**Biosynthesis of Vitamin B₆**

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**SUMMARY**

The biosynthesis of vitamin B₆ was studied in an *Escherichia coli* mutant, blocked between pyridoxal and pyridoxal. Specific incorporation of glycerol, pyruvate, serine, and glucose was shown by chemical degradation of labeled samples of pyridoxal isolated from the medium of cultures which had been incubated with various ¹⁴C-labeled radiomers of these substrates.

The observed pattern of incorporation of ¹⁴C shows that pyridoxal is derived from 3 triose units. One of these is incorporated by way of pyruvate, as a 3-carbon unit at the oxidation level of acetaldehyde. The other 2 triose units are incorporated intact.

A hypothetical sequence for the biogenesis of pyridoxal is advanced on the basis of the tracer evidence. 5-Deoxyxylulose 1-phosphate and a branched chain 8-carbon sugar are postulated as intermediates.

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Past attempts to identify the primary precursors of vitamin B₆ and to define the steps of its biosynthesis have failed to yield clear-cut results. Neither investigations with organisms containing metabolic blocks (3-17) nor tracer studies (10, 17-25) have led to firm conclusions. Inferences drawn by different investigators were, in some cases, mutually contradictory.

D-L-Alanine, L-aspartate, L-cysteine, D-glutamate, and DL-leucine each promoted the growth of a pyridoxal auxotroph of *Escherichia coli* (7). Resting cells of a soil microorganism, identified as a strain of *Flavobacterium*, generated approximately 3 times as much pyridoxal on incubation with glycerol alone, or with glycerol in the presence of other amino acids, *e.g.* L- or D-alanine, L-cysteine, L-serine, or glycine (9, 10, 17).

An early view, attractive on structural grounds, that alanine might serve as a precursor of pyridoxal, was based on the observation that, in *Streptococcus faecalis* and in *Lactobacillus casei*, D-alanine replaced the vitamin as a growth factor (3, 4). This view had to be abandoned when it was shown that cells grown with D-alanine did not synthesize the vitamin (5), and that vitamin B₆ was required for formation of D-alanine, rather than the reverse (6).

Of a series of α-amino acids whose effectiveness as a sole carbon source for the yeast, *Candida albicans*, was investigated, aspartate and glutamate induced maximal accumulation of pyridoxal (11). Of the Krebs cycle acids tested, malic acid and citric acid were the most effective in supporting pyridoxal production (12). With a mixture of glyoxylate and acetate as the sole carbon source, the growth rate of the yeast as well as the yield of pyridoxal was high, whereas glyoxylate alone did not support growth. These results were interpreted to indicate that the glyoxylate cycle played a part in the biosynthesis of pyridoxal from dicarboxylic acids (13).

Glycolaldehyde, together with either glycine or serine, replaced vitamin B₆ in promoting the growth of two B₆-requiring strains of *E. coli*, and supported the synthesis of the vitamin (8). On the basis of a recent study of serine-requiring mutants of *E. coli* it was suggested that it was 3-phosphoserine, rather than serine, which may be a precursor of pyridoxal (14-16), and that activity from [3-¹³C]serine might be incorporated by way of 1-carbon intermediates. However, label from [¹³C]formic acid or from [methyl-¹³C]methionine did not enter the product in this system. Nor did [¹³C]aspartic acid serve as a precursor (23).

Radioactivity from [2-¹⁴C]succinic acid entered pyridoxal in the yeast, *C. albicans* (29), and label from [3-¹⁴C]serine, [1-¹⁴C]alanine, and [1-¹⁴C]- and [2-¹⁴C]acetate acid was reported to be incorporated into pyridoxamine in *Candida utilis* (18) (but see below). No attempt was made to determine the sites of labeling in these samples. Pyridoxamine, isolated from cultures of *C. utilis* which had been incubated with [1, 3-¹⁴C]glyceraldehyde, [3-¹⁴C]pyruvic acid, [4-¹³C]aspartic acid, [1-¹⁴C]succinic acid, [¹³C]formic acid, [7-¹⁴C]nicotinic acid (16), [U-¹⁴C]aspartic acid, [7-¹⁴C]tryptophan, or [8-¹⁴C]quinolinic acid failed to show activity beyond the limits of confidence of the determination.


In the strain of *Flavobacterium*, already referred to (see above), label from DL-[2-¹⁴C]aspartic acid entered pyridoxal. The product was shown, by partial degradation, to be nonrandomly labeled. Its aminomethyl (C-4') and hydroxymethyl (C-5') carbon atoms were essentially free of activity (19). Surprisingly, on the other hand, it was reported very recently by the same authors that in the pyridoxal, derived from [2-¹⁴C]aspartic acid in the same organism, these 2 carbon atoms contained 50% of the total activity (25).

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Aspartic acid, oxalacetic acid, or homoserine.

Such as aspartic acid, oxalacetic acid, or homoserine.

Theoretical models for the biogenesis of the pyridoxine skeleton, nitrogen source (Scheme 3) (28).

Be derived from mevalonic acid, isoleucine, leucine, or valine.

Again, in Scheme 3, the CL unit might be related to citric acid, and the branched C5 unit might originate from alanine or from any of the C3 precursors excluding the isoleucine precursor, o-dihydroxy-p-methylvaleric acid.

Structural formulation of this scheme was not attempted (20).

The major difficulty in testing these schemes or other hypothetical models for the biogenesis of the pyridoxine skeleton, which can be arbitrarily constructed on the basis of structural relations of putative precursors to the product, and by analogy with the known origin of other naturally occurring pyridine derivatives (29), is the minute quantity of vitamin B₆ (40 to 200 ng per mg of dry cell material) (30, 31) which is present in systems that produce it. Success in biosynthetic tracer studies of the origin of B₆ is thus contingent on a high radiochemical yield in the incorporation of radioactive tracer, such that the specific activity of the product, even after many-fold dilution with inactive carrier, is still high enough for degradation studies.

The choice of carrier is dictated mainly by the relative abundance in the experimental organism of the various forms of vitamin B₆ (32). Since in yeasts pyridoxamine appears to be the most abundant form of the vitamin (33, 34) and since its isolation from the yeast C. utilis has been reported in detail (18), our early experiments were carried out with this yeast. Samples of pyridoxine hydrochloride isolated from C. utilis (A.T.C.C. 9950) (18) after incubation in the presence of [1,3-14C]glycerol or of [α-14C]alanine appeared to maintain a high and constant level of radioactivity after repeated recrystallization. However, when these purified samples were subjected to sublimation in a high vacuum as a final purification step, the sublimed samples of pyridoxamine hydrochloride so obtained were totally inactive. In each case all activity remained associated with minute amounts of nonvolatile impurities. C. utilis thus appeared to be unsuitable for a study of vitamin B₆ biosynthesis.

In 1965 to 1966, W. B. Dempsey described a mutant of E. coli B (BG-2) (35, 36) blocked at the oxidation step between pyridoxol 5'-phosphate and pyridoxal 5'-phosphate. This mutant is capable of synthesizing pyridoxol 5'-phosphate and pyridoxal, but does not grow unless pyridoxal is supplied. When a growing culture is deprived of pyridoxal, growth ceases, but under these conditions the organism continues, for about 3 hours, to produce pyridoxol 5'-phosphate and pyridoxal, at a rate 4 times that found in wild type E. coli B. The newly synthesized pyridoxol and pyridoxal 5'-phosphate are excreted into the culture medium (37).

The tracer experiments, here described, made use of this E. coli B mutant. Labelled substrate was added to cultures at the onset of pyridoxal starvation and incubation was continued for 4 to 5 hours. Samples of labeled pyridoxal, isolated by carrier dilution from the culture medium, were chemically degraded by unambiguous steps in such a way that the distribution of 14C within the carbon skeleton could be deduced.

The pattern of incorporation of 14C which was observed shows that pyridoxal is derived from 3 triose units. Two of these are incorporated intact, yielding the C3 units of pyridoxol, C-3,-4,-5 and C-5′-5′-6′, respectively. The third, which yields the C4 unit, C-2′-2′, is incorporated via pyruvate, as a 2-carbon unit at the oxidation level of acetaldehyde.

Materials and Methods

Organism

The organism used in this investigation was a pyridoxal auxotroph of E. coli, obtained from W. B. Dempsey. Its original designation, E. coli B-B₆-2 (36) was recently changed and the mutant has now been reclassified as E. coli B-WG2 (38). The mutant grows well on a medium supplemented with pyridoxal, but cannot utilize pyridoxol.

We are greatly indebted to Dr. W. B. Dempsey for supplying an isolate of E. coli B mutant WG2.
Stock cultures were maintained on monthly slants of minimal medium containing glycerol (0.5%) as a carbon source, supplemented with pyridoxal hydrochloride (6 × 10⁻⁴ M). These slants were incubated for about 48 hours at 37°C and then stored at 4°C.

**Media**

All bacterial stocks were maintained on a minimal medium containing glycerol (0.5%) as the carbon source, and inorganic salts (KH₂PO₄, 7 g; K₂HPO₄, 3 g; (NH₄)₂SO₄, 1 g; MgSO₄, 0.1 g; CaCl₂, 0.01 g, made up to 1000 ml with glass-distilled water) (39), supplemented with pyridoxal hydrochloride (final concentration 8 × 10⁻⁷ M). This medium will be referred to as supplemented glycerol (0.5%) medium. When solid medium was required Bacto agar (20 g) was added. Pyridoxal solutions were sterilized by filtration.

In all tracer experiments the same salts medium was used, but the glycerol concentration was reduced to 0.2 or 0.1% (see Table I), and pyridoxal supplementation was omitted. These media will be called unsupplemented glycerol (0.2%) medium and unsupplemented glycerol (0.1%) medium, respectively.

In one experiment (Experiment 1) glycerol was replaced by glucose (0.2%) and in another (Experiment 14) by glucose (0.1%). These media will be referred to as unsupplemented glucose (0.2%) and (0.1%) medium, respectively.

**Reisolation Procedure**

Mutant stocks were reisolated before each tracer experiment. Stock cells were suspended in sterile distilled water (10 ml) and the absolute count of this suspension was estimated by means of a hemocytometer. Successive dilutions in sterile distilled water yielded a final suspension containing approximately 200 cells per ml. Samples of this suspension (0.5 ml) were spread on plates of supplemented glycerol (0.5%) medium. The plates were incubated at 37°C to yield single colonies of approximately 2-mm diameter.

Two series of plates were prepared, one of supplemented glycerol (0.5%) medium, the other of unsupplemented glycerol (0.5%) medium. Each plate was allotted 20 inoculation sites, 80 sites were used on each medium. Both media were inoculated at a given site with cells from a single colony. The plates were incubated (37°C) and inspected for growth after 36 hours. Cells from supplemented sites, which had no growth at the corresponding unsupplemented site, were subcultured onto slants of supplemented glycerol (0.5%) medium. These slants were incubated (37°C, 24 hours) before use in a tracer experiment.

**Growth Experiment**

Supplemented glycerol (0.5%) medium (250 ml) was placed in a 1-liter Erlenmeyer flask. The medium was inoculated with freshly isolated cells and the culture incubated on a rotary shaker (37°C, 100 rpm). The optical density of the culture was measured by means of a Klett Summerson photoelectric colorimeter (model 800-3, fitted with a green filter) at zero time and every hour until a constant value was observed and stationary phase was thus evident. Distilled water was used as a blank in every measurement. The resultant growth curve was used to determine the period of logarithmic growth in all subsequent incubations.

**Labeled Compounds**

The labeled compounds used in individual tracer experiments are listed in Table I. Sixteen tracer experiments were carried out. In fourteen of these (Experiments 1 to 5, 7 to 12, 14 to 16) the ¹⁴C-labeled substrate was dissolved in sterile distilled water (10 ml), and this solution was added to the culture fluid by means of a disposable syringe. Mixtures of ¹³C-labeled compounds were used in the two remaining experiments. In Experiment 13 a mixture of L-iso-leucine and L-allo-iso-leucine was used. In Experiment 6 intermolecularly doubly labeled sodium [1,³¹³C]pyruvate of known distribution of activity was used which was prepared as follows.

**Sodium [1,³¹³C]pyruvate (Experiment 6)—Samples of sodium [¹³C]pyruvate (150 µCi) and sodium [³¹³C]pyruvate (150 µCi) were each dissolved in sterile distilled water (10 ml). Samples (4 × 10 µl) were withdrawn from each solution for liquid scintillation counting. The solutions were then mixed and made up to a volume of 25 ml with sterile water. This solution of intermolecularly labeled [1,³¹³C]pyruvate was added to the culture fluid in Experiment 6.**

Two samples (2 × 100 µl) were withdrawn from the solution of intermolecularly labeled [1,³¹³C]pyruvate. Each 100 µl was added to a solution containing sodium pyruvate (110 mg) in distilled water (100 ml). To each of these solutions were added 20 µl of a solution of phenylhydrazine hydrochloride, which had been prepared from 0.25 ml of phenylhydrazine, made up to 50 ml with 0.1 M hydrochloric acid. The reaction mixture was allowed to stand overnight, when the crystals of [1,³¹³C]pyruvic acid phenylhydrazone were filtered off and dried. For Kuhn-Roth oxidation this doubly labeled material was diluted with unlabeled carrier as follows.

[1,³¹³C]Pyruvic acid phenylhydrazone (11 mg) was dissolved in aqueous sodium hydroxide (0.1 M, 1 ml) and this solution was added to a solution of intermolecularly labeled [1,³¹³C]pyruvate was added to the culture fluid. The mixture was allowed to stand overnight. Crystals of the diluted [1,³¹³C]pyruvic acid phenylhydrazone (123 mg) were filtered off and dried. A portion of this product (60 mg, specific activity, (3.47 ± 0.02) × 10⁴ cpms mmole⁻¹) was subjected to Kuhn Roth oxidation by a method analogous to that described below for the Kuhn-Roth oxidation of pyridoxol hydrochloride) and the acetic acid so obtained was converted (see below) into acetyl-α-naphthylamide (specific activity, (1.65 ± 0.01) × 10⁴ cpms mmole⁻¹; relative specific activity (intact [1,³¹³C]pyruvic acid phenylhydrazone = 100 ± 1), 48 ± 1%).

**Incubations in Presence of Labeled Substrates**

Freshly isolated cells from a 24-hour slope were used to inoculate two 250-ml samples of supplemented glycerol (0.5%) medium in 1-liter Erlenmeyer flasks. The cultures were incubated on a rotary shaker (30°, 400 rpm) until the optical density indicated that growth was well into the exponential phase (approximately 12 hours). The cells were harvested by centrifugation for 10 min at 7000 × g were then washed with sterile distilled water (3 × 100 ml).

The cells were divided into four equal parts and each was resuspended in 250 ml of unlabeled glycerol (0.2%) medium in 1-liter Erlenmeyer flasks (Experiments 2 to 7, 9 to 13). The solution of radioactive tracer was equally divided among these flasks which were then incubated for 6 hours on a rotary shaker (30°, 400 rpm).

In Experiments 8, 15, and 16 the unsupplemented glycerol
(0.2%) medium was replaced by unsupplemented glycerol (0.1%) medium. In Experiment 1 unsupplemented glucose (0.2%) medium and in Experiment 14 unsupplemented glucose (0.1%) medium were used. In Experiment 6 four 250-ml samples of supplemented glycerol (0.5%) medium were inoculated and incubated, the harvested cells were then resuspended in six 300-ml samples of unsupplemented glycerol (0.2%) medium. Incubation conditions remained the same.

Isolation of Pyridoxol

Work-up of Bacterial Cultures—The contents of the culture flasks from each of the incubation experiments with labeled substrate were centrifuged 10 min at 7000 × g, and the supernatant solution decanted.

Cells remaining in the combined decanted solution were removed by filtration (0.2 μm membrane filter, Nagle Co.). The filtrate was concentrated to 200 ml in vacuo on a rotary evaporator and the concentrated solution was subjected to acid hydrolysis. Sulfuric acid (1 M, about 80 ml) was added until the final pH of the solution was 1.5, and the mixture was autoclaved (121°C, 3 hours). The hydrolysate was lyophilized and the residue stored at 4°C.

Chromatography—The lyophilized residue was dissolved in potassium acetate-acetic acid buffer (0.2 M, pH 4.5, 200 ml) and the solution was filtered through a fine sintered glass filter. Pyridoxol hydrochloride (5 mg) was added to the filtrate as a carrier, and this solution was applied to a cation exchange column, which had been prepared as follows: Dowex 50-X8 (200 to 400 mesh) was washed, in succession, with water, hydrochloric acid (3 M), water, potassium hydroxide (6 M), and water, until free of fine particles. This material was loaded into a column (15 × 1.5 cm) which was then washed with potassium hydroxide (6 M), followed by water, until the pH of the eluate was approximately 9.

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) (200 ml), adjusted to pH 6.6 by addition of sodium hydroxide (0.2 M).

Fractions (10 ml) were collected and assayed by ultraviolet spectroscopy in order to determine the position of pyridoxol in the elution sequence. Pyridoxol was eluted in the first six fractions following the addition of the final buffer (pH 6.6).

A second Dowex 50-X8 (200 to 400 mesh) column (6 × 1 cm) was prepared and washed with hydrochloric acid (0.1 M) until the eluate was acidic. The pyridoxol fractions from the first column were pooled, evaporated under reduced pressure, and redissolved in hydrochloric acid (0.1 M, 30 ml). Pyridoxol hydrochloride (15 mg) was added to this solution which was then applied to the second column. The column was eluted with distilled water until the eluate was neutral, and then with dilute ammonia (3%).

The ammoniacal eluate (10 ml) was evaporated under reduced pressure and the solid residue dissolved in a small volume of anhydrous methanol (2 ml). This solution was applied to a preparative plate for thin layer chromatography (thickness 2 mm, Silica Gel G according to Stahl). Development was for 15 cm in the solvent system tert-butyl alcohol-methyl ethyl ketone-ammonia (0.880)-water (4:3:2:1). The plate was dried and the pyridoxol band was identified by its characteristic blue fluorescence under ultraviolet light. The band was removed from the plate and the pyridoxol extracted from the silica gel by stirring overnight at 40°C in anhydrous methanol (40 ml). The resultant slurry was filtered through a fine sintered glass filter. The filtrate was reduced to a small volume (1 to 2 ml) and pyridoxol hydrochloride (10 to 20 mg) was added, together with a few drops of hydrochloric acid (0.1 M). On addition of anhydrous ether pyridoxol hydrochloride crystallized.

The product was repeatedly recrystallized from the same solvent system until successive crystal batches did not change in specific radioactivity. In several of the experiments (e.g., Experiments 2, 4, 6, 8, 14 to 16) a further quantity of inactive pyridoxol hydrochloride was added as a carrier at this stage. Final purification was effected by sublimation at 125-130°C and 2 × 10⁻³ mm. The yield of sublimed pyridoxol monohydrochloride, melting point 205-206°C (with decomposition) (reported melting point 204-205°C (with decomposition) (40, 41); 206-208°C (12)), corresponded, in general, to 70 to 80% of the total weight of inactive carrier (Table I) which had been added. The sublimed product was used for chemical degradation.

Systematic Degradation of ¹⁴C-Labeled Samples of Pyridoxol

Each of the labeled samples of pyridoxol, isolated from the tracer experiments (Table I), was degraded in order to determine radioactivity at individual carbon atoms. The methods employed for this purpose for the most part utilized known chemical reactions, which were adapted to small scale work and improved yields.

Carbon Atoms 2' and 2 as Acetic Acid (Scheme 4)

Kuhn-Roth Oxidation of Pyridoxol Hydrochloride (cf. Reference 48)—Pyridoxol hydrochloride (60 mg) was dissolved in dilute sulfuric acid (10 ml, 10% v/v), and chromium trioxide (2 g) was added. A slow stream of nitrogen was then passed through the solution which was slowly distilled, while its volume was maintained by repeated addition of 5-ml portions of water. Over approximately 4 hours 80 ml of distillate were collected. The distillate, containing acetic acid, was titrated to pH 7 with sodium hydroxide (0.1 M), and the solution was then evaporated at 90°C, yielding sodium acetate (24 mg, 72%).

Acetyl-α-naphthylamide (C-2',2) (44)—A portion of the sodium acetate (approximately 5 mg) was dissolved in water (1 ml) and a solution containing a slight molar excess of α-naphthylamine hydrochloride (15 mg) in water (1 ml) was added, followed by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (approximately 40 mg). Acetyl-α-naphthylamide, which solidified on stirring, was filtered off, washed with water, recrystallized from a mixture of benzene and hexane, and sublimed at 80-90°C and 10⁻³ mm. Yield was 5 mg. Melting point was 158-160°C (reported melting point 159-160°C (44)).

Schmidt Reaction of Kuhn-Roth Acetate and Conversion of Resulting Methylamine (C-2') into 2,4-Dinitro-N-methylalanine—A portion of the sodium acetate (approximately 15 mg) was heated with sodium azide (30 mg) and concentrated sulfuric acid (1 ml) on the steam bath in a flask attached to a system of gas traps. The carbon dioxide which evolved was passed by means of a stream of nitrogen into potassium hydroxide solution (10%). When gas evolution had ceased, the potassium hydroxide solution in the traps was replaced by methanol (15 ml) to which
2,4-dinitrofluorobenzene (0.1 ml) and sodium bicarbonate (0.5 g) had been added. The acidic reaction mixture was cooled to near freezing point in a Dry Ice-acetone bath, basified (pH > 12) by addition of potassium hydroxide solution (10%), and was then heated 3 hours on the steambath while the system was being flushed with nitrogen.

The methanol solutions from the traps were combined and evaporated. The residue was dissolved in ether (5 ml), and the ether solution washed with water (3 x 1 ml), and concentrated. The mixture was cooled to room temperature and filtered, the filtrate was evaporated in vacuo, the residue was dissolved in methanol and anhydrous hydrazine (95%, 5 ml) was refluxed 18 hours with exclusion of moisture. The solution was evaporated in vacuo at 100°, and the residue was dissolved in a mixture of methanol and tetrahydrofuran, from which hydrazine hydrochloride crystallized. The filtrate was evaporated, the residue dissolved in tetrahydrofuran, and a solution of hydrogen chloride in anhydrous ether was added. 4'-Deoxypyridoxol hydrochloride which precipitated was recrystallized from methanol-ether. Yield was 45 mg (81%). Melting point was 267-269° (reported melting point 274° (46)).

Kuhn-Roth Oxidation of 4'-Deoxypyridoxol—4'-Deoxypyridoxol hydrochloride (42 mg) was oxidized, by the method described above for the Kuhn-Roth oxidation of pyridoxol hydrochloride. The sodium acetate (22 mg, 60%) so obtained was converted into acetyl-a-naphthylamide (C-2',-4',2,-4) and into 2,4-Dimethyl-5-hydroxy-4-hydroxymethyl-5-o-hydroxybenzyl-3,4'-0-isopropylidene-2-methylpyridine (3,4'-0-Isopropylidene-5'-phenylpyridoxal) (25 mg) was dissolved in anhydrous tetrahydrofuran (2 ml), and phenyl magnesium bromide (60 mg) in tetrahydrofuran (1 ml) was added. The solution was refluxed 2 hours and dilute hydrochloric acid (0.1 M, 5 ml) was then added. The acid solution was extracted with chloroform (3 x 25 ml) and was then carefully basified with saturated sodium bicarbonate solution. The basic solution was extracted with chloroform (3 x 25 ml) and the extract dried (anhydrous potassium carbonate) and evaporated. The residue was sublimed in vacuo at 100-120° and 3 x 10⁻³ mm to give the 5'-phenyl derivative as a colorless solid, melting point 159-160°. Yield was 29 mg (70%). Melting point was 61-62° (reported melting point 62-63° (52), 64-65° (51)).

Benzoic Acid (C-5') by Oxidation of the 5'-Phenyl Derivative—The 5'-phenyl derivative (25 mg) was suspended in aqueous potassium permanganate (250 mg in 10 ml) and the mixture was heated 12 hours at 100° with stirring. Excess permanganate was destroyed by addition of sodium bisulfite and sulfuric acid (10%, v/v). The colorless solution was extracted with ether (3 x 25 ml), the ether extract was washed with water (10 ml), dried (anhydrous sodium sulfate), and evaporated. The residue was sublimed at 60-70° and 3 x 10⁻³ mm, to yield benzoic acid, 9 mg (70%), melting point 121-122°.

Carbon 4' (Scheme 7)

3,4'-O-Isopropylideneisopyridoxal (3) (48)—Anhydrous hydrogen chloride was passed through a cooled (0°) stirred suspension of pyridoxol hydrochloride (50 mg) in dry acetone (5 ml) until the solid had dissolved (approximately 5 min). The solution was kept 3 hours at -10°, anhydrous ether (10 ml) was then added, and the mixture was kept at -10° for a further 30 min. The product, 3,4'-O-Isopropylideneisopyridoxal hydrochloride, was washed with ether and dried. Yield was 55 mg (94%). Melting point was 209-211° (reported melting point 217-219° (49); 210-212° (with decomposition) (48)). The hydrochloride was converted into the free base by precipitation with sodium bicarbonate. Yield was 44 mg (87%). Melting point was 109-110° (reported melting point 108-109° (50), 111-112° (48), 113-115° (49)).

5-Formyl-3-hydroxy-4-hydroxymethyl-3,4'-O-isopropylidene-2-methylpyridine (3,4'-O-Isopropylideneisopyridoxal) (4) (51)—3,4'-O-Isopropylideneisopyridoxal (40 mg) was carefully dried in vacuo and dissolved in anhydrous pyridine (5 ml) containing acetyl-cu-naphthylamide (C-2'-4';2,-4) and into 2,4-Dinitro-N-methylaniline, which crystallized on standing, basified (pH > 12) by addition of potassium hydroxide solution (lo%), and was cooled to near freezing point in a Dry Ice-acetone bath, basified (10%, v/v). The colorless solution was extracted with ether (3 x 25 ml), the ether extract was washed with water (3 x 1 ml), and concentrated. The solution was refluxed 2 hours and dilute hydrochloric acid (5 M). Elution was continued until the eluate no longer showed absorption at 280 nm. The eluate was evaporated.
in vacuo and the residue crystallized from hot water, yielding colorless crystals of 3-methoxy-2-methylpyridine-4,5-dicarboxylic acid. Yield was 43.5 mg (61%). Melting point was 223-224° (reported melting point 224° (55)). Mass spectrum: M+ at m/e 167; nuclear magnetic resonance ((CD3)2SO): δ 2.40 (s, 3H), 3.86 (s, 3H), 7.62 (d (J = 0.5), 1H), 8.52 (d (J = 0.5), 1H) ppm. The samples of pyridoxol hydrochloride which were isolated weighed to the nearest microgram on a Micro Gramatic balance (Mettler Instrument Corp.) was weighed on aluminum planchets (diameter 3.2 cm) and then dissolved in suitable solvents. Aqueous sucrose solution (10% w/v) was used for pyridoxol hydrochloride. Dimethylformamide containing colloidon (1%) was found to be suitable for all other substances. Even distribution of the samples was ensured by the lens tissue technique, and the planchets were thoroughly dried before counting.

Radioactivity was assayed, on samples of finite thickness on aluminium planchets, with a low background gas flow Geiger counting system (Nuclear Chicago Corp., model 4342). The counting efficiency for 14C was approximately 30%. Corrections for background and self-absorption were applied. Samples for counting were prepared as follows. Solid material (0.5 to 3 mg, by carrier dilution and not of the original biosynthesized product. Confidence limits shown in the tables are standard deviation of the mean.

### RESULTS AND CRITERIA FOR THEIR INTERPRETATION

The samples of pyridoxol hydrochloride which were isolated by the carrier dilution technique from the medium of cultures of the E. coli B mutant W29 after incubation with various 14C-labeled substrates, maintained radioactivity after vigorous purification in all but 2 of the 16 tracer experiments. Details of these experiments are presented in Table I. It should be noted that the specific activity of pyridoxol hydrochloride given in Table I is that of the purified sample isolated by carrier dilution and not of the original biosynthesized product.

<table>
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<th>Table I</th>
<th>Incorporation of labeled substrates into pyridoxol</th>
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<td><strong>Substrate</strong></td>
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<tr>
<td>10</td>
<td>DL-[2-14C]Leucine</td>
</tr>
<tr>
<td>10</td>
<td>DL-[3-14C]Leucine</td>
</tr>
<tr>
<td>13</td>
<td>DL-[3,3',4,5-14C]Isoleucine</td>
</tr>
<tr>
<td>9</td>
<td>Sodium [14C]formate</td>
</tr>
<tr>
<td>12</td>
<td>L-[methyl-14C]Methionine</td>
</tr>
</tbody>
</table>
Table II

Specific activity of pyridoxol and its degradation products. C-2' and C-3, derived from various substrates

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Substrate</th>
<th>Pyridoxol SAd</th>
<th>Acetic acid (C-2',-2) SAd</th>
<th>Methylamine (C-2') SAd</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>[3-14C]Pyruvic acid</td>
<td>1.20 ± 0.03</td>
<td>1.15 ± 0.03</td>
<td>0.47 ± 0.01</td>
</tr>
<tr>
<td>4</td>
<td>[3-14C]Pyruvic acid</td>
<td>0.54 ± 0.01</td>
<td>0.68 ± 0.02</td>
<td>0.87 ± 0.02</td>
</tr>
<tr>
<td>5</td>
<td>[2-14C]Pyruvic acid</td>
<td>0.72 ± 0.02</td>
<td>0.64 ± 0.03</td>
<td>0.84 ± 0.02</td>
</tr>
<tr>
<td>6</td>
<td>[1,3-14C]Pyruvic acid</td>
<td>1.05 ± 0.02</td>
<td>0.27 ± 0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>7</td>
<td>DL-[3-14C]Serine</td>
<td>0.60 ± 0.01</td>
<td>0.12 ± 0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>8</td>
<td>[2-14C]Acetic acid</td>
<td>0.30 ± 0.01</td>
<td>0.13 ± 0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>9</td>
<td>DL-[3-14C]Aspartic acid</td>
<td>0.20 ± 0.01</td>
<td>0.08 ± 0.004</td>
<td>24 ± 2</td>
</tr>
<tr>
<td>9</td>
<td>DL-[3-14C]Glucose</td>
<td>0.31 ± 0.02</td>
<td>0.04 ± 0.002</td>
<td>24 ± 2</td>
</tr>
</tbody>
</table>

a Assayed as pyridoxol hydrochloride.
b Assayed as acetyl-α-naphthylamide.
c Assayed as 2,4-dinitro-N-methylaniline.
d SA = specific activity (counts min⁻¹ mmole⁻¹) × 10⁻⁴.

e RSA = relative specific activity (per cent) (pyridoxol = 100).

For calculation of the latter value an accurate assay of the amount of pyridoxol generated in each experiment (approximately 150 ± 50 µg) would have been required. Since knowledge of the pyridoxol content of the cultures was not crucial to the present investigation and since the requisite microbiological assay is time-consuming and tedious, such determinations were not carried out. The specific activity of the newly biosynthesized pyridoxol is therefore unknown, and without this value meaningful radiochemical yields cannot be calculated.⁴

Another index of incorporation efficiency is available, however. Since the weight of added carrier was at least two orders of magnitude larger than the approximate weight of pyridoxol originally present in the culture, and since the specific activity of the labeled pyridoxol isolated after carrier dilution is independent of the chemical yield obtained in the isolation, the total activity (counts min⁻¹) entering the product from a given substrate (i.e., carrier added (milligrams) × specific activity of product (counts min⁻¹ mmole⁻¹) / molecular weight of pyridoxol hydrochloride (205.5 mg mmole⁻¹)) serves as an approximate measure of the relative efficiency of different substrates as pyridoxol precursors. On this basis, glyceraldehyde (Experiments 1, 2, 15, 16) and glucose (Experiment 14) are the most efficient precursors (total activity recovered in the product per 0.1 mCi of substrate: 2.8 × 10⁸ to 5.4 × 10⁹ cpm). Pyruvate (Experiments 3 to 6) and serine (Experiment 8) fall into a second group (1.1 × 10³ to 2.4 × 10⁹ cpm). From acetate (Experiment 7), leucine (Experiment 10), aspartate (Experiment 11) or formate (Experiment 9) little activity entered the product ( < 1.0 × 10⁶ cpm). The product from the experiments with isoleucine (Experiment 13) and with methionine (Experiment 12) was inactive.

Distribution of label within the product was investigated (Table II) by partial degradation (Scheme 4) of the radioactive samples of pyridoxol by Kuhn-Roth oxidation. The acetic acid (isolated as the 2,4-dinitrophenyl derivative). Random incorporation of label into pyridoxol would have supplied one-eighth of the total activity to each of its carbon atoms, and would have resulted in the recovery of one-quarter of the total activity in the Kuhn-Roth acetate (C-2',-2) and one-eighth in the methylamine (C-2') obtained therefrom.

The Kuhn-Roth acetate derived from pyridoxol from the incubations with [2-14C]leucine (Experiment 10) and formate (Experiment 9) did indeed contain one-quarter of the total activity. Lack of material precluded further degradation of the acetate samples.

The result of Kuhn-Roth oxidation showed that distribution of label in all other samples of pyridoxol was nonrandom. The acetic acid (C-2',-2) isolated from the samples of pyridoxol derived from pyruvate (Experiments 3, 5, and 6) and serine (Experiment 8) contained 90% or more of the activity of the intact vitamin. Further degradation of the acetic acid to methylamine showed that almost all the activity of the pyridoxol derived from [3-14C]pyruvate (Experiment 4) and from [1,3-14C]glycerol (Experiment 6) was confined to C-2'. The pyridoxol derived from [2-14C]glucose (Experiment 7) or from [3-14C]aspartate (Experiment 11) contained approximately 60% of its total activity within the C₂ unit, C-2',-2.

More extensive degradation of the samples of pyridoxol derived from [1,3-14C]glycerol (Experiment 1, 2, 15), [2-14C]glycerol (Experiment 16), and [1-14C]glucose (Experiment 14) permitted assay of activity not only at C-2' and C-2 (Scheme 4), but also at C-4' (Scheme 7), C-5' (Scheme 6), and, somewhat indirectly, at C-4. The results of these degradations are pre-
presented in Tables III and IV. Pyridoxol derived from \([1,3-^{14}C]\)-glycerol contained approximately one-fifth of its activity at each of C-2', C-4', and C-5', whereas C-2 and C-4 were essentially without label (Tables III and IV). Pyridoxol derived from \([2J4C]\)glycerol, on the other hand, contained approximately one-third of its activity at each of C-2 and C-4, while C-2', C-4', and C-5' were inactive, within experimental error (Table IV). The entire activity of the sample of pyridoxol derived from \(n-[1-^{13}C]\)glucose is accounted for in terms of the activities of C-2', C-4', and C-5' (Table IV). Each of C-2' and C-4' contain approximately 37% of the total activity, whereas C-5' accounts for 26% of label.

Since less than 66% of that of the intact vitamin (Table V), at least one-third of the acetate, obtained in this way, must have been derived from the C_2 unit, C-4', C-4. It is evident that if the labeling pattern in the C_2 unit, C-4', C-4, of a given pyridoxol sample is significantly different from that of the C_2 unit, C-3', C-2, the specific activities of the samples of acetate and methylamine obtained from it by Schemes 4 and 5 must differ. Conversely, if these specific activities do not differ significantly, the labeling patterns of the C_2 units, C-4', C-4, and C-3', C-2, must be similar. Since degradations are available permitting individual assay of activity at C-2', C-2, and C-4', it follows that activity at C-4 can be deduced.

**DISCUSSION**

**General**—When deprived of exogenous pyridoxal an exponentially growing culture of *E. coli* B mutant WG2 ceases to grow but continues to generate pyridoxol at an enhanced rate for 3 to 4 hours. This newly formed pyridoxol is shed into the culture medium (37).

These characteristics made the organism a potentially valuable tool in the investigation of the biosynthesis of pyridoxol by tracer methods, and in the search for the primary precursors of the vitamin, in particular. Since, in the absence of added pyridoxal, there is no growth, little if any radioactive label should be dissipated into macromolecules if tracer is added at the onset of pyridoxal starvation. Since the duration of metabolic activity following pyridoxal deprivation is short, randomization of label should be minimal. Since pyridoxol is synthesized at an unusually high rate, the chances of incorporation of label into the desired product are enhanced. Since pyridoxol is excreted into the culture medium, labeled product would be removed from the site of metabolic activity. Lastly, the isolation of pyridoxol from the medium is far less tedious than its isolation from intact cells.
Distribution of activity in pyridoxol derived from [1,3-14C]glycerol. Relative specific activity at C-2' and C-3

<table>
<thead>
<tr>
<th>Product (carbon atoms of pyridoxol)</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SA</td>
<td>RSA</td>
</tr>
<tr>
<td>Pyridoxol (all)</td>
<td>1.78 ± 0.05</td>
<td>100 ± 3</td>
</tr>
<tr>
<td>Acetic acid (C-2',-2)</td>
<td>0.59 ± 0.01</td>
<td>22 ± 1</td>
</tr>
<tr>
<td>Methylamine (C-2')</td>
<td>0.39 ± 0.01</td>
<td>22 ± 1</td>
</tr>
</tbody>
</table>

* SA = specific activity (counts min⁻¹ mmole⁻¹) X 10⁻⁴.
* RSA = relative specific activity (per cent) (pyridoxol = 100).
* Assayed as pyridoxol hydrochloride.
* Obtained from the sample of pyridoxol, specific activity (2.38 ± 0.06) X 10³ cpm mmole⁻¹ (Experiment 2), by further dilution with inactive pyridoxol.
* Assayed as 2,4-dinitro-N-methylaniline.

Distribution of activity in pyridoxol, derived from glycerol and glucose

<table>
<thead>
<tr>
<th>Product (carbon atoms of pyridoxol)</th>
<th>Substrate</th>
<th>Experiment 15</th>
<th>Experiment 16</th>
<th>Experiment 14</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SA</td>
<td>RSA</td>
<td>SA</td>
<td>RSA</td>
</tr>
<tr>
<td>Pyridoxol (all)</td>
<td>1.67 ± 0.03</td>
<td>100 ± 2</td>
<td>1.09 ± 0.03</td>
<td>100 ± 3</td>
</tr>
<tr>
<td>Acetic acid (C-2',-2)</td>
<td>0.30 ± 0.01</td>
<td>22 ± 1</td>
<td>0.35 ± 0.01</td>
<td>32 ± 1</td>
</tr>
<tr>
<td>Methylamine (C-2')</td>
<td>0.31 ± 0.01</td>
<td>19 ± 1</td>
<td>0.35 ± 0.01</td>
<td>32 ± 1</td>
</tr>
<tr>
<td>4'-Deoxypyridoxol (2) (all)</td>
<td>1.63 ± 0.04</td>
<td>98 ± 3</td>
<td>1.10 ± 0.03</td>
<td>101 ± 4</td>
</tr>
<tr>
<td>Acetic acid (C-2',-4',-2,-4)</td>
<td>0.30 ± 0.01</td>
<td>22 ± 1</td>
<td>0.35 ± 0.01</td>
<td>32 ± 1</td>
</tr>
<tr>
<td>Methylamine (C-2',-4')</td>
<td>0.31 ± 0.01</td>
<td>19 ± 1</td>
<td>0.35 ± 0.01</td>
<td>32 ± 1</td>
</tr>
<tr>
<td>Pyridoxol (all)</td>
<td>1.67 ± 0.03</td>
<td>100 ± 2</td>
<td>1.09 ± 0.03</td>
<td>100 ± 3</td>
</tr>
<tr>
<td>5'-Phenyl derivative (5) (all)</td>
<td>1.64 ± 0.02</td>
<td>98 ± 2</td>
<td>1.08 ± 0.05</td>
<td>99 ± 5</td>
</tr>
<tr>
<td>Benzoic acid (C-5')</td>
<td>0.37 ± 0.01</td>
<td>22 ± 1</td>
<td>0.006 ± 0.002</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>Pyridoxol (all)</td>
<td>0.94 ± 0.01</td>
<td>100 ± 1</td>
<td>0.76 ± 0.04</td>
<td>100 ± 5</td>
</tr>
<tr>
<td>0-Methylpyridoxol (6) (all)</td>
<td>0.93 ± 0.02</td>
<td>99 ± 2</td>
<td>0.36 ± 0.01</td>
<td>100 ± 5</td>
</tr>
<tr>
<td>4,5-Dicarboxylic acid (7) (all)</td>
<td>0.94 ± 0.03</td>
<td>100 ± 3</td>
<td>0.36 ± 0.01</td>
<td>100 ± 5</td>
</tr>
<tr>
<td>5-Mono-carboxylic acid (8) (all but C-4')</td>
<td>0.73 ± 0.02</td>
<td>78 ± 2</td>
<td>0.79 ± 0.03</td>
<td>100 ± 6</td>
</tr>
</tbody>
</table>

* SA = specific activity (counts min⁻¹ mmole⁻¹) X 10⁻⁴.
* RSA = relative specific activity (per cent) (pyridoxol = 100).
* Assayed as pyridoxol hydrochloride.
* Assayed as acetyl-α-naphthylamide.
* Assayed as 2,4-dinitro-N-methylaniline.
* Obtained from the sample of pyridoxol, specific activity (1.67 ± 0.03) X 10³ cpm mmole⁻¹ (Experiment 15), by further dilution with inactive pyridoxol.
* Obtained from the sample of pyridoxol, specific activity (1.09 ± 0.03) X 10³ cpm mmole⁻¹ (Experiment 16), by further dilution with inactive pyridoxol.
* Obtained from the sample of pyridoxol, specific activity (0.46 ± 0.01) X 10³ cpm mmole⁻¹ (Experiment 14) by methylation, followed by dilution with inactive 0-methylpyridoxol.

The preliminary experiments confirmed these expectations. Radioactive pyridoxol, which maintained high and constant activity after repeated crystallization and resublimation, was obtained when [1,3-14C]glycerol served as the sole carbon source (Experiment 2), as well as when [1,3-14C]glycerol served as the tracer while glucose served as the major carbon source (Experiment 1).

Having found conditions for incorporation of activity into pyridoxol from a labeled substrate which, at this early stage of the investigation, was regarded solely as a general carbon source, entry of label from a series of putative specific precursors (c.f. Schemes 1 to 3) was then examined (Experiments 3, 7 to 13).

As a result of these experiments several of the substrates were dismissed from further consideration, since, on the basis of the criteria already discussed (see "Results and Criteria for Their Interpretation"), incorporation of their activity was minimal and random (Tables I and II). Thus, hypotheses demanding 1-carbon units (e.g. Scheme 1) (methionine (Experiment 12, no
of pyruvate serves as a source of C-3 of pyridoxol, i.e. whether the intact 3-carbon chain of pyruvate enters the vitamin (Schemes 1 and 2). Since a chemical degradation of pyridoxol, capable of extracting C-3, has not yet proved practicable, an experiment with [1-14C]pyruvate would have yielded an inconclusive result. Instead, a doubly labeled sample, [1,3-14C]pyruvate, containing 48 ± 1% of its label at the methyl group (C-3) and 52 ± 1% at the carboxyl group (C-1), was used as the tracer (Experiment 6). Intact incorporation of this sample would have led to pyridoxol containing 48% of its total label at C-2' and 52% at C-3. On Kuhn-Roth degradation such a sample of pyridoxol would have yielded acetic acid (C-2',2) and thence methylamine (C-2') containing 48% of the activity of vitamin. In fact (Table II) the acetic acid which was obtained contained 90% of the activity of pyridoxol, most of which was located at C-2' and was therefore derived (cf. Experiments 3 and 4) from the methyl group of the doubly labeled precursor. It follows that the carboxyl group of this pyridoxol had not been incorporated into pyridoxol.

Thus, it is a 2-carbon unit, corresponding to the methyl and the carboxyl group of pyruvate, which supplies the 2 carbon unit, C-2',2, of pyridoxol. The route, which leads from pyruvate into this 2-carbon fragment remains to be established. A working hypothesis, consistent with all available experimental evidence, will be presented later in this paper.

Incorporation of Glycerol—The samples of pyridoxol obtained from the experiments with [1,3-14C]glycerol (Experiment 1 and 2) were among those showing the highest radiochemical recovery. Kuhn-Roth oxidation of these samples yielded acetic acid (C-2',2), accounting for 22 and 21%, respectively, of the activity of the intact vitamin (Table III). These values were sufficiently close to that expected for random distribution of activity (25%) to appear to indicate that incorporation of label from glycerol may have been nonspecific, as expected for a general carbon source. Further degradation of the Kuhn-Roth acetate proved that such an inference was entirely unwarranted. Almost all of this activity (18%) was confined to the C-methyl group (C-2') (Table III), showing that incorporation of glycerol had been nonrandom.

The distribution, within pyridoxol, of activity derived from [1,3-14C]glycerol as well as from [2-14C]glycerol was therefore examined in greater detail (Experiments 10 and 16), by means of more extensive controlled chemical degradation of the labeled samples (Schemes 5 to 7). The distribution of radioactivity established by the results of these degradation experiments (Table IV) is summarized in Figs. 1 and 2. It appears that, of

4 The samples of [1,3-14C]glycerol which were used had been prepared by a chemical synthesis (59) (J. R. Catch, Radiochemical Centre, Amersham, England, personal communication) which places radioisotope at only one of the primary carbon atoms of each labeled molecule. However, since glycerol is a prochiral compound (60) whose enzymic phosphorylation takes place exclusively at the pro-R-hydroxymethyl group, metabolic phosphorylation of the labeled sample of glycerol must yield an enantiomeric mixture of (2E)-[1-14C]- and (2R)-[1-14C]glycerol 3-phosphate (stereospecific numbering (60)) (i.e. L- or [1-14C]- and L- or [3-14C]glycerolphosphate (G).

- **Table V**

<table>
<thead>
<tr>
<th>Product (carbon atoms of pyridoxol)</th>
<th>SA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>RSA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>SA</th>
<th>RSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyridoxol&lt;sup&gt;1&lt;/sup&gt;.............</td>
<td>0.29 ± 0.01</td>
<td>100 ± 3</td>
<td>0.118 ± 0.004</td>
<td>100 ± 3</td>
</tr>
<tr>
<td>Acetic acid&lt;sup&gt;2&lt;/sup&gt; (C-2')</td>
<td>0.27 ± 0.01</td>
<td>92 ± 4</td>
<td>0.117 ± 0.005</td>
<td>100 ± 5</td>
</tr>
<tr>
<td>4'-Deoxypyridoxol (all).........</td>
<td>0.074 ± 0.002</td>
<td>63 ± 3</td>
<td>0.074 ± 0.002</td>
<td>63 ± 3</td>
</tr>
</tbody>
</table>

<sup>a</sup> SA = specific activity (counts min<sup>-1</sup> mmole<sup>-1</sup>) × 10<sup>4</sup>. RSA = relative specific activity (per cent) (pyridoxol = 100).

<sup>b</sup> Assayed as pyridoxol hydrochloride.

<sup>c</sup> Obtained from the sample of pyridoxol, specific activity (0.29 ± 0.01) × 10<sup>4</sup> cpm mmole<sup>-1</sup>, by further dilution with inactive pyridoxol.

<sup>d</sup> Assayed as acetyl-O-3-naphthylamide.
the 8 carbon atoms of pyridoxol, five (of which three are C-2', C-4', and C-5', each of which contains approximately 20% of the total activity) are derivable from the terminal carbon atoms of glycerol (Fig. 1), and three (of which two are C-2 and C-4, each of which contains approximately 33% of the total activity) from the central carbon atom of this substrate (Fig. 2). The data lead to the inference that 3 3-carbon units related to glyceraldehyde 3-phosphate are implicated in the biosynthesis of pyridoxol and that, on route to the product, one of these units loses a terminal carbon atom. From the mode of incorporation of pyruvate (see above) it follows that the truncated glycerol unit supplies C-2' and C-2 of pyridoxol. The labeling pattern of glycerol-derived pyridoxol and the structural correspondence of precursor units and product, deduced from the experimental data, are shown in Fig. 3.

Glycerol enters the glycolytic pathway by way of L-α-glycerophosphate, phosphodihydroxyacetone and α-glyceraldehyde 3-phosphate and is hence convertible into pyruvate. Glycerol can thus serve as the source of the pyruvate-derived carbon atoms of pyridoxol (C-2', 2). Since the final step on this route from glycerol into pyruvate, the dephosphorylation of phosphoenolpyruvate, is essentially irreversible and since reconversion of pyruvate into triose phosphate by alternative pathways is unlikely to be important under the chosen experimental conditions, pyruvate cannot replace glycerol as a complete source of the carbon skeleton of pyridoxol. Since pyruvate thus leads to singly labeled pyridoxol whereas glycerol leads to multiply labeled product, the lower recovery of radioactivity in the pyruvate-derived samples (see "Results and Criteria for Their Interpretation") is explained.

The distribution of label within the pyridoxol sample derived from D-[3-14C]serine (Experiment 8) closely resembles that of the pyruvate-derived samples (Table II). Since serine is convertible into pyruvate (56, 57) by an enzymic process which is essentially irreversible (58) it follows that entry of serine into pyridoxol takes place via pyruvic acid. The low radiochemical yield (Experiment 8, Table I) is consistent with this conclusion. The enzymes catalyzing the conversion, in E. coli, of L- and D-serine into pyruvate are known to require pyridoxal phosphate (59). This is the cofactor which is limiting in a pyridoxal-less mutant under conditions of pyridoxal deprivation, the present experimental conditions.

**Biosynthesis of Pyridoxol: Working Hypothesis**

The tracer evidence suggests that glycerol or closely related compounds are the primary precursors of pyridoxol. Whereas glycerol itself lacks the reactivity demanded by the condensation reactions which must occur in the construction of the 8-carbon skeleton of pyridoxol from 3-carbon precursors, the triose phosphates, phosphodihydroxyacetone, and α-glyceraldehyde 3-phosphate, would appear to be likely substrates of such reactions. A hypothetical biosynthetic sequence which is consistent with
Biosynthesis of Vitamin B₆

**Scheme 8. Biosynthesis of pyridoxol. Structural correspondence of glycerol, pyruvate, and pyridoxol (box) and hypothetical biosynthetic sequence.**

All available experimental data and is also mechanistically rational is presented in Scheme 8. In the scheme the predicted labeling pattern of pyridoxol, as well as that of the three postulated primary precursors, phosphodihydroxyacetone, n-glyceraldehyde 3-phosphate, and the pyruvate-derived 2-carbon unit (shown at the oxidation level of acetaldehyde), when derived from chemically labeled glycerol, is indicated. (The interrelationship of the precursor units, through the Embden-Meyerhof pathway, is not shown.)

The distribution of activity of glycerol-derived pyridoxol, deduced from the experimental data (Fig. 3), corresponds to that demanded by the hypothesis (Scheme 8).

An early intermediate predicted by the scheme is a 5-deoxypentulose phosphate, originating by condensation of acetaldehyde and phosphodihydroxyacetone. Depending on the mode of this postulated condensation, the compound would be either a 1-phosphate (as shown in Scheme 8) or a 3-phosphate (1). 5-Deoxypentulose 3-phosphates appear to be unknown. The intermediacy of such a compound would nevertheless be attractive. The irreversible loss of the S-phosphate group at a later stage of the sequence, during the conversion of an enol phosphate to a ketone (c.f. the conversion of phosphoenolpyruvate into pyruvate) would explain why pyridoxol diphosphate has not been detected.

Formation of 5-deoxypentulose 1-phosphate on the other hand, by aldolase-catalyzed condensation of acetaldehyde and phosphodihydroxyacetone, is known to occur in a variety of tissues, e.g. muscle and yeast (92). Since, in aldolase-catalyzed reactions, the hydroxy groups astride the newly formed carbon-carbon bond are almost invariably trans to each other (65), the two possible products of this reaction are 5-deoxy-d-xylulose 1-phosphate or 5-deoxy-l-xylulose 1-phosphate. Aldolase preparations from pea seedlings (64) and from human erythrocytes (65) yield the d-enantiomer. An aldolase from an E. coli mutant catalyzes formation of the l-enantiomer (66). The d-enantiomer is arbitrarily shown in Scheme 8.

The experiment with n-[1-14C]glucose (Experiment 14) provides further evidence in support of the biosynthetic hypothesis outlined in Scheme 8 and, in particular, serves to distinguish between the two possible modes of entry of phosphodihydroxyacetone. Glycolysis of n-[1-14C]glucose leads, via n-[1-14C]fructose 1,6-diphosphate, to [1-14C]phosphoenolpyruvate, to [1-14C]dihydroxyacetone 1-phosphate and unlabeled n-glyceraldehyde 3-phosphate. Equilibration of the two triose phosphates, catalyzed by triose phosphate isomerase, eventually leads to n-[3-14C]glyceraldehyde 3-phosphate, which on further degradation yields [3-14C]pyruvate and hence [2-14C]acetaldehyde. Incorporation of these fragments into pyridoxol (Scheme 9), would place activity from [1-14C]glucose at C-2' (via acetaldehyde), at C-5' (via n-glyceraldehyde 3-phosphate) and at either C-4' or at C-3 (via phosphodihydroxyacetone), depending on whether the 1-phosphate or the 3-phosphate of 5-deoxypentulose had served as intermediate. Three carbon...
atoms of the pyridoxol sample derived from [1-14C]glucose, C-2', C-5', and either C-4' or C-3, would thus account for its entire activity. The observed distribution of label (Fig. 4) agrees with this prediction: within experimental error all activity is accounted for in terms of the activities of C-2', C-4', and C-5'. The presence of activity at C-4' is consistent with the intermediacy of 5-deoxyxylulose 1-phosphate, but not with that of the 3-phosphate.

Although the location of label from D-[1-14C]glucose is entirely according to prediction, the quantitative distribution of activity among the predicted positions is puzzling. Equilibration of label among the glycolytic C₃ units would lead to pyridoxol carrying 33% of its total activity at each of C-2', C-4', and C-5'. This was not observed (Fig. 4). Since glycolytic breakdown of [1-14C]glucose yields, in the first instance, labeled phosphodihydroxyacetone and unlabeled glyceraldehyde 3-phosphate, and since the equilibrium of the triose phosphate isomerase reaction favours the former, equilibration of label need not take place in a short term incubation. Phosphodihydroxyacetone would then show a higher specific activity than glyceraldehyde 3-phosphate, and the site of pyridoxol derived from [1-14C]-dihydroxyacetone 1-phosphate (C-4') would be expected to carry a greater share of the total activity of the intact vitamin than the site derived from D-[3-14C]glyceraldehyde 3-phosphate (C-5'). This is indeed observed (Fig. 4).

Since pyruvate arises glycolytically from phosphoglyceraldehyde, activity at C-2' of pyridoxol should be similar to that at C-5'. In fact (Fig. 4) the observed activity at C-2' resembles that of C-4' and is unlike that of C-5'. Taken at face value this result appears to indicate that the pyruvate-derived 2-carbon unit which serves as the precursor of the C₃ unit, C-2'-2, of pyridoxol, is derived from phosphodihydroxyacetone or directly from a hexose rather than from phosphoglyceraldehyde.

This and other aspects of the present results require further study. Although the observed distribution of activity from acetate and from aspartate (60% of the total activity of pyridoxol within the C₃ unit, C-2'-2) (Experiments 7 and 11, Table II) may carry a greater share of the total activity of the intact vitamin than the site derived from D-[3-14C]glyceraldehyde 3-phosphate, the site derived from D-[3-14C]glyceraldehyde 3-phosphate, and the site of pyridoxol derived from [1-'14C]-dihydroxyacetone 1-phosphate (C-4') would be expected to carry a greater share of the total activity of the intact vitamin than the site derived from D-[3-14C]glyceraldehyde 3-phosphate (C-5'). This is indeed observed (Fig. 4).

Nonetheless, the foundations have been laid to an understanding, long overdue (c.f. 61, 67, 68), of the process which leads from primary precursors to vitamin B₆.

REFERENCES
7. Amano, T. (1958) Vitamins (Kyoto) 14, 595
10. Suzuki, R., Haruna, Y., Hataishi, O., Yamamoto, S., and Ogata, K. (1964) Vitamins (Kyoto) 29, 569
21. Hataishi, O. (1965) Vitamins (Kyoto) 31, 320
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40. Kuhn, R., and Wendt, G. (1938) Ber. 71, 1118
42. Harris, S. A., and Folkens, K. (1939) J. Amer. Chem. Soc. 61, 1245
43. Kuhn, R., and Wendt, G. (1939) Ber. 72, 305
47. Harris, S. A. (1940) J. Amer. Chem. Soc. 62, 3203
53. Kuhn, R., and Wendt, G. (1939) Ber. 71, 1504
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