Biosynthesis of Vitamin B_6. Direct Evidence for the Mode of Incorporation of C_3-Units derived from D-[U-$^{13}C_6$]Glucose

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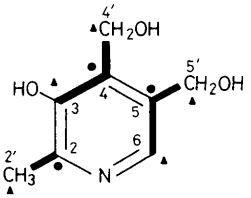
The ^{13}C n.m.r. spectrum of a sample of pyridoxol, derived biosynthetically from D-[U-$^{13}C_6$]glucose in *Escherichia coli*, shows that C-2–C-3 and C-4–C-5 of the pyridine nucleus are the only two carbon–carbon bonds of pyridoxol which are generated *de novo* in the course of its biosynthesis from glucose, constituting direct evidence in support of the biogenetic scheme which we had proposed on the basis of earlier investigations with radioactive tracers.

It is known from tracer experiments with [1-^{14}C]-, [1,3-$^{13}C_2$]-, and [2-^{14}C]-glycerol[1—3] that, in *Escherichia coli* B mutant WG 2, the entire C_8-skeleton of pyridoxol (**1**) can originate from the carbon atoms of glycerol in a specific manner: five of the eight C-atoms (C-2′, -3, -4′, -5′, -6) are derived from the primary carbon atoms of glycerol (▲), the other three (C-2, -4, -5) from the secondary carbon atom (●). On the basis of these findings and from the mode of incorporation of label from [1-^{14}C]- and [6-^{14}C]-glucose[1,4] we advanced a chemically



(**1**)

rational scheme for the origin of pyridoxol from three triose precursors.[1] In particular, we inferred[1—3] that two intact triose units, derived either from glycerol or from glucose, entered the C_3-segments, C-3,-4,-4′ and C-5′,-5,-6 of pyridoxol, whereas a C_2 precursor, derived from a third triose unit by loss of a terminal carbon atom,[1—5] supplies the C_2-fragment, C-2′,-2. It is a corollary of this biogenetic scheme that the only new carbon–carbon bonds which are generated in the course of the biosynthesis of pyridoxol from glucose or glycerol are those destined to become C-2–C-3 and C-4–C-5 of the pyridoxol skeleton, whereas all other carbon–carbon bonds of pyridoxol represent intact C–C bonds present in the triose precursors. We now present direct evidence that C-2–C-3 and C-4–C-5 are indeed the only two carbon–carbon bonds which are formed *de novo* in the biosynthesis of pyridoxol from glucose.

Each of five 1 l cultures of *E. coli* B WG 2 was incubated, as described earlier,[1] with D-glucose (1 g/l) as the general carbon source. In each incubation a mixture of 200 mg D-[U-$^{13}C_6$]glucose (99% ^{13}C per C atom, Los Alamos Stable Isotope Resource) and 800 mg natural abundance D-glucose was used. Pyridoxol hydrochloride was isolated[1] from each culture after addition of natural abundance pyridoxol hydrochloride (2.5 mg) as carrier, and the isolated samples were combined and purified by column and thin layer chromatography, followed by high vacuum sublimation.

The ^{13}C n.m.r. spectrum of a saturated solution of the isolated pyridoxol hydrochloride (6.5 mg in 60 μl D_2O) was recorded on a Bruker AM 300 spectrometer (Figure 1). With the signals in the ^{13}C n.m.r. spectrum of pyridoxol[6] now correctly assigned,† the biosynthetic connectivity of enriched carbon atoms should be evident from the spin coupling patterns of the individual signals due to these enriched carbon atoms.

The sample of pyridoxol whose ^{13}C n.m.r. spectrum was determined was a composite mixture of biosynthetically enriched and natural abundance material: the biosynthetic pyridoxol was generated from a sample of glucose which consisted of a mixture of 80% natural abundance glucose (1.1% ^{13}C per C atom) and of 20% [U-$^{13}C_6$]glucose (99% ^{13}C per C atom). The biosynthetic sample derived from this glucose thus consisted of a mixture of enriched and nonenriched molecules. This biosynthetically produced pyridoxol (*ca.* 80 μg per 1 l culture) was isolated after carrier dilution with 2.5 mg of natural abundance pyridoxol.

The signal of each of the individual carbon atoms in the ^{13}C n.m.r. spectrum of the isolated pyridoxol sample should be 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 multiplet (due to enriched ^{13}C attached to enriched ^{13}C). Thus, the signal corresponding to each of C-2′, C-4′, and C-5′, carbon atoms which are attached to one carbon only, should appear as a central line straddled by a doublet. Assuming that each of these carbon atoms enters the biosynthetic product as a multicarbon unit, the predicted ratio, area of central line/total area of outer doublet, which can be calculated for these three carbon atoms is 64 : 36,‡ *i.e.* the central line

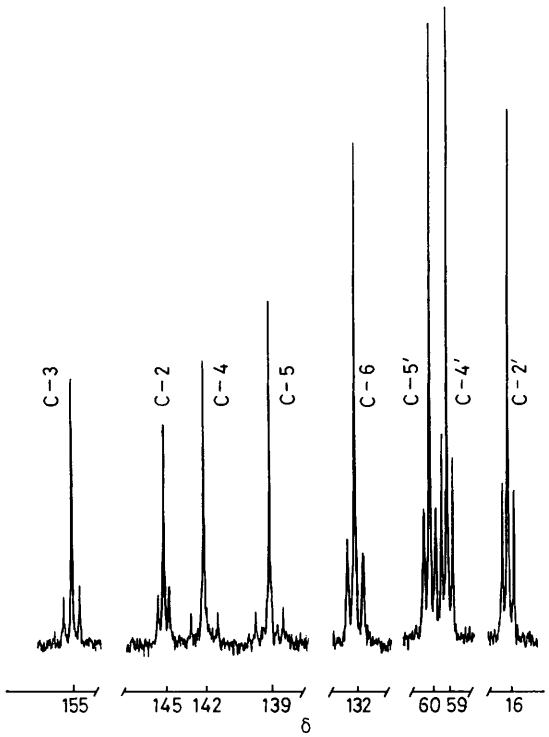


Figure 1. 75.47 MHz proton decoupled ^{13}C n.m.r. spectrum of pyridoxol, derived biosynthetically from D-[U-$^{13}C_6$]glucose. Determined on a Bruker AM 300 spectrometer. Spectral parameters: 180 234 transients, spectral width 18 500 Hz, pulse flip angle 25° acquisition time 0.88 s, line broadening 0.3 Hz, memory size 32 K zero filled to 128 K, final digital resolution 0.28 Hz per data point.

contributes 64% to the total signal area, whereas each line of the outer doublet contributes 18%.

Correspondingly, a carbon atom entering the biosynthetic product as the central secondary carbon atom of a three carbon unit should give rise to a signal which consists of a central line (64% of total signal area) straddled by a set of four lines of equal intensity (36% of total signal area, *i.e.* 9% per line), while a carbon atom entering as the central tertiary carbon atom of a four carbon unit should give rise to a signal consisting of a central line (64% of total signal area) straddled by a more complex multiplet (36% of total signal area).

The relative signal areas of the central line and of one of the outer lines, for the signals of all carbon atoms in the spectrum of the sample of pyridoxol derived from D-[U-$^{13}C_6$]glucose, are given in Table 1. It is evident that whereas C-2′, C-2, C-3, C-4′, C-5′, and C-6 each show direct coupling to only one other carbon atom, C-4 and C-5 must each be the central carbon atom of a C_3 unit. The separation of the lines contributing to the signals of C-4 and C-5 can be calculated from the measured values of the coupling constants, $^1J_{3,4}$ 64.5, $^1J_{4',4}$ 45.1 Hz and $^1J_{6,5}$ 64.9, $^1J_{5',5}$ 48.8 Hz, respectively (Table 1). In each case four lines, symmetrically distributed around the central line, would be expected. Their separation would be 64.5 + 45.1 = 109.6 Hz for the outer pair of lines and 64.5 − 45.1 = 19.4 Hz for the inner pair of lines in the signal of C-4, and 64.9 + 48.8 = 113.7 Hz and 64.9 − 48.8 = 16.1 Hz for the corresponding pairs in the signal of C-5.

The observed separation of the outer lines in the signal of C-4 is 109.6 Hz, in that of C-5, 112.8 Hz, in excellent agreement with the calculated values. The inner pair of lines in the signals due to C-4 and C-5 are not resolved (spectral width at the base of the central line, 22 Hz). The relatively simple

† Assignments of the signals for C-4′ and C-5′ were inverted in our earlier paper (ref. 3). The revised assignment is confirmed by long range coupling, through an intact $^{13}C_3$ unit, between C-6 and C-5′, ($^2J_{6,5'}$ 3.7 Hz), observable in the doublet portion of the signals due to C-6 and C-5′ (δ 131.8, 60.1).

‡ % of total signal area in the doublet: 100×80×99×0.2/[(80×99×0.2) + (80×1.1×0.8) + (2500×1.1)] = 36%.

Table 1. Chemical shifts, percentage of the total signal area of the most and least intense lines of each signal, and ^{13}C–^{13}C coupling constants (in Hz) in the 75.47 MHz proton decoupled ^{13}C n.m.r. spectrum of pyridoxol derived from D-[U-$^{13}C_6$]glucose.

Carbon atom	Chemical shift, δ	% of total signal area: Central line	% of total signal area: One of the outer lines	^{13}C–^{13}C Coupling constants, Hz	
C-2′	16.5	60 ± 3	20 ± 1	$^1J_{2',2}$	46.4
C-2	144.7	62 ± 3	19 ± 1		
C-3	154.7	67 ± 4	17 ± 1	$^1J_{3,4}$	64.5
C-4	142.5	84 ± 4	8 ± 0.5	$^1J_{4',4}$	45.1
C-4′	59.0	60 ± 3	20 ± 1		
C-5′	60.1	58 ± 3	21 ± 1	$^1J_{5',5}$	48.8
C-5	138.8	82 ± 4	9 ± 0.5	$^1J_{6,5}$	64.9
C-6	131.8	64 ± 3	18 ± 1	$^2J_{6,5'}$	3.7

appearance of the ^{13}C n.m.r. spectrum indicates, furthermore, that no carbon fragment larger than C_3 is implicated in the biosynthesis of pyridoxol.

From the coupling constants and from the relative signal areas (Table 1) it follows 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′ has biosynthetic connectivity to a neighbour. Therefore, the C_3 moieties, C-3,-4,-4′ and C-5′,-5,-6 of pyridoxol are derived intact from a biosynthetic precursor. Conversely, there is no measurable coupling between C-4 and C-5 (predicted *ca.* 65 Hz; *cf.* $^1J_{3,4}$ and $^1J_{6,5}$, Table 1) or between C-3 and C-6 (predicted *ca.* 14 Hz; *cf.* pyridine,[7] $^3J_{2,5}$ 14 Hz). Thus, there is no connectivity between C-4 and C-5. It follows that the bond, C-4–C-5, is newly formed in the course of biosynthesis.

These results establish the biosynthetic connectivity of pyridoxol derived from glucose to be as shown in (**1**). They provide confirmation of the inferences which were drawn from the results of our earlier tracer work[1—3] and serve to invalidate alternative biogenetic schemes.[8]

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