Biosynthesis of Thiamine: 5-Aminoimidazole Ribotide as the Precursor of All the Carbon Atoms of the Pyrimidine Moiety

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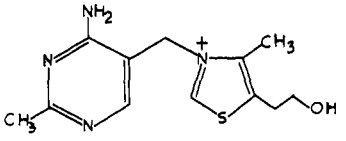
Abstract: It is an established fact that three carbon atoms of the pyrimidine moiety of thiamine orginate from the imidazole ring of 5-aminoimidazole ribotide (AIR) in bacteria. But as yet, no experimental evidence has been obtained about the origin of the other three carbon atoms. Experiments with labeled biosynthetic samples of 5-aminoimidazole riboside (AIR_s) were carried out. The incorporation of label was studied in a *Salmonella typhimurium* strain able to synthesize the pyrimidine part of thiamine from glucose and a minute amount of AIR_s. No incorporation of ^{13}C was found in the pyrimidine synthesized from [U-^{13}C]glucose and natural AIR_s. By contrast, the isotopic composition of the pyrimidine synthesized from natural glucose and [U-^{13}C]AIR_s was found close to that of the labeled AIR_s. From AIR_s labeled mainly in its ribose part by ^{14}C and inactive glucose was obtained a pyrimidine labeled mainly in the three carbon atoms that do not derive from the imidazole part. These carbons were found approximately as radioactive as those of the ribose part of AIR_s. The conclusion is that three carbon atoms of the pyrimidine moiety of thiamine originate from the ribose part of AIR which is thus the precursor of all the carbon atoms of this pyrimidine.

The higher plants, a number of fungi, and many bacteria can synthesize thiamine (**1**) (vitamine B_1) from a carbon source and inorganic salts. There is strong evidence that the phosphoric and pyrophosphoric esters of the pyrimidine (**2**) (pyramine) act as normal precursors.[1] A very important contribution to the problem



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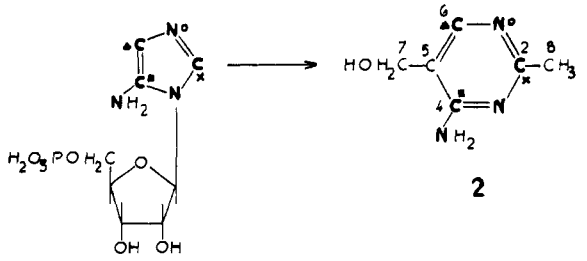
of the biosynthesis of pyramine itself in the enterobacterium *Salmonella typhimurium* was given by Newell and Tucker in 1968,[2] but still, many problems remain unsolved. Furthermore, there is evidence for the operation of at least two distinct pathways. In yeast, ^{14}C formate incorporates into carbon C-4 of pyramine **2** almost molar activity dilution,[3] while in *S. typhimurium*[4] and *Escherichia coli*,[5] the same precursor incorporates efficiently in carbon C-2. The study of six other microorganisms let Yamada et al. to put forward the generalization that these two distinct modes of incorporation were characteristic of eucaryotes and procaryotes, respectively.[6] A confirmatory indication for the existence of two distinct pathways may also be found in the observations that glycine is a specific precursor of pyramine in *S. typhimurium*[4,7] and *E. coli*[8] but is not incorporated into this unit in yeast.[9]

Various labeled D-glucose molecules are incorporated into pyramine, in yeast grown in the presence of ethanol as main carbon source, but the distribution of the label has not yet been reported.[10] All other information concerning yeast is negative. On the other hand, it has been known for some time[2] that in enterobacteria pyramine **2** shares with purine nucleotides a common precursor,

Scheme I. Metabolic Connection between the Imidazole of AIR and the Pyrimidine of Thiamine in *E. coli* and *S. typhimurium*



3 **2**

5-aminoimidazole ribotide (AIR, **3**) (Scheme I). All the atoms of the imidazole ring of AIR incorporate into pyramine. The connection between atoms of both rings has been indirectly determined in *Salmonella typhimurium*[4,7] and *Escherichia coli*.[8] These data allow us to write a scheme for the conversion of the imidazole ring of AIR to the pyrimidine ring of pyramine (Scheme I). As well as imidazole atoms, a two-carbon unit and a one-carbon unit (light faced in the scheme) are involved in pyramine biosynthesis. Concerning the origin of these two units, only negative information has been reported so far, except the recent observation[11] that the label from [6-^{14}C] glucose incorporates exclusively into C-7 of pyramine, a fact that, however, gives in itself no proof of the nature of involved intermediates.

We wish to report now experimental evidence showing that in fact all the carbons of pyramine including C-8 (the methyl carbon) derive from AIR.

Methods, Results, and Discussion

Resting cell suspensions of the *S. typhimurium* (strain thi 10/T $ath_{32\beta}^{2b}$) can synthesize thiamine from exogenous glucose (10 mM), 5-aminoimidazole riboside (AIR_s, the riboside related to AIR) (10 μM), and the thiazole precursor of thiamine (1 μM). Within 1.5 h, the intracellular level of thiamine rises from 0.06 to 0.6 nmol/mg of dry weight cells. When AIR_s is omitted in the medium, the concentration of thiamine remains at the initial low level. From previous results,[2,4,7] it can be asserted that the imidazole ring of AIR_s is the exclusive precursor of C-2, C-4, and C-6 of pyramine. To study the origin of the other carbon atoms, we supplied glucose or AIR_s, labeled with ^{13}C or ^{14}C.

Biosynthetic samples of AIR_s were used in these studies. Solutions of this labile compound were prepared by incubating resting

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Table I. Theoretical and Observed Distributions of ^{13}C within the Pyrimidine Moiety of Thiamine Derived from [U-^{13}C]AIR_s and Unlabeled Glucose

no. of ^{13}C atoms in the pyrimidine moiety of thiamine	% of the total molecules of the thiamine pyrimidine containing the indicated no. of ^{13}C atoms					
	calculated[a]					
	(3)	(3) + 1	(3) + 2	(3) + 2 + 1[b]	(3) + 3[c]	found
6	2×10^{-5}	7×10^{-4}	6×10^{-2}	2.20	4.44	1.8
5	5×10^{-3}	0.13	5.29	7.74	10.18	6.4
4	0.47	6.51	11.33	15.53	13.60	13.9
3	15.18	19.74	21.55	24.14	22.08	23.2
2	26.23	27.53	27.73	24.14	19.01	26.1
1	29.36	29.24	19.01	17.43	15.91	21.1
0	28.75	16.85	15.04	8.81	14.77	7.4
av abundance of ^{13}C, %	21.54	28.37	35.21	42.02	42.02	40.2

[a] Besides the three C atoms of the imidazole part, further C atoms are assumed to be incorporated from the labeled AIR_s. The number of these further atoms is indicated by the figures following the (3). [b] The 3 further atoms from AIR_s are assumed to derive from two different labeled C units. [c] The 3 further atoms from AIR_s are assumed to derive from a single labeled C unit.

cells of *E. coli* (strain W-11) and extracting the excreted riboside from the incubation medium. The purification on Dowex 50 (NH_4^+) according to Love and Levenberg[12] was followed by chromatography on Dowex-1 (borate).

In the following discussions, the measured figures relative to the labeling of pyramine have been corrected to account for the presence of endogeneous thiamine at the beginning of the experiment.

Experiments with ^{13}C. In the presence of natural AIR_s and [U-^{13}C]glucose (a sample in which $^1/_3$ of the molecules were D-[U-^{13}C]glucose, 85 atom % ^{13}C, and $^2/_3$ were natural glucose) *Salmonella* cells gave thiamine, the pyrimidine part of which was analyzed according to White and Rudolph:[8] cleavage by ethanethiol affforded 2-methyl-4-amino-5-(ethylthiomethyl)pyrimidine which was examined by gas chromatography–mass spectrometry. Incorporation of ^{13}C was evaluated from the molecular ion (M^+, m/e 183) as well as from the main fragment ($M^+ - SC_2H_5$, m/e 122). The average abundance of ^{13}C in the pyrimidine was calculated from the intensity of successive mass peaks of these ions. The pyrimidine was found to contain ^{13}C with only 1.3% abundance, very close to the natural one (1.07%). Incorporation of a single carbon atom from glucose would have led to a 5.73% abundance. This result suggests that although glucose was the main carbon source for cells, it supplied no carbon atoms of pyramine. This appeared to derive completely from AIR_s, already a known precursor of three of its six carbon atoms, or from some compound stored in derepressed *Salmonella* cells.

Starting from [U-^{13}C]glucose, a sample obtained by mixing 48.8% molecular part of [U-^{13}C]glucose (85 atom % ^{13}C) with 51.2% of natural glucose, we prepared a sample of AIR_s with all the carbon atoms of the imidazole and ribose moieties evenly labeled. The pyrimidine part of thiamine synthesized in the presence of this labeled AIR_s and of natural glucose was found to contain ^{13}C with 40.2% average abundance. This figure compares well with the ^{13}C abundance in the precursor AIR_s which should be the same as that of [U-^{13}C]glucose utilized in its biosynthesis, i.e., 42.03%. By contrast, the calculated figures corresponding to the introduction into the pyrimidine of one or three unlabeled carbon atoms from a foreign source would be respectively 35.2 and 21.5%. A confirmation may be obtained from the spectrum of the ^{13}C-labeled pyrimidine molecules. As several molecules of glucose contributed to the biosynthesis of AIR_s and as the precursor of AIR_s was a mixture of labeled (48.8%) and natural (51.2%) glucose, there was a sharp decrease in the proportion of molecules with six ^{13}C atoms. However, the theoretical repartition may be calculated by assuming successively that three, two, one, or no carbon atoms are supplied to the pyrimidine by an unlabeled source foreign to AIR_s. In these calculations, the number of glucose molecules involved in the synthesis of AIR_s should be postulated to take into account the recombinations between labeled and unlabeled carbon units. It was assumed, according to the known biosynthetic pathway of AIR, that the three carbon atoms of the imidazole derived from two different glucose molecules. To obtain the calculated figures of Table I, these three C atoms were combined successively with the following sets of atoms: three unlabeled atoms; two unlabeled and a single labeled atom; a single unlabeled and two labeled atoms from a single glucose molecule; three labeled atoms from two different glucose molecules; and three labeled atoms from a single glucose molecule. The involvement of at least two glucose molecules in the biosynthesis of the ribose part of AIR may occur by operation of the pentose–heptose pathway which cannot be ruled out.

In the observed repartition (Table I, last column), the percentage of molecules found with five ^{13}C atoms leaves no doubt that at least five carbon atoms of pyramine derived from the labeled source. As regards the percentage of molecules with six ^{13}C atoms, the observed repartition contrasts markedly with the calculated by assuming the incorporation of only five carbon atoms of AIR_s into pyramine. The bound figures are closest to those corresponding to the hypothesis that the three carbons of pyramine of unknown origin derive from the ribose part of AIR_s, while this sugar has been mainly biosynthesized by the pentose–heptose pathway.

These results are significant only if the purity of the labeled AIR_s as ^{13}C carbon source is ascertained. The purification of AIR_s is difficult because of its lability, especially in the dry state or as a concentrated solution. Successive thin-layer chromatographies on cellulose had to be avoided, as about 70% was lost at each step. With regard to the purity of AIR_s, the following evidences may be pointed out. Dowex-50 chromatography yielded a sample which was already free of heavy contaminants such as glucose or its main fermentation products. The next purification step, a Dowex-1 (borate) chromatography, would remove, if present, any traces of thiamine, pyramine, or their phosphates. However, the medium from which AIR_s was extracted contained no appreciable amounts of these very inconvenient possible contaminants. Thus, after these two steps, it was very unlikely that the AIR_s solution would contain any important, foreign carbon source.

However, since no quantitative evidence concerning the purity of AIR_s labeled with ^{13}C was available, the next experiments were made with radioactive carbon.

Experiments with ^{14}C-Labeled AIR_s. Incubation of *E. coli* (strain W11) cells in the presence of inactive glucose and [U-^{14}C]ribose (20×10^6 DPM/μmol) allowed the preparation of a purified solution of [^{14}C]AIR_s (74 000 ± 400 DPM/mL). The estimation of the molar activity of AIR_s in this solution is fraught with uncertainties. Love and Levenberg reported[12] ϵ 24 600 at 500 nm for the dye obtained in a Bratton–Marshall test.[13] This value is based on the assay of the ribose released by the acidic hydrolysis of the riboside, assuming that 1 mol of ribose was effectively released per mol of riboside. On the other hand, another group reported ϵ 17 000, a value based on direct measurements

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Table II. Incorporation of Radioactivity from AIR_s Labeled[a] with ^{14}C into the Pyrimidine Moiety of Thiamine

products	SA[b]
"pyrimidine thioether"	397 ± 4.5
4-amino-2,5-dimethylpyrimidine	382 ± 8
2,5-dimethyl-4-hydroxypyrimidine	387 ± 8

[a]The sample of labeled AIR_s was biosynthesized from inactive glucose and [U-^{14}C]ribose. It was mainly labeled in its ribose part. Specific radioactivity of the ribose part of AIR_s is 590×10^3 DPM/μmol. [b]Specific activity (DPM/μmol $\times 10^{-3}$) of the pyrimidines obtained by successive chemical transformations of the pyrimidine part of thiamine synthesized from labeled AIR_s. SA = measured specific activity × dilution rate by carrier. The biosynthesized extracted thiamine diphosphate was diluted with 15140 parts of inactive cocarboxylase as carrier. Labeled 4-amino-2,5-dimethylpyrimidine was diluted by 2.84 parts of inactive compound.

on samples of a model compound.[14] These authors pointed out that AIR_s in solution is accompanied by formylamidine riboside, the product of hydrolytic opening of the imidazole ring, which is not detected by the Bratton–Marshall reaction. The reported extinction coefficients are thus apparent values. In our experiments, a value of 21000 generally fitted the amount of ribose released by acidic hydrolysis from freshly prepared AIR_s solutions. The result of our assays, given as amount of AIR_s, includes in fact all its transformation ribosides.

Fortunately, a precise knowledge of the amounts of AIR_s was not critical to our purpose. A value of 590000 ± 20000 DPM/μmol was obtained for the specific radioactivity of the ribose (0.11 μmol) released by acidic hydrolysis of the AIR_s solution. These results show that 84–90% of the radioactivity in the AIR_s solution was located in the ribose part of AIR_s and less than 20% in the imidazole part and eventually in impurities. This labeling pattern was expected a priori for a sample of AIR_s biosynthesized from labeled ribose because it is known that ribose is poorly metabolized by *E. coli* in the presence of glucose, its carbon atoms being mainly incorporated into direct metabolites of ribose phosphate.[15]

Thiamine phosphate biosynthesized from this radioactive AIR_s and inactive glucose by *Salmonella* cells was extracted, diluted by inactive cocarboxylase as carrier, and cleaved by ethanethiol to 4-amino-5-(ethylthiomethyl)-2-methylpyrimidine which was isolated, purified, and converted successively to 4-amino-2,5-dimethylpyrimidine and to 2,5-dimethyl-4-hydroxypyrimidine. This last compound was degraded to a mixture of formic (C-6), acetic (C-2, C-8), and propionic acid (C-4, C-5, C-7). Schmidt degradation of acetic and propionic acids gave carbon dioxide and respectively methylamine and ethylamine which were isolated as *N*-methyl- and *N*-ethylphtalimide.

A large part of the label of AIR_s (about 60%) was found in the pyrimidine moiety of thiamine; it is beyond doubt that this radioactivity was effectively incorporated in pyramine, for it followed the chemistry of this pyrimidine (Table II) and was fully recovered in the degradation products of 2,5-dimethyl-4-hydroxypyrimidine (Table III). As expected, the three carbon atoms (C-2, C-4, and C-6) which originated from the imidazole part of AIR_s were poorly labeled (8–12% the radioactivity of one carbon atom of the ribose part of AIR_s). On the other hand, C-8 and (C-5, C-7) were found approximately as radioactive as respectively one and two carbons of the ribose part of AIR_s.

The radioactivity of the imidazole part of AIR_s can be deduced a posteriori from that of C-2, C-4, and C-6. In estimating the distribution of radioactivity in the AIR_s solution a further 8–12% activity is to be added to the 84–90% contribution of the ribose part. Thus, it can be asserted that 92–100% of the radioactivity found in the AIR_s solution was effectively located in AIR_s (or ribosides derived from AIR_s). The radioactivity of the methyl carbon of *N*-methylphtalimide (C-8) was found significantly higher than the average value for the ethyl carbons of *N*-ethylphtalimide

Table III. Distribution within the Pyrimidine Moiety of Thiamine of the Radioactivity Incorporated from AIR_s Labeled[a] with ^{14}C

products	SA[b]	RSA[c]	(activity in product)/(activity per C in ribose)[d]
formic acid (C-6)	9.25 ± 2.8	2.4	0.08
acetic acid (C-2, C-8)	161.5 ± 4.5	41.7	
propionic acid (C-4, C-5, C-7)	213 ± 6.2	54.9	
N-methylphthalimide (C-8)	141.9 ± 4.3		1.2
N-ethylphthalimide (C-5, C-7)	208.5 ± 6		1.77
$BaCO_3$ (C-2)	14.5 ± 4.3		
$BaCO_3$ (C-4)	15.5 ± 4.3		0.13

[a]This sample of AIR_s was mainly labeled in its ribose part. Specific activity of the ribose part is 590×10^3 DPM/μmol. [b]Specific activity (DPM/μmol $\times 10^{-3}$) of the products. SA = measured specific activity × dilution rate by carrier. [c]Relative specific activity, % (2,5-dimethyl-4-hydroxypyrimidine = 100%). [d]5 × SA/specific activity of ribose of AIR_s.

(C-5, C-7). While this might have been a consequence of an imperfect separation of acetic and propionic acids after the degradation of 2,5-dimethyl-4-hydroxypyrimidine, a careful TLC examination of the crude, phtalimido derivatives indicated that the extent of cross contamination could not exceed 5%. Furthermore, both derivatives were separately purified by preparative TLC. Any contamination would be definitely removed in the process. Another possible explanation is that the precursor ribose was not evenly labeled in all its carbon atoms. This point is now under examination.

Conclusion

The experiments with [U-^{13}C]AIR_s have shown that AIR_s supplied all of the carbon atoms of pyramine. It was known, from previous experimental evidence,[4,7] that only three carbon atoms of pyramine derive from the imidazole ring of AIR. Therefore, it can be concluded that as well as the whole imidazole part of AIR three carbon atoms of the ribose part contribute to pyramine biosynthesis. In complete agreement with these results, radioactivity from AIR_s labeled mainly with ^{14}C in its ribose part was found to incorporate into the three carbon atoms of pyramine, the origin of which was yet unknown. Owing to the minute amount of supplied AIR_s as compared with that of glucose, in both experiments, the incorporation of label from AIR_s after metabolic degradation is ruled out.

Thus, in enterobacteria the pyrimidine moiety of thiamine appears to be the product of a most puzzling intramolecular conversion of 5-aminoimidazole ribotide. Our results together with the observations of Yamada and Kumaoka[11] and the evidence of chemical structure make it very likely that the hydroxymethyl of ribose in AIR is the precursor of the hydroxymethyl in pyramine; moreover this biosynthesis from the ribotide probably generates the monophosphate of pyramine as the direct precursor of thiamine.

Experimental Section

Organisms. Two strains were used: *Escherichia coli* (W-11) was a purine requiring strain which excreted AIR_s when incubated in a medium free of purine. It was kindly supplied by Dr. H. S. Love. *Salmonella typhimurium* thi 10/T $ath_{38\beta}{}^{2b}$ exhibited a double growth requirement for a purine and for the pyrimidine part of thiamine (pyramine). Unlike other *ath* mutant, it was able to use exogenous AIR_s to synthesize pyramine. Moreover, it required tryptophan and the thiazolic precursor of thiamine for growth. It was kindly supplied by Dr. P. C. Newell.

Media. The organisms were grown and incubated in media containing the inorganic salts of the medium of Davis and Mingioli[16] and the carbon sources indicated for each experiment.

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Materials. D-[U-^{13}C]Glucose (85 atom % ^{13}C) and D-[U-^{14}C]ribose (250 mCi/mmol) were obtained from C.E.A., Saclay (France). Samples of labeled glucose were diluted with natural glucose as indicated, before feeding. Radioactive ribose was diluted to a specific radioactivity of 10 mCi/mmol.

Assay of Thiamine. Thiamine phosphates in aliquots of extracts were converted to free thiamine by hydrolysis in the presence of Mylase SR (Société Rapidase, Séclin, France) (10 mg/mL) at pH 5 (45 °C, 4 h). Thiamine was then assayed with *Lactobacillus fermenti* with use of the procedure of MaciasR.[17]

Gas Chromatography–Mass Spectrometry. A Ribermag R 10–10 gas chromatograph–mass spectrometer equipped with a 25 m × 0.34 mm Girdel column packed with CPSIL 5 was used. The oven was set at 140 °C. The retention time of 4-amino-5-(ethylthiomethyl)-2-methylpyrimidine was 4 min under these conditions. The mass spectra were recorded after ionization by electron impact at 70 eV.

Radioactivity Measurements. All the measurements were performed by liquid scintillation counting with a SL 20 Intertechnique liquid scintillation spectrometer. The samples of organic compounds were dissolved in water, ethanol, or cyclohexane and dispersed in Ready-Sol HP (Beckman) (10 mL). The efficiency of counting was determined by external standardization. It was generally higher than 90%. Samples of carefully dried baryum carbonate were counted in the same counting liquid with 85% efficiency.

Preparation of AIR_s. Solutions of AIR_s were prepared according to Love and Levenberg[12] without major modification. Washed cells of *E. coli* (strain W-11) were incubated in a medium (50 mL) containing initially glucose (22 mM), glycine, and formate (1 mM) and eventually radioactive ribose (0.5 mM). When glucose was labeled, glycine and formate were omitted. When stored at 4 °C, the solutions of AIR_3 (about 0.2 mM in 0.01 M ammonium hydroxide) do not change appreciably (Bratton–Marshall assay) within a day. A decrease in aminoimidazole concentration of ca. 30% occurs within a month . In consequence, all the purification steps were conducted at 4–6 °C. After incubation (3 H), the cells were separated by centrifugation and the clear, pale yellow medium was passed through a Dowex 50 (NH_4^+) (200–400 mesh) column (1.4 × 18 cm); the column was washed with 0.1 M sodium citrate buffer (pH 6.5) (100 mL); AIR_s was eluted with 0.01 M ammonium hydroxyde; it was detected by the Bratton–Marshall assay[14] in an aliquot of fractions. The solution of AIR_s was then concentrated to 8 mL at 30 °C under diminished pressure; 0.1 M borax was added (1 mL) and the solution was passed through a Dowex 1 (borate) (200–400 mesh) column (0.9 × 4 cm) which retained the riboside. Elution with 0.01 M borate (10-mL fractions) yielded AIR_s, generally in fractions 6–8. The riboside solution was finally desalted by fixation on a Dowex 50 (NH_4^+) column (4 mL) and elution with 0.01 M ammonium hydroxyde (15 mL). From 50 mL of incubation medium, a solution containing about 4 μmol of AIR_s was obtained.

Estimation of the Specific Radioactivity of the Ribose Part of AIR_s. Unlabeled AIR_s (0.5 μmol) was heated for 1 h at 90 °C in acid solution (1.2 M HCl; 1.5 mL). The solution was then passed through an Amberlite IR 45 (basic form) column (4 mL) and a Dowex 50 (H^+) column (1 mL). The Bial's orcinol method,[18] which also responds to furaldehyde, was considered as an acceptable method for the estimation in an aliquot of the total (and partially degraded) ribose released during the hydrolysis. The concentration of free ribose, necessary for the calculation of the dilution rate, was estimated by gas chromatography of the trimethylsilyl ether as 0.89 times the figure found in the colorimetric assay. Labeled AIR_s was hydrolyzed in the same conditions, and the amount of free ribose in the deionized hydrolysate was calculated as 0.89 times the figure derived from the orcinol test. Cold ribose (96 mg) was added as carrier. Reduction with $NaBH_4$ gave ribitol (62 mg) which was recrystallized from ethanol. The specific activity of the ribose from AIR_s was calculated from the measured specific activity of ribitol and the dilution rate.

Biosynthesis of Thiamine. Washed cell suspensions of cells derepressed for thiamine biosynthesis were prepared according to Newell and Tucker[2a] by cultivating the strain thi 10/T ath_{38} in the presence of a suboptimal amount of thiamine: cells were grown in a medium (500 mL) containing initially glucose (0.3%), hypoxanthine (0.4 mM), tryptophane (0.3 mM), and thiamine (20 nM). As soon as the concentration of cells in the culture was 0.55 mg/mL of dry weight of bacteria, cells were separated by centrifugation, washed twice with cold saline, and resuspended in a medium containing glucose (0.2%) and the thiazole precursor of thiamine (2 μM). The concentration of cells was 1 mg/mL. In a 500-mL Erlenmeyer flask, a 120-mL fraction of the cell suspension was incubated after addition of AIR_s (to a 10 μM concentration) without stirring for 1.5 h at 37 °C. At the same time, a 20-mL fraction of the suspension was incubated without AIR_s as control.

Extraction and Degradation of Thiamine. The cells were separated by centrifugation (5000 g, 15 min) and resuspended in 0.1 M citric acid (6 mL); the suspension was placed in a boiling water bath for 15 min and then adjusted to pH 4.8 with 2 M NaOH. Cell debris was removed by centrifugation, and the clean supernatant solution was diluted to 10 mL. An aliquot (0.5 mL) was saved for thiamine assay. In experiments with ^{13}C, thiamine phosphates in the solution were subjected to the cleavage with ethanethiol according to White and Rudolph[8] which allowed the isolation of the pyrimidine part as 2-methyl-4-amino-5-(ethylthiomethyl)pyrimidine, extracted in dichloromethane from the aqueous solution. This pyrimidine was assayed by GC–MS to determine its ^{13}C content.

In experiments with radioactive carbon, a known amount of inactive cocarboxylase was added as carrier (about 300 mg). The pyrimidine thioether afforded by the cleavage with ethanethiol was purified by column chromatography on silica gel (10% ethanol in ether) and then recrystallized in cyclohexane to constant specific radioactivity: mp 115 °C; UV (ethanol) λ_{max} (ϵ) 239 (9300), 278 nm (5300).

The thioether was desulfurized[3] with Raney nickel to 2,5-dimethyl-4-aminopyrimidine, which was isolated and purified by sublimation (120 °C, 1 mmHg) and crystallization (acetone); after dilution by carrier, it was converted by acid hydrolysis[3] to 2,5-dimethyl-4-hydroxypyrimidine which was purified by recrystallization from ethylacetate.

Degradation of the Pyrimidine Moiety of Thiamine. 2,5-Dimethyl-4-hydroxypyrimidine (ca. 20 mg) was degraded to a mixture of formic, acetic, and propionic acids by the known method.[3] The three acids were separated on a column of silica gel (4 g) deactivated with 0.25 M H_2SO_4 (5 mL);[19] the mixture of organic salts was dissolved in 3 M H_2SO_4 (0.2 mL), mixed with dry silica gel (0.5 g), and poured at the top of the column. The eluent was 3% *tert*-butyl alcohol in dichloromethane; acids were titrated in the fractions (2 mL) with 0.01 M NaOH in the presence of phenol red. Propionic acid (ca. 100 μmol) was eluted in fractions 6–9 and acetic acid (ca. 100 μmol) in fractions 13–18. Then formic acid (ca. 90 μmol) was eluted with 6% *tert*-butyl alcohol in dichloromethane. The specific radioactivity of each acid was measured in aliquots of the solution of its sodium salt.

The dried propionate and acetate were subjected to the Schmidt degradation according to Phares[20] yielding ethylamine and methylamine respectively and baryum carbonate. The amines were isolated as *N*-ethyl- and *N*-methylphthalimide after reaction with carbethoxyphtalimide in a solution buffered at pH 9 by sodium carbonate.[21] The phtalimido derivatives were purified by silica gel TLC (cyclohexane:ethylacetate, 5:1). Analytical TLC showed that each derivative was free of the other, and starting carbethoxyphtalimide. *N*-methylphtalimide: mp 135 °C (lit.[u0] mp 134–135 °C); UV (cyclohexane), 290 nm (1520). *N*-Ethylphtalimide: mp 78–79 °C (lit.[22] mp 79 °C); UV (cyclohexane) 290 nm (1520).

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