Comparative Biogenetic Anatomy of Vitamin B_1: A ^{13}C NMR Investigation of the Biosynthesis of Thiamin in *Escherichia coli* and in *Saccharomyces cerevisiae*

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Abstract: The mode of incorporation into thiamin of label from [1,2,3,4,5,6-$^{13}C_6$]-D-glucose and other bond-labeled substrates, in a prokaryote, *Escherichia coli*, and in a eukaryote, *Saccharomyces cerevisiae*, was determined by means of high field ^{13}C NMR spectroscopy. The success of this approach to the study of thiamin biosynthesis was based on the development of a procedure for the separation of μg amounts of ^{13}C enriched thiamin. The differences, in *E. coli* and in *S. cerevisiae*, in the mode of incorporation of multiatom units derived from these bond-labeled precursors into the pyrimidine and the thiazole nucleus of thiamin are compared and contrasted. The results confirm and extend findings of earlier studies that employed mass spectrometric analysis of the incorporation pattern of ^{13}C- and ^{2}H-labeled substrates and studies with radioactive tracers.

Introduction

Thiamin (Vitamin B_1) (**3**) is generated (Scheme 1) by the union of two subunits, the pyrimidine unit, 4-amino-5-hydroxymethyl-2-methylpyrimidine (**1**), and the thiazole unit, 5-(2-hydroxyethyl)-4-methylthiazole (**2**). The steps whereby the vitamin and its pyrophosphate ester, cocarboxylase, the coenzyme of transketolase (EC 2.2.1.1) and several other enzymes, is formed from its subunits, and the enzymology and genetics of the union of the subunits are well understood.[1−4]

The biosynthetic origin of each of the two subunits is not yet fully clarified, however. Current work on their origin, more than half a century after the isolation of thiamin,[5] the determination of its structure and its synthesis,[6] and of the elucidation of its biochemical function,[7] is still at the first stage of biosynthetic investigations, i.e., the determination of precursor−product relationships.

A substantial obstacle to the elucidation of these biosynthetic precursor−product relationships was the minute amount of thiamin biosynthesized by microorganisms (e.g., 5−27 μg/g dry weight by yeasts grown in a thiamin-free medium[8]). Another reason for the slow progress was the influence of the dogma of biochemical unity which led to conceptual difficulties in accepting the notion that the pathways to thiamin might be different in different organisms. Apparently contradictory results which could not be accommodated by a single pathway were reconciled only when it was recognized that there was biochemical diversity in the biosynthesis of thiamin in different organisms. It is now known that prokaryotes, such as the bacterium *Escherichia coli*, and eukaryotes, such as the yeast *Saccharomyces cerevisiae*, differ substantially in the routes whereby the two subunits originate. In each case primary building blocks of the subunits have now been recognized, but the mechanism and the enzymology and genetics of the steps of their formation are entirely unknown.

Glycine, formate, and glucose have been reported to serve as precursors of thiamin in bacteria (*E. coli* and *Salmonella typhimurium*) as well as in yeasts. However, in each case their mode of entry into thiamin, in the two types of microorganism, is different: In particular it was reported that, in bacteria, label from [^{14}C]glycine was incorporated into the pyrimidine unit of thiamin in such a way that the carboxyl group yielded P-4 (*i.e.*, C-4 of the pyrimidine unit),[9,10] and the methylene group entered P-6.[10,11] In yeast, on the other hand, [^{14}C]glycine did not enter the pyrimidine but the thiazole unit. The glycine methylene group yielded T-2 (i.e., C-2 of the thiazole unit),[12−14] but its carboxyl group was not incorporated. The nitrogen atom of [^{15}N]glycine entered N-1 of the thiamin pyrimidine in bacteria[10] but N-3 of the thiamin thiazole in yeast.[13] In each case it was

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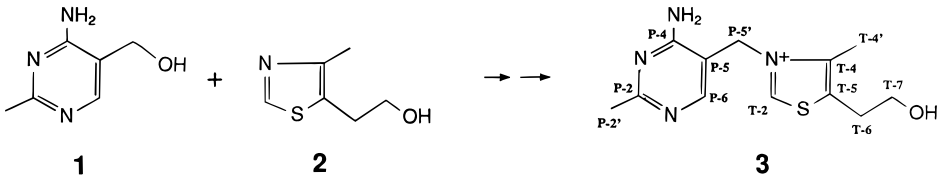
Scheme 1



1 **2** **3**

suggested but not shown unequivocally, that the C−N fragment of glycine entered as an intact unit.

The site of entry into thiamin of [^{14}C]formate also differs in bacteria and in yeast. It was reported that this substrate supplies P-2 (that is C-2 of the pyrimidine unit) in *E. coli*[15] and other prokaryotes[9,16] but P-4 in yeast[17,18] and other eukaryotes.[16] In a single instance it was reported[19] that, in a strain of *S. cerevisiae*, formate entered both P-2 (25%) and P-4 (75%).

Another set of results, divergent in yeast and bacteria, concerns the mode of entry of glucose. ^{14}C incorporation data were interpreted to show that, in yeast, the C_5 chain, T-4′,4,5,6,7 (*i.e.*, C-4′,4,5,6,7 of the thiazole unit), originated intact from glucose via a pentulose.[20,21] In *E. coli* B, on the other hand, it was concluded on the basis of mass spectrometric fragmentation data, that the carbon atoms derived from [$^{13}C_6$]glucose entered the thiazole moiety in two separate fragments, as a C_3 unit (yielding T-5,6,7) plus a C_2 unit (yielding T-4′,4), rather than as an intact C_5 unit. Results of experiments[22] with deuterium-labeled samples of glucose, glycerol and pyruvate were consistent with such an interpretation. The glucose-derived C_3 unit was thought to originate via glyceraldehyde phosphate, whereas the C_2 unit was thought to arise from the CH_3CO moiety of pyruvate.[22] It was predicted that in *E. coli* condensation of these two substrates yielded a 1-deoxypentulose phosphate from which the thiazole C_5 unit was derived.[22] The experimental evidence to show that such a deoxypentulose, 1-deoxy-D-xylulose, did indeed serve as a precursor of the C_5 unit of the thiazole moiety was supplied by S. David and his collaborators.[23−25]

Glucose carbon atoms enter not only the thiazole unit but also the pyrimidine unit. In *E. coli* label from [6-^{14}C]glucose was reported[26,27] to enter P-5′, and it was inferred that P-5,5′ arose by an intramolecular rearrangement from the ribose unit of 5-aminoimidazole ribotide. This compound, a purine precursor, had been shown earlier to serve as an intermediate on the route from glycine plus formate into the thiamin pyrimidine in *S. typhimurium*.[28] Evidence was subsequently presented[29,30] that in *S. typhimurium* not only P-5,5′ but also P-2′ was derived by an intramolecular process from this ribose unit.

In yeast, label from glucose enters every one of the pyrimidine carbon atoms.[19] The mode of entry of these glucose carbon atoms remains to be clarified.

Most past investigations of the origin of thiamin reported results from a single microbiological system. Only one group of investigators[16,31,32] attempted a direct comparison of the mode incorporation into thiamin of various substrates, in a series of prokaryotes and eukaryotes. All investigations so far have employed either radioactive tracers or stable isotopes in conjunction with mass spectrometric analysis.

We have now been able to achieve incorporation into thiamin of ^{13}C from bond-labeled substrates, at a level that permitted analysis of the distribution of label within the isolated thiamin by high resolution ^{13}C NMR spectroscopy. The power of this method and its advantages over other techniques for the determination of biosynthetic incorporation patterns has been repeatedly outlined.[4,33] To apply ^{13}C NMR in the investigation of thiamin biosynthesis, a method had to be developed whereby μg amounts of enriched thiamin could be recovered. This method is described in detail in the Experimental Section.

We were thus able to compare and contrast the mode of incorporation into thiamin of multiatom fragments derived from ^{13}C−^{13}C and from ^{13}C−^{15}N bond-labeled substrates, in a prokaryote, *E. coli* mutant WG2, and in a eukaryote, *S. cerevisiae* strain ATTC 7752 (= IFO 1234). ^{13}C NMR chemical shifts and coupling constants for thiamin chloride hydrochloride are summarized in Table 1.

The *E. coli* mutant is the same organism that we employed in our investigations of pyridoxine biosynthesis.[34] The yeast strain ATCC 7752, which had been employed by K. Yamada and her collaborators in their investigations of thiamin biosynthesis,[35] was chosen for this work in preference to strain ATCC 24903 that we had used in our earlier investigations of thiamin biosynthesis,[13] since, as already mentioned, it had been shown that, whereas formate entered two sites, P-4 (75%) and P-2 (25%) of the pyrimidine unit in the latter,[19] incorporation of formate was confined to to C-4 of the pyrimidine nucleus in the former.[17,18]

A preliminary report of the result of one of the 9 experiments here presented has appeared.[36]

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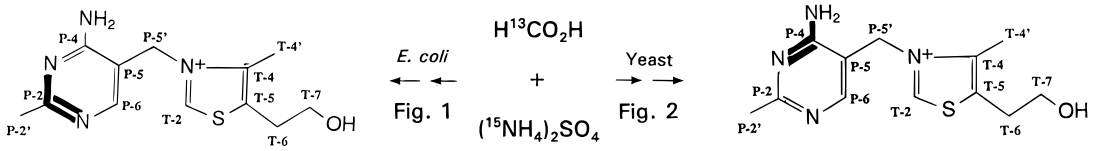
Table 1. ^{13}C NMR Spectrum of Thiamin (**3**): Chemical Shifts and Coupling Constants[a]

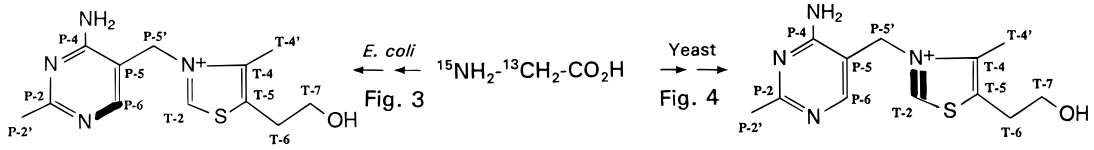
chemical shift (δ)	carbon atom no.		coupling constants ($\pm$0.5 Hz) $^{13}C-^{13}C$		$^{13}C-^{15}N$	
11.2	T-4′		$^1J_{T-4',4}$	49.3		
21.1		P-2′	$^1J_{P-2',2}$	53.5		
29.3	T-6		$^1J_{T-5,6}+{}^1J_{T-7,6}$	83.6		
50.0		P-5′	$^1J_{P-5',5}$	50.4		
60.3	T-7		$^1J_{T-7,6}$	35.4		
106.3		P-5	$^1J_{P-6,5}+{}^1J_{P-5',5}$	120.3		
136.7	T-5		$^1J_{T-5,6}$	47.8		
			$^1J_{T-4,5}+{}^1J_{T-5,6}$	122.1		
142.9	T-4		$^1J_{T-4,5}$	73.4		
			$^1J_{T-4',4}+{}^1J_{T-4,5}$	123.0		
144.7		P-6	$^1J_{P-6,5}$	69.0	$^1J_{P-6,N-1}$	12.4
154.8	T-2				$^1J_{T-2,N-3}$	15.6
163.1		P-4			$^1J_{P-4,N-3}$, $^1J_{P-4,NH2}$	18−23
163.3		P-2			$^1J_{P-2,N-1}$, $^1J_{P-2,N-3}$	14−17

[a] Chemical shift (ppm) relative to dioxane as internal standard (66.6 ppm with respect to TMS at 0 ppm).

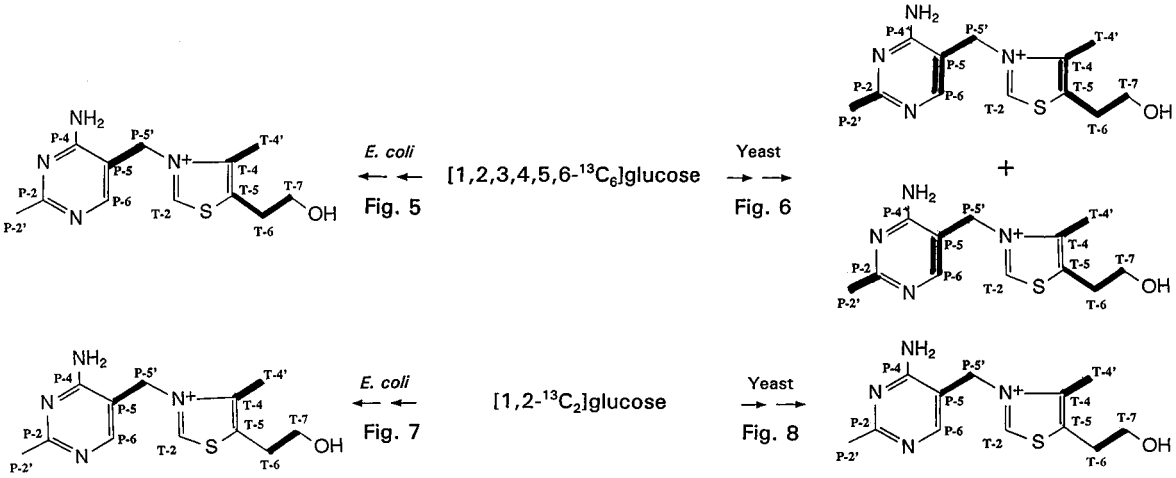
Scheme 2



Scheme 3



Scheme 4

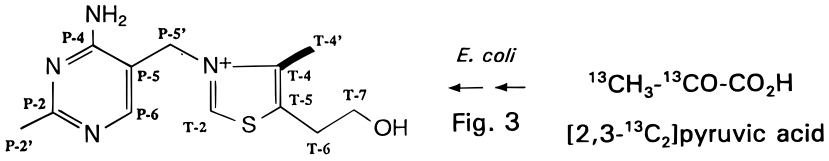


Results and Discussion

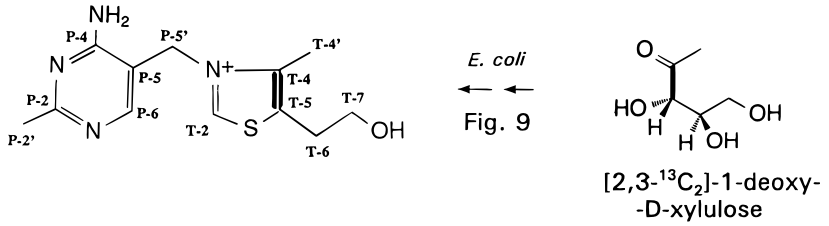
In this investigation the mode of incorporation into thiamin of [^{13}C]formate in the presence of [^{15}N]ammonium sulfate (Scheme 2) and of bond-labeled samples of glycine (Scheme 3), of glucose (Scheme 4), and of other substrates (Schemes 5 and 6) was determined in a bacterium, *E. coli* B WG2, and a yeast, *S. cerevisiae* ATTC 7752, by ^{13}C NMR spectroscopy.

Because of the minute amount of thiamin that is generated in microbiological cultures,[8] NMR spectroscopy has not hitherto been successfully employed in tracing the transfer of enriched atoms from a substrate into thiamin. In attempting to obtain a level of enrichment amenable to detection by ^{13}C NMR, formate was the substrate of choice in our first experiments. Sodium [^{13}C]formate is one of the cheapest ^{13}C enriched compounds available. Furthermore, as is well documented (see above) the incorporation of formate is confined to a single carbon atom within the thiamin pyrimidine nucleus, P-4 in yeast[17,18] and P-2 in *E. coli*.[15] Experiments with sodium [^{13}C]formate did not lead to detectable enrichment. However, when cultures of *E. coli* B WG2 and of *S. cerevisiae* ATTC 7752 were incubated with sodium [^{13}C]formate in the presence of excess [^{15}N]ammonium sulfate, ^{13}C enrichment within thiamin was detectable: The NMR spectra of the samples of thiamin chloride hydrochloride isolated from these two experiments are shown in Figures 1 and 2. An expansion of the spectral region, 162−164 ppm, is also shown in Figures 1 and 2. In the expanded spectrum of

Scheme 5



Scheme 6



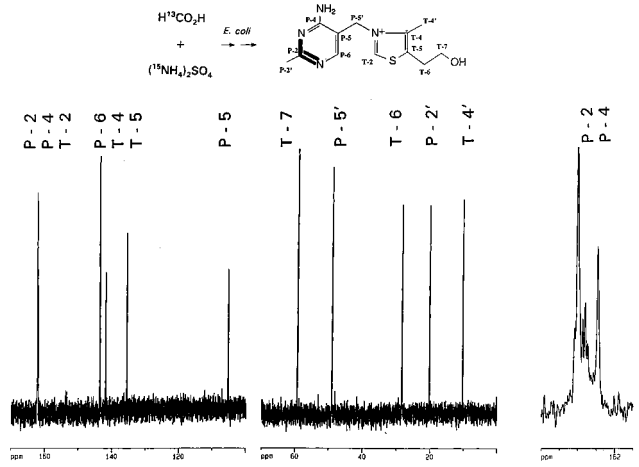


Figure 1. ^{13}C NMR spectrum of thiamin chloride hydrochloride (in D_2O) isolated from *E. coli* B mutant WG2, after incubation in the presence of sodium [^{13}C]formate plus [^{15}N]ammonium sulfate. An expansion of the spectral region 162−164 ppm, showing the signals due to P-2 and P-4, appears on the right.

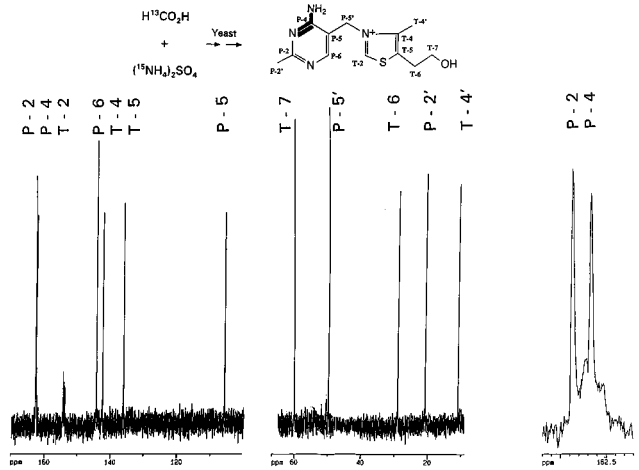


Figure 2. ^{13}C NMR spectrum of thiamin chloride hydrochloride (in D_2O) isolated from *S. cerevisiae* ATTC 7752, after incubation in the presence of sodium [^{13}C]formate plus [^{15}N]ammonium sulfate. An expansion of the spectral region 162−164 ppm, showing the signals due to P-2 and P-4, appears on the right.

E. coli thiamin (Figure 1) satellites are visible at the signal due to P-2 (163.3 ppm), while P-4 appears as a singlet. In the spectrum of yeast thiamin (Figure 2) the signal due to P-4 (163.1 ppm) shows satellites, whereas P-2 appears as a singlet.

These incorporation patterns (Scheme 2) confirm the results obtained by the earlier tracer experiments with sodium [^{14}C]-formate. The satellites show a complex pattern (in theory a doublet of doublets), since in each case the carbon atom is coupled to two nitrogen atoms. In *E. coli*, bond-labeling within the thiamin pyrimidine occurs at N-1,P-2 and at P-2,N-3 ($^1J_{P-2,N-1}$, $J_{P-2,N-3}$ ca. 14−17 Hz) (Figure 1). In yeast, bond-labeling within the thiamin pyrimidine occurs at N-3,P-4 and at P-4,NH_2 ($^1J_{P-4,N-3}$, $^1J_{P-4,NH2}$ ca. 18−23 Hz) (Figure 2). Since the two incubations were carried out in the presence of 98% [^{15}N]ammonium sulfate, with no other nitrogen source present, it is likely that in each case ^{13}C is coupled to both nitrogen atoms.

The two spectra were determined in D_2O solution. For this reason the signal due to T-2 (154.8 ppm) appears as a triplet, due to proton/deuterium exchange.[37]

Having demonstrated the applicability of ^{13}C NMR in investigations of thiamin biosynthesis when satellites due to bond-labeling are present within the spectrum of the biosynthetic product, we selected [2-^{13}C,^{15}N]glycine as the next substrate to be tested.

The sites of labeling due to glycine are known (see above). In *E. coli* B, label from [2-^{14}C]glycine enters P-6 of the thiamin pyrimidine,[10] and ^{15}N from [^{15}N]glycine enters the adjacent nitrogen atom, N-1.[10] In yeast label from [2-^{14}C]glycine enters T-2 of the thiamin thiazole,[12−14] and ^{15}N from [^{15}N]glycine enters the adjacent nitrogen atom, N-3.[13,31] In each case intact incorporation of the C−N bond of glycine into thiamin had been suggested but not established experimentally. If successful, our experiments would provide evidence in support of this prediction.

It was by no means certain a priori that transfer of the ^{13}C−^{15}N bond into thiamin would be readily detectable, since the coupling constants of the pair of satellites in the thiamin spectrum so generated would be small (^1J = 10−20 Hz). For this reason sodium [2,3-$^{13}C_2$]pyruvate was administered together with the [2-^{13}C,^{15}N]glycine in the *E. coli* experiment, the first one to be carried out. It had been shown[22] that label from [3-$^2H_3$]pyruvic acid entered T-4′ and postulated, but not proven by direct experiment, that the CH_3CO moiety of pyruvic acid generates the C_2 unit, T-4′,4 of the thiamin thiazole in *E. coli*. Transfer into thiamin of the intact ^{13}C−^{13}C bond of [2,3-$^{13}C_2$]-pyruvate, providing direct evidence for the derivation of the C_2 unit, T-4′,4 of the thiazole from the CH_3CO group of pyruvate, would be indicated by NMR signals with readily detectable satellites (^1J ∼ 50 Hz). Sodium [2,3-$^{13}C_2$]pyruvate was

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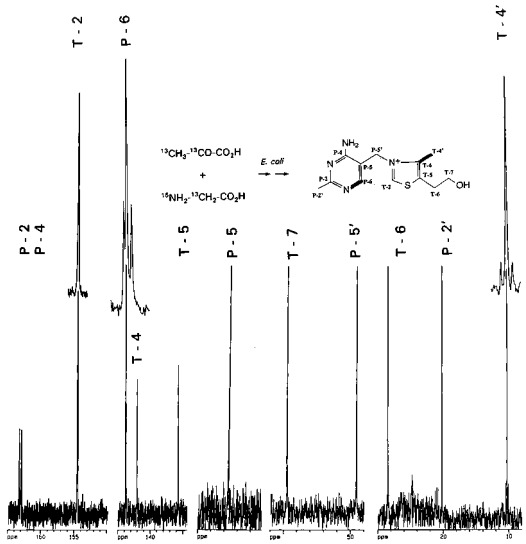

Figure 3. ^{13}C NMR spectrum of thiamin chloride hydrochloride (in DMSO-d_6) isolated from *E. coli* B mutant WG2, after incubation in the presence of [2-^{13}C,^{15}N]glycine plus sodium [2,3-$^{13}C_2$]pyruvate. Expansions of the signals due to T-4, P-6, and T-4′ are shown at the top.

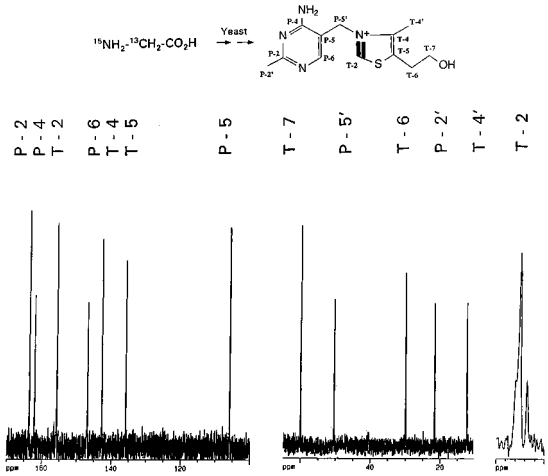

Figure 4. ^{13}C NMR spectrum of thiamin chloride hydrochloride (in DMSO-d_6) isolated from *S. cerevisiae* ATTC 7752, after incubation in the presence of [2-^{13}C,^{15}N]glycine. An expansion of the spectral region 143−145 ppm, showing the signal due to T-2, appears on the right.

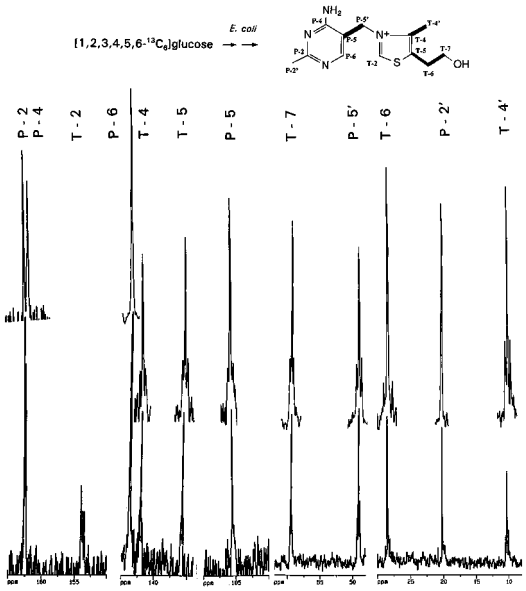

Figure 5. ^{13}C NMR spectrum of thiamin chloride hydrochloride (in D_2O) isolated from *E. coli* B mutant WG2, after incubation in the presence of [1,2,3,4,5,6-$^{13}C_6$]-D-glucose. Expansions of the signals are shown at the top.

therefore added as an additional substrate, to serve as an internal standard, signaling that thiamin synthesis had indeed taken place, in the event that enrichment due to [2-^{13}C,^{15}N]glycine could not be detected. As it turned out, this precaution proved to be unnecessary.

The spectrum (Figure 3) of a DMSO-d_6 solution[38] of thiamin, isolated in this experiment, establishes the intact incorporation of both bond-labeled precursor fragments, 2-^{13}C-^{15}N of glycine and 2-^{13}C-3-^{13}C of pyruvic acid. Satellites (^1J = 12.4 Hz) are clearly detectable at the signal due to P-6 (144.7 ppm) and at no other pyrimidine carbon, showing that the C−N unit, P-6,N-1, is derived intact from the C−N fragment of glycine. Furthermore, the thiazole carbon atom, T-4′ (11.2 ppm) shows satellites (^1J = 49.3 Hz), substantiating that the C_2 fragment, T-4′,4, is derived intact from pyruvic acid. Satellites at the signal due to T-4 were not clearly observable, due to the lower intensity of the signal.

The [2-^{13}C,^{15}N]glycine experiment with *S. cerevisiae* was carried out next. Sodium [2,3-$^{13}C_2$]pyruvate was not added in this case. Intact incorporation of the C−N bond of glycine into thiamin is demonstrated by the ^{13}C NMR spectrum (Figure 4) of a DMSO-d_6 solution of thiamin. The expanded spectral region (150−165 ppm) shows satellites (^1J = 15.6 Hz) around the signal due to T-2 (154.8 ppm, indicating ^{15}N,^{13}C coupling. Intact incorporation of the C−N bond of glycine into T-2,N-3 of the thiazole unit is thereby demonstrated. None of the other thiamin carbon atoms adjacent to nitrogen (T-4,P-2,P-4,P-5′,P-6) show satellite peaks.

The two experiments, Figures 3 and 4, establish intact incorporation of the C−N bond of glycine, into two entirely different regions of thiamin, P-6,N-1 in *E. coli*, and T-2,N-3 in yeast (Scheme 3).

The final group of experiments was intended to confirm and extend data on the mode of entry of glucose into the thiamin subunits. Incorporation of label from [1,2,3,4,5,6-$^{13}C_6$]-D-glucose and [1,2-$^{13}C_2$]-D-glucose was investigated.

The ^{13}C spectrum of the sample of thiamin chloride hydrochloride that was isolated from a culture of *E. coli* B mutant WG2, after incubation with [1,2,3,4,5,6-$^{13}C_6$]-D-glucose, is shown in Figure 5. Expansion of individual regions of the spectrum (Figure 5) shows that glucose-derived multicarbon units enter thiamin: The C_2 unit, P-5,5′ of the pyrimidine moiety, the C_2-unit, T-4′,4 of the thiazole moiety, and the C_3-unit, T-5,6,7 of the thiazole moiety, are so derived. The signals due to P-5′ (50.0 ppm) and P-5 (106.3 ppm) each show a doublet of satellites (^1J = 50.4 Hz), whereas the signal due to P-6 (144.7 ppm) does not. Thus, P-5,5′ is derived as an intact C_2 unit from glucose. Similarly, a doublet of satellites (^1J = 49.3 Hz) is apparent at the signals due to T-4′ (11.2 ppm) and T-4 (142.9 ppm), showing the intact derivation from glucose of this pair of carbons, T-4′,4. If T-4 had been part of a larger glucose-derived unit, less intense satellites, due to a doublet of doublets, would have appeared (cf., Figure 6). Such a doublet of doublets (outer doublet, 83.6 Hz) is apparent at the signal due to T-6 (29.3 ppm), which is thus coupled to T-5 (136.7 ppm) (^1J =

(38) In the NMR determination of thiamin from the experiments with labeled glycine as the substrate, the use of D_2O as the solvent was avoided in order to preclude deuterium/proton exchange at T-2, leading to multiplicity of the T-2 signal.

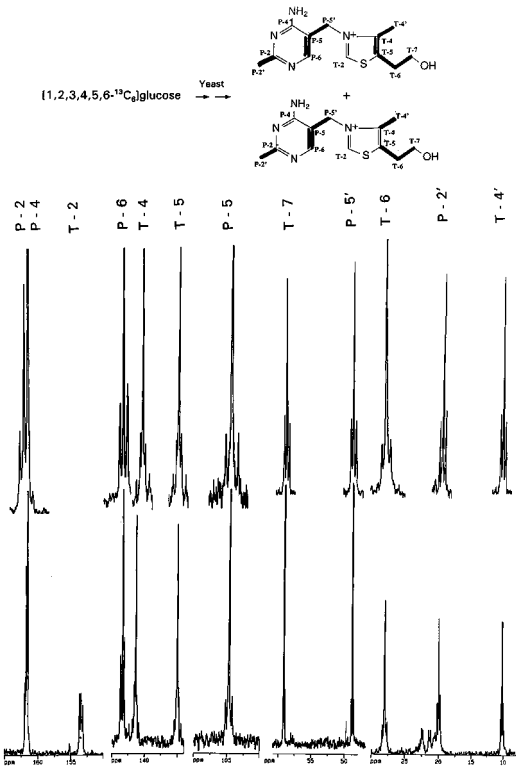

Figure 6. ^{13}C NMR spectrum of thiamin chloride hydrochloride (in D_2O) isolated from *S. cerevisiae* ATTC 7752, after incubation in the presence of [1,2,3,4,5,6-$^{13}C_6$]-D-glucose. Expansions of the signals are shown at the top.

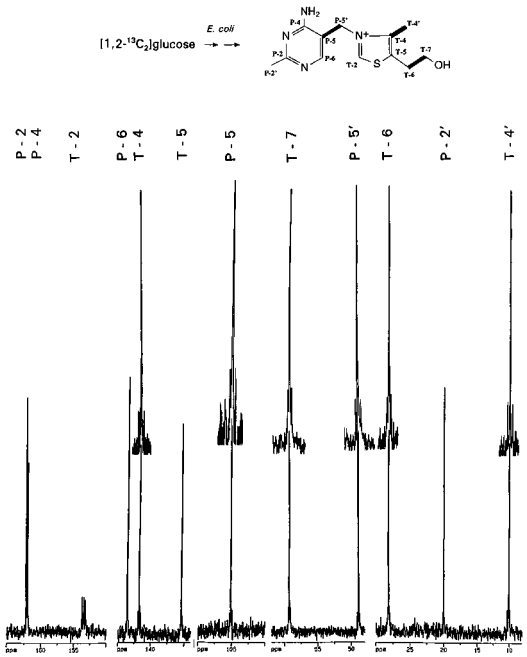

Figure 7. ^{13}C NMR spectrum of thiamin chloride hydrochloride (in D_2O) isolated from *E. coli* B mutant WG2, after incubation in the presence of [1,2-$^{13}C_2$]-D-glucose. Expansions of the signals are shown at the top.

47.8 Hz) as well as to T-7 (60.3 ppm) ($^1J = 35.4$ Hz), each of whose signals appears as a doublet of satellites, indicating the intact derivation of the C_3 unit, T-5,6,7, from glucose.

The remaining carbon atoms of the pyrimidine unit, P-2 (163.6 ppm), P-2′ (21.1 ppm), P-4 (163.1 ppm), and P-6 (144.7 ppm), appear as natural abundance singlets.

The spectrum thus shows that the fragments P-5,5′, T-4,4′, and T-5,6,7 are derived from glucose as intact multicarbon units but does not provide information concerning the identity of the glucose carbon atoms from which these multicarbon units are derived.

An experiment with [1,2-$^{13}C_2$]-D-glucose (Figure 7) shows that all three of these multicarbon units are derived from the terminal pair of carbon atoms of glucose: Three intact C_2 units, P-5′,5 (50.0, 106.3 ppm, $^1J = 50.4$ Hz), T-4′,4 (11.2, 142.9 ppm, $^1J = 49.3$ Hz), and T-7,6 (60.3, 29.3 ppm, $^1J = 35.4$ Hz), arise from C-1,2 of glucose. Since glycolysis and phosphotriose isomerase interconversion leads to equilibration of C-1,2 with C-6,5 of glucose, the experiment does not pinpoint the precise origin, C-1,2 or C-6,5, of these units.

The incorporation pattern (Scheme 4) is consistent with and confirms earlier tracer results, in particular the entry of label from [6-^{14}C]glucose into P-5′ of thiamin in *E. coli* B.[26,27] In view of the present results it is difficult to understand the reason why in this earlier tracer work label from [1-^{14}C]-D-glucose failed to enter thiamin in *E. coli*.

It was Yamada and Kumaoka who first suggested that in *E. coli* the C_2 unit, P-5,5′ of the thiamin pyrimidine was derived from C-5,6 of glucose via C-4,5 of the ribose moiety of 5-aminoimidazole ribotide.[26,27] Later this notion was substantiated by David et al.[29,30]

The entry of a C_2 unit, derived from C-1,2 (or C-6,5) of glucose into T-4′,4 of the thiamin thiazole, and of a C_3 unit, derived from C-1,2,3 (or from C-6,5,4) of glucose into T-7,6,5 of the thiazole unit, is consistent with the results of R. H. White[22] who deduced, on the basis of mass spectrometric analysis of enriched samples of thiamin obtained from *E. coli* after incorporation of ^{13}C labeled samples of glucose and ^2H-labeled samples of pyruvate and of glycerol, that the C_2 unit, T-4′,4 of the thiazole, was derived intact from pyruvate and that the C_3 unit, T-7,6,5, was derived from glycerol, via phosphoglyceraldehyde.

The incorporation of sodium [2,3-$^{13}C_2$]pyruvate into *E. coli* thiamin was described above (see Figure 3). It resulted in thiamin labeled at T-4′,4 of the thiazole unit (Scheme 5). This substantiates the earlier inference[22] that the intact CH_3CO unit of pyruvate serves as the progenitor of T-4′,4 of the thiazole unit. It was suggested that a 1-deoxypentulose 5-phosphate of unspecified stereochemistry, arising by condensation of pyruvic acid with phosphoglyceraldehyde, serves as an intermediate. That the nonphosphorylated D-threo compound was indeed involved in the biosynthesis of the thiamin thiazole unit in *E. coli* was shown by David and his group.[23−25] It is now shown that the compound does indeed serve as a direct precursor. A sample of [2,3-$^{13}C_2$]-1-deoxy-D-xylulose was synthesized in 14 steps from ethyl bromo[1,2-$^{13}C_2$]acetate (99% ^{13}C) in 16% overall yield.[39] As shown in Figure 9, this bond-labeled precursor entered the thiazole unit intact, yielding T-4,5 (Scheme 6). This is the bond that is generated by condensation of glyceraldehyde with pyruvate. Thus, 1-deoxy-D-xylulose serves as the C_5 precursor from which that the C_5 unit, T-4′,4,5,6,7, is generated.

The incorporation pattern of [1,2,3,4,5,6-$^{13}C_6$]-D-glucose into the pyrimidine moiety of thiamin in *S. cerevisiae* (Figure 6) differs substantially from that observed in *E. coli* (Figure 5). Whereas in *E. coli* glucose yielded P-5,5′ and no other fragment as an intact C_2 unit, within the yeast thiamin pyrimidine glucose yields P-2′,2 (21.1, 163.3 ppm, $^1J_{P-2',2} = 53.5$ Hz) as an intact

(39) Kennedy, I. A.; Hemscheidt, T.; Britten, J. F.; Spenser, I. D. *Can. J. Chem.* **1995**, *73*, 1329−1337.

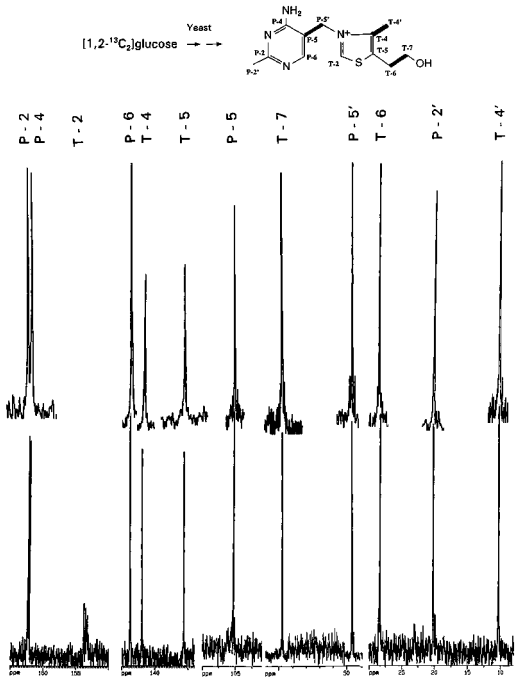

Figure 8. ^{13}C NMR spectrum of thiamin chloride hydrochloride (in D_2O) isolated from *S. cerevisiae* ATTC 7752, after incubation in the presence of [1,2-$^{13}C_2$]-D-glucose. Expansions of the signals are shown at the top.

C_2 unit and P-5′,5,6 as an intact C_3 unit (P-5′:50.0 ppm, $^1J_{P-5',5}$ = 50.4 Hz; P-5:106.3 ppm, outer doublet 120.3 Hz; P-6:144.7 ppm, $^1J_{P-5,6}$ = 69.0 Hz).

The *S. cerevisiae* experiment with [1,2-$^{13}C_2$]-D-glucose (Figure 8) shows that P-5,5′ is derived from the terminal C_2 unit of glucose (C-1,2 or C-6,5), so that the C_3 unit, P-6,5,5′ originates from a glycolytic triose phosphate. Label from [1,2-$^{13}C_2$]glucose did not enter P-2′,2 of the thiamin pyrimidine. This suggests that this C_2 unit originates from C-2,3 of glucose. On the basis of the incorporation patterns obtained in earlier tracer work with ^{14}C labeled radiomers of glucose, which showed that there was substantial incorporation of label from [2-^{14}C]glucose into C-2′, we suggested[19] that the C_5 unit, P-2′,2,6,5,5′, of yeast thiamin pyrimidine was generated by nitrogen insertion into a pentose which in turn was derived from glucose, mainly by the oxidative route. This idea is consistent with the present result.

The incorporation pattern of [1,2,3,4,5,6-$^{13}C_6$]-D-glucose into the yeast thiamin thiazole **(**Figure 6) supports such an inference. As will be shown below, the C_5 unit, T-4′,4,5,6,7 of the thiazole is generated from glucose in two ways: Its major source is an intact C_5 unit derived presumably from C-2,3,4,5,6 of glucose. Such a mode of entry was indicated earlier in another yeast, *Candida utiliz*, by the incorporation of label from [2-^{14}C]-D-glucose mainly into T-4′ and of label from [6-^{14}C]glucose mainly into T-7 of the yeast thiamin thiazole unit.[21]

This mode of entry is deduced from the satellite pattern in the NMR spectrum. The signals due to T-4′ (11.2 ppm) and T-7 (60.3 ppm) show doublets (^1J = 49.3 Hz and ^1J = 35.4 Hz, respectively). The signal due to T-6 (29.3 ppm) shows a doublet of doublets (of which only the outer pair, 83.6 Hz, is detectable), indicating coupling to T-7 as well as to T-5. The signals due to T-4 (142.9 ppm) and T-5 (136.7 ppm), on the other hand, show a more complex pattern: Each shows not only a doublet (^1J = 49.3 Hz and ^1J = 47.8 Hz, respectively) (indicating coupling of T-4 to T-4′ only, and coupling of T-5 to T-6, only) but also a doublet of doublets (coupling of T-4 to T-4′ as well as to T-5, 123.0 Hz, outer doublet, and coupling of T-5 to T-4 as well as to T-6, 122.1 Hz, outer doublet). Thus, the C_5 chain, T-4′,4,5,6,7 can arise from glucose in two ways (Scheme 4), either as an intact C_5 unit (presumably via an intact pentulose, formed from glucose C-2,3,4,5,6 by the oxidative route) or by union of a glucose-derived C_2 unit with a glucose-derived C_3 unit (presumably via a pentulose, formed from glucose by the transketolase route).

The fact that this C_5 unit can arise as an intact five-carbon chain from glucose serves as clear evidence that the mode of biosynthesis of the thiamin thiazole differs in *E. coli* and in yeast. The fact that a pentulose can arise from glucose by another pathway, which happens to lead to an NMR coupling pattern which cannot be differentiated from that found in the spectrum of *E. coli* thiamin, complicates the analysis of the mode of incorporation but does not weaken the evidence that there is a difference of origin.

The current results confirm earlier data and fill some of the gaps in the biogenetic anatomy of the pyrimidine and the thiazole units of thiamin in *E. coli* (and presumably in other prokaryotes) and in *S. cerevisiae* (and presumably in other eukaryotes). The major contributions of this work are (i) the development of a method to study thiamin biosynthesis by application of ^{13}C NMR spectroscopy and (ii) the direct and concurrent comparison, by experiments employing similar methods, of the incorporation patterns of identical substrates into thiamin, in a prokaryote and a eukaryote.

Future experiments with appropriately bond-labeled samples of glucose and pentoses will readily lead to a complete identification of the mode of their conversion into the pyrimidine and the thiazole nucleus of thiamin.

Experimental Section

Labeled Compounds. The following labeled compounds were acquired from a commercial source (Cambridge Isotope Laboratories, Inc.) (CIL): [1,2,3,4,5,6-$^{13}C_6$]-D-glucose (98% ^{13}C); [1,2-$^{13}C_2$]-D-glucose (99% ^{13}C); sodium [2,3-$^{13}C_2$]pyruvate (99% ^{13}C); sodium [^{13}C]formate (99% ^{13}C); [2-^{13}C,^{15}N]glycine (99% ^{13}C, 98% ^{15}N); [^{15}N]ammonium sulfate (98% ^{15}N). [2,3-$^{13}C_2$]-1-Deoxy-D-xylulose was synthesized[39] from ethyl bromo[1,2-$^{13}C_2$]acetate (99% ^{13}C) (CIL) in 14 steps with an overall yield of 16%.

Experiments with *S. cerevisiae*. Organism. The yeast employed in experiments 2, 4, 6, and 8 (see Table 2) was *S. cerevisiae* strain ATTC 7752 (= IFO 1234).

Medium. A slightly modified Olson & Johnson medium[42] of the following composition (per L) was used: glucose 10 g (in experiments 2 and 4) or 6.5 g (in experiments 6 and 8), L-asparagine 2.5 g,[43] $(NH_4)_2HPO_4$ 6 g,[44] KH_2PO_4 1 g, $MgSO_4$ (anhydrous) 500 mg, NaCl 100 mg, $CaCl_2$ 100 mg, *myo*-inositol 10 mg, biotin 0.02 mg, Ca pantothenate 0.5 mg, $ZnSO_4$ 0.4 mg, $CuSO_4 \cdot 5H_2O$ 0.04 mg, $Fe(NH_4)_2(SO_4)_2$ 0.15 mg. The pH of the solution was adjusted to pH 5, by addition of 85% orthophosphoric acid. The presence of asparagine in the medium greatly enhances the cell mass produced in the incubation. It was shown that the presence of asparagine does not affect the incorporation pattern of label from [1,2,3,4,5,6-$^{13}C_6$]-D-glucose into thiamin.

Growth. The labeled compound (see Table 2) was dissolved in the medium (1 L), and the solution was filter sterilized (0.2 μm filter). The solution was distributed in equal portions in 10 sterile 500 mL Erlenmeyer flasks. The flasks were inoculated with yeast cells that had been grown at 30 °C for 24 h on an agar slant (20 g agar per L of the above medium). Incubation on a shaking incubator at 30 °C was

(40) 4-Hydroxy-L-threonine partially inhibits the growth of *E. coli* B on a pyridoxal supplemented growth medium,[41] but addition of L-threonine permits the mutant to grow.

(41) Westley, J. W.; Pruess, D. L.; Volpe, L. A.; Demny, T. C.; Stempel, A. *J. Antibiot.* **1971**, *24*, 330−1.

(42) Olson, B. H.; Johnson, M. J. *J. Bacteriol.* **1949**, *57*, 235−246.

(43) In experiments 2 and 4 L-asparagine was omitted from the medium.

(44) In experiment 2 this was replaced by K_2HPO_4 6.7 g/L.

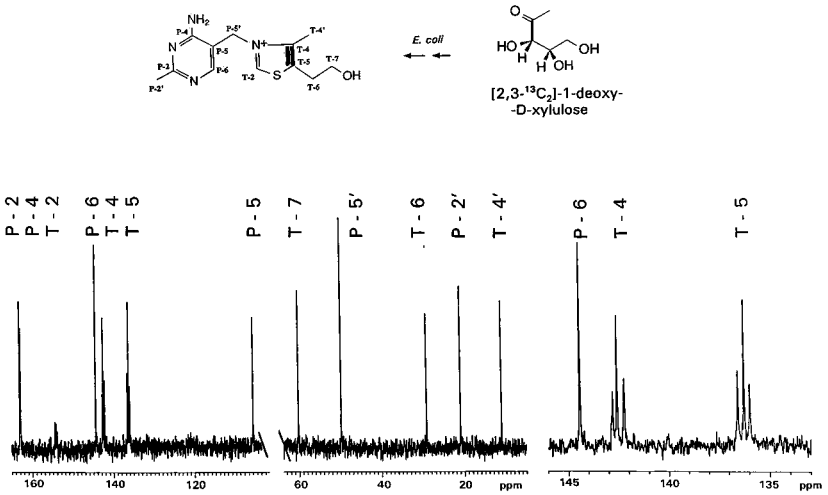

Figure 9. ^{13}C NMR spectrum of thiamin chloride hydrochloride (in D_2O) isolated from *E. coli* B mutant WG2, after incubation in the presence of [2,3-$^{13}C_2$]-1- deoxy-D-xylulose. An expansion of the spectral region 135−145 ppm, showing the signals due to P-6, T-4, and T-5, appears on the right.

Table 2. Experimental Details

expt. no.	substrates	weight [mg/L (mmol)]	no. of incubations	^{13}C NMR spectrum of isolated thiamin
		E. coli B WG2		
1	sodium [^{13}C]formate	325 (4.7)	5 × 1 L	Figure 1
	[^{15}N]ammonium sulfate	1000 (7.5)		
	D-glucose	1000 (5.6)		
3	sodium [2,3-$^{13}C_2$]pyruvate	200 (1.8)	5 × 1 L	Figure 3
	[2-^{13}C,^{15}N]glycine	200 (2.6)		
	D-xylose	500 (3.3)		
	glycolaldehyde	50 (0.8)		
5	[1,2,3,4,5,6-$^{13}C_6$]-D-glucose	100 (0.54)	5 × 1 L	Figure 5
	D-glucose	1000 (5.6)		
7	[1,2-$^{13}C_2$]-D-glucose	300 (1.7)	5 × 1 L	Figure 7
	D-glucose	700 (3.9)		
9	[2,3-$^{13}C_2$]-1-deoxy-D-xylulose	200 (1.5)	5 × 1 L	Figure 9
	D-xylose	500 (3.3)		
	4-hydroxy-L-threonine[40]	100 (0.74)		
	L-threonine	20 (0.17)		
		S. cerevisiae ATTC 7752 (= IFO 1234)		
2	sodium [^{13}C]formate	2000 (29.0)	10 × 0.1 L	Figure 2
	[^{15}N]ammonium sulfate	2000 (14.9)		
	D-glucose	10000 (55.6)		
4	[2-^{13}C,^{15}N]glycine	1000 (13.0)	10 × 0.1 L	Figure 4
	D-glucose	10000 (55.6)		
6	[1,2,3,4,5,6-$^{13}C_6$]-D-glucose	1250 (6.7)	80 × 0.1 L	Figure 6
	D-glucose	6500 (36.1)		
8	[1,2-$^{13}C_2$]-D-glucose	1500 (8.3)	10 × 0.1 L	Figure 8
	D-glucose	6500 (36.1)		

continued for 32 h (into late log phase). Details of the components used in each of the experiments are presented in Table 2.

Workup. Extraction. The cells from each 1 L incubation were separated from the medium by centrifugation (1 h, 4000 rpm, 5 °C). The separated cells were suspended in dilute hydrochloric acid (0.1 M, 50 mL), thiamin pyrophosphate (3 mg) was added, and the mixture was heated 1 h at 100 °C. After cooling, acetate buffer (1 M, pH 5.5, 12.5 mL) was added to adjust the pH of the suspension to pH 4.7. The suspension was centrifuged (4000 rpm, 5 °C, 1 h), the cell debris was washed with acetate buffer (0.2 M, pH 4.7, 20 mL), and the cells were again removed by centrifugation. **Acid Phosphatase Treatment.** Taka-diastase[45] (Sankyo, 150 mg) was added to the combined supernatant and washings. The mixture was incubated overnight at 37 °C, when hydrolysis of thiamin pyrophosphate into thiamin was complete, as determined by TLC (CH_3CN/0.1 M HCl 2:1) and subsequent transformation of the thiamin spot into the fluorescent compound, thiochrome, by immersion of the TLC plate into a solution of $K_3Fe(CN)_6$ (40 mg) in aqueous NaOH (15% w/v, 40 mL) and irradiation with UV light (360 nm).

Column Chromatography. Amberlite-CG50, Sephadex-G10,[46,47] and silica gel columns were used in succession. Amberlite-CG50 (100−200 mesh, 25 g) was washed in a beaker in sequence with NaOH solution (1 M, 4 × 300 mL), distilled water (2 × 300 mL), and aqueous HCl (1 M, 3 × 300 mL) and applied to a column (19 × 300 mm). The column was washed with water until the eluate was neutral. After application of the cell extract, gradient elution was accomplished by filling the mobile phase reservoir with aqueous HCl (0.001 M, 100

(45) Presumably as a result of a high degree of purification, the enzyme preparations sold by Fluka and by Sigma as Taka-Diastase did not show acid phosphatase activity. Such activity stems from a minor enzymic contaminant of the α-amylase enzyme preparation. The Fluka product, Clara-diastase, showed phosphatase activity and can be used in place of the Sankyo product.

(46) Gelotte, B. *J. Chromatogr.* **1960**, *3*, 330−342.

(47) Davidek, J.; Pudil, F.; Seifert, J. *J. Chromatogr.* **1977**, *140*, 316−318.

mL) and maintaining the solvent level in the reservoir by addition of aqueous HCl (0.1 M). The fraction containing thiamin (total volume ca. 25 mL) was eluted after ca. 350 mL. This fraction was cautiously evaporated (foaming) at ca. 30 °C on a rotary vacuum evaporator, yielding a solid residue (ca. 200 mg).

The Amberlite-CG50 column was regenerated with aqueous HCl (0.1 M) and was used for 10 separations, before being discarded.

Sephadex-G10 (100 g) was washed in sequence with aqueous HCl (0.01 M, 3 × 300 mL), distilled water (2 × 300 mL), and aqueous HCl (0.001 M, 3 × 300 mL) and applied to a column (19 × 900 mm) which was allowed to settle for 3 h and was then eluted with aqueous HCl (0.001 M, 200 mL). The product from the Amberlite-CG50 column, dissolved in aqueous HCl (0.001 M, ca. 4 mL) and was then applied, and elution with 0.001M HCl continued. Cellular material started to emerge after ca. 90 mL. Thiamin chloride hydrochloride emerges after ca. 130 mL in a 25 mL fraction. The rate of elution should be very low, so that the desired fractions emerge in no less than ca. 10 h. The Sephadex-G10 column was regenerated by washing with aqueous HCl (0.001 M, 300 mL) and was used for five separations before being discarded.

Isolation of Thiamin Chloride Hydrochloride. The thiamin fraction from the Sephadex-G10 column was evaporated, and the residue was dissolved in the solvent mixture acetonitrile/0.1 M HCl 1:1. The two phase system that formed was applied to a column (12 × 280 mm) charged with silica gel. The column was then eluted with the same solvent mixture. This system separates the desired product from most of the cellular contaminants. The product was once again applied to a similar silica gel column, and elution was repeated, yielding thiamin chloride hydrochloride, contaminated with a little silica gel. The latter can be removed by Amberlite-CG50 chromatography (vide supra). For NMR studies this final purification step was found to be unnecessary.

Experiments with *E. coli*. Organism. The organism used in these investigations was *E. coli* B strain WG2. This is a pyridoxine auxotroph (*pdxH*$^-$) that lacks pyridoxol phosphate oxidase (E.C. 1.1.1.65 or E.C. 1.4.3.65).

Media. Nutrient Medium. This was a nutrient broth medium (Oxoid Ltd., London, England) prepared according to the supplier's instructions.

Minimal Salts Medium. The minimal salts medium contained the following salts: KH_2PO_4 7 g/L, K_2HPO_4 3 g/L, $(NH_4)_2SO_4$ 1 g/L,[48] $MgSO_4$ 0.1 g/L and $CaCl_2$ 0.01 g/L. D-Glucose (experiments 1, 5, 7) or D-xylose (experiments 3, 9) served as the general carbon source. Pyridoxal hydrochloride was added to a concentration of 6×10^{-7} M when the minimal medium was used to grow the *pdxH*$^-$ mutant, *E. coli* B WG2. All media were prepared in distilled water and were sterilized by autoclaving. The pyridoxal hydrochloride supplement solution was sterilized by filtration.

Stock Cultures. Stock cultures of *E. coli* B WG2 were maintained on monthly slants of the nutrient broth medium. After subculturing from the previous month's stock, fresh slants were incubated 24 h at 37 °C and were then stored at 4 °C. Every time fresh stock slants were prepared, slants of minimal salts medium, with and without pyridoxal supplementation, were inoculated and incubated 24 h at 37 °C in order to monitor for the presence of wild-type revertants.

Incubation of *E. coli* in the Presence of Labeled Compounds. Each tracer experiment was started by subculturing from the current month's nutrient broth stock slant onto a pyridoxal-supplemented minimal medium slant, which had been prepared with either 0.5% w/v D-glucose (experiments 1, 5, 7) or 0.5% w/v D-xylose (experiments 3, 9) as the carbon source. The slant was incubated 24 h at 37 °C. Cells from this slant were then used to inoculate 2 × 500 mL samples of the same medium, one of which was supplemented with pyridoxal hydrochloride, the other without pyridoxal supplementation. These cultures were incubated on a rotary shaker (New Brunswick Scientific) at 37 °C, until an optical density measurement at 600 nm indicated that growth in the supplemented culture was well into the exponential phase (approximately 12 h). Any growth in the unsupplemented culture served to indicate the presence of wild-type revertants or possible contaminants. If this were observed, the experiment could have been aborted at this stage without any wastage of labeled substrate. Fortunately, in our hands the *pdxH*$^-$ mutation in strain WG2 is very stable, and so far such an occasion has not arisen. Nevertheless, the control unsupplemented culture was included in every experiment as a precautionary measure.

The cells from the pyridoxal-supplemented culture were harvested by centrifugation (10 min at 7000 rpm) and washed with sterile 0.9% saline (3 × 100 mL). The washed cells were divided into two equal portions, each one of which was resuspended in minimal salts medium (500 mL) without pyridoxal but containing the appropriate carbon source, the labeled substrate, and other addends. These cultures were incubated 6 h on the rotary shaker (400 rpm, 37 °C). To obtain sufficient material for NMR investigation, each incubation was repeated five times. Details of the components used in each of the experiments are presented in Table 2.

Workup of Bacterial Cultures. The contents of each 500 mL culture flask from each of the 2 × 500 mL incubation experiments with ^{13}C-labeled substrate were centrifuged 10 min at 7000 rpm, and the supernatant solutions decanted. Cells remaining in the combined decanted solution were separated by filtration (0.2 μm membrane filter, Nagle Co.).

Isolation of Thiamin from the Cells of *E. coli* WG2. Cells from five 1 L incubations were collected by centrifugation and combined. The separated cells were suspended in dilute hydrochloric acid (0.1 M, 50 mL), thiamin pyrophosphate (3 mg) was added and the mixture heated 1 h at 100 °C. Further workup followed the procedure described above for the isolation of thiamin from *S. cerevisiae*.

NMR Spectroscopy. Instrumentation. NMR spectra were acquired on a Bruker DRX 500 spectrometer, operating at 11.74 T, using a Bruker 2.5 mm microprobe: pulse width (90°, 8 μs), spectral width 28 985.5 Hz, recycle time 4.6 s, digital resolution 0.88 Hz per data point.

Spectra: 125.776 Hz proton decoupled ^{13}C NMR spectra of thiamin chloride hydrochloride in 100 μL of D_2O (Figures 1, 2, 5−9) or 100 μL of DMSO-d_6 (Figures 3, 4). Approximately 90 000 transients were required in order to generate the ^{13}C NMR spectra showing satellites.

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(48) In experiment 1 this was replaced by $(^{15}NH_4)_2SO_4$ 1 g/L.