Biosynthesis of Vitamin B_6 in Yeast: Incorporation Pattern of Glucose

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Received May 30, 2001

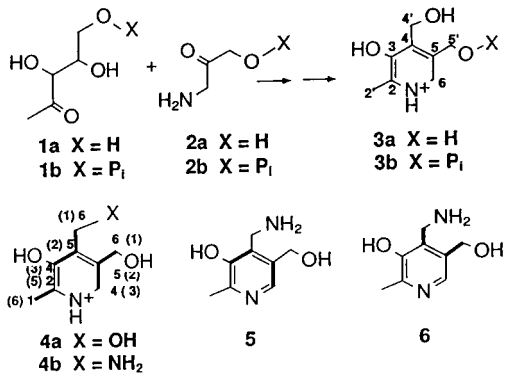
Abstract: Two yeasts, *Saccharomyces cerevisiae* ATCC 7752 and *Candida utilis* ATCC 9256, were incubated in the presence of variously multiply ^{13}C-labeled samples of D-glucose. The ^{13}C incorporation pattern within pyridoxamine dihydrochloride, established by ^{13}C NMR spectroscopy, differed from that which had previously been found within pyridoxine, isolated from *Escherichia coli*. Thus, the origin of the carbon skeleton of vitamin B_6 in yeast differs substantially from its origin in *E. coli*. In particular, in yeast the distribution of ^{13}C within the C_5 chain C-2′,2,3,4,4′ of pyridoxamine corresponds to the distribution of ^{13}C within the C_5 chain C-1,2,3,4,5 of the ribose component of cytidine. It follows that the C_5 chains of pyridoxamine and of ribose originate from a common glucose-derived pentulose or pentose intermediate. By contrast, in *E. coli* the C_5 chain of pyridoxine is derived from 1-deoxy-D-xylulose 5-phosphate which, in turn, originates by condensation of pyruvic acid with glyceraldehyde 3-phosphate.

Recent reports indicate that the biosynthesis of vitamin B_6 differs in different types of microorganisms. Genes that have been found to be essential for pyridoxine biosynthesis in several fungi, for example, *Aspergillus nidulans* and *A. flavus*, that is, eukaryotic organisms, show no homology with any of the pyridoxine (pdx) genes of the bacterium, *Escherichia coli*, that is, a prokaryote.[1,2]

Incorporation studies with ^{15}N-labeled substrates support the existence of two mutually exclusive pathways to pyridoxine, even though the dividing line between prokaryotic and eukaryotic organisms is somewhat blurred. Whereas glutamic acid, but not the amide group of glutamine, serves as the source of the pyridoxine nitrogen atom in *E. coli* and two other bacteria, in the yeast *Saccharomyces cerevisiae* and three other eukaryotes, as well as in two bacterial species, the nitrogen source is the amide group of glutamine and not glutamic acid.[3,4]

The biogenetic anatomy[5] of the skeleton of vitamin B_6 (**3a**) in *E. coli*, and the genetics of its formation,[6] are now well understood, and current investigations address the mechanism and the enzymology[7−9] of its biosynthesis. By contrast, no information is available on the origin of the carbon skeleton of the vitamin in yeast. We now report the first evidence that the biogenetic origin of the carbon skeleton of vitamin B_6 in yeast does indeed differ from that in *E. coli*.

In *E. coli* the two ultimate acyclic building blocks that combine to yield the pyridoxine skeleton are 1-deoxy-D-xylulose 5-phosphate (**1b**) and 1-amino-3-hydroxyacetone 3-phosphate (**2b**).[5,10,11] Both are derived from glucose in now transparent fashion: The former (**1b**) arises by condensation, accompanied by decarboxylation, of pyruvic acid (derived from C-1,2, with loss of C-3, of glucose), with phosphoglyceraldehyde (C-4,5,6 of glucose) giving rise to C-2′,2,3,4,4′ of the pyridoxine skeleton. The latter precursor (**2b**), which gives rise to N-1,C-6,5,5′ of the pyridoxine skeleton, is generated by oxidative decarboxylation (i.e., with loss of C-3 of glucose) of 4-hydroxythreonine



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10.1021/ja0113201 CCC: $20.00
Published on Web 10/30/2001

Table 1. ^{13}C NMR Spectrum of Pyridoxamine: Chemical Shifts and Coupling Constants

chemical shift (ppm)	carbon atom no	coupling constants (± 0.5 Hz) $^{13}C-^{13}C$	
15.2	C-2′	$^1J_{C-2',C-2}$	46.2
34.9	C-4′	$^1J_{C-4',C-4}$	45.8
58.8	C-5′	$^1J_{C-5',C-5}$	48.4
		$^2J_{C-5',C-6}$	5.0
131.1	C-6	$^1J_{C-6,C-5}$	64.4
		$^2J_{C-6,C-5'}$	4.5
136.2	C-4	$^1J_{C-4,C-3}$	66.2
		$^1J_{C-4,C-4'}$	45.9
		$^1J_{C-4,C-3} + {}^1J_{C-4,C-4'}$	114.1
138.2	C-5	$^1J_{C-5,C-6}$	64.0
		$^1J_{C-5,C-5'}$	48.7
		$^1J_{C-5,C-5'} + {}^1J_{C-5,C-6}$	113.4
143.2	C-2	$^1J_{C-2,C-2'}$	46.9
		$^1J_{C-2,C-2'} + {}^1J_{C-2,C-3}$	118.0
153.9	C-3	$^1J_{C-3,C-4}$	66.7
		$^1J_{C-3,C-2}$	72.6
		$^1J_{C-3,C-2} + {}^1J_{C-3,C-4}$	138.4

4-phosphate[9] which, in turn, is derived from erythronic acid 4-phosphate[6] (i.e., from C-3,4,5,6 of glucose). The correspondence in *E. coli* of the pyridoxine carbon atoms with those of glucose is shown in **4a**. (Numbers in **4** refer to the carbon atoms of D-glucose from which the carbon atoms of pyridoxine are derived in *E. coli*.[5])

We have investigated the mode of incorporation of ^{13}C-labeled samples of glucose into pyridoxamine in two yeasts, *Saccharomyces cerevisiae* ATCC 7752 (= IFO 1234) and *Candida utilis* ATCC 9256. It was shown by liquid chromatography with fluorescence detection that pyridoxamine was the most abundant B_6 vitamer in the cells of each of the two yeasts. The NMR data of pyridoxamine dihydrochloride are summarized in Table 1.

The first experiments were carried out with *S. cerevisiae*. A 2 L yeast culture was incubated in minimal medium[12] in the presence of [$^{13}C_6$]glucose (expt 1) until the cell density had reached an absorbance higher than 1.6 at 620 nm (16−20 H). Unlabeled pyridoxamine di-HCl (2.5 mg) was then added, and the labeled pyridoxamine dihydrochloride (ca. 50−70 μg), diluted with the carrier, was isolated by HPLC (semipreparative Ultrasphere octadecylsilyl (ODS) column, 25 cm × 10 mm i.d., Beckman, Fullerton, CA). The isolated sample gave a ^{13}C NMR spectrum that showed incorporation of three glucose-derived multicarbon units, a C_2 unit yielding C-2′,2, and two C_3 units, yielding C-3,4,4′ and C-6,5,5′ of pyridoxamine (**4b**)[13a] This result, which is similar to that obtained from the corresponding experiment with *E. coli* (**4a**) (see Figure 1 in ref 5), appeared to indicate that the mode of construction of the B_6 carbon skeleton in yeast is similar to that in *E. coli*.

The result of an experiment with D-[4,5-$^{13}C_2$]glucose (expt 11) as the substrate appeared to be consistent with this conclusion. Bond-labeling of pyridoxamine was observed at C-3,4 and at C-5,6 (**5**). All other pyridoxamine carbon atoms appeared as singlets.[13b]

However, two other experiments dispelled the notion that the route to vitamin B_6 in yeast was the same as in *E. coli*: Thus, unlike in *E. coli* (see Figure 2c in ref 5), label from sodium [2,3-$^{13}C_2$]pyruvate (expt 13) did not enter C-2′,2 of pyridoxamine in *S. cerevisiae*, nor did unlabeled sodium pyruvate (expt 3) displace entry of a glucose-derived C_2 unit into C-2′,2. This difference in the modes of entry of label from glucose into the C_5 chain C-2′,2,3,4,4′ of pyridoxine observed in yeast and in *E. coli* was highlighted by a further experiment.

D-[1,2-$^{13}C_2$]Glucose (expt 10) gave a sample of pyridoxamine whose ^{13}C NMR spectrum showed that it was bond-labeled at C-4′,4 and at C-5′,5 but not at C-2′,2 (**6**).[13c] In *E. coli*, by contrast, all three of these C_2 units had been bond-labeled (see Figure 2B in ref 5). Thus, unlike in *E. coli*, in yeast the C_2 unit C-2′,2 cannot be derived from C-1,2 (or from C-2,1) of glucose.

Careful comparison of the relative heights of the signals due to C-2′ and C-6 in the spectrum of pyridoxamine from expt 10 (18% w/w D-[1,2-$^{13}C_2$]glucose/D-glucose) with those in the spectrum of natural abundance pyridoxamine dihydrochloride (Figure 1D), in which these two signals are of approximately equal intensity, suggested that C-2′ might be ^{13}C-enriched in the sample derived from D-[1,2-$^{13}C_2$]glucose. To substantiate that this was indeed so, incubations were carried out with carrier-free D-[1-^{13}C]glucose (expt 8) (Figure 1C) and with 58% w/w D-[2-^{13}C]glucose/D-glucose (expt 9) (Figure 1B).

Enrichment was not detectable at C-2′ in the pyridoxamine spectrum derived from the former experiment.[13d] However, in the spectrum of pyridoxamine dihydrochloride[13e] derived from D-[2-^{13}C]glucose (expt 9, Figure 1B) the signal due to C-2′ showed significant (ca. 1.66-fold) enrichment. Thus, C-2′ of pyridoxamine is derived from C-2 of glucose, whereas C-1 of glucose is not incorporated into the C_2-unit, C-2′,2.

This result suggested that the C_5 unit C-2′,2,3,4,4′ of pyridoxamine may be derived intact from a pentulose or pentose that is generated from glucose by the oxidative route. Two approaches were taken to examine this possibility.

S. cerevisiae does not utilize pentoses as a general carbon source[14] and ^{13}C-labeled pentoses cannot therefore be employed as substrates. The first approach to examine the possible derivation of the C_5 unit via a pentose was to determine the distribution of ^{13}C within a pentose that was generated from ^{13}C-labeled glucose concurrently with pyridoxamine in the

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(13) Incorporation patterns within pyridoxamine dihydrochloride: (a) *S. cerevisiae*, [$^{13}C_6$]glucose, expt 1: C-2′ 15.2 ppm, $^1J_{C-2C-2'}$ = 46.5 Hz; C-2 143.2 ppm, $^1J_{C-2,C-2'}$ = 46.4 Hz; C-3 153.9 ppm, $^1J_{C-3,C-4}$ = 65.1 Hz, C-4 136.2 ppm, $^1J_{C-4,C-3} + {}^1J_{C-4,C-4'}$ = 114.1 Hz; C-4′ 34.9 ppm, $^1J_{C-4',C-4}$ = 46.2 Hz; C-6 131.1 ppm, $^1J_{C-6,C-5}$ = 64.6 Hz; C-5 138.2 ppm, $^1J_{C-5,C-6} + {}^1J_{C-5,C-5'}$ = 112.2 Hz; C-5′ 58.8 ppm, $^1J_{C-5',C-5}$ = 48.0 Hz, $^2J_{C-5',C-6}$ = 3.3 Hz. Some C-3,4 and C-5,6 coupling ($^1J_{C-4,C-3}$ 66.2 Hz, $^1J_{C-5,C-6}$ 63.0 Hz) was also detectable. (b) *S. cerevisiae*, [4,5-$^{13}C_2$]glucose, expt 11 (Figure 3C): C-3 153.9 ppm, $^1J_{C-3,C-4}$ = 66.2 Hz; C-4 136.2 ppm, $^1J_{C-4C-3}$ = 66.2 Hz; C-5, 138.2 ppm, $^1J_{C-5,C-6}$ = 64.0 Hz; C-6 131.1 ppm, $^1J_{C-6,C-5}$ = 63.3 Hz. (c) *S. cerevisiae*, [1,2-$^{13}C_2$]glucose, expt 10: C-4′ 34.9 ppm, $^1J_{C-4',C-4}$ = 45.0 Hz; C-4 136.2 ppm, $^1J_{C-4,C-4'}$ not detectable; C-5′ 58.8 ppm, $^1J_{C-5',C-5}$ = 48.0 Hz; C-5 138.2 ppm, $^1J_{C-5,C-5'}$ = 48.7 Hz. (d) *S. cerevisiae*, [1-^{13}C]glucose, expt 8 (Figure 1C): No enrichment at C-2′ (15.2 ppm); enrichment at C-4′ (34.9 ppm) 1.23 x NA.; at C-5′ (58.8 ppm) 1.45 x NA. (e) *S. cerevisiae*, [2-^{13}C]glucose, expt 9 (Figure 1B): Enrichment at C-2′ (15.2 ppm) 1.66 × NA.; no detectable enrichment at C-4 (136.2 ppm) and at C-5 (138.2 ppm). (f) *C. utilis*, [1, 2-$^{13}C_2$]glucose, expt 17 (Figure 3B): C-2′ 15.2 ppm, enrichment 1.33 × NA.; C-5′ 58.8 ppm, $^1J_{C-5,C-5'}$ = 48.3 Hz; C-5 138.2 ppm, $^1J_{C-5,C-5'}$ = 48.5 Hz; C-4′ 34.9 ppm, $^1J_{C-4',C-4}$ = 45.8 Hz; C-4 136.2 ppm, $^1J_{C-4,C-4'}$ = 45.9 Hz. (g) *C. utilis*, [$^{13}C_6$]glucose, expt 16 (Figure 3A): C-2′ 15.2 ppm, $^1J_{C-2',C-2}$ = 46.5 Hz; C-2 143.2 ppm, $^1J_{C-2,C-2'}$ = 46.4 Hz; $^1J_{C-2,2'} + {}^1J_{C-2,C-3}$ = 118.6 Hz; C-3 153.9 ppm, $^1J_{C-3,C-2} + {}^1J_{C-3,C-4}$ = 138.4 Hz; $^1J_{C-3,C-4}$ = 66.8 Hz; C-4 136.2 ppm, $^1J_{C-4,C-3}$ = 66.3 Hz; $^1J_{C-4,C-3} + {}^1J_{C-4,C-4'}$ = 112.2 Hz; C-4′ 34.9 ppm, $^1J_{C-4',C-4}$ = 45.7 Hz; C-6 131.1 ppm, $^1J_{C-6,C-5}$ = 64.1; $^2J_{C-6,C-5'}$ = 4.5 Hz; C-5 138.2 ppm, $^1J_{C-5,C-6}$ = 64.6 Hz; $^1J_{C-5,C-5'}$ = 48.5 Hz; $^1J_{C-5,C-6} + {}^1J_{C-5,C-5'}$ = 112.3 Hz; C-5′ 58.8 ppm, $^1J_{C-5',C-5}$ = 48.5 Hz; $^2J_{C-5',C-6}$ = 4.9 Hz. (h) *C. utilis*, [1,2-$^{13}C_2$]xylose, expt 18 (Figure 3D): C-2′ 15.2 ppm, $^1J_{C-2',C-2}$ = 46.3 Hz; C-2 143.2 ppm, $^1J_{C-2,C-2'}$ = 46.0 Hz; C-4′ 34.9 ppm, $^1J_{C-4',C-4}$ = 45.4 Hz; C-4 136.2 ppm, $^1J_{C-4,C-4'}$ = not detectable; C-5′ 58.8 ppm, $^1J_{C-5',C-5}$ = 48.5 Hz; C-5 138.2 ppm, $^1J_{C-5,C-5'}$ = 47.6 Hz; $^1J_{C-5,C-6}$ = not detectable; C-6 131.1 ppm, $^1J_{C-6,C-5}$ = 62.6.

(14) See, e.g.: Rose, A. H.; Harrison, J. S. *The Yeasts*, 2nd ed.; Academic Press: London, 1987; Vol. 2, p 10: "The traditional ethanol-producing yeast, *Sacch. cerevisiae* is, however, unable to utilize pentoses."

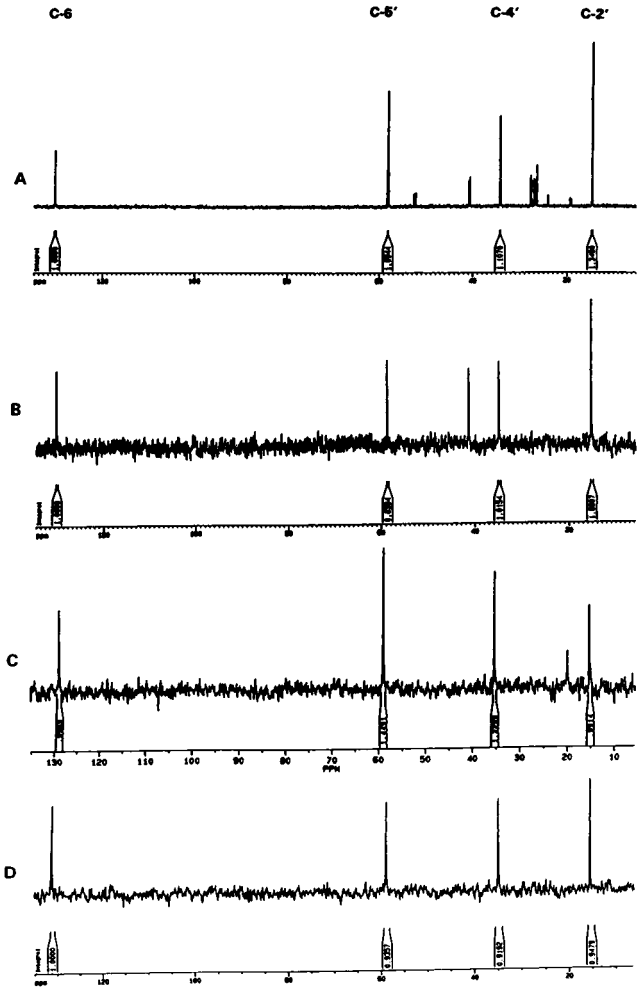

Figure 1. Upfield region (0−140 ppm) of quantitative ^{13}C NMR spectra of pyridoxamine dihydrochloride, showing the signals due to C-2′, C-4′, C-5′ and C-6 and signal integrals (arbitrary units, relative to the C-6 signal set at 1.00): (A) Pyridoxamine di-HCl derived from [1,2-$^{13}C_2$]glucose (expt 17). (B) Pyridoxamine di-HCl derived from [2-^{13}C]glucose (expt 9). (C) Pyridoxamine di-HCl derived from [1-^{13}C]-glucose (expt 8). (D) Unenriched reference sample of pyridoxamine di-HCl.

course of the experiments with [$^{13}C_6$]-, [1,2-$^{13}C_2$]-, and [4,5-$^{13}C_2$]glucose (expts 1, 10, and 11). The pentose selected for this purpose was the ribose component of cytidine. Cytidine was isolated from the cell hydrolysate, without addition of carrier, by strong cation exchange (SCX) extraction, followed by HPLC, using the same mobile phase that had been used in the case of pyridoxamine.

The NMR spectrum of the ribose component of the nucleoside was determined and the ^{13}C incorporation pattern compared with that shown by the NMR spectrum of the corresponding ^{13}C-labeled pyridoxamine. Table 2 shows the NMR data of the ribose component of cytidine.

The ribose from the experiment with [1,2-$^{13}C_2$]glucose (expt 10) gave a spectrum that showed significant enrichment at C-1 (ca. 6× natural abundance) as well as strong C-4,5 coupling.[15c] The ribose from [4,5-$^{13}C_2$]glucose (expt 11) (Figure 2C) showed bond-labeling at C-3,4.[15b] As is evident from the comparison of the incorporation patterns of D-glucose observed in the cytidine−ribose[16] and in pyridoxamine in expts 10 and 11 (Table 3), these results are consistent with the intermediacy of a pentose or pentulose between glucose and pyridoxamine.

Table 2. ^{13}C NMR Spectrum of the Ribose Fragment of Cytidine[15] Chemical Shifts and Coupling Constants

chemical shift (ppm)	carbon atom no	coupling constants (± 0.5 Hz) $^{13}C-^{13}C$	
61.9	C-5	$^1J_{C-5,C-4}$	42.1
70.6	C-3	$^1J_{C-3,C-4}$	39.0
		$^1J_{C-3,C-2} + {}^1J_{C-3,C-4}$	76.4
75.1	C-2	$^1J_{C-2,C-1}$	42.7
		$^1J_{C-2,C-3} + {}^1J_{C-2,C-1}$	80.4
85.3	C-4	$^1J_{C-4,C-3}$	39.3
		$^1J_{C-4,C-5}$	41.7
		$^1J_{C-4,C-3} + {}^1J_{C-4,C-5}$	81.0
90.1	C-1	$^1J_{C-1,C-2}$	42.7

A somewhat different picture emerged in the case of the incorporation pattern from [$^{13}C_6$]glucose (expt 1). The cytidine−ribose[15a] showed major bond labeling at C-1,2 and at C-3,4,5, as was also found in the pyridoxamine from this experiment,[13a] but in addition the ribose spectrum showed significant bond-labeling at C-1,2,3 and at C-4,5 (ref 15a, cf. Figure 16 in ref 20) indicating the incorporation of an intact glucose-derived C_5 chain. Incorporation of such an intact C_5 chain had not been observed in any of the spectra of the pyridoxamine samples obtained from the experiments with [$^{13}C_6$]glucose carried out thus far (expts 1−4).

This apparent discrepancy prompted us to reexamine one of the samples of pyridoxamine derived from [$^{13}C_6$]glucose (expt 1) by a more sensitive NMR technique, to determine whether a

(15) Incorporation patterns within the ribose unit of cytidine: For assignments of the NMR signals in the ^{13}C NMR spectrum of cytidine see Kalinowski, H.-O.; Berger, S.; Braun, S. *Carbon-13 NMR Spectroscopy*; John Wiley & Sons: Chichester, 1988; p 440. Assignments of the C-2 and C-3 carbon atoms of the ribose moiety, there given, must be reversed. (a) *S. cerevisiae*, [$^{13}C_6$]glucose, expt 1: Major coupling: C-1,2 (C-1 90.1 ppm, $^1J_{C-1,C-2}$ = 42.8 Hz; C-2 75.1 ppm, $^1J_{C-2,C-1}$ = 42.6 Hz; C-3,4,5: C-3 70, 6 ppm, $^1J_{C-3,C-4}$ = 39.0 Hz; C-4 85.3 ppm, $^1J_{C-4,C-3} + {}^1J_{C-4,C-5}$ = 81.0 Hz; C-5 61.9 ppm, $^1J_{C-5,C-4}$ = 42.1 Hz. Minor coupling: C-1,2,3: $^1J_{C-2,C-1} + {}^1J_{C-2,C-3}$ = 80.1 Hz; C-2,3,4: $^1J_{C-3,C-2} + {}^1J_{C-3,C-4}$ = 76.3 Hz; C-3,4: $^1J_{C-3,C-4}$ = 39.0 Hz, $^1J_{C-4,C-3}$ = 39.7 Hz. (b) *S. cerevisiae*, [4,5-$^{13}C_2$]glucose, expt 11 (Figure 2C): C-3 70.6 ppm, $^1J_{C-3,C-4}$ = 38.7 Hz; C-4 85.3 ppm, $^1J_{C-4,C-3}$ = 39.3 Hz. (c) *S. cerevisiae*, [1,2-$^{13}C_2$]glucose, expt 10: C-1 90.1 ppm ^{13}C enrichment ca. 6 × natural abundance (NA); Major coupling: C-4 85.3 ppm, $^1J_{C-4,C-5}$ = 41.4 Hz; C-5 61.9 ppm, $^1J_{C-5,C-4}$ = 42.0 Hz; minor coupling: C-1 90.1 ppm, $^1J_{C-1,C2}$ = 42.3 Hz; C-2 75.1 ppm, $^1J_{C-2,C-1}$ = 42.0 Hz. (d) *C. utilis*, [$^{13}C_6$]glucose, expt 16 (Figure 2B): Major coupling: C-1,2 (C-1 90.1 ppm, $^1J_{C-1,C-2}$ = 42.9 Hz; C-2 75.1 ppm, $^1J_{C-2,C-1}$ = 42.8 Hz; C-3,4,5: C-3 70, 6 ppm, $^1J_{C-3,C-4}$ = 39.0 Hz; C-4 85.3 ppm, $^1J_{C-4,C-3} + {}^1J_{C-4,C-5}$ = 81.0 Hz, $^1J_{C-4,C-5}$ = 41.8 Hz; C-5 61.9 ppm, $^1J_{C-5,C-4}$ = 42.1 Hz. Minor coupling: C-1,2,3: $^1J_{C-2,C-1} + {}^1J_{C-2,C-3}$ = 80.4 Hz; C-2,3,4: $^1J_{C-3,C-2} + {}^1J_{C-3,C-4}$ = 76.6 Hz; C-3, 4: $^1J_{C-3,\,C-4}$ = 39.0 Hz, $^1J_{C-4,\,C-3}$ = 40.0 Hz. (e) *C. utilis*, [1, 2-$^{13}C_2$]glucose, expt 17 (Figure 2A): C-1 90.1 ppm, enrichment ca. 5 x NA.; major coupling: C-4 85.3 ppm, $^1J_{C-4,C-5}$ = 41.9 Hz; C-5 61.9 ppm, $^1J_{C-5,C-4}$ = 42.1 Hz. Minor coupling: C-1 90.1 ppm, $^1J_{C-1,C-2}$ = 42.5 Hz; C-2 75.1 ppm, $^1J_{C-2,C-1}$ = 42.9 Hz. (f) *C. utilis*, [1,2-$^{13}C_2$]xylose, expt 18 (Figure 2D): Major coupling: C-1 90.1 ppm, $^1J_{C-1,C-2}$ = 42.7 Hz; C-2 75.1 ppm, $^1J_{C-2,C-1}$ = 42.8 Hz; C-4 85.3 ppm, $^1J_{C-4,C-5}$ = 41.7 Hz; C-5 61.6 ppm, $^1J_{C-5,C-4}$ = 42.1 Hz. Minor coupling: C-3,4,5: $^1J_{C-4,C-3} + {}^1J_{C-4,C-5}$ = 81.4 Hz; C-2,3,4: $^1J_{C-3,C-2} + {}^1J_{C-3,C-4}$ = 74.4 Hz.

(16) It is clear from the observed distribution of ^{13}C within the cytidine−ribose and correspondingly within the C_5 unit, C-2′,2,3,4,4′, of pyridoxamine that the path of ^{13}C from the variously labeled samples of glucose (expts 1, 9, 10, 11, 16, 17) into the two products follows a complex route. An investigation of the steps of this route is beyond the scope of the present study of pyridoxine biosynthesis. However it is possible to rationalize the observed labeling patterns, including not only the major sites of bond-labeling, but also most of the minor sites, on the basis of an analysis of the complexity of the processes leading from glucose to pentoses, including the irreversible oxidative and the reversible non-oxidative pentose phosphate pathways as well as glycolysis and triose isomerization.[17−19]

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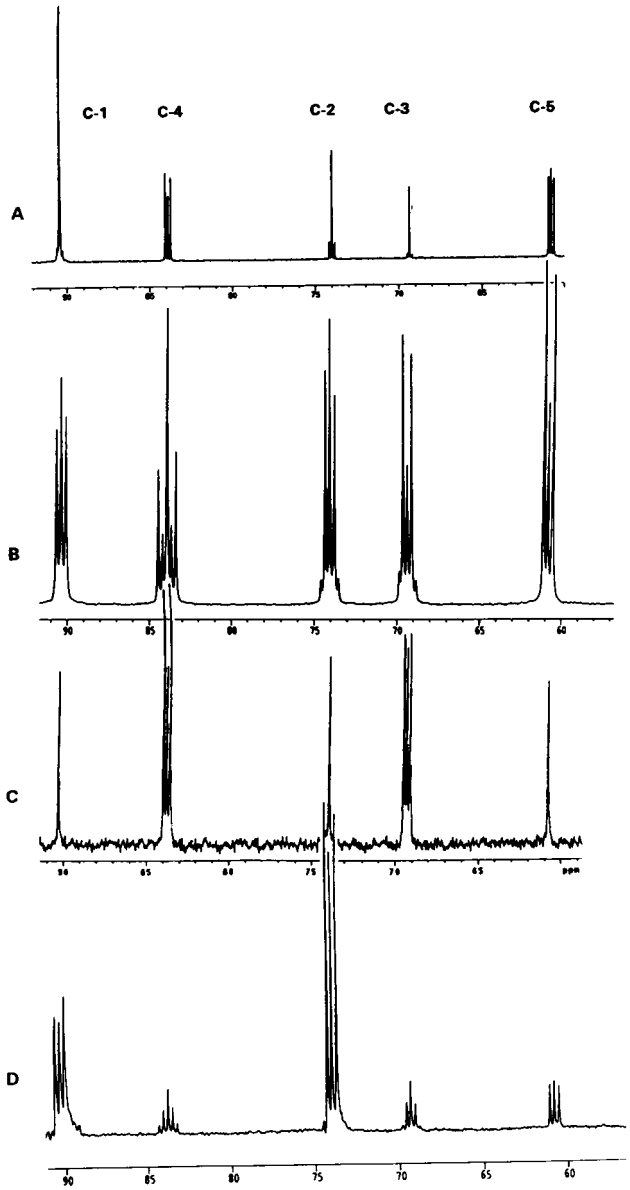

Figure 2. ^{13}C NMR spectra of the ribose unit of cytidine: (A) Cytidine−ribose derived from [1,2-$^{13}C_2$]glucose (expt 17). (B) Cytidine−ribose derived from [$^{13}C_6$]glucose (expt 16). (C) Cytidine−ribose derived from [4,5-$^{13}C_2$]glucose (expt 11). (D) Cytidine−ribose derived from [1,2-$^{13}C_2$]xylose (expt 18)

similar labeling pattern was present, even if only as a minor contributor. To do so we exploited the modified gHMBC sequence which one of us had previously employed in a study[21] of the incorporation, in *Gingko biloba*, of [$^{13}C_6$]glucose into gingkotoxin (4′-*O*-methylpyridoxol). Coupling was indeed evident between the protons attached to ^{13}C at C-2′ and the ^{13}C nucleus residing at C-3 (Supporting Information). It follows that the C_3 unit, C-2′,2,3, of pyridoxamine was derived intact from [$^{13}C_6$]glucose. Since an intact C_3 unit, C-3,4,4′, was shown to be present earlier,[13a] the gHMBC result provided the first evidence that an intact glucose-derived C_5 unit is incorporated into pyridoxamine, albeit to a minor extent. This was subsequently confirmed when pyridoxamine derived from [$^{13}C_6$]-glucose was isolated from the yeast *Candida utilis* (see below).

In the second approach to confirm the derivation of the pyridoxamine C_5 unit C-2′,2,3,4,4′ via a 5-carbon sugar,

(21) Fiehe, K.; Arenz, A.; Drewke, C.; Hemscheidt, T.; Williamson, R. T.; Leistner, E. *J. Nat. Prod.* **2000**, *63*, 185−189.

Table 3. Comparison of the ^{13}C and ^{2}H Incorporation Patterns within Cytidine−Ribose and Pyridoxamine

Precursor	Expt. No	Organism	Labeling pattern
[$^{13}C_6$]glucose	1 16	S. cerevisiae C. utilis	major (ca 9/10) minor (ca 1/10)
[6,6-$^{2}H_2$]glucose	16	C. utilis	
[1-^{13}C]glucose	8	S. cerevisiae	
[2-^{13}C]glucose	9	S. cerevisiae	
[1,2-$^{13}C_2$]glucose	10 17	S. cerevisiae C. utilis	
[4,5-$^{13}C_2$]glucose	11	S. cerevisiae	
[1,2-$^{13}C_2$]xylose	18	C. utilis	

incubations were carried out with a yeast, *Candida utilis* ATCC 9256, that utilizes D-xylose as a general carbon source. A 2 L culture of this yeast yielded 80−100 μg of pyridoxamine. D-[$^{13}C_6$]- and D-[1,2-$^{13}C_2$]glucose (expts 16 and 17) were again employed as substrates to verify that the mode of incorporation of these substrates into pyridoxamine corresponded to that which had been observed with *S. cerevisiae*, and incubations were carried out also with D-[6,6-$^{2}H_2$]glucose (expt 16) and with D-[1,2-$^{13}C_2$]xylose (expt 18). As before, pyridoxamine and cytidine were isolated.

Pyridoxamine obtained from D-[1,2-$^{13}C_2$]glucose (expt 17) gave a spectrum (Figure 3B) that, as before, showed coupling at C-5′,5 and at C-4′,4, but none at C-2′,2. However, the signal at C-2′ was enriched approximately 1.3-fold, relative to the C-6 signal (Figure 1A).[13f] The spectrum (Figure 2A) of the corre-

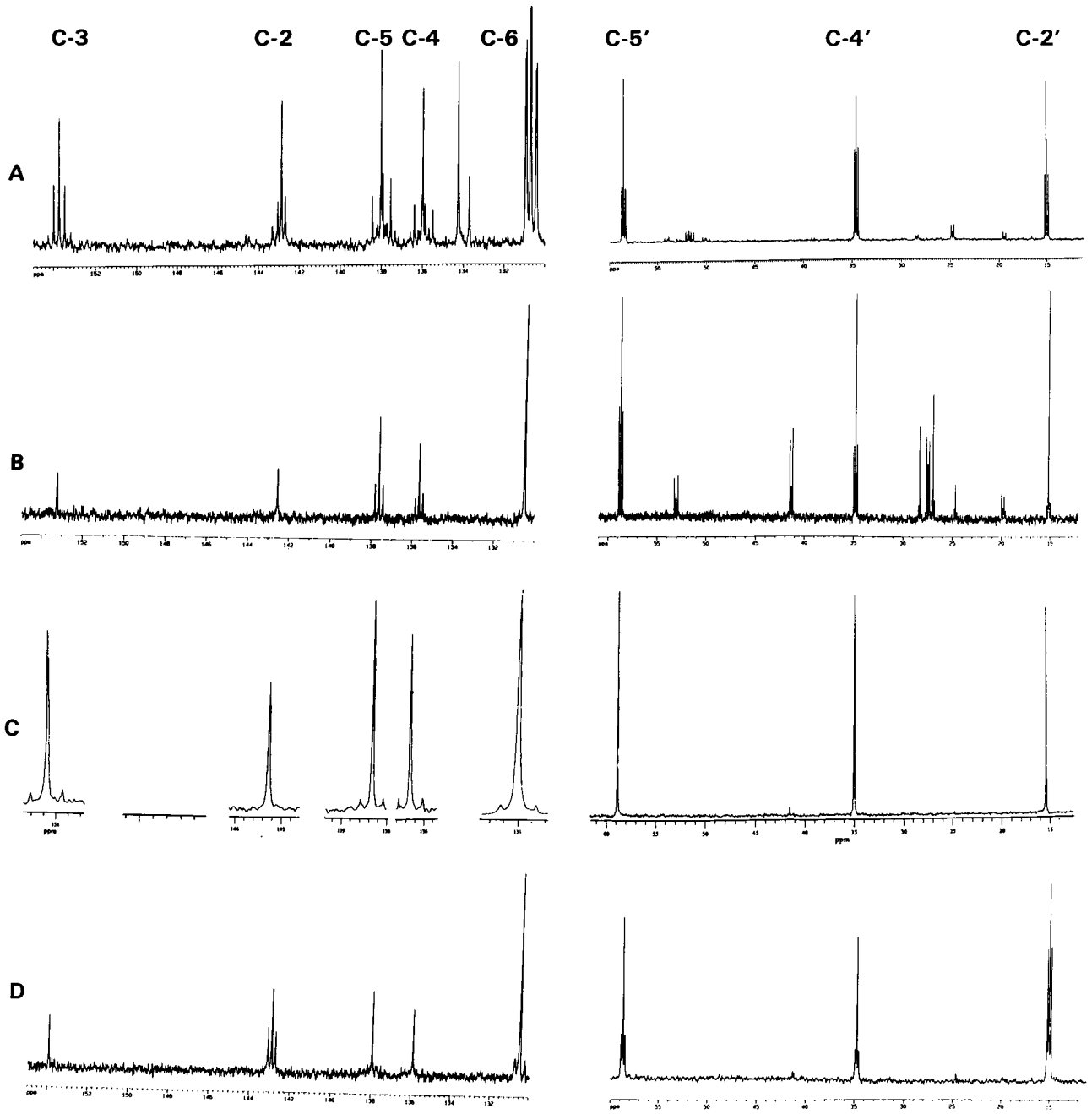

Figure 3. ^{13}C NMR spectra of pyridoxamine: (Spectra determined on different spectrometers were adjusted in size, so that they can be presented on the same scale): (A) Pyridoxamine di-HCl derived from [$^{13}C_6$]glucose (expt 16). (B) Pyridoxamine di-HCl derived from [1,2-$^{13}C_2$]glucose (expt 17). (C) Pyridoxamine di-HCl derived from [4,5-$^{13}C_2$]glucose (expt 11). (D) Pyridoxamine di-HCl derived from [1,2-$^{13}C_2$]xylose (expt 18).

sponding cytidine−ribose[15e] showed enrichment at C-1 corresponding to ca. 5× natural abundance, as well as strong coupling at C-4,5.

The cytidine−ribose from the *Candida* experiment with D-[$^{13}C_6$]glucose + D-[6,6-$^2H_2$]glucose (expt 16) gave a ^{13}C NMR spectrum (Figure 2B)[15d] essentially identical with that of the sample from the D-[$^{13}C_6$]glucose experiment with *S. cerevisiae*[15a] (expt 1). The pyridoxamine from expt 16 gave a ^{13}C NMR spectrum (Figure 3A)[13g] which, like that from expt 1,[13a] showed strong coupling at C-2,2′ and at 3,4,4′, but in addition showed coupling at C-2′,2,3 and at C-2,3,4.[22] Thus, an intact C_5 unit derived from glucose had entered the C_5 chain C-2′,2,3,4,4′.

The ^2H NMR spectrum of the cytidine−ribose from this experiment (expt 16) (as well as from the corresponding experiment with *S. cerevisiae* (expt 2)), showed deuterium enrichment at the two diastereotopic deuterium atoms at C-5 (3.77 and 3.88 ppm, relative to the signal of DOH at 4.72 ppm). The ^2H NMR spectrum of the pyridoxamine from this experiment showed deuterium enrichment at the C-4′- and the C-5′-CH_2OH groups (4.40 and 4.85 ppm, respectively, relative to HOD at 4.72). Incorporation of deuterium was not detectable either at the C-2′-CH_3 group (2.67 ppm) or at C-6 (8.2 ppm).

Comparison of the labeling pattern of each of the pyridoxamine samples with that within the cytidine−ribose samples isolated from the same experiment (Table 3) shows a cor-

(22) The differences in the NMR spectra of the pyridoxamine samples derived from [$^{13}C_6$]glucose isolated from *S. cerevisiae* (expt 1) and from *C. utilis* (expt 16) are quantitative in nature. The higher yield of pyridoxamine in expt 16 permitted detection of C-2,3,4 coupling. Furthermore, the pyridoxamine spectra from expts 16−18 were determined using a Shigemi tube, leading to improved sensitivity.

respondence in the labeling of the C_5 unit C-2′,2,3,4,4′ with that of the ribose. This strongly suggests that the C_5 unit C-2′,2,3,4,4′ and the cytidine−ribose are of common origin.

In the final experiment (expt 18) *C. utilis* was incubated in the presence of D-[1,2-$^{13}C_2$]xylose. The cytidine−ribose[15f] (Figure 2D) was found to be bond-labeled at C-1,2 and also at C-4,5.[23] The pyridoxamine sample from this experiment[13h] (Figure 3D) was strongly bond-labeled at C-2′,2. Coupling was present also at C-4′,4, at C-5′,5, and at C-5,6.

The transfer of bond-label from C-1,2 of the pentose substrate into C-2′,2 of pyridoxamine shows that the C_2-unit, C-2′,2, of pyridoxamine originates from the C_2-unit C-1,2 of a pentulose or pentose and provides further support for the view that a pent-(ul)ose serves as an intermediate between glucose and the C_5 unit.

While it is not yet possible to advance a route to the pyridoxamine skeleton in *S. cerevisiae* and *C. utilis*, the results that are now presented provide compelling evidence in support of the view that the vitamin B_6 carbon skeleton in yeasts arises by a route that is different from that in *E. coli*.

Experiments are in progress to provide answers to the remaining questions concerning the biosynthetic anatomy of vitamin B_6 in yeast, that is, the origin of the segment N-1,C-6,5,5′ of pyridoxamine, the stage of the biosynthetic process at which the C-2′ hydroxy group is eliminated, and the manner whereby ring formation takes place.

Table 4. Incubation of Yeast Cultures with ^{13}C-Labeled Substrates

expt	labeled substrate	weight mg/L (mM)	incubation volume (L)	incubation duration (H)	isolated product
		Saccharomyces cerevisiae			
1	D-[$^{13}C_6$]glucose	1000 (5.4)	2 × 1	12	Pyr (Supp. Inf.)
	+ D-glucose	4000 (22.2)			Cyt
2	D-[$^{13}C_6$]glucose	1000 (5.4)	2 × 1	17	Pyr
	+ D-[6,6-$^2H_2$]glucose	5000 (27.8)			Cyt
3	D-[$^{13}C_6$]glucose	1250 (6.7)	2 × 1	8	Pyr
	+ D-glucose	4000 (22.2)			
	+ Na^+ pyruvate	1250 (11.4)			
4	D-[$^{13}C_6$]glucose	1000 (5.4)	2 × 1	24	Pyr
	+ D-glucose	4000 (22.2)			
	+ D-erythronic lactone	1000 (8.5)			
8	D-[1-^{13}C]glucose	5000 (27.6)	1	12	Pyr (Figure 1C)
9	D-[2-^{13}C]glucose	2000 (11.0)	2 × 1	24	Pyr (Figure 1B)
	+ D-glucose	1500 (8.3)			
10	D-[1,2-$^{13}C_2$]glucose	1000 (5.5)	2 × 1	12	Pyr
	+ D-glucose	4500 (25.0)			Cyt
11	D-[4,5-$^{13}C_2$]glucose	500 (2.8)	2 × 1	18	Pyr (Figure 3C)
	+ D-glucose	3500 (19.4)			Cyt (Figure 2C)
13	Na^+ [2,3-$^{13}C_2$]pyruvate	1000 (8.9)	2 × 1	12	Pyr
	+ D-glucose	6200 (34.7)			
		Candida utilis			
16	D-[$^{13}C_6$]glucose	1000 (5.4)	2 × 1	16	Pyr (Figure 3A)
	+ D-[6,6-$^2H_2$]glucose	5000 (27.5)			Cyt (Figure 2B)
17	D-[1,2-$^{13}C_2$]glucose	1000 (5.5)	2 × 1	16	Pyr (Figure 1A, 3B)
	+ D-glucose	4000 (22.2)			Cyt (Figure 2A)
18	D-[1,2-$^{13}C_2$]xylose	1000 (6.5)	2 × 1	26	Pyr (Figure 3D)
	+ D-xylose	4000 (26.7)			Cyt (Figure 2D)

Experimental Section

^{13}C-Labeled Compounds. All but one of the labeled compounds were purchased from Cambridge Isotope Laboratories, Andover, MA. The exception was D-[4,5-$^{13}C_2$]glucose, which was obtained from Isotech Inc., Miamisburg, Ohio.

Microorganisms. *Saccharomyces cerevisiae* ATTC 7752 (IFO 1234) was cultured and worked up as described earlier.[12] Experiments with *Candida utilis* ATTC 9256 were carried out in a similar manner. The yeast cultures were grown to a cell density of 1.6 or greater (12−24 h). Details of the experiments are summarized in Table 4.

Isolation of Pyridoxamine and Cytidine. Cells isolated by centrifugation from the incubation medium (2 L) were suspended in 0.1 M HCl (20 mL). The mixture was spiked with pyridoxamine dihydrochloride (2.5 mg) and autoclaved at 120° C for 70 min. After cooling the mixture was centrifuged and the supernatant collected. The cell mass was washed twice with water (10 mL) with ultrasonic mixing, and the washings were collected by centrifugation. The combined supernatant and washings were neutralized with 0.1 M NaOH, and the pH was adjusted to pH 4.7 with 0.2 M sodium acetate buffer. The mixture was treated with Takadiastase (Sankyo) (50 mg) and incubated overnight at 37° C.

The clear liquid was passed at room temperature through a 5 g SCX column (Varian, Harbor City, CA) at a slow rate, using mild water pump suction. The column had been prepared by successive washings with one-column volumes of 1 M NH_4OH, methanol, 1 M HCl, methanol, and finally with water. The column was then washed (1×) with water and methanol, and the drainings and washings were saved for the isolation of cytidine. The column was then eluted with 2.5% NH_4OH in methanol (75 mL).

Isolation of Pyridoxamine. For liquid chromatography (LC) a semi-prep column (25 cm × 10 mm i.d.), packed with 5 μm Ultrasphere octadecylsilane (ODS) silica particles (Beckman, Fullerton CA) and protected with an ODS guard column, was used. The mobile phase containing 400 μL of formic acid plus 800 μL of concentrated NH_4OH per L of water was pumped at a flow rate of 2.5 mL/min, using a model LC 10 AT pump (Shimadzu, Columbia, MD). The peaks were monitored by a 254 nm model 450 UV detector (Alltech, Dearfield, IL) and recorded on a model CR-5A integrator (Shimadzu). Samples were injected with a Rheodyne model 7725i injector, fitted with a 1 mL loop.

The SCX extract was evaporated to dryness, and the residue was dissolved in the formic acid/ammonia mobile phase (1 mL) and centrifuged. The clear supernatant was injected into the LC column in 200 μL aliquots, and the peak eluting at the retention time of pyridoxamine (ca. 7 min) was collected. At about 20 min the mobile phase was replaced by methanol, and at about 30 min methanol was again replaced by the mobile phase. The next injection was made after the column had reequilibrated with the mobile phase.

The fractions containing pyridoxamine were combined and evaporated. The residue was dissolved in water (2 mL) and applied to a washed (see above) 500 mg SCX column at a slow rate. The column was washed with 2 mL of water and 1 mL of methanol and eluted with a mixture of dichloromethane (20 mL), methanol (2 mL), and concentrated NH_4OH (0.2 mL). The elution was carried out by gravity or under mild positive pressure of nitrogen. The eluate was evaporated and the residue placed in a vacuum desiccator to remove all traces of ammonia. The residue was dissolved in 1% HCl in methanol which converts pyridoxamine into its di-HCl salt, the solution was evaporated, yielding a residue which is ready for NMR determination. As shown by the NMR spectra (Figures 1 and 3) most of the samples were pure. Minor highly ^{13}C-enriched nonaromatic contaminants were present in the samples from expt 8 (Figure 1C), expt 16 (Figure 3A), and expt 17 (Figures 1A and 3B). Their presence did not interfere with the interpretation of the spectra.

Isolation of Cytidine. Cytidine was isolated from the cell hydrolysate without addition of carrier. About 20−30% of the cytidine present in the cell hydrolysate is retained by the SCX column together with pyridoxamine. The rest of the cytidine passes through the column. The drainings and washings of the SCX column were saved and processed with the regenerated 5 g SCX column (regenerated as in the preparation of the column used for the first extraction, see above). The adsorbed bases were eluted with 2.5% ammoniacal methanol (70 mL). The residue of the eluate was subjected to HPLC.

The peak corresponding to the retention time of cytidine (ca. 15 min) was collected from the pyridoxamine extract as well as from the

(23) The bond labeling in the cytidine−ribose derived from D-[1,2-$^{13}C_2$]-xylose is readily understood on the basis of the metabolism of xylose reported to occur in *C. utilis*.[24]

(24) (a) Horitsu, H.; Tomoeda, M. *Agr. Biol. Chem.* **1966**, *30*, 962−966. (b) Horitsu, H.; Tomoeda, M.; Kumagai, K. *Agr. Biol. Chem.* **1968**, *32*, 514−517.

second extract. The cytidine fractions were combined and evaporated. The residue, dissolved in ca. 2−3 mL water was passed through a washed 2 g fast SCX column. The column was washed with water (2 mL) followed by methanol (1 mL) and was then eluted with 2.5% NH_4-OH in methanol (25 mL). The eluate was evaporated to yield cytidine, ready for NMR spectroscopy. The NMR spectra (Figure 2) showed that the samples were pure.

NMR Spectroscopy. Instrumentation.NMR spectra were acquired at McMaster and at the University of Hawaii. At McMaster: on a Bruker DRX 500 spectrometer, operating at 11.74 T, using a Bruker 2.5 mm microprobe (expts 1−15) or a Shigemi tube (expts 16−18) with 100 μL of solution; pulse width 90° (13.5 μs), spectral width 28 985.5 Hz, recycle delay 4.6 s, digital resolution 0.88 Hz per data point. At the University of Hawaii: on a GE Omega 500 spectrometer, using a Nalorac 3 mm microtube, pulse width 60°, recycle delay 2 s, 32K data points.

Spectra: 125.776 Hz proton decoupled ^{13}C NMR spectra of pyridoxamine and of cytidine in 100 μL of D_2O. Approximately 50 000 transients were required for the pyridoxamine samples, and approximately 15000 scans were needed for the cytidine samples to generate the ^{13}C spectra showing satellites.

^{2}H Spectra were obtained on a Bruker Avance 300 instrument, operating at 46.07 MHz at 7.05 T. Spectra were acquired at 30° C using a 30° pulse with a 4.55 s recycle time with a digital resolution of 0.114 Hz per point. Signals were referenced to H_2O at 4.72 ppm.

Acknowledgment. This work was supported by the National Institute of General Medical Sciences, U.S. Public Health Service (Grant GM 50778, to I.D.S.). An upgrade of the NMR instrumentation in Hawaii that was used for this investigation was funded by the NSF (CHE 9974921) and by the Pardee Foundation. We thank Richard M. Pauloski for skilled technical assistance.

Supporting Information Available: Modified Gradient HMBC spectrum (two-dimensional) of pyridoxamine dihydrochloride, derived from D-[$^{13}C_6$]glucose in *S. cerevisiae* (expt 1) (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA0113201