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# Biosynthesis of Vitamin $B_6$ in Yeast. Incorporation Pattern of Trioses

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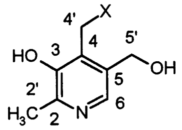
The biosynthetic origin of the $C_3$ unit, C-6,5,5′, of pyridoxamine was investigated in two yeasts, *Candida utilis* ATCC 9256 and *Saccharomyces cerevisiae* ATCC 7752. The incorporation patterns within pyridoxamine bishydrochloride derived from variously multiply $^{13}C$- and $^{2}H$-labeled samples of glycerol and glyceraldehyde, established by NMR spectroscopy, indicate that the three-carbon unit C-6,5,5′ of pyridoxamine is derived intact from a triose.

## Introduction

It is now well-documented on the basis of genetic investigations[1−3] as well as by tracer studies with $^{15}N$[4,5] and $^{13}C$[6,7] that two distinct routes exist for the biosynthesis of Vitamin $B_6$ (pyridoxine, **1**) from primary precursors.

Investigations of the origin of the carbon skeleton of pyridoxine in the bacterium *Escherichia coli*[6] as well as in yeasts[7] (e.g., *Saccharomyces cerevisiae*), employing glucose as the general carbon source, established by tracer studies with $^{13}C$-labeled substrates that the intermediates on the route from glucose to the vitamin are different in the two types of microorganism (Schemes 1 and 2).

In *E. coli* the $C_5$ unit C-2′,2,3,4,4′ of the vitamin originates via 1-deoxy-D-xylulose 5-phosphate[8,9] (**4a**), which is formed from two glucose catabolites: condensation accompanied by decarboxylation (i.e., loss of C-3 of glucose) of pyruvic acid (**3**) (derived from C-1,2,3 of glucose) with phosphoglyceraldehyde (**2**) (i.e., C-4,5,6 of glucose) gives rise to C-2′,2,3,4,4′ of the pyridoxine skeleton, from C-1,2,4,5,6 of glucose (Scheme 1). Label from [2,3-$^{13}C_2$]pyruvate enters C-2′,2 of pyridoxol and label from [2,3-$^{13}C_2$]-1-deoxy-D-xylulose enters the $C_5$ unit C-2′,2,3,4,4′ of pyridoxol (**1a**), and unlabeled 1-deoxy-D-xylulose (**4b**) displaces entry of label from glucose into this unit.[6] A kinase catalyzing the phosphorylation of 1-deoxy-D-xylulose (**4b**) has been identified in *E. coli*.[10]



**1a** X = OH
**1b** X = $NH_2$

In the yeast *S. cerevisiae*, by contrast, label from [2,3-$^{13}C_2$]pyruvate did not enter the vitamin, nor did unlabeled sodium pyruvate displace entry of a glucose-derived $C_2$ unit into C-2′,2.[7] Furthermore, tracer experiments with variously $^{13}C$-labeled samples of glucose in this yeast, and of glucose, xylose and ribose in *Candida utilis*, showed that the $C_5$ unit C-2′,2,3,4,4′ of the vitamin is derived from the $C_5$ fragment, C-2,3,4,5,6 of glucose, via a pent(ul)ose[7] (**5**) (Scheme 1). Thus, label from [1,2-$^{13}C_2$]xylose[7] and from [1,2-$^{13}C_2$]ribose (expt 14, Table 1) is incorporated into C-2′,2 of pyridoxamine (Figure 1B).

The evidence is compelling that the derivation of the $C_5$ unit C-2′,2,3,4,4′ of pyridoxine from glucose takes different routes in *E. coli*[6] and in yeasts.[7]

The present investigation concerns the route from glucose into the remaining $C_3$ unit, C-6,5,5′, of pyridoxine. In *E. coli* this unit, together with the N atom, is derived from glucose via 1-amino-3-hydroxyacetone 3-phosphate (**9a**).[11] This precursor, which thus gives rise to the $C_3N$ unit 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-hydroxy-L-threonine 4-phosphate[11] (**8a**),

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(1) (a) Lam, H. M.; Tancula, E.; Dempsey, W. B.; Winkler, M. E. *J. Bacteriol.* **1992**, *174*, 1554−1567. (b) Roa, B. B.; Connolly, D. M.; Winkler, M. E. *J. Bacteriol.* **1989**, *171*, 4767−4777.

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(5) Tanaka, K.; Tazuya, K.; Yamada, K.; Kumaoka, H. *J. Nutr. Sci. Vitaminol.* **2000**, *46*, 55−57.

(6) Hill, R. E.; Himmeldirk, K.; Kennedy, I. A.; Pauloski, R. M.; Sayer, B. G.; Wolf, E.; Spenser, I. D. *J. Biol. Chem.* **1996**, *271*, 30426−30435.

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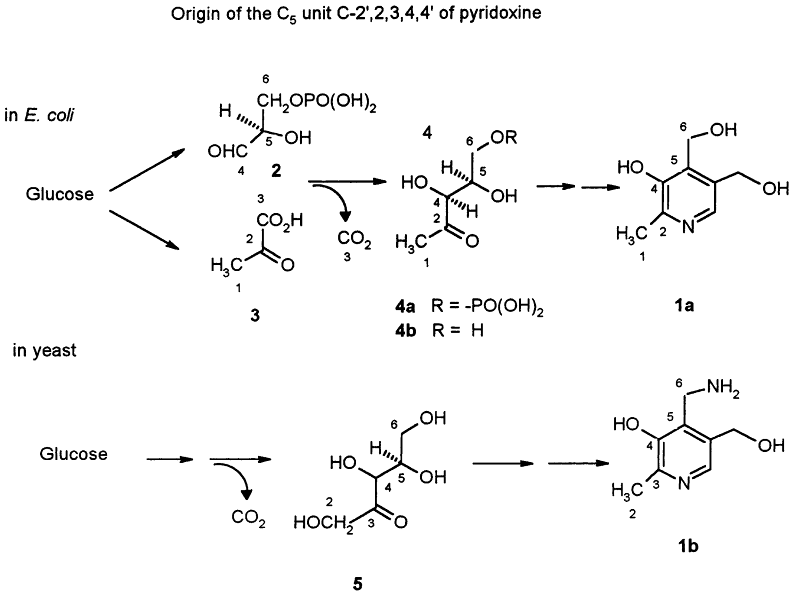
(9) Cane, D. E.; Du, S.; Spenser, I. D. *J. Am. Chem. Soc.* **2000**, *122*, 4213−4214.

(10) Wungsintaweekul, J.; Herz, S.; Hecht, S.; Eisenreich, W.; Feicht, R.; Rohdich, F.; Bacher, A.; Zenk, M. H. *Eur. J. Biochem.* **2001**, *268*, 310−316.

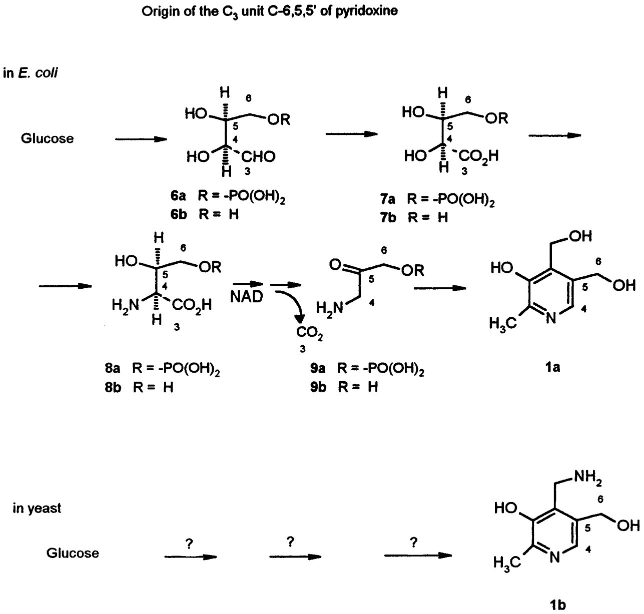
(11) Cane, D. E.; Hsiung, Y.; Cornish, J. A.; Robinson, J. K.; Spenser, I. D. *J. Am. Chem. Soc.* **1998**, *120*, 1936−1937.

10.1021/jo020730k CCC: $25.00

**SCHEME 1. Dissimilar Derivations from Glucose of $C_5$ Unit C-2′,2,3,4,4′ of Pyridoxine in *E. coli* and in Yeast**



**SCHEME 2. Derivation of $C_3$ Unit C-6,5,5′ of Pyridoxine in *E. coli* and in Yeast**



which in turn is derived from D-erythronic acid 4-phosphate[12] (**7a**) (i.e., from C-3,4,5,6 of glucose). Unlabeled 4-hydroxy-L-threonine (**8b**) displaces incorporation of label from [$^{13}C_6$]glucose into C-6,5,5′, and label from [2,3-$^{13}C_2$]-4-hydroxy-L-threonine and from D-[2,3-$^{13}C_2$]erythronic acid (**7b**) entered this site[6] (Scheme 2). Phosphorylation of these substrates must thus have taken place. A kinase catalyzing the conversion of **8b** into **8a** has been characterized in *E. coli*.[13]

In yeast the $C_3$ unit C-6,5,5′ of pyridoxamine is likewise derived from C-4,5,6 of glucose as an intact unit[7] (Scheme 2).

The correspondence, in yeast, of the pyridoxine carbon atoms with those of glucose is shown in Schemes 1 and 2. (Numbers in the structures in Schemes 1 and 2 refer to the carbon atoms of D-glucose from which the carbon atoms of pyridoxine and of the intermediates are derived.)[14]

The mode of entry of label from glucose into the $C_3$ unit C-6,5,5′ of pyridoxine thus appears to be similar in *E. coli* and in yeast (Scheme 2). Whereas the steps in *E. coli* are now well-understood, no information on the process in yeast is available. The present study was carried out to gain information on the intermediates on the route from glucose into this $C_3$ unit in yeast. The evidence here presented shows that there are significant differences in the derivation of this unit from glucose in the two types of microorganism.

## Results and Discussion

The first experiments that were carried out tested the incorporation into pyridoxamine (**1b**) in two yeasts, *S. cerevisiae* ATCC 7752 (= IFO 1234) and *C. utilis* ATCC 9256, of compounds that were known to serve as precursors in *E. coli*. It was found (expt 1, Table 1) that in *S. cerevisiae* label from [2,3-$^{13}C_2$]-4-hydroxy-L-threonine (**8b**) did not enter pyridoxamine, nor did unlabeled D-erythrose (**6b**) (expt 2), D-erythronic acid,[7] (**7b**) or 1-amino-3-hydroxyacetone (**9b**) (expt 3) displace [$^{13}C_6$]glucose-derived label from C-6,5,5′. In *C. utilis* incubation with [$^{13}C_6$]glucose in the presence of 1-amino-3-hydroxyacetone (**9b**) (expt 9) did not lead to displacement of label from this $C_3$ unit. Since in *E. coli* [2,3-$^{13}C_2$]-4-hydroxy-L-threonine,[6] [2,3-$^{13}C_2$]-D-erythronic acid[6] (**7b**) and aminohydroxyacetone phosphate[11] (**9a**) each serves as a precursor of the $C_3$ unit, C-6,5,5′, it is clear that in yeast, unlike in *E. coli*, uptake or phosphorylation of these compounds is inhibited or that the route from glucose into this unit is different.

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[12] (a) Lam, H.-M.; Winkler, M. E. *J. Bacteriol.* **1990**, *172*, 6518–6528. (b) Man, T.-K.; Zhao, G.; Winkler, M. E. *J. Bacteriol.* **1996**, *178*, 2445–2449 and references quoted therein.

[13] Zhao, G.; Winkler, M. E. *FEMS Microbiol. Lett.* **1996**, *135*, 275–280.

[14] As a result of the triose phosphate isomerase and aldolase reactions, carbon atoms derived from C-1,2,3 of glucose also show labeling from C-6,5,4 and vice versa.

**TABLE 1. Incubation of Yeast Cultures with $^{13}$C-Labeled Substrates**

| expt no. | labeled substrate | weight (mg/L) | incubation (h) | |
|---|---|---|---|---|
| | *Saccharomyces cerevisiae* | | | |
| 1 | D-glucose | 4000 | 17 | no incorp |
| | [2,3-$^{13}C_2$]-4-hydroxy-L-threonine | 360 | | |
| 2 | D-[$^{13}C_6$]glucose | 1000 | 18 | no displ |
| | D-glucose | 2000 | | |
| | D-erythrose | 1900 | | |
| 3 | D-[$^{13}C_6$]glucose | 1000 | 18 | no displ |
| | D-glucose | 4000 | | |
| | 1-amino-3-hydroxyacetone | 1750 | | |
| 4 | D-[$^{13}C_6$]glucose | 1000 | 12 | no displ |
| | D-glucose | 4000 | | |
| | L-aspartic acid | 1500 | | |
| 5 | D-[$^{13}C_6$]glucose | 1000 | 17 | no displ |
| | D-glucose | 4000 | | |
| | glycolaldehyde | 500 | | |
| 6 | D-[$^{13}C_6$]glucose | 1000 | 44 | no displ |
| | D-glucose | 4000 | | |
| | DL-hydroxyaspartic acid | 2000 | | |
| 7 | D-[$^{13}C_6$]glucose | 1000 | 17 | no displ |
| | D-glucose | 4000 | | |
| | DL-isoserine | 2000 | | |
| | *Candida utilis:* D-Glucose as General Carbon Source | | | |
| 8 | D-[$^{13}C_6$]glucose | 1000 | 17 | no displ |
| | D-glucose | 4000 | | |
| | fumaric acid | 2000 | | |
| 9 | D-[$^{13}C_6$]glucose | 1000 | 22 | no displ |
| | D-glucose | 4000 | | |
| | 1-amino-3-hydroxyacetone | 850 | | |
| 10 | D-[$^{13}C_6$]glucose | 1000 | 17 | no displ |
| | D-glucose | 4000 | | |
| | DL−1-amino-2,3-dihydroxy-propane | 2000 | | |
| 11 | D-[$^{13}C_6$]glucose | 1000 | 17 | no displ |
| | D-glucose | 4000 | | |
| | L-homoserine | 1000 | | |
| 12 | D-[$^{13}C_6$]glucose | 1000 | 16 | no displ |
| | D-glucose | 4000 | | |
| | DL-glyceraldehyde | 2000 | | |
| 13 | D-[$^{13}C_6$]glucose | 1000 | 20 | no displ |
| | D-glucose | 4000 | | |
| | D-glyceraldehyde | 1800 | | |
| 19 | D-glucose | 3750 | 22 | incorp (Figure 3C) |
| | [1,1,2,3,3-$^{2}H_5$]glycerol | 1250 | | |
| | *Candida utilis:* D-Xylose as General Carbon Source | | | |
| 14 | D-xylose | 1000 | 72 | incorp (Figure 1B) |
| | D-ribose | 3000 | | |
| | D-[1,2-$^{13}C_2$]]ribose | 1000 | | |
| 15 | D-xylose | 4000 | 18 | no incorp |
| | [2,3-$^{13}C_2$]succinic acid | 1500 | | |
| 16 | D-xylose | 3500 | 21 | incorp (Figure 2B) |
| | [1,3-$^{13}C_2$]glycerol | 1500 | | |
| 17 | D-xylose | 4000 | 20 | incorp (Figure 3B) |
| | D-[3,3-$^{2}H_2$]- glyceraldehyde | 1000 | | |
| 18 | D-xylose | 3750 | 22 | incorp |
| | [1,1,2,3,3-$^{2}H_5$]glycerol | 1250 | | |
| 20 | D-xylose | 3600 | 20 | incorp (Figure 3D) |
| | (*R*)-[1,1-$^{2}H_2$]glycerol | 1400 | | |
| 21 | D-xylose | 3600 | 20 | no incorp (Figure 3E) |
| | (*S*)-[1,1-$^{2}H_2$]glycerol | 1450 | | |
| 22 | D-xylose | 5000 | 23 | incorp (Figure 3F) |
| | $D_2O$ | 50% | | |
| 23 | D-xylose | 3750 | 23 | incorp |
| | glycerol | 1250 | | |
| | $D_2O$ | 50% | | |

In further experiments, compounds were tested that are derivable from glucose and whose incorporation into pyridoxine in yeast had been reported in early work: label from [2,3-$^{13}C_2$]succinic acid[15] (expt 15) was not incorporated, while fumaric acid[15] (expt 8), L-aspartic acid[16,17] (expt 4), or glycolaldehyde[17] (expt 5) did not displace entry of label from [$^{13}C_6$]glucose into C-6,5,5′ of pyridoxamine. Other compounds whose potential incorporation was examined appeared to have structural features analogous to those required to generate the segment N-1,C-6,5,5′ of the pyridoxine skeleton (Scheme 3). These were DL-1-amino-2,3-dihydroxypropane (**10**)

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[15] Sato, K. *Vitamins (Jpn)* **1966**, *33*, 357−360.

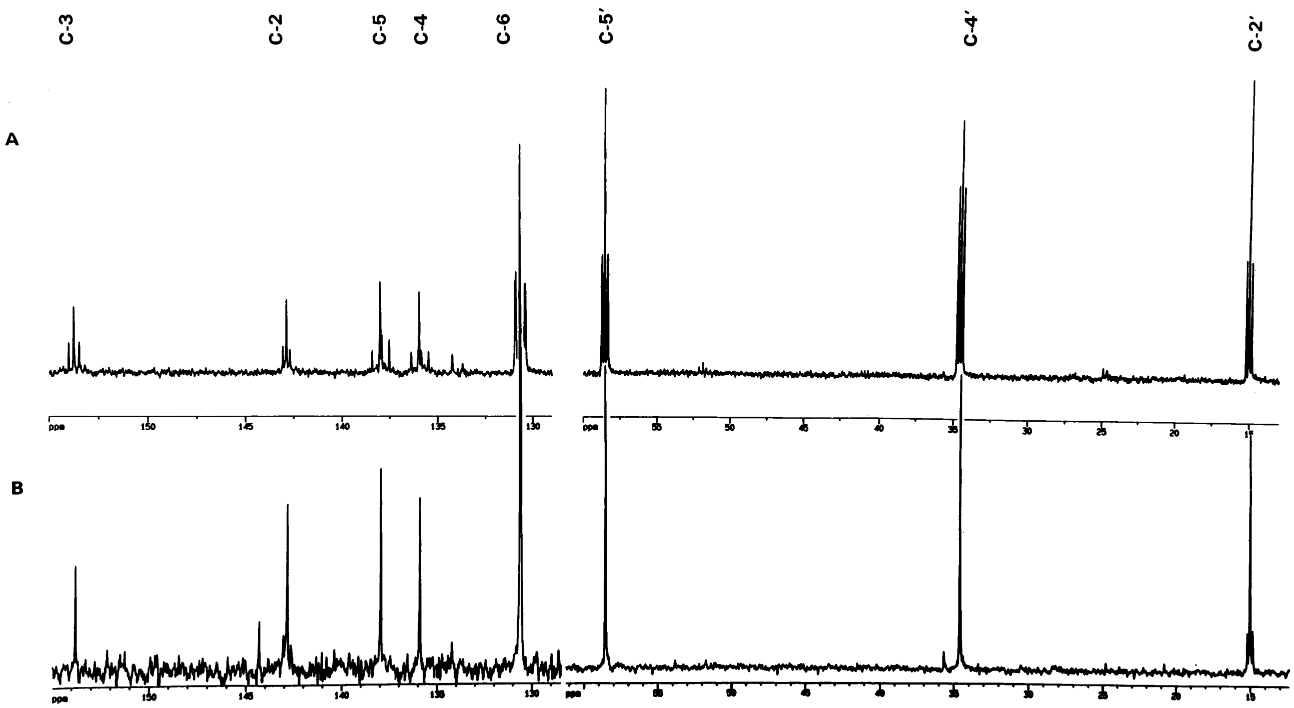

**FIGURE 1.** $^{13}C$ NMR spectra of samples of pyridoxamine bis-HCl derived from incubation of *Candida utilis* in the presence of (A) D-[$^{13}C_6$]glucose (ref 7, Figure 3A) and (B) D-[1,2-$^{13}C_2$]ribose (expt 14).

(expt 10), L-homoserine (**11**) (expt 11), DL-*threo*-3-hydroxyaspartic acid (**13**) (expt 6), and DL-isoserine (**14**) (expt 7). None of them displaced entry of $^{13}C$ from [$^{13}C_6$]glucose into pyridoxamine.

Since in yeast an intact glucose-derived $C_3$ fragment had been found to generate C-6,5,5′ of pyridoxamine,[7] the possible derivation of this fragment from a triose must be examined. Experiments with $C_3$ compounds related to glucose were performed with *C. utilis*, a yeast that has much higher permeability than *S. cerevisiae* to glycerol and glyceraldehyde.[18] Attempts to demonstrate incorporation of DL- or D-glyceraldehyde (expts 12 and 13) by displacement of label from [$^{13}C_6$]glucose failed. The close metabolic relationship of glucose and glyceraldehyde 3-phosphate presumably results in nonspecific displacement of label from [$^{13}C_6$]glucose. Alternatively, uptake of glyceraldehyde into the cells may be inhibited in the presence of excess glucose in the medium.

In further experiments D-glucose was replaced by D-xylose, which *C. utilis* can utilize as general carbon source. No displacement experiments were carried out on uniformly $^{13}C$-labeled xylose, whose cost is astronomical, whereas the price of commercially available $^{13}C$-labeled and particularly of $^{2}H$-labeled samples of glycerol and glyceraldehyde is affordable.

The result of an experiment with [1,3-$^{13}C_2$]glycerol (expt 16), with D-xylose as the general carbon source, showed that this $C_3$ compound entered the $C_3$ unit C-6,5,5′ of pyridoxamine: comparison of the $^{13}C$ NMR spectrum of this sample (Figure 2B) with that of natural abundance $^{13}C$ pyridoxamine (Figure 2A) showed that in the former the signals due to C-6 (131.1 ppm) and C-5′ (58.8 ppm), as well as that due to C-4′ (34.9 ppm), were enriched, relative to the signal due to C-2′ (15.2 ppm), by a factor of 2. Since it is most unlikely that glycerol should have lost one of the $^{13}C$-enriched terminal carbon atoms, leaving the resulting two carbon fragment to be incorporated separately and equally into C-6,5 and C-5,5′ of pyridoxamine, this result, together with the earlier finding[7] that a $C_3$ unit from [$^{13}C_6$]glucose was incorporated intact, provides evidence for the derivation of C-6,5,5′ of pyridoxamine from a compound related to glycerol.[19]

With D-xylose as the general carbon source and D-[3,3-$^{2}H_2$]glyceraldehyde as the labeled substrate (expt 17), deuterium was detected at C-5′ of pyridoxamine (Figure 3B). This observation is consistent with the earlier finding that label from [6,6-$^{2}H_2$]glucose had entered this site.[7] Glycolysis converts C-4,5,6 of [6,6-$^{2}H_2$]glucose into C-1,2,3 of [3,3-$^{2}H_2$]glyceraldehyde 3-phosphate (**2**).

Surprisingly, a similarly labeled pyridoxamine was obtained when [1,1,2,3,3-$^{2}H_5$]glycerol (expt 18) served as

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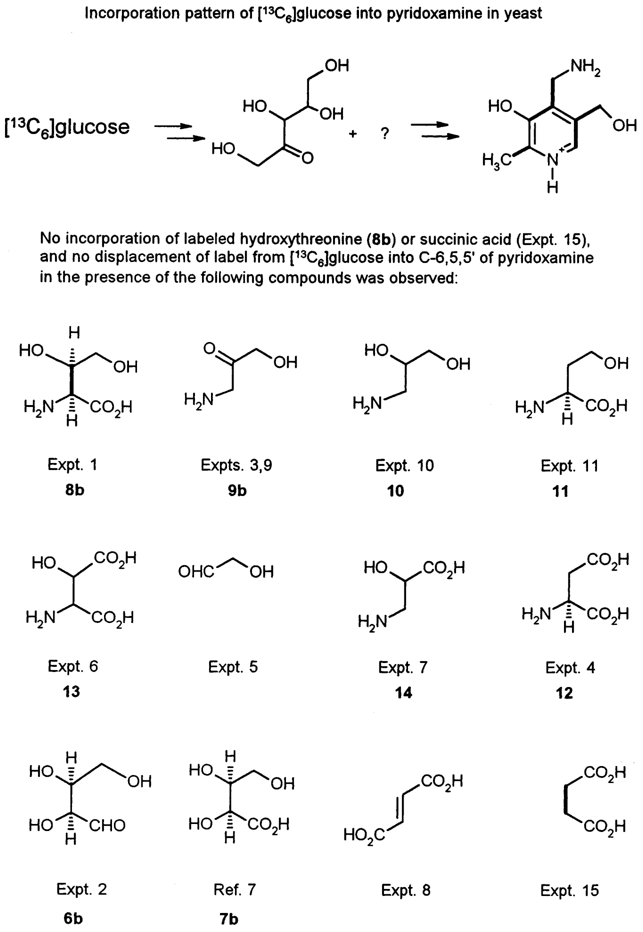
[16] Sato, K.; Suzuki, T.; Sahashi, Y. *Vitamins (Jpn)* **1965**, *31*, 356–358.

[17] Schroer, R. A.; Frieden, E. H. *Proc. Soc. Exp. Biol.* **1973**, *142*, 369–373.

[18] Gancedo, C.; Gancedo, J. M.; Sols, A. *Eur. J. Biochem.* **1968**, *5*, 165–172.

[19] Since glycerol serves as a precursor also of pentoses, i.e., of the source of the $C_5$ unit C-2′,2,3,4,4′ of pyridoxamine, it was not unexpected that enrichment was detectable also at C-4′. Since signal strength due to the aromatic carbon atoms other than C-6 is much less intense than signal strength due to the peripheral C atoms of pyridoxamine, it was not possible to evaluate enrichment at C-3. In an earlier study of the incorporation of label from [1,3-$^{14}C$]glycerol into the pentose-derived $C_5$ unit C-4′,4,5,6,7 of the thiazole unit of thiamin in yeast, label was shown to be present at the thiazole C atoms that are derived from C-1 (28%), C-2 (12%), C-3 + C-4 (30%), and C-5 (30%), respectively, of the pentose. A similar distribution of label into the pentose-derived segment of pyridoxamine in yeast leads to predicted labeling within pyridoxamine at C-2′ (28%), C-2 (12%), C-3 + C-4 (30%), and 4′ (30%).[20]

**SCHEME 3. Compounds That Did Not Spare Incorporation of Label from D-[$^{13}C_6$]Glucose into $C_3$ Unit C-6,5,5′ of Pyridoxine in Yeast and Are Therefore Unlikely To Be Intermediates in the Biosynthesis**



the labeled substrate. Deuterium was confined to the C-5′ site. None was present at C-6. Similarly, with [1,1,2,3,3-$^{2}H_5$]glycerol as the tracer and D-glucose as the general carbon source (expt 19, Figure 3C), deuterium was located at C-5′ of pyridoxamine, with minor incorporation into C-4′ and none into C-6. Metabolic oxidation of [1,1,2,3,3-$^{2}H_5$]glycerol, via stereospecific phosphorylation, oxidation to dihydroxyacetone phosphate, followed by isomerization, would have been expected to lead to [1,-(2),3,3-$^{2}H_3$]glyceraldehyde, whose intact incorporation should have yielded [5′,5′,6-$^{2}H_3$]pyridoxamine. Yet, no label was present at C-6.

To probe whether this finding was authentic or an experimental artifact, two further experiments with glycerol were performed, this time with chirally labeled samples. In separate experiments, (*R*)-[1,1-$^{2}H_2$]- and (*S*)-[1,1-$^{2}H_2$]glycerol[21] (expts 20 and 21, respectively) were administered in the presence of D-xylose as the general carbon source. The sample of pyridoxamine derived from (*R*)-[1,1-$^{2}H_2$]glycerol carried label at C-5′ (Figure 3D). The sample of pyridoxamine from the (*S*)-[1,1-$^{2}H_2$]-derivative (Figure 3E) was free of deuterium.

There are several possibilities for the timing of the loss of deuterium from the triose carbon atom destined to become C-6 in the course of the conversion of glycerol into glyceraldehyde phosphate and the incorporation of the latter into the pyridoxine ring system.

One possibility, trivial from the point of view of the biosynthetic process, is nonenzymic deuterium/proton exchange, either in the course of the incubation or during workup.

That exchange takes place during the incubation was shown by two experiments in which *C. utilis* was grown on xylose in the presence of 50% $D_2O$, either in the absence (expt 22, Figure 3F) or in the presence (expt 23) of unlabeled glycerol. In each case the pyridoxamine that was isolated contained deuterium, not only at C-6 but also at the other sites, C-2′, C-4′, and C-5′. Thus, non-site-specific exchange takes place during incubation and deuterium is maintained during workup, even though the solution containing pyridoxamine is exposed to rigorous conditions of pH, temperature, and pressure in the course of its isolation (see Experimental Section).

Since the lack of incorporation of deuterium from the precursor carbon atom destined to become C-6 of pyridoxamine is site-specific, we favor the view that this loss of deuterium is not due to nonenzymic D/H exchange but is the result of a specific enzyme-catalyzed metabolic process.

An investigation of the precise cause of the loss of the C-6 hydrogen is beyond the scope of the present investigation. However, it is likely that this loss results from a metabolic event in the course of conversion of glycerol into glyceraldehyde 3-phosphate. The final step in this 3-step process, i.e., the conversion of dihydroxyacetone 3-phosphate into (*R*)-glyceraldehyde 3-phosphate, catalyzed by triose phosphate isomerase (E.C. 5.3.1.1), is accompanied by loss of the *re* proton from C-1 of dihydroxyacetone 3-phosphate.[22] The aldolase reaction, i.e., reversible equilibration of dihydroxyacetone phosphate plus (*R*)-glyceraldehyde 3-phosphate and fructose 1,6-bisphosphate, catalyzed by yeast aldolase (E.C. 4.1.2.13), is accompanied by loss of the *si* proton from C-1 of dihydroxyacetone 3-phosphate.[23] Thus, deuterium at each of the two sites would be lost and replaced by protium when these processes occur in the course of triose metabolism during the incubation period (ca. 20 H).

It is our view that the carbon skeleton of pyridoxine in yeast is generated by union of a pentulose with a triose. We have shown earlier[24] that 2′-hydroxypyridoxol is an intermediate of this process. A hypothetical, chemically rational scheme for union of a pentulose with glyceraldehyde (plus a nitrogen source represented as ammonia) leading to 2′-hydroxypyridoxol and thence to pyridoxal is shown in Schemes 4 and 5.

## Experimental Section

**$^{2}$H- and $^{13}$C-Labeled Compounds.** D-[$^{13}C_6$]glucose, [2,3-$^{13}C_2$]succinic acid, [1,3-$^{13}C_2$]glycerol, D-[1,2-$^{13}C_2$]ribose, D-[3,3-$^{2}H_2$]glyceraldehyde, and [$^{2}H_5$]glycerol were obtained from

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[20] White, R. L.; Spenser, I. D. *J. Am. Chem. Soc.* **1982**, *104*, 4934−4943.

[21] Hill, R. E.; Iwanow, A.; Sayer, B. G.; Wysocka, W.; Spenser, I. D. *J. Biol. Chem.* **1987**, *262*, 7463−7471.

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[23] Kadonaga, J. T.; Knowles, J. R. *Biochemistry* **1983**, *22*, 130−136.

[24] Zeidler, J.; Ullah, N.; Gupta, R. N.; Pauloski, R. M.; Sayer, B. G.; Spenser, I. D. *J. Am. Chem. Soc.* **2002**, *124*, 4542−4543.

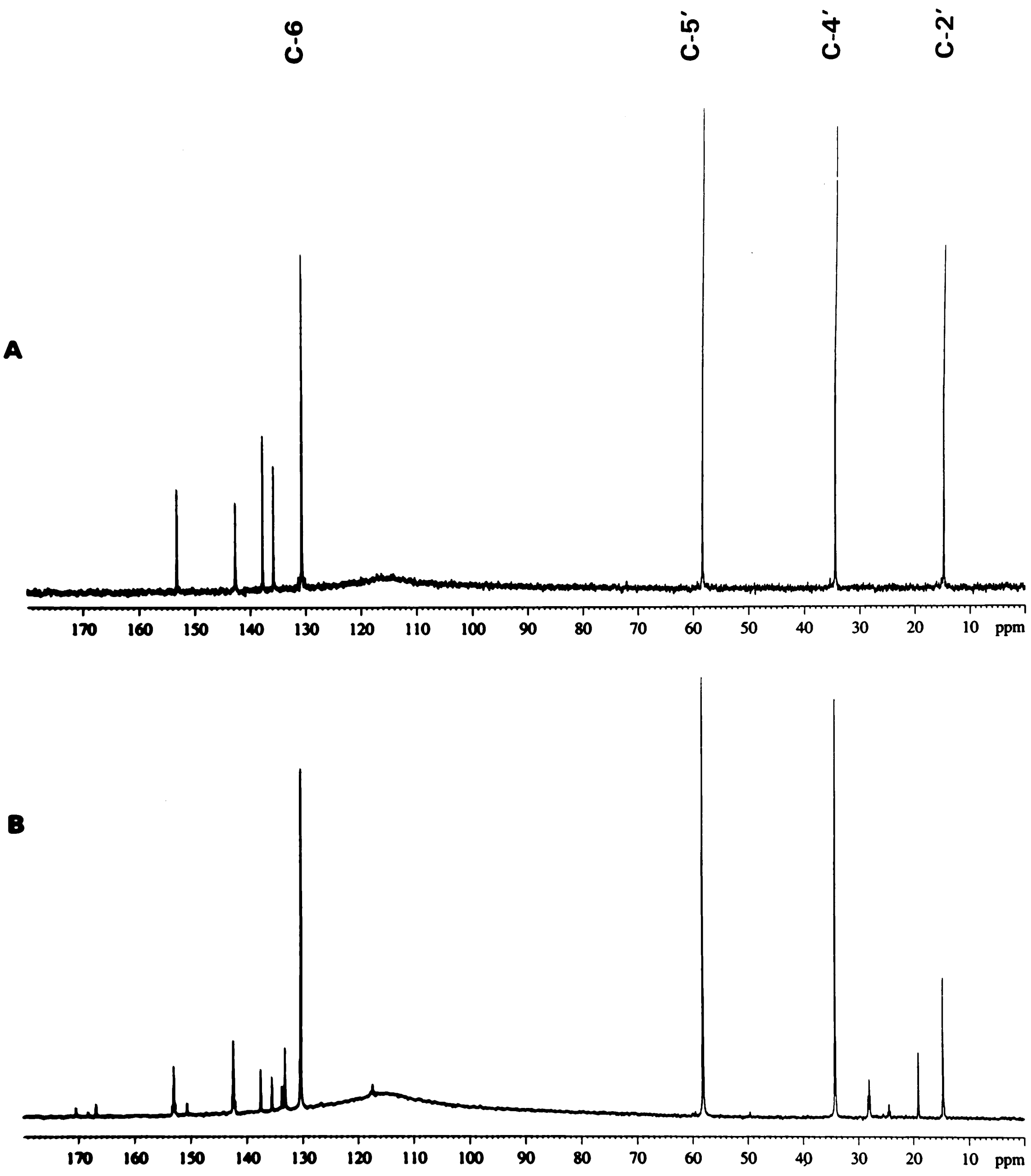

**FIGURE 2.** $^{13}$C NMR spectra of (A) a natural abundance $^{13}$C sample of pyridoxamine bis-HCl and of (B) a sample derived from incubation of *Candida utilis* in the presence of [1,3-$^{13}C_2$]glycerol (expt 16).

commercial sources. [2,3-$^{13}C_2$]-4-Hydroxy-L-threonine[25] and (*R*)- and (*S*)-[1,1-$^2H_2$]glycerol[21] were synthesized as previously described.

**Microorganisms.** *Saccharomyces cerevisiae* ATTC 7752 (IFO 1234) and *Candida utilis* ATTC 9256 were cultured in a synthetic minimal medium.[26] The yeast cultures (2 × 1 L) in 2-L conical flasks on a shaking incubator at 32 °C were grown to a cell density of 1.6 OD (629 nm) or higher (12−24 h). Details of the experiments are summarized in Table 1.

**Isolation of Pyridoxamine. Hydrolysis.** The cells were separated from the culture by centrifugation (15 min, 7200 rpm, 5 °C) and suspended in HCl (0.1 M, 30−35 mL). Pyridoxamine bis-HCl (2.5 mg) and thiamin diphosphate (3 mg)[27] were added to serve as carriers, and the mixture was heated (1.5 h, 121 °C) in a steam autoclave. After cooling to room temperature the suspension was centrifuged (6000 rpm) in 15-mL conical centrifuge tubes, and the supernatant was decanted and saved. The pellet was resuspended, washed with $H_2O$ (10−15 mL), and centrifuged as before, and the washings were added to the extract. The pH of the solution was raised to 4.7 by addition of potassium acetate buffer (1.0 M, pH 5.5, 3 mL, followed by 0.1 M, pH 4.7, 6 mL).

Takadiastase (150 mg) was added to the combined supernatant and washings, and the solution was incubated at 37 °C overnight.

**Chromatography.** Amberlite CG-50 (3 g), a weak cation-exchange resin, was washed with HCl (1 M, 3 × 20 mL) by

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[25] Wolf, E.; Spenser, I. D. *J. Org. Chem.* **1995**, *60*, 6937−6940.

[26] Himmeldirk, K.; Sayer, B. G.; Spenser, I. D. *J. Am. Chem. Soc.* **1998**, *120*, 3581−3589.

[27] Incorporation of labeled substrates into thiamin (Vitamin $B_1$) will be reported in a separate publication.

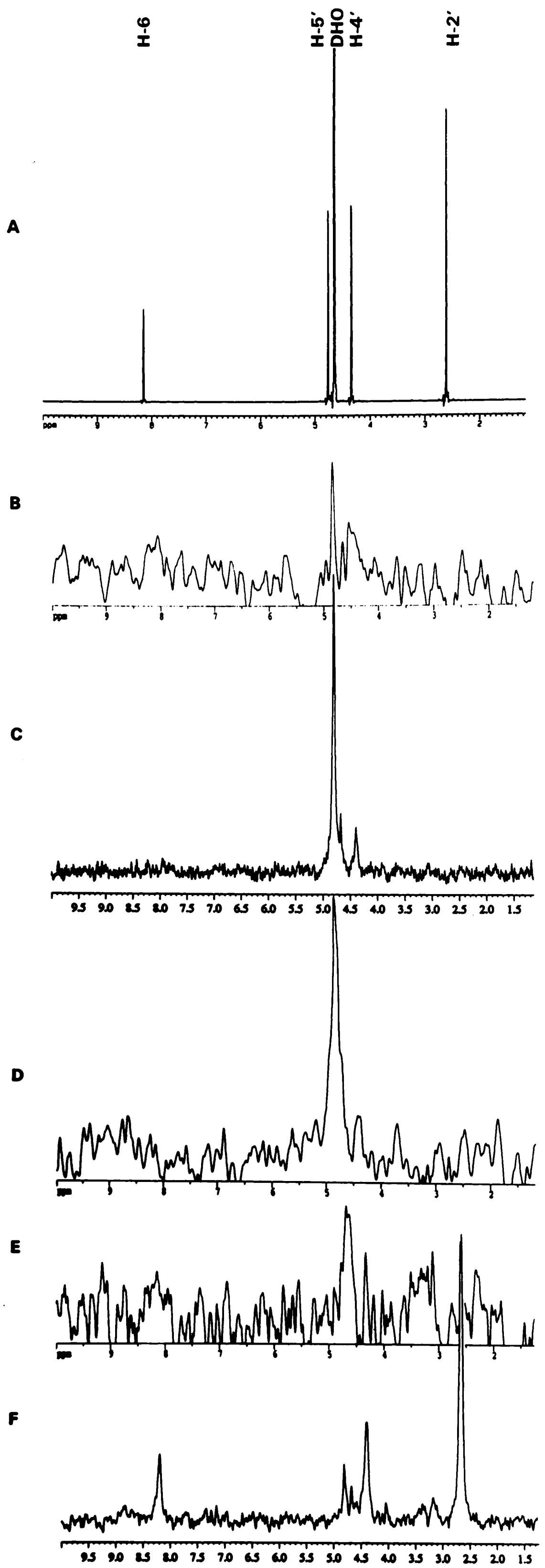
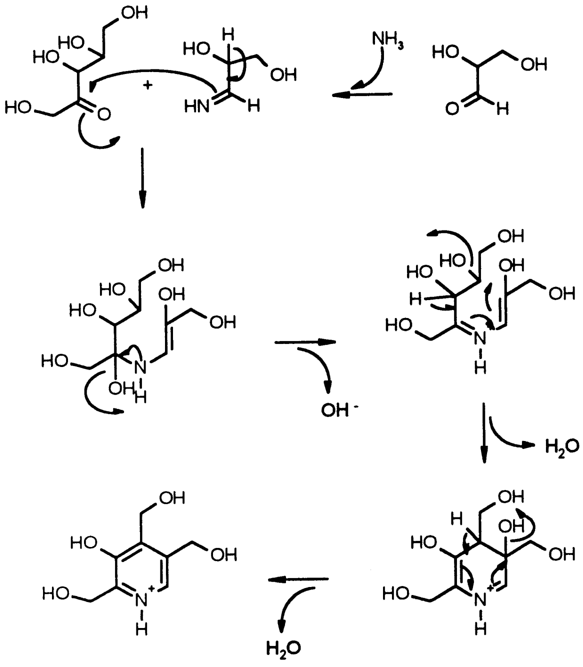
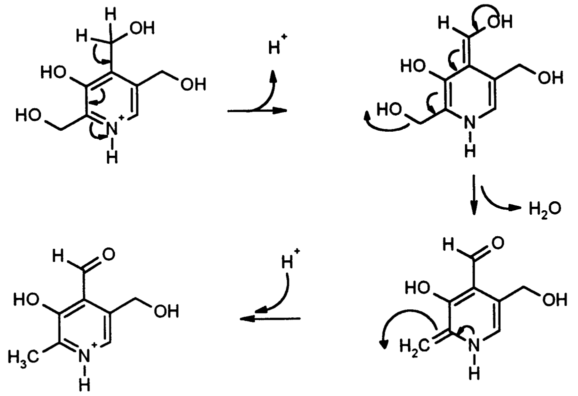


**FIGURE 3.** (A) $^1$H NMR spectrum of pyridoxamine bis-HCl. (B−F) $^2$H NMR spectra of samples of pyridoxamine bis-HCl derived from incubation of *Candida utilis* in the presence of (B) D-[3,3-$^2H_2$]glyceraldehyde (expt 17); (C) [1,1,2,3,3-$^2H_5$]-glycerol (expt 19); (D) (*R*)-[1,1-$^2H_2$]glycerol (expt 20); (E) (*S*)-[1,1-$^2H_2$]glycerol (expt 21), and (F) 50% $D_2O$ (expt 22).

**SCHEME 4. Biosynthesis of Pyridoxine in Yeast. Phase 1: Hypothetical Mechanism for Construction of 2′-Hydroxypyridoxol from a Pentulose and a Triose**



**SCHEME 5. Biosynthesis of Pyridoxine in Yeast. Phase 2: Hypothetical Mechanism for Conversion of 2′-Hydroxypyridoxol into Pyridoxal**



decantation to remove fine particles. The resin was suspended in deionized water, poured into a glass column (1 cm i.d.) and washed with water until the eluate was neutral. The cell hydrolysate (50 mL) was passed through the column by gravity, followed by an equal volume of water. The column was then eluted with HCl (0.1 M), and fractions were collected every 4 min. The fractions containing pyridoxamine were identified by spotting 1 $\mu$L of each fraction on filter paper, brief exposure to ammonia vapor, and examination under long wavelength UV light (blue fluorescence). Positive fractions were combined, evaporated at 30 °C, and dried overnight in vacuo over NaOH at room temperature. The Amberlite column was regenerated by washing with HCl (1 M) and water. The dried residue from the pyridoxamine fractions was dissolved in methanol (3 × 250 $\mu$L) and applied to a column (1 cm i.d.) packed with silica gel in methanol (silica gel 60, 40−63 microns, 230−400 mesh, 15 mL). The column was eluted with methanol containing concentrated HCl (1% v/v). Fractions were collected every 4 min and examined for pyridoxamine as before. Positive fractions were combined, evaporated at 30 °C, and dried over NaOH in vacuo at room temperature for several

H. This column separates pyridoxamine from thiamin,[27] which elutes later. The pyridoxamine containing residue was dissolved in water and passed through an SCX cartridge (strong cation-exchange, 500 mg) at a slow rate under mild suction. The cartridge had been prepared by successive washing with $NH_4OH$ (1 M), methanol, HCl (1 M), methanol, and water. After passage of the pyridoxamine fraction the cartridge was washed with water and methanol and then eluted by gravity with a mixture of dichloromethane (20 mL), methanol (2 mL), and concentrated $NH_4OH$ (0.2 mL). The eluate was evaporated to dryness, and the residue was purified by HPLC.[7] After another SCX treatment (see above) the product was dissolved in methanolic HCl (1% v/v), the solution was evaporated, and the residue was dried in vacuo over NaOH. The pure pyridoxamine bishydrochloride so obtained was dissolved in $D_2O$ or deuterium-depleted water (70 $\mu$L) and transferred to a Shigemi tube for NMR spectroscopy.

**NMR Spectroscopy.** Proton decoupled $^{13}C$ NMR spectra of pyridoxamine were acquired at 125.776 and 150.92 MHz, respectively, using a Shigemi sample tube; pulse width 90° (13.5, 13.8 $\mu$s); spectral width 28,985.5 Hz, 36,231 Hz; recycle delay 4.6 s, 4.9 s; digital resolution 0.88 Hz, 0.55 Hz per data point, respectively. Approximately 50 000 transients were required in order to generate the $^{13}C$ spectra showing satellites.

$^2H$ spectra were acquired at 46.07 and 92.12 MHz, respectively, using a 30° pulse and a digital resolution of 0.114 and 0.158 Hz per point, respectively. The spectra were obtained at 35 °C and referenced to the residual HDO signal at 4.67 ppm.

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