}$C]glycerol (2, 5) that all eight carbon atoms of pyridoxol are derived from the carbon atoms of glycerol in nonrandom fashion. Glycerol is a prochiral molecule. As a consequence of this prochirality, there are several modes whereby glycerol can yield the two C3 units and the C4 unit which combine to yield the carbon skeleton of pyridoxol.

These three units are derived from glycerol in such a manner that label from D-[1-$^{14}$C]glyceraldehyde and from D-[6-$^{14}$C]glycerol enters C-4' of the C3 unit, C-4'/C-4/C-3, C-5' of the C3 unit, C-5'/C-5/C-6, and C-2' of the C2 unit, C-2'/C-2 (2, 3). This mode of incorporation of label, together with the now proven maintenance of the integrity of bonds present within glucose on the route into pyridoxol, is consistent with the intermediacy between glucose and pyridoxol of the normal glycolytic triose phosphate intermediates, 3-phosphoglycerate and dihydroxyacetone 1-phosphate, whose phosphorylated primary carbon atoms then give rise to C-4' and C-5' and, via the methyl group of pyruvate, to C-2' of the C2 unit, C-2'/C-2.

If glycerol entered pyridoxol by way of the same intermediates, its prochiral primary carbon atoms must be incorporated into pyridoxol in a specific and predictable manner. This supposition can be tested by means of incorporation experiments with (S)-[1,1-$^{3}$H$_2$]glycerol and (R)-[1,1-$^{3}$H$_2$]glycerol. Samples of these compounds were prepared by standard methods (see "Materials and Methods").

In order to confirm that experiments with these chiral samples of deuterium-labeled glycerol would yield unequivocal results, it was necessary to determine which of the hydrogen atoms of pyridoxol are derived directly from glycerol. It is clear that only seven of the eight hydrogens at C-2', C-4', C-5', and C-6 of the vitamin, can be so derived since the eighth, one of the three methyl protons at C-2', must have entered the course of the conversion of a C$_2$H$_4$OH group of glycerol into the C$_2$OH group of pyruvate. Furthermore, the results of a tracer experiment with [2-$^{14}$C, 1,3-$^{3}$H$_2$]glycerol as the precursor (6) showed that one of these seven protons is lost in the course of the biosynthesis of the vitamin. The sample of [2-$^{14}$C, 1,3-$^{3}$H$_2$]glycerol that was administered to the E. coli B WG2 culture had a $^3$H/$^{14}$C ratio of 15.4 ± 0.4. Retention within pyridoxol of seven tritium atoms/three glycerol units, the theoretical maximum, should have yielded a sample of pyridoxol, $^3$H/$^{14}$C = 9.0. The observed ratio, $^3$H/$^{14}$C = 7.7, indicated that one of these seven tritium atoms had not been incorporated into the product (6).

Degradation of the pyridoxol sample ($^3$H/$^{14}$C = 7.7) by the reaction sequence shown in Scheme 2 established the site of the missing tritium. 6-Chlororotri-O-acetylpipridoxol (17) was obtained from the pyridoxol sample by way of tri-O-acetylpyridoxol 1-oxide (16). 3,4'-O-Isopropyldeneoxydipyrrol (14) was also prepared (2). These three derivatives showed the same $^3$H specific activity, the same $^{14}$C specific activity, and therefore the same $^3$H/$^{14}$C ratio as the pyridoxol hydrochloride (13) from which they were obtained (Table II). Since 6-chlororotri-O-acetylpipridoxol (17) showed the same $^3$H/$^{14}$C ratio as the other three compounds, even though it lacks the proton at C-6 that is present within the other compounds, C-

### Table III

| Carbon atom | Chemical shift (δ) | % of total signal area | $^1$C/$^{13}$C coupling constants | $^1$C/$^{13}$C coupling reactions |
|-------------|------------------|-----------------------|-----------------------------------|---------------------------------|
| C-2'        | 16.5             | 60 ± 3                | 20 ± 1                            | $^1$J_{c,5} = 64.6             |
| C-2         | 144.7            | 62 ± 3                | 19 ± 1                            | $^1$J_{c,4} = 64.5             |
| C-3         | 154.7            | 67 ± 4                | 17 ± 1                            | $^1$J_{c,4} = 64.5             |
| C-4         | 142.5            | 84 ± 8                | 6 ± 0.5                           | $^1$J_{c,4} = 45.1             |
| C-4'        | 69.0             | 60 ± 3                | 20 ± 1                            | $^1$J_{c,5} = 48.8             |
| C-5'        | 66.1             | 55 ± 3                | 21 ± 1                            | $^1$J_{c,5} = 48.8             |
| C-5         | 138.8            | 82 ± 4                | 9 ± 0.5                           | $^1$J_{c,5} = 46.9             |
| C-6         | 131.8            | 64 ± 3                | 18 ± 1                            | $^1$J_{c,5} = 3.7              |

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6 of the pyridoxol generated from [2-13C, 1,3-3H]glycerol must have been the site into which tritium of the precursor had not been incorporated in the course of biosynthesis. Another degradation, Kuhn-Roth oxidation (2), that localized the tritium label at C-2' (Scheme 2) further substantiated this conclusion. These results, which are summarized in Table II, prepared the ground for the experiments with (R) [1,1-'H2] and (S)-[1,1-'H2]glycerol.

In two separate experiments, cultures of E. coli B W2G were incubated in the presence of (R)-[1,1-'H2]glycerol (1 g/liter) (Experiment 2) and (S)-[1,1-'H2]glycerol (1 g/liter) (Experiment 3). Tracer amounts of [2-13C]glycerol (250 µCi/liter) were also added to each incubation. Pyridoxol hydrochloride was isolated from each culture after carrier dilution and a derivative, isopropylidene-pyridoxol, was prepared in each case. The samples were radioactive. The samples from the experiment with (R)-glycerol (Experiment 2) had specific activities of 7.5 x 10^5 dpm/mmol (pyridoxol hydrochloride) and 7.4 x 10^5 dpm/mmol (isopropylidene-pyridoxol hydrochloride), respectively. The corresponding samples from (S)-glycerol (Experiment 3) had specific activities of 3.0 x 10^5 and 3.1 x 10^5 dpm/mmol, respectively. Each of the two samples of pyridoxol hydrochloride was dissolved in methanol (~50 µl) and examined for deuterium content by 1H NMR spectroscopy. The spectra of the two samples (Fig. 4, B and C) were different.

The spectrum in methanol of the sample of pyridoxol hydrochloride obtained from the incubation with (R)-deuterio-glycerol (Fig. 4B) showed, after only 600 scans, three signals in addition to the natural abundance signals of the solvent (3.30 ppm, CDH2; 4.95 ppm, OD) (Fig. 4D). The three signals, of equal intensity, at 2.54, 4.65, and 5.00 ppm, are assigned to deuterium at C-2', C-5', and C-4' of the vitamin, respectively. These carbon atoms are therefore derived from the re-hydroxymethyl carbon atom of glycerol, which is incorporated into three sites of the vitamin with retention of its proton pair.

Even after 28,000 scans, the spectrum (Fig. 4C) of the pyridoxol obtained from the incubation with (S)-deuterio-glycerol did not differ from that of the solvent (Fig. 4D). Two signals, at 3.30 and 4.95 ppm, i.e. the natural abundance signals of the solvent, were apparent and were the only signals in both spectra. Thus, no deuterium, above natural abundance, was present within the pyridoxol that had been isolated from the incubation with (S)-[1,1-'H2]glycerol. Since this sample was radioactive (3.0 x 10^5 dpm/mmol, see above), biosynthesis of pyridoxol had occurred in this incubation. Since no deuterium was present, it follows that C-2', C-4', and C-5', the three pyridoxol carbon atoms derived from the re-hydroxymethyl carbon of glycerol, are not derived from the si-hydroxymethyl carbon of glycerol. Thus, glycerol enters the pyridoxol skeleton in a stereospecific manner. C-3 and C-6 of glucose or from the re-hydroxymethyl group of glycerol, whereas the other terminal carbon atom originates stereospecifically from the si-hydroxymethyl group of glycerol.

The observed stereochemistry in the stereospecific incorporation of label from prochirally labeled samples of glycerol into the C2 segments of pyridoxol is thus entirely consistent with the observed regiochemistry in the regiospecific incorporation of label from specifically labeled samples of glucose.

Similar consistency is observed with respect to the origin of the C2 segment, C-2'/C-2', of pyridoxol. This moiety arises from a C2 unit derived from pyruvate by decarboxylation. The methyl group of pyruvate is known to be derived from either C-1 and C-6 of glucose or from the re-hydroxymethyl group of glycerol. This is the incorporation pattern into C-2' of pyridoxol that is indeed observed.

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