and the most active material (167 g), as determined by bioassay, was collected near the middle of each run or during elution with $CHCl_3$-MeOH (9:1). Further chromatography of this material was carried out in six portions on a 6-cm i.d. column, 280 g of silica per run, by using 1.5 L of each of the following solvents: hexane-ethyl acetate (1:4), ethyl acetate, ethyl acetate-MeOH (19:1), ethyl acetate-MeOH (9:1), and ethyl acetate-MeOH (1:1). Similar fractions were again combined on the basis of TLC analysis, and 17 g of highly cytotoxic material was selected for further study. The concentrate was split into four portions, and each was then chromatographed on a reversed phase column. Each sample was deposited on approximately 90 cm^3 of C_{18} silica packed in a precolumn, which was placed in a preparative HPLC instrument ahead of a C_{18} cartridge. Columns were then eluted with 3.5 l of MeOH-H_2O (7:3), and the complex of *Trewia* maytansinoids emerged as a broad peak at column volumes of 1.5-3.5. Samples were arbitrarily collected across the peak, yielding 12 fractions and a total of 8.4 g of material, mainly mixtures of **1-6**. Individual compounds were obtained by semipreparative HPLC on a C_{18} μ-Bondapak column in 15-mg portions eluting with MeOH-H_2O (65:35) and operating at 2 mL/min; each fraction was further purified by preparative TLC. Elution times and yields obtained under these conditions were as follows: **5**, 12.5 min, 743 mg ((6.5×10^{-4})%); **6**, 15.0 min, 127 mg ((1.1×10^{-4})%); **4**, 25.5 min, 207 mg ((1.8×10^{-4})%); **2**, 27.5 min, 191 mg ((1.6×10^{-4})%); **3**, 31.0 min, 25 mg ((2.0×10^{-5})%); **1**, 33.0 min, 3700 mg ((3.2×10^{-3})%).

Treflorine (4). After preparative TLC and recrystallization from CH_2Cl_2-hexane, **4** had the following properties: mp 205-208 °C dec; IR ($CHCl_3$) 3600, 3440, 1760, 1715, 1675, 1640, 1610, 1590 cm^{-1}; UV max (EtOH) 233 nm (ϵ 24000), 243 (sh, 18500), 253 (19850), 282 (5060), 288 (5060); $[\alpha]^{23}_D$ -138° (*c* 0.045, $CHCl_3$); ^1H and ^{13}C NMR values, Tables I and II; mass spectrum (70 eV), *m/z* (relative intensity) 688 (M^+ - a, 2.7), 188 (5.7), 149 (4.2), 69 (14.7), 58 (32.1), 55 (13.4), 44 (100); $C_{35}H_{45}ClN_2O_{10}$ requires *m/z* 688.2762, found ($M^+ - (H_2O +$ HNCO)) found 688.2751.

Conversion of 4 to 7a and Characterization as the Methyl Ester 7b. A solution of **4** (162 mg) and sodium carbonate (120 mg) in 16 mL of 50% aqueous methanol reacted at room temperature for 96 h. The reaction mixture was acidified with 40 mL of 5% aqueous tartaric acid solution and extracted with $CHCl_3$, yielding 160 mg of $CHCl_3$-soluble material. This product was partially purified by preparative TLC on silica plates developed with $CHCl_3$-MeOH (4:1) to provide 54 mg of crude **7a**. Crude **7a** was treated with diazomethane, and the methylated product was subjected to preparative TLC on plates developed with CH_2Cl_2-MeOH (9:1) which yielded methyl ester **7b**: 3.5 mg; mp 135-140 °C; ^1H NMR spectrum of **7b**, summarized in Table I; mass spectrum (70 eV), *m/z* (relative intensity) 702 (M^+ - a, 1.2), 667 (1.8), 572 (2.7), 528 (1.0), 511 (1.6), 202 (45), 149 (11.0), 111 (18.6), 97 (24.1), 85 (25.3), 83 (39.0), 75 (28.0), 72 (45), 69 (46), 57 (69), 55 (67), 44 (100), 43 (56); $C_{36}H_{47}ClN_2O_{10}$ requires *m/z* 702.2919, found 702.2880 ($M^+ - (H_2O +$ HNCO)).

Trenudine (5). After preparative TLC and recrystallization from CH_2Cl_2-hexane, **5** gave the following properties: mp 200-205 °C dec; IR ($CHCl_3$) 3600, 3450, 3360, 1760, 1715, 1665, 1610, 1590 cm^{-1}; UV max (EtOH) 233 nm (ϵ 26400), 248 (sh, 21500), 253 (22600), 282 (6130), 288 (6130; $[\alpha]^{23}_D$ -114° (*c* 0.24, $CHCl_3$); ^1H and ^{13}C NMR values, Tables I and II; mass spectrum (70 eV), *m/z* (relative intensity) 704 (M^+ - a, 3.9), 672 (2.4), 669 (2.6), 593 (2.6), 204 (3.2), 185 (3.1), 171 (5.5), 95 (10.2), 85 (10.5), 83 (11.7), 81 (11.5), 69 (16.9),, 55 (23.9), 44 (100); $C_{35}H_{45}ClN_2O_{11}$ requires *m/z* 704.2711, found 704.2711 ($M^+ - (H_2O +$ HNCO)).

Conversion of 5 to 8a and Characterization as the Methyl Ester 8b. A solution of **5** (80 mg) and sodium carbonate (60 mg) in 8 mL of 50% aqueous methanol reacted at room temperature for 72 h. The reaction mixture was acidified with 40 mL of 2% aqueous tartaric acid solution and extracted with $CHCl_3$, yielding 63 mg of $CHCl_3$-soluble material. This material was partially purified by preparative TLC on silica plates developed with $CHCl_3$-MeOH (4:1), yielding 9.6 mg of crude **8a**. Crude **8a** was treated with diazomethane, and the methylated product was subjected to preparative TLC on silica plates developed with CH_2Cl_2-MeOH (19:1): yield of methyl ester **8b**, 2.7 mg; mp 150-160 °C; ^1H NMR spectrum of **8b**, summarized in Table I; mass spectrum (70 eV), *m/z* (relative intensity) 718 (M^+ - a, 0.5), 683 (0.3), 588 (0.2), 570 (0.3), 544 (0.2), 149 (31.3), 111 (31.3), 97 (45), 83 (36), 71 (59), 69 (52), 57 (67), 55 (55), 44 (100), 43 (61); $C_{36}H_{47}ClN_2O_{11}$ requires *m/z* 718.2868, found 718.2859 ($M^+ - (H_2O +$ HNCO)).

***N*-Methyltrenudone (6).** After preparative TLC and recrystallization from dichloromethane-hexane, **6** gave the following properties: mp 192-197 °C dec; IR ($CHCl_3$) 3600, 3450, 1760, 1720, 1675, 1640, 1590 cm^{-1}; UV max (EtOH) 233 nm (ϵ 27000), 247 (sh, 21300), 252 (21900), 282 (5270), 289 (5470); $[\alpha]^{23}_D$ -110° (*c* 0.183, $CHCl_3$); ^1H and ^{13}C NMR values, Tables I and II; mass spectrum (70 eV), *m/z* (relative intensity) 716 (M^+ - a, 12.3), 681 (4.4), 605 (2.6), 542 (7.5), 292 (10.0), 109 (15.1), 75 (47.7), 71 (18.3), 58 (100), 55 (27.7), 44 (74.6), 43 (48.1); $C_{36}H_{45}ClN_2O_{11}$ requires *m/z* 716.2711, found 716.2662 ($M^+ - (H_2O +$ HNCO)).

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Registry No. 1, 78987-26-5; **2**, 78987-27-6; **3**, 78987-28-7; **4**, 82390-93-0; **5**, 82390-94-1; **6**, 82400-19-9; **7b**, 82390-95-2; **8b**, 82390-96-3; **7a**, 82390-97-4; **8a**, 82390-98-5.

Thiamin Biosynthesis in Yeast. Origin of the Five-Carbon Unit of the Thiazole Moiety

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Abstract: Radioactivity from D-[1-^{14}C]-, D-[2-^{14}C]-, and D-[6-^{14}C]glucose, from D-[1-^{14}C]fructose, and from [1-^{14}C]glycerol is incorporated nonrandomly into the C_5 chain of the thiazole moiety of thiamin in *Saccharomyces cerevisiae*. The incorporation pattern leads to the inference that the C_5 chain is derived from a 2-pentulose, which is generated from the hexose precursors by the oxidative as well as by the nonoxidative pentose phosphate pathway. A chemically rational scheme for the biogenesis of the thiazole moiety of thiamin is presented.

The ultimate steps in the biosynthesis of thiamin (**1**) (vitamin B_1), and, in particular, the mode of construction of its skeleton, by condensation of the intact pyrimidine unit (**29**) with the intact thiazole unit (**3**), are well documented.[1] The routes leading to

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Table I. Tracer Experiments with *S. cerevisiae* (ATCC 24903) Growing on a Thiamin-Free Medium with D-Glucose as Carbon Source[a]

	labeled substrate				fraction of administered radioact. remaining in the medium after harvesting of cells, %	thiamin chloride hydrochloride	
expt[b]	substrate	nominal total radioact., μCi	nominal spec radioact., mCi/mmol	culture vol, mL		wt of inactive carrier added, mg	spec act. of isolated product, 10^{-4} dpm/mmol
6	DL-[3-^{14}C]cysteine[c]	100	52	700	93	0[e]	inactive
7	L-[3-^{14}C]serine[c]	125	56	700	13	204	0.28 ± 0.01
8	[1-^{14}C]succinic acid[d]	100	26	700	71	31	inactive
9	sodium 2-keto[5-^{14}C]glutarate[c]	32	10	450	66	0[e]	inactive
10	D-[1-^{14}C]ribose[d]	50	50	450	100	0[e]	inactive
11	sodium [1-^{14}C]acetate[d]	223	58	1250	13	55	1.31 ± 0.02[f]
12	sodium [3-^{14}C]pyruvate[d]	50	19	500	87	58	0.08 ± 0.01
13	sodium L-[U-^{14}C]lactate[d,g]	100	139	1400	7	60	0.29 ± 0.02

[a] For growth conditions, see ref 5. [b] Experiments 1–5; see ref 5. [c] Amersham/Searle. [d] New England Nuclear. [e] Thiamin-containing chromatographic fractions were inactive, and the experiment was not continued. [f] This activity was due to a persistent radioactive impurity, as shown by chemical degradation. Neither the pyrimidinesulfonic acid (6) nor the 5-(β-hydroxyethyl)-4-methylthiazole (2) (isolated as phenylurethane derivative (structure 7 in ref 5)) was radioactive. [g] Grown on a thiamin-free medium with ethanol as carbon source. For growth conditions, see Experimental Section.

Table II. Tracer Experiments with *S. cerevisiae* (ATCC 24903) Growing on a Thiamin-Free Medium with Ethanol as Carbon Source[a]

	labeled substrate				fraction of administered radioactivity remaining in medium after harvesting of cells, %	thiamin chloride hydrochloride	
expt	substrate	nominal total radioact., μCi	nominal specific radioact., mCi/mmol	vol, mL		wt of inactive carrier added, mg	specific activity of isolated product, 10^{-4} dpm/mmol
14	D-[U-^{14}C]glucose[b]	250	313	1400	11	125	1.96 ± 0.04
15	D-[1-^{14}C]glucose[b]	500	56	2800	6	277	2.22 ± 0.03
16	D-[1-^{14}C]fructose[c]	500	57	2800	9	290	2.38 ± 0.03
17	D-[6-^{14}C]glucose[b]	250	53	1400	14	79	1.42 ± 0.04
18	D-[6-^{14}C,6-^{3}H]-glucose[b]	500 (^{14}C) 5000 (^{3}H)	53 34×10^3	2800	15 39	226	2.91 ± 0.07[d]
19	D-[2-^{14}C]glucose[b]	500	53	2800	9	310	5.21 ± 0.08
20	[1-^{14}C]glycerol[b]	500	56	2800	11	258	3.35 ± 0.04

[a] For growth conditions, see Experimental Section. [b] New England Nuclear. [c] Amersham Searle. [d] Activity due to ^{14}C.

each of the two subunits are unknown, however, and not even their primary precursors have been fully identified.

The thiazole moiety has different sets of precursors in yeasts and in bacteria. In each case the nitrogen atom and the adjacent CH carbon atom (C-2) are derived as an intact unit from an amino acid. In yeast it is glycine,[2-5] in bacteria tyrosine,[6-8] which supplies its C_α–N fragment to serve as the source of the –C=N– segment of the thiazole ring. Earlier results, which had been interpreted as showing that this carbon atom, C-2 of the thiazole ring, was derived from the *S*-methyl group of methionine and that the C_3 unit, C-5,6,7 of the thiazole, also originated from methionine, have been shown to be in error.[5,7,9]

We now report that, in yeast, radioactivity from specifically labeled glycerol, glucose, and fructose enters the C_5 unit of the thiazole moiety, C-4′,4,5,6,7, in nonrandom fashion. The distribution of label permits the inference that the C_5 unit originates from a pentose precursor. A rational hypothesis accounting for the derivation of the thiazole unit of thiamin in yeast, from glycine and a 2-pentulose, is presented. A preliminary account of part of this work has appeared.[10]

In bacteria, the C_5 unit has very recently been reported to originate by combination of a C_2 unit derived from pyruvate (C-4′,4)[11] with a C_3 unit, derived from a triose[11,12] (C-5,6,7), possibly via a 1-deoxy-2-pentulose.[13]

In bacteria, the S atom of the thiazole nucleus is derived from cysteine.[9,14] Cysteine does not supply carbon atoms to the thiazole ring system.[9,14]

The origin of the pyrimidine moiety of thiamin is unknown. Incorporation studies[1] with various substrates, in yeasts and bacteria, suggest that the derivation of this pyrimidine differs markedly from that of the nucleic acid pyrimidines but lead to results that are in some cases mutually contradictory and that cannot be interpreted in terms of a single set of precursor–product relationships.

We now report the incorporation of radioactivity from specifically labeled glycerol and hexoses and advance a new proposal for the origin of the pyrimidine unit of thiamin in yeast.

Methods and Results

In 15 tracer experiments cultures of *Saccharomyces cerevisiae* (ATCC 24903), growing on a thiamin-free medium with either

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Table III. Distribution of ^{14}C from Labeled Substrates between the Pyrimidine and Thiazole Moities of Thiamin

		product					
		thiamin chloride hydrochloride (1)		pyrimidine sulfonic acid (2)		phthalimido-thiazole (6)	
expt	substrate	SA[a]	RSA[b]	SA[a]	RSA[b]	SA[a]	RSA[b]
7	L-[3-^{14}C]serine	0.28 ± 0.01	100 ± 4	0.25 ± 0.01	89 ± 5	inactive	
11	sodium [1-^{14}C]acetate	1.31 ± 0.02	100 ± 2	inactive		inactive[c]	
14	D-[U-^{14}C]glucose	1.96 ± 0.04	100 ± 2	1.10 ± 0.01	56 ± 1	1.06 ± 0.03	54 ± 2
15	D-[1-^{14}C]glucose	2.22 ± 0.03	100 ± 1	1.34 ± 0.02	60 ± 1	0.97 ± 0.02	44 ± 1
16	D-[1-^{14}C]fructose	2.38 ± 0.03	100 ± 1	1.30 ± 0.01	55 ± 1	1.06 ± 0.02	45 ± 1
17	D-[6-^{14}C]glucose	1.42 ± 0.04	100 ± 3	0.69 ± 0.02	49 ± 2	0.72 ± 0.02	51 ± 2
18	D-[6-^{14}C,6-^{3}H]glucose	18.38 ± 0.31[d]	100 ± 2	7.94 ± 0.10	43 ± 1	10.93 ± 0.13	59 ± 1
		2.91 ± 0.07[e]	100 ± 2	1.43 ± 0.04	49 ± 2	1.46 ± 0.03	50 ± 2
19	D-[2-^{14}C]glucose	5.21 ± 0.08	100 ± 2	3.10 ± 0.03	60 ± 1	2.09 ± 0.02	40 ± 1
20	[1,3-^{14}C]glycerol	3.35 ± 0.04	100 ± 1	1.92 ± 0.03	57 ± 1	1.49 ± 0.03	45 ± 1

[a] Specific activity (dpm $mmol^{-1}$) × 10^{-4}. [b] Relative specific activity, % (thiamin chloride hydrochloride = 100). [c] Counted as the thiazole phenylurethane (structure 7 in ref 5). [d] ^{3}H results. [e] ^{14}C results.

D-glucose (experiments 6–12) or ethanol (experiments 13–20) as the carbon source, were incubated with ^{14}C-labeled substrates. Radioactive tracer was added at the onset of logarithmic growth. The cells were collected when maximum cell density had been attained. Thiamin chloride hydrochloride (**1**) was isolated from the cells, mixed with nonradioactive thiamin chloride hydrochloride, and then purified to constant radioactivity. The details of these experiments are presented in Tables I and II.

The samples of thiamin isolated from the experiments with DL-[3-^{14}C]cysteine (experiment 6), [1-^{14}C]succinic acid (experiment 8), sodium 2-keto[5-^{14}C]glutarate (experiment 9), D-[1-^{14}C]ribose (experiment 10), and sodium [1-^{14}C]acetate (experiment 11) were inactive. The specific activity of the thiamin samples from the experiments with sodium [3-^{14}C]pyruvate (experiment 12) and sodium L-[U-^{14}C]lactate (experiment 13) was not high enough to permit the further carrier dilution required to yield sufficient material for degradation.

The samples of thiamin chloride hydrochloride, which maintained a significant level of radioactivity after several recrystallizations, were degraded by bisulfite cleavage to the pyrimidinesulfonic acid (**2**) and the thiazole (**3**). The latter, an oil at room temperature, was converted, via 5-(β-chloroethyl)-4-methylthiazole (**5**), into the phthalimido derivative (**6**) for purification and assay of radioactivity. The results are summarized in Table III.

The sample of thiamin derived from [1-^{14}C]acetate (experiment 11) yielded degradation products that were completely inactive. The sample derived from L-[3-^{14}C]serine (experiment 7) contained all of its label in the pyrimidine moiety. The samples of thiamin derived from glucose, fructose, and glycerol (experiments 14–20) each yielded samples of the pyrimidinesulfonic acid and the phthalimidothiazole derivative which were labeled and which, in each case, accounted for all the activity of the intact thiamin. In general, the pyrimidinesulfonic acid contained somewhat more than half and the thiazole derivative somewhat less than half of the activity of the intact thiamin.

The distribution of radioactivity within the C_5 chain of the thiazole unit was determined by the reactions outlined in Scheme I. Oxidation of the thiazole (**3**) with pyridinium dichromate gave 5-formyl-4-methylthiazole (**4**) (loss of C-7), which was isolated as the semicarbazone derivative. Acid permanganate oxidation of the phthalimido derivative (**6**) gave *N*-phthaloyl-β-alanine (**7**) (C-5,6,7), and Kuhn–Roth oxidation of **6** gave acetate (C-4′,4), isolated as acetyl-α-naphthylamine (**8**). Schmidt degradation of the Kuhn–Roth acetate gave methylamine (C-4′), which was converted into *N*-methylphthalimide (**9**) by treatment with *N*-carbethoxyphthalimide (Nefkens' reagent).

The results of the degradation of the labeled samples of 4-methyl-5-(β-hydroxyethyl)thiazole (**3**) derived from thiamin from the experiments with D-glucose (experiments 14, 15, 17–19), D-fructose (experiment 16), and glycerol (experiment 20) are summarized in Table IV.

The samples of 4-methyl-5-(β-phthalimidoethyl)thiazole (the "thiazole unit") from the thiamin derived from D-[1-^{14}C]glucose (experiment 15) and from D-[1-^{14}C]fructose (experiment 16) each contained most of the label (>90%) at the *C*-methyl group, C-4′ (*N*-methylphthalimide) (**9**). The thiazole unit from thiamin from D-[6-^{14}C]glucose (experiments 17 and 18), on the other hand, contained most of the ^{14}C label (93%, 87%) at C-7 (*N*-phthaloyl-β-alanine (**7**) (experiment 17) (assuming C-5,6 to be free of activity as in experiment 18), 4-methyl-5-(β-phthalimidoethyl)thiazole (**6**) minus 5-formyl-4-methylthiazole (**4**) (experiment 18)), while the rest of the activity was located at C-4′ (*N*-methylphthalimide (**9**), experiment 18). In each of these samples C-4 of the thiazole unit was free of activity (acetyl-α-naphthylamine (**8**) minus *N*-methylphthalimide (**9**) (experiments 15 and 16); **6** minus *N*-phthaloyl-β-alanine (**7**) minus *N*-methylphthalimide (**9**) (experiment 18)).

A different distribution was found in the thiazole unit of thiamin from D-[2-^{14}C]glucose (experiment 19). The entire activity of the sample was present within the C_2 unit, C-2′,2 (acetyl-α-naphthylamine (**8**)). Label was approximately equally divided

Scheme I. Chemical Degradation of Thiamin Chloride Hydrochloride to Separate the Carbon Atoms of the C_5 Chain of the Thiazole Moiety

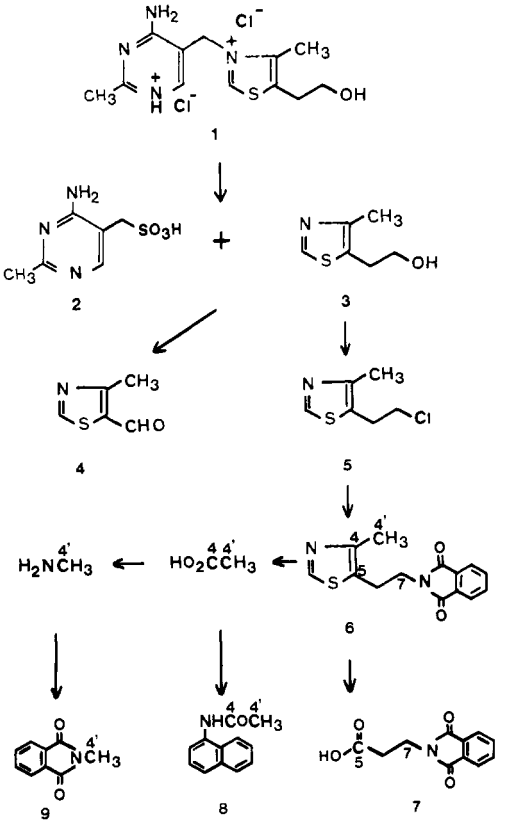

Table IV. Incorporation of Hexoses and Glycerol into the Thiazole Unit of Thiamin

	C atoms of thiazole moiety	precursors											
		expt 15 D-[1-^{14}C]glucose		expt 16 D-[1-^{14}C]fructose		expt 17 D-[6-^{14}C]glucose		expt 18 D-[6-^{14}C,6-^{3}H]glucose			expt 19 D-[2-^{14}C]glucose		expt 20 [1-^{14}C]glycerol
		SA[a]	RSA[b]	SA[a]	RSA[b]	SA[a]	RSA[b]	SA[a,c]	RSA[b,c]	SA[a,d]	RSA[b,d]	SA[a]	RSA[b]	SA[a]	RSA[b]
4-methyl-5-(β-phthalimidoethyl)thiazole (6)	all	0.97 ± 0.02	100 ± 2	1.06 ± 0.02	100 ± 2	0.72 ± 0.02	100 ± 3	10.9 ± 0.1	100 ± 1	1.46 ± 0.03	100 ± 2	2.09 ± 0.02	100 ± 1	1.49 ± 0.03	100 ± 2
5-formyl-4-methylthiazole (4)	all but C-7							0.05 ± 0.02	0.5 ± 0.2	0.25 ± 0.01	17 ± 1			1.04 ± 0.01	70 ± 2
acetyl-α-naphthylamine (8)	C-4,4′	0.89 ± 0.02	92 ± 3	0.89 ± 0.02	84 ± 2							2.00 ± 0.02	96 ± 1	0.59 ± 0.03	40 ± 2
N-methylphthalimide (9)	C-4′	0.90 ± 0.01	93 ± 2	0.96 ± 0.02	91 ± 3			0.07 ± 0.04	0.6 ± 0.4	0.24 ± 0.02	16 ± 2	0.96 ± 0.02	46 ± 1	0.41 ± 0.01	28 ± 1
N-phthaloyl-β-alanine (7)	C-5,6,7	0.05 ± 0.01	6 ± 1	0.05 ± 0.01	5 ± 1	0.64 ± 0.03	93 ± 5	11.6 ± 0.1	106 ± 2	1.27 ± 0.02	87 ± 2			0.89 ± 0.02	60 ± 2

[a] Specific activity (dpm mmol^{-1}) × 10^{-4}. [b] Relative specific activity, % (4-methyl-5-(β-phthalimidoethyl)thiazole = 100%). [c] ^{3}H results. [d] ^{14}C results.

Scheme II. Distribution of Activity (% of Total Activity at Indicated C Atoms) in the Thiazole Moiety of Thiamin Derived from Hexoses and from Glycerol

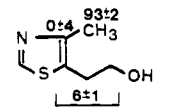

Figure 1
from 1-^{14}C-Glucose
Experiment 15

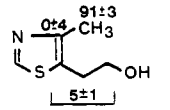

Figure 2
from 1-^{14}C-Fructose
Experiment 16

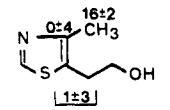

Figure 3
from 6-^{14}C-Glucose
Experiments 17, 18

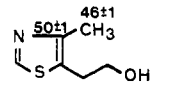

Figure 4
from 2-^{14}C-Glucose
Experiment 19

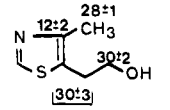

Figure 5
from 1,3-^{14}C-Glycerol
Experiment 20

Table V. Distribution of ^{3}H and ^{14}C in D-[6-^{14}C,6-^{3}H] Glucose (Experiment 18)

	spec act. (^{14}C) dpm per mmol × 10^{-6}	^{3}H/^{14}C
feeding solution containing mixture of D-[6-^{3}H]- and D-[6-^{14}C]glucose		9.09 ± 0.24
β-D-[6-^{14}C,6-^{3}H]glucose pentaacetate	2.55 ± 0.04	8.96 ± 0.24
formaldehyde dimethone from C-6 of glucose	2.55 ± 0.01	9.09 ± 0.04
mean value		9.05 ± 0.11

between C-4′ (*N*-methylphthalimide (**9**)) and C-4 (acetyl-α-naphthylamine (**8**) minus *N*-methylphthalimide (**9**)).

All activity of the thiazole unit of thiamin derived from [1-^{14}C]glycerol (experiment 20) was accounted for within the five carbon atoms of the C_5 chain (acetyl-α-naphthylamine plus *N*-phthaloyl-β-alanine). Of this activity 28% is present at C-4′ (*N*-methylphthalimide), 12% at C-4 (acetyl-α-naphthylamine minus *N*-methylphthalimide), 30% at C-7 ((**6**) minus (**4**)), and the remaining 30% at C-5 plus C-6 (*N*-phthaloyl-β-alanine minus C-7).

The distribution of label within these samples is summarized in Scheme II.

The distribution of tritium, relative to ^{14}C, in the sample of D-[6-^{14}C,6-^{3}H]glucose, used as substrate in experiment 18 (Table V), and in the sample of thiamin derived from it (Table VI), was also determined. A small portion (<1%) of the solution (^{3}H/^{14}C ratio 9.09 ± 0.24) containing the mixture of D-[6-^{3}H]- and D-[6-^{14}C]glucose was added to a solution of nonradioactive D-glucose. Part of this mixture was used to prepare a sample of β-D-glucose pentaacetate, which after repeated crystallization showed a ^{3}H/^{14}C ratio of 8.96 ± 0.24 and a ^{14}C specific activity of 2.55 (±0.04) × 10^{6} dpm per mmol, and part was subjected to periodate oxidation to liberate C-6 of glucose as formaldehyde. The formaldehyde was trapped as the dimedone derivative, which, after purification, had a ^{3}H/^{14}C ratio of 9.09 ± 0.04 and a ^{14}C specific activity of 2.55 (±0.01) × 10^{6} dpm per mmol, values identical with those shown by the glucose from which the formaldehyde was derived. Within the limits of error, the ^{3}H/^{14}C ratio of the feeding solution, glucose pentaacetate, and formaldehyde dimethone were identical (mean value of ^{3}H/^{14}C ratio, 9.05 ± 0.11). This ratio was maintained in the *N*-phthaloyl-β-alanine (**7**) (^{3}H/^{14}C ratio 9.1 ± 0.2) derived from the thiazole moiety of

Table VI. Distribution of ^3H, Relative to ^{14}C, within Thiamin, Derived from D-[6-^{14}C,6-^3H] Glucose (Experiment 18)

	RSA[a] (%)				$^3H/^{14}C$	
	from Table III		from Table IV			
	^3H	^{14}C	^3H	^{14}C	obsd	calcd from $^3H/^{14}C$ of the degradation products
precursor						
D-[6-^{14}C,6-^3H] glucose (from Table V)					9.1 ± 0.1	
product						
thiamin chloride hydrochloride (1)	100 ± 2	100 ± 2			6.3 ± 0.2	6.5 ± 0.2[b]
degradation products of thiamin						
pyrimidinesulfonic acid (2)	43 ± 1	49 ± 2			5.6 ± 0.2	
4-methyl-5-(β-phthalimidoethyl)thiazole (6)	59 ± 1	50 ± 2	100 ± 1	100 ± 2	7.5 ± 0.2	8.0 ± 0.3[c]
5-formyl-4-methylthiazole (4) semicarbazone			0.5 ± 0.2	17 ± 1	0.2 ± 0.1	
N-methylphthalimide (9)			0.6 ± 0.4	16 ± 2	0.3 ± 0.2	
N-phthaloyl-β-alanine (7)			106 ± 2	87 ± 2	9.1 ± 0.2	

[a] Relative specific activity, %. Left columns (from Table III), intact thiamin = 100%; right columns (from Table IV), intact thiazole = 100%. [b] [[(5.6 ± 0.2)(49 ± 2)]/100] + [[(7.5 ± 0.2)(50 ± 2)]/100]. [c] [[(9.1 ± 0.2)(87 ± 2)]/100] + [[(0.3 ± 0.2)(16 ± 2)]/100].

thiamin by degradation and representing the C_3 fragment, C-5,-6,-7, and the H atoms attached to C-6 and C-7. No tritium, relative to ^{14}C, was present at C-4′ (*N*-methylphthalimide, $^3H/^{14}C$ ratio 0.3 ± 0.2) or at C-6 (5-formyl-4-methylthiazole semicarbazone, $^3H/^{14}C$ ratio 0.2 ± 0.1) (Table VI).

Discussion

In examining the possible sources of the C_5 unit, C-4′,4,5,6,7, of the thiazole nucleus of thiamin, it was an obvious first step in selecting labeled substrates that might deliver radioactivity into this unit to consider primary metabolites from which an intact C_5 unit, appropriately functionalized for ring formation by interaction with glycine and a sulfur source, might be derivable.

Of the various possibilities the one that was most attractive, on structural grounds, was a pentose derivative.[15] However, this was not regarded as a promising choice as a substrate to be used in a tracer experiment, since yeasts, in general, do not utilize pentoses that are supplied to the medium.[16] Nevertheless, an experiment with D-[1-^{14}C]ribose (experiment 10, Table I) was performed, but not surprisingly, and in agreement with earlier work,[4] all radioactivity was recovered from the culture medium, and thiamin obtained from the cells after carrier dilution was not radioactive.

A second possibility was a C_5 chain derived from a polyketide source. Indeed, 4-oxopentan-1-ol, a compound that might be so derived, had been suggested as a possible precursor.[17] Incorporation of label from [1-^{14}C]acetate was tested (experiment 11, Table I). While only little radioactivity remained in the culture medium, none of it entered the thiamin (Table III).

Glutamate is a further compound that had been invoked as a possible source of the C_5 unit.[18] The corresponding α-keto acid, 2-ketoglutarate, was tested as a substrate (experiment 9), as was another Krebs cycle intermediate, succinate (experiment 8). In each case most of the activity (ca. 70%) was recovered from the medium, and in each case the thiamin remained nonradioactive. Succinate (together with glycine) and glutamate are the precursors, in bacteria[19] and higher plants,[20] respectively, of δ-aminolevulinic acid. This was yet another five-carbon metabolite, which, on the basis of chemical functionality, might have deserved consideration as a possible precursor of the thiazole C_5 chain.

Several other experiments, with substrates that on a structural basis were regarded as potential sources of a segment of the C_5 chain, met with similar lack of success.

Neither pyruvate (experiment 12, Table I) nor lactate (experiment 13, Table I), possible precursors of the C_2 unit, C-4′,4, of the thiazole moiety, supplied significant radioactivity to thiamin, even though, in the latter experiment, little radioactivity remained in the culture medium. Cysteine, which was suggested to be a source of a segment of the C_5 unit,[21] is not taken up by *S. cerevisiae* (experiment 6, Table I). Serine, a precursor of the carbon skeleton of cysteine,[22] appears to be taken up (experiment 7, Table I) but does not supply radioactivity into the thiazole nucleus of thiamin (Table III and ref 4). The small amount of label within the pyrimidine moiety is very probably due to incorporation of C-3 of serine via the one-carbon pool, as formate, which is known to enter the pyrimidine unit nonrandomly.[23,24]

The lack of success in achieving incorporation of label from exogenously supplied samples of Krebs cycle intermediates and other compounds that lie at the core of primary metabolism forced us to reexamine the possibility that the C_5 unit in question was, after all, derived from a five-carbon sugar, the negative result in the experiment with D-[1-^{14}C]ribose (experiment 10) notwithstanding.

A rational scheme for the derivation of the thiazole unit from such a precursor is shown in Scheme III. The thiazole is generated, after formation of a Schiff base between glycine and a phosphorylated 2-ketopentose, by a series of steps comprising dehydration, decarboxylation, and addition of sulfur. No oxidation or reduction steps are included in the sequence. The participation of glycine in the biosynthesis of the thiazole nucleus of thiamin in yeast has been fully documented.[5] If the other starting material were a 2-pentulose 5-phosphate, then the thiazole phosphate (**14**), a known precursor of thiamin,[1] would be generated directly.

Pentoses are not taken up by *S. cerevisiae* (e.g., D-ribose (experiment 10)) or, if taken up, are not utilized (e.g., D-xylose[25]), and the nonfermentability of pentoses serves as a criterion for the classification of yeasts.[26,27] Nonetheless, the pentose phosphate pathway is a component of yeast metabolism.[28] Pentose deriv-

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(23) S. David, B. Estramareix, and H. Hirshfeld, *Biochim. Biophys. Acta*, **148**, 11–21 (1967).
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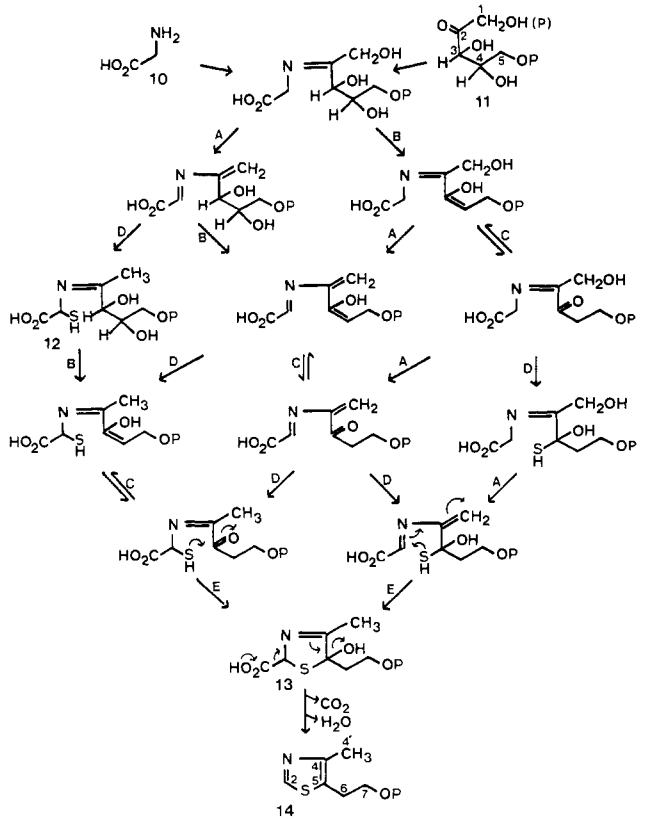
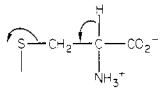
(26) J. A. Barnett in "The Fungi. An Advanced Treatise", Vol. 3, G. C. Ainsworth and A. S. Sussman, Eds., Academic Press, New York, 1968, pp 557–595.
(27) J. P. van der Walt in "The Yeasts. A Taxonomic Study", J. Lodder, Ed., North Holland Publishing Co., London, 1970, pp 595–604.
(28) H. J. Blumenthal in "The Fungi. An Advanced Treatise", Vol. 1, G. C. Ainsworth and A. S. Sussman, Eds., Academic Press, New York, 1965, pp 229–268.

Scheme III. Biogenesis of the Thiazole Unit of Thiamin, in Yeast, from Glycine and a 2-Pentulose 5-Phosphate[a]



[a] (A) Dehydration (or elimination of phosphate; P ≡ $-PO(OH)_2$); (B) dehydration; (C) tautomerization; (D) addition of sulfur; the S donor, which, for simplicity, is represented as H_2S, is more likely to be cysteine; if so, ring closure (E) would be accompanied by a fragmentation process



catalyzed by a C–S lyase (EC 4.4.1).

atives are generated from hexoses within the cell. The distribution of ^{14}C in pentose derivatives, generated from labeled hexoses, has been intensively studied.[29] Distribution, within the thiazole unit, of label derived from specifically ^{14}C-labeled samples of glucose could, in principle, serve as a test for the derivation of the C_5 unit of the thiazole moiety from a pentose precursor, provided that incorporation of label into the carbon atoms of the C_5 unit is sufficiently high to permit the labeling pattern to be established by controlled chemical degradation and provided that the labeling pattern that is observed is interpretable in terms of established patterns of hexose–pentose interconversions.

To enhance the probability of obtaining adequate incorporation of label, a change in experimental procedure was necessary. In our experiments thus far (experiments 1–12), glucose had served as the general carbon source of the medium that was employed. A different carbon source would be required in experiments designed to test incorporation of label from [^{14}C]glucose in order to maintain as high a specific activity as possible in the ^{14}C tracer. A low specific activity in the tracer, in combination with the carrier dilution, necessary in the isolation of the product, would lead to a dilution of the concentration of label in the isolated thiamin beyond the limits of detection, even in an experiment in which optimal ^{14}C incorporation into newly synthesized thiamin had occurred. What was required to enhance the chances of success was a carbon source that, in terms of metabolism, was not closely related to glucose. It was found that ethanol after a 24-h lag period served as a carbon source for *S. cerevisiae*, strain ATCC 24903. With ethanol as the carbon source, feeding experiments with ^{14}C-labeled samples of glucose and related hexoses became feasible and, as it turned out, were successful in yielding thiamin of significant specific activity.

Controlled degradation of the thiazole moiety of each of the radioactive samples of thiamin, obtained from the experiments with labeled hexoses (experiments 14–19), was carried out by the reaction sequences shown in Scheme I. In each case label was found to be incorporated nonrandomly (Table IV; Scheme II). The distribution pattern that was revealed was characteristic and interpretable in terms of the intermediacy of pentoses.

The routes from D-glucose 6-phosphate (**15**) into D-ribulose 5-phosphate (**17**) and into D-xylulose 5-phosphate (**22**) are shown in Scheme IV. The predicted incorporation pattern of the carbon atoms of glucose, via each of the two pentose derivatives, into the thiazole unit via Scheme III, is also indicated.

D-Ribulose 5-phosphate (**17**) is derived from D-glucose 6-phosphate (**15**) via D-gluconic acid 6-phosphate (**16**) with loss of C-1, by the "oxidative pentose phosphate pathway",[30] a route that involves an oxidative decarboxylation. Incorporation of a 2-pentulose, generated in this fashion, into the C_5 unit of the thiazole nucleus of thiamin would deliver C-2 to C-6 of glucose into C-4′,4,5,6,7 of the thiazole.

Another route from D-glucose 6-phosphate, via the "nonoxidative limb" of the pentose phosphate pathway, yields D-xylulose 5-phosphate (**22**) as the primary product. The key reaction of this route[31] is the transfer, catalyzed by the enzyme transketolase (EC 2.2.1.1), of a C_2 unit ("active glycolaldehyde"), C-1,2, of D-fructose 6-phosphate (**18**), onto D-glyceraldehyde 3-phosphate (**21**) (generated from D-glucose 6-phosphate (**15**) by the reactions of glycolysis). Incorporation of a 2-pentulose generated in this fashion into the thiazole C_5 unit would deliver C-1,2,4,5,6 of glucose into C-4′,4,5,6,7.

Since D-ribulose 5-phosphate (**17**) and D-xylulose 5-phosphate (**22**) are interconvertible (EC 5.1.3.1), feeding experiments with labeled hexoses cannot determine which of the two 2-pentuloses is the immediate precursor of the thiazole unit. Since pentose phosphate carbon recycles into hexose phosphate,[32,33] a prolonged metabolic period would generate a more complex labeling pattern whose interpretation would not be clear-cut. This eventuality did not arise.

The participation, in the biosynthesis of the C_5 unit of the thiazole, of a pentose precursor, generated by the nonoxidative as well as by the oxidative pentose phosphate pathway, can be deduced from the labeling pattern obtained in the experiments with D-[1-^{14}C]glucose (experiment 15) and D-[1-^{14}C]fructose (experiment 16) on the one hand and with D-[2-^{14}C]glucose (experiment 19) on the other. The experiments with D-[6-^{14}C]-glucose (experiments 17 and 18) and [1-^{14}C]glycerol (experiment 20) provide additional confirmatory evidence. In each case the distribution of label is fully interpretable in terms of well-established enzymic processes.

Activity from D-[1-^{14}C]glucose (experiment 15) and D-[1-^{14}C]fructose (experiment 16) is located almost entirely (>90%) in the *C*-methyl group (C-4′) of the thiazole unit (Table IV; Scheme II, Figures 1 and 2). This is the distribution predicted by the nonoxidative pathway. The remainder of the activity (<10%) was located at C-5, C-6, and/or C-7. Lack of material precluded further degradation to determine the exact location of label. Incorporation of label into a second position of the C_5 chain (presumably C-7) can be readily rationalized (see below).

A complementary result was obtained when D-[6-^{14}C]glucose (experiments 17 and 18) was used as a tracer (Table IV, Scheme

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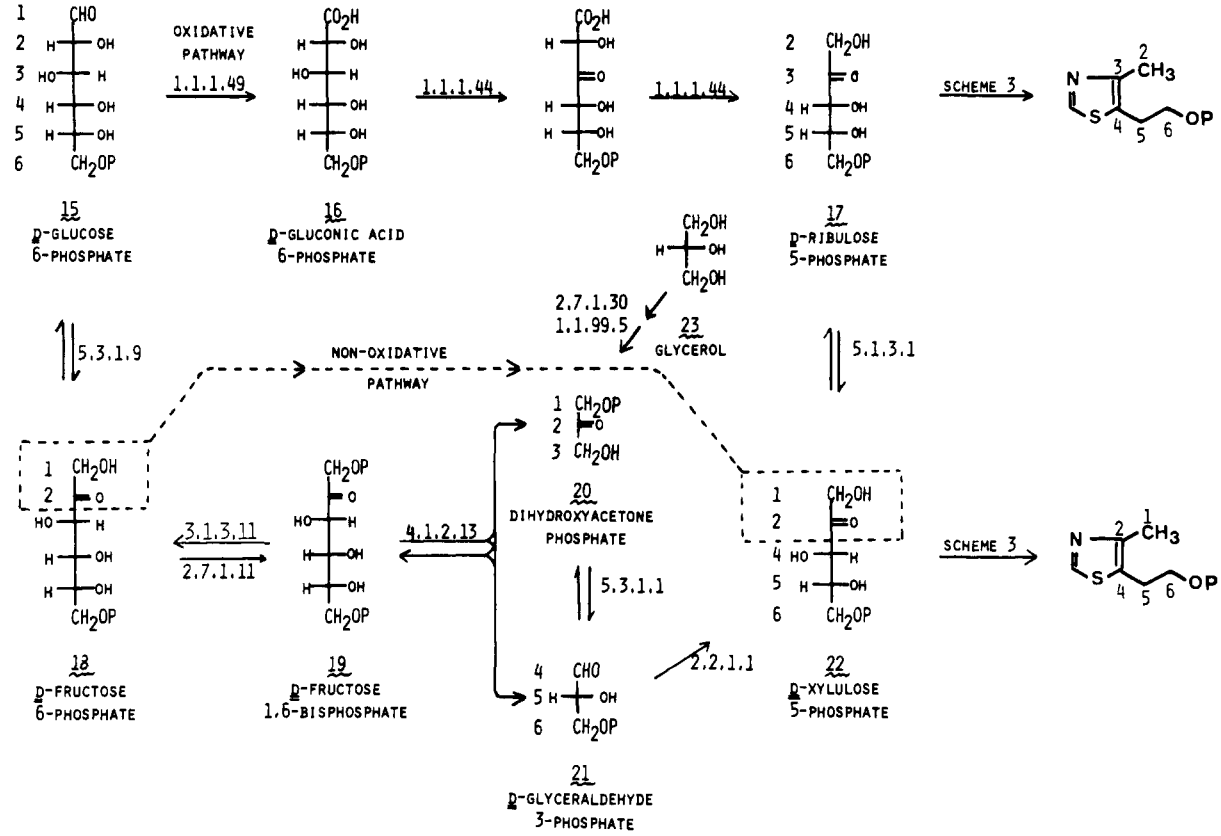
Scheme IV. Predicted Incorporation of Label, from the Carbon Atoms of D-Glucose 6-Phosphate, into the Thiazole Moiety of Thiamin, by the Oxidative Pentose Phosphate Pathway, via D-Ribulose 5-Phosphate, and by the Nonoxidative Pentose Phosphate Pathway, via D-Xylulose 5-Phosphate[a]



[a] Carbon numbering refers to the carbon atoms of glucose, throughout. For numbering of thiazole carbon atoms, see formula **6**, Scheme I.

II, Figure 3). The label was located mainly at C-7 of the C_5 unit (>80%). The rest was present at the *C*-methyl group (C-4′) (<20%).

The presence of label at a second site of the C_5 chain (at C-4′ in addition to C-7 in the case of [6-^{14}C]glucose, at C-7 in addition to C-4′ in the case of [1-^{14}C]glucose and [1-^{14}C]fructose) in each of these experiments is explained on the basis of the interconversion of the triose phosphates **20** and **21** (EC 5.3.1.1), the subsequent resynthesis of D-fructose 1,6-bisphosphate (**19**) (EC 4.1.2.13), and the hydrolysis of the latter to D-fructose 6-phosphate (**18**) (EC 3.1.3.11). The phosphatase catalyzing this hydrolysis is present in yeast that is grown on a medium low in glucose.[34,35] When the fructose phosphates **19** and **18**, resynthesized in this way, are then utilized in the nonoxidative pentose phosphate pathway, the observed distribution of activity results. It is of interest that a somewhat larger amount of label is found at the second site in the experiment with [6-^{14}C]glucose compared to that in the experiment with [1-^{14}C]glucose.

The results discussed so far indicate that a pentose intermediate, generated from glucose or fructose by the nonoxidative pentose phosphate pathway, serves as a precurser of the C_5 unit of the thiazole moiety of thiamin. The result of the experiment with D-[2-^{14}C]glucose (experiment 19) leads to the conclusion that not only the nonoxidative but also the oxidative pentose phosphate pathway serves to generate this intermediate. Approximately half the radioactivity derived from [2-^{14}C]glucose was present at C-4, the other half at C-4′ (Table IV; Scheme II, Figure 4). The distribution of activity between C-4 and C-4′ serves as a measure of the relative contribution to the C_5 chain of label from pentose intermediates generated by the nonoxidative (label at C-4) and the oxidative pathway (label at C-4′). It would appear that the two routes make an approximately equal contribution under the conditions of the experiment.

The conclusion that both pathways are implicated is further borne out by the result of the experiment with [1-^{14}C]glycerol (experirment 20) (Table IV; Scheme II, Figure 5), which enters the metabolic chain by phosphorylation and conversion into dihydroxyacetone phosphate. Under the conditions of this experiment the oxidative pathway (predicted sites of labeling, C-4,5,7) makes a smaller contribution than the transketolase route (predicted sites of labeling, C-4′,5,7) (ca. 1:2).

The observed pattern of distribution of label, from ^{14}C-labeled hexoses, within the thiazole moiety of thiamin, permits the conclusion that the C_5 unit, C-4′,4,5,6,7, of this moiety is derived from a 2-pentulose intermediate as predicted by Scheme III. It is likely that the 2-pentulose derivative (**11**) that condenses with glycine (**10**) is the 5-phosphate of D-ribulose (**17**) or of D-xylulose (**22**), or the corresponding 1,5-bisphosphate derivative of one of them.

It is an entertaining thought that one of the two pathways giving rise to the 2-pentulose, the nonoxidative pentose phosphate pathway, includes an enzymic reaction, the transketolase step, which is known to require thiamin as a cofactor.[36] Thiamin is thus required in one of the reactions of its own biosynthesis! This may explain why a second route, the oxidative pathway, which does not require thiamin, is also used as a source of the 2-pentulose precursor of the C_5 unit.

The distribution of label from D-[1-^{14}C]-, D-[2-^{14}C]-, and D-[6-^{14}C]glucose within D-ribose[29] and D-arabitol[37] and within the ribitol moiety of riboflavin[38] has, likewise, been taken as evidence in support of the concurrent operation of the oxidative and the nonoxidative pentose phosphate pathways in generating inter-

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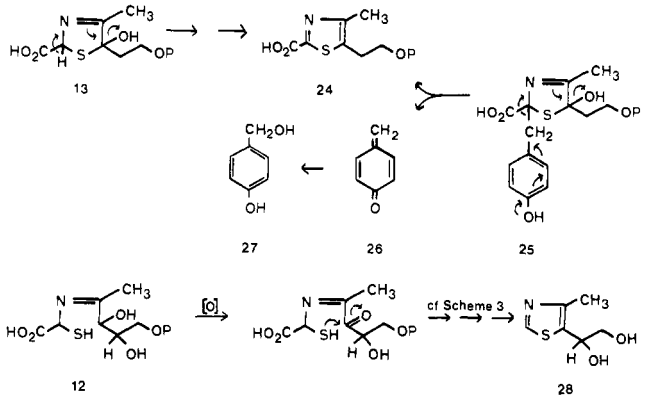
(35) A. Sols in "Aspects of Yeast Metabolism", A. K. Mills and H. Krebs, Eds., F. A. Davis Co., Philadelphia, PA, 1968, pp 47–70.
(36) L. O. Krampitz, *Annu. Rev. Biochem.*, **38**, 213–240 (1969).
(37) J. F. T. Spencer, A. C. Neish, A. C. Blackwood, and H. R. Sallans, *Can. J. Biochem. Physiol.*, **34**, 495–501 (1956).
(38) G. W. E. Plaut and P. L. Broberg, *J. Biol. Chem.*, **219**, 131–138 (1956).

Scheme V. Hypothetical Derivations of the Thiazolecarboxylic acid (**24**) and of the Diol (**28**) in *E. coli*



mediates in the biosynthesis of these C_5 sugars, in microorganisms. The contribution from each of the two pathways depends on the manner in which the organism is grown, on the stage of development of the culture, and on the carbon source.[39]

In bacteria, e.g., in *E. coli*, the route to the thiazole nucleus of thiamin appears to be somewhat different from that in yeast. Thus, in place of glycine, it is tyrosine which supplies a C–N unit from its α-carbon atom[6,7] and its amino group[8] to yield the C-2–N unit of the thiazole nucleus. The fact that the thiazolecarboxylic acid (**24**) and the diol (**28**) are generated as byproducts of thiamin biosynthesis in *E. coli*[40,41] suggests that biosynthesis takes place by a sequence of steps analogous to those outlined in Scheme III. The thiazolecarboxylic acid (**24**) may be formed (Scheme V) by loss of water from the intermediate (**13**) of Scheme III, followed by hydrolysis of the phosphate ester, or by loss of the *p*-hydroxybenzyl moiety from **25**, followed by hydrolysis. The diol **28** may be formed, i.a., from the intermediate **12**, by oxidation at the carbon atom destined to become C-5 of the thiazole, followed by ring closure, decarboxylation, and elimination of water (Scheme V).

Scheme III may be readily adapted to accommodate tyrosine as a precursor. Elimination of the *p*-hydroxybenzyl moiety at an early stage generates intermediates identical with those on the left side of Scheme III. Its elimination at a later stage would generate, by steps analogous to those on the right side of Scheme III, a new set of intermediates, **25** among them. Elimination of the *p*-hydroxybenzyl moiety leads to an unstable quinone methide (**26**), which would be trapped by water to yield *p*-hydroxybenzyl alcohol (**27**) (Scheme V). Formation of *p*-hydroxybenzyl alcohol does, in fact, accompany thiamin biosynthesis in *E. coli*.[42]

Phenylalanine inhibits the biosynthesis of the thiazole unit of thiamin in *E. coli*.[43] If phenylalanine instead of tyrosine were to form a Schiff base with a 2-pentulose, the further transformation of such an adduct would be blocked at some stage, since there is no facile mechanism for the elimination of the benzyl moiety.

The origin of the C_5 unit of the thiazole moiety of thiamin in bacteria has been investigated by use of stable isotopes. The distribution of label in the thiazole unit was determined by gas chromatography–mass spectrometry.[11-13] Most, but not all, of the incorporation data are interpretable in terms of the intermediacy of a 2-pentulose.

Thus, the distribution of label from L-(*RS*-3-$^2H_1$)(*sn*)glycerol[11] and from L-(*RS*-3-$^2H_1$,3-^{18}O)(*sn*)-glycerol[12] and the nonincorporation of label from L-(*RS*-1-$^2H_1$)glycerol[11] and from (2-^2H)-glycerol[11] may be rationalized on the basis of the interconversions shown in Scheme IV. The incorporation of label from D-(1-^2H)glucose, D-(6,6-$^2H_2$)glucose, and D-(5,6,6-$^2H_3$)glucose[11] is similarly accounted for.

In each of the experiments with samples of glucose carrying deuterium at C-6, label was present not only at C-7 of the thiazole but also at C-4′, as expected if, in the conversion of glucose into the C_5 unit, the steps in the nonoxidative pentose phosphate route responsible for this partial randomization of label (see above) take place with maintenance of the integrity of one or both of the H–C-6 bonds.

In yeast this integrity is not maintained. In the experiment with D-[6-^{14}C,6-^{3}H]glucose (experiment 18), 87 ± 2% ^{14}C was present at C-7 of the thiazole, 16 ± 2% at C-4′ (Table IV, Scheme II, Figure 3). Yet, all the tritium within the thiazole unit was present at C-7 (106 ± 2%, *N*-phthaloyl-β-alanine (**7**), Table IV). Thus, while the $^3H/^{14}C$ ratio of the original glucose (9.05 ± 0.11, Table V) was maintained at C-7 of the thiazole (*N*-phthaloyl-β-alanine (**7**), $^3H/^{14}C$ 9.1 ± 0.2, Table VI), the *C*-methyl group, C-4′, contained ^{14}C only (*N*-methylphthalimide (**9**), $^3H/^{14}C$ 0.3 ± 0.2; 5-formyl-4-methylthiazole (**4**), $^3H/^{14}C$ 0.2 ± 0.1).

Since it is known that >90% ^{3}H, relative to ^{14}C, is maintained in the conversion of acetate to methylamine by the Schmidt reaction[44] and since three degradation products, two containing C-4 only and one containing C-7 only, all obtained under entirely different reaction conditions, yielded consistent results, it is unlikely that loss of tritium, relative to ^{14}C, occurred in the course of degradation. If this loss of tritium, relative to ^{14}C, does indeed take place in the course of the conversion of hexose into the C_5 unit of the thiazole, this would indicate a significant difference in the origin of the C_5 precursor in bacteria and in yeast.

The results of two other experiments underline this difference. Firstly, in an experiment with (3-$^2H_3$)pyruvate[11] several species containing trideuteriomethyl at C-4′ of the thiazole nucleus were found to make a significant contribution to the thiamin that was isolated.[11] This result cannot be explained on the basis of the obligatory intermediacy of a 2-ketopentulose in generating the C_5 unit. It was interpreted as evidence for the direct incorporation of the C_2 unit, C-3,2, of pyruvate into the C_5 chain. A hitherto unknown acyloin-type condensation between a pyruvate-derived C_2 unit and D-glyceraldehyde phosphate, analogous to known biochemical acyloin condensations between a pyruvate-derived C_2 unit and other aldehydes,[36] was proposed for the formation of the C_5 unit. Such condensations are known to require thiamin as cofactor.

Secondly, in apparent confirmation of this postulate, a trideuteriomethyl unit from 1-deoxy-D-*threo*-(1-$^2H_3$)-2-pentulose entered the *C*-methyl group of the thiazole unit of thiamin.[13] Label from 1-deoxy-D-*erythro*-(1-$^2H_3$)-2-pentulose was not significantly incorporated, indicating that the precursor–product relationship was stereospecific. 1-Deoxy-D-*threo*-2-pentulose (1-deoxy-D-xylulose) has recently been reported to occur naturally in a *Streptomyces* species.[45,46]

Pyruvate cannot be a direct precursor of the thiazole unit in yeast. This conclusion is suggested not only by the negative results in the experiments with pyruvate (experiment 12) and lactate (experiment 13) but also by the mode of incorporation of radioactivity from labeled hexoses. The incorporation pattern is not consistent with the direct incorporation of pyruvate. If the *C*-methyl group (C-4′) of the thiazole nucleus were derived from C-3 of pyruvate and C-7 from C-3 of glyceraldehyde phosphate, as is suggested in the case of bacterial thiamin,[11] these two carbon atoms would have had to be equally labeled in each of the experiments with D-[1-^{14}C]- and D-[6-^{14}C]glucose and D-[1-^{14}C]-fructose, since in yeast D-glyceraldehyde phosphate is an obligatory intermediate in the conversion of D-glucose into pyruvate. In fact, in each of these experiments (experiments 15–18) the ^{14}C content at C-4′ was substantially different from that at C-7.

The available evidence suggests that the route to the thiazole moiety differs in yeast and bacteria with respect to the amino acid

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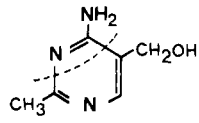
precursor of the C-2–N unit as well as with respect to the carbohydrate substrates yielding the C_5 chain.

The origin of the pyrimidine moiety of thiamin is still in dispute.[1,47] Evidence, such as exists, is fragmentary and contradictory. As in the case of the thiazole moiety, different paths appear to operate in yeast and in bacteria. Thus, glycine is a specific precursor of the pyrimidine moiety in *E. coli*[48,49] but is not incorporated into this unit in yeast.[5]

The observation that significant incorporation of label into the pyrimidine unit takes place from hexose precursors (Table III), together with the obvious structural similarities between the pyrimidine unit of thiamin (**29**) and another B vitamin, pyridoxol



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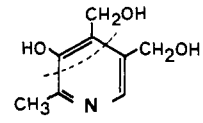

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(**30**), which is derived from carbohydrate precursors,[50,51] leads us to suspect analogy in the biogenetic origin of the two compounds. The incorporation pattern, in pyridoxol, of activity from D-[1-^{14}C] and D-[6-^{14}C]glucose,[50,51] as well as from [1-^{14}C]glycerol,[50,52] has been fully elucidated. The investigation of the distribution of label within the pyrimidine unit of hexose-derived thiamin is in hand.

Experimental Section

Materials. Microorganism. *Saccharomyces cerevisiae* (ATCC 24903) was maintained on malt extract/yeast extract/peptone/glucose slants as described previously.[5]

Media. In the experiments with DL-cysteine (experiment 6), L-serine (experiment 7), succinic acid (experiment 8), α-ketoglutarate (experiment 9), ribose (experiment 10), acetate (experiment 11), and pyruvate (experiment 12) (see Table I), the glucose-based thiamin-free medium, described in our earlier paper,[5] was used.

In the experiments with glucose (experiments 14, 15, 17–19), fructose (experiment 16), glycerol (experiment 20), and lactate (experiment 13), a medium was used that contained neither thiamin nor glucose but used ethanol as the general carbon source.

The details of the thiamin-free medium are as follows.

The inorganic salts [$(NH_4)_2SO_4$ (5 g), KH_2PO_4 (1 g), $MgSO_4 \cdot 7H_2O$ (0.5 g), NaCl (0.1 g), $CaCl_2 \cdot 2H_2O$ (0.1 g)] and the amino acids [L-histidine monohydrochloride (10 mg), DL-methionine (20 mg), and DL-tryptophan (20 mg)] were dissolved in water (93 mL). A stock vitamin solution (1.0 mL, containing *myo*-inositol (2000 μg/mL), calcium DL-pantothenate (400 μg/mL), D-biotin (2 μg/mL)), and a trace element solution (1.0 mL, containing boric acid (500 μg/mL), $CuSO_4 \cdot 5H_2O$ (40 μg/mL), KI (100 μg/mL), $FeCl_3$ (200 μg/mL), $MnSO_4 \cdot H_2O$ (400 μg/mL), $Na_2MoO_4 \cdot 2H_2O$ (200 μg/mL), and $ZnSO_4 \cdot 7H_2O$ (400 μg/mL)) were added. Ethanol (95%, 5.0 mL) was added to bring the volume of the medium to the standard size (100 mL) before filter sterilization.

Methods. Growth in Thiamin-Free Media. The procedure for the growth of *S. cerevisiae* (ATCC 24903) described in an earlier publication[5] was employed with the ethanol-based medium as well as the glucose-based medium.

Growth Curve Measurements. Growth was monitored by measuring the optical density of the culture at 400–465 nm. A Klett–Summerson photoelectric colorimeter, Model 900-3, which was fitted with a blue filter (No. 42), was used.

Radioactive Tracer Experiments. Tracer experiments with glucose-based thiamin-free medium (experiments 6–12) were described in an earlier publication.[5]

Tracer experiments with ethanol-based thiamin-free medium proceeded as follows: Radioactive tracer was added at the onset of logarithmic growth (i.e., after approximately 30 h of incubation), and the cells were harvested 40–45 h later when maximum growth had been attained. The radioactive tracer was dissolved in sterilized water (50 mL) and the solution divided equally among the 250-mL Erlenmeyer flasks, which contained the growing yeast cultures, when the cell density had reached approximately 10^6 cells/mL or 10 Klett units.

Isolation of Thiamin. The procedure for the centrifugation and extraction of the yeast cells and for the isolation of thiamin was described previously.[5]

Degradation of Thiamin. See Scheme I.

Bisulfite Cleavage. Thiamin was cleaved to (4-amino-2-methylpyrimidin-5-yl)methanesulfonic acid (**2**) and 5-(β-hydroxyethyl)-4-methylthiazole (**3**) by the published procedure.[5]

Degradation of 5-(β-Hydroxyethyl)-4-methylthiazole (3). Oxidation of 3 with Pyridinium Dichromate. C-7 by Difference. Pyridinium dichromate (380 mg, 1 mmol, prepared by the method of Corey and Schmidt[53]) was suspended in methylene chloride (1.5 mL). One drop of water (approximately 50 mg) was added, and the mixture was stirred until the reagent and the water formed a homogeneous phase. 5-(β-Hydroxyethyl)-4-methylthiazole (24 mg, 0.16 mmol) in methylene chloride (1.5 mL) was added, and stirring was continued at room temperature for 24 h. Ether (10 mL) was added, and the reaction mixture was decanted. The black, gummy residue was extracted with ether (3 × 10 mL). All extracts were combined and filtered through silica gel (60–200 mesh). The colorless filtrate was evaporated to dryness in vacuo. Sublimation at room temperature (2×10^{-2} mmHg) (2.7 Pa) yielded 5-formyl-4-methylthiazole (**4**) (11 mg, 52%): mp 66–70 °C (lit. mp 72.5 °C[54] and 75 °C[55]); ^{1}H NMR ($CDCl_3$) δ 2.79 (s, 3 H), 9.02 (s, 1 H), 10.18 (s, 1 H); MS, *m/e* 127 (M^+).

5-Formyl-4-methylthiazole Semicarbazone. 5-Formyl-4-methylthiazole (**4**) (7 mg), along with sodium acetate (10 mg) and semicarbazide hydrochloride (8 mg), was dissolved in water (0.5 mL). A white precipitate formed immediately. The solvent was removed and the precipitate was dissolved in hydrochloric acid (0.1 M). Neutralization with ammonia (10% v/v) gave 5-formyl-4-methylthiazole semicarbazone (7 mg, 69%): mp 240–241 °C (lit.[54] mp 241 °C); ^{1}H NMR (CD_3SOCD_3) δ 2.41 (s, 3 H), 6.28 (s, 2 H), 8.13 (s, 1 H), 8.96 (s, 1 H), 10.26 (s, 1 H); MS, *m/e* 184 (M^+).

5-(β-Chloroethyl)-4-methylthiazole (5).[56,57] 5-(β-Hydroxyethyl)-4-methylthiazole (**3**) (60 mg) was dissolved in chloroform (2 mL) at 0 °C. Thionyl chloride (0.5 mL) was added dropwise over a period of a few minutes with stirring, and stirring was continued for 1 h. The solution was warmed to room temperature and evaporated to dryness in vacuo. The residue was freed from excess thionyl chloride by the addition of two portions of ether and evaporation to dryness in vacuo. The solid residue was dissolved in water (5 mL), neutralized with solid sodium bicarbonate, and extracted with ether (4 × 5 mL). The extracts were combined, dried over anhydrous magnesium sulfate, and evaporated in vacuo to give 60 mg (88%) of colorless oil. Analysis by thin-layer chromatography (silica gel/ether) indicated that the product (R_f 0.45) was free from starting material (R_f 0.25); ^{1}H NMR ($CDCl_3$) δ 2.42 (s, 3 H), 3.23 (t, 2 H), 3.70 (t, 2 H), 8.64 (s, 1 H).

4-Methyl-5-(β-phthalimidoethyl)thiazole (6).[58] The above oil was dissolved in dry dimethylformamide (1 mL), and potassium phthalimide (105 mg) was added. The mixture was stirred at 100–105 °C for 2 h. At this point, thin-layer chromatography (silica gel/ether) indicated that the starting material (R_f 0.45) had completely reacted and that the major spot (R_f 0.30) was due to the desired product. After the mixture had cooled to room temperature, water (5 mL) was added and the product was collected by filtration. Recrystallization from 1:1 water/methanol and sublimation at 120 °C (2×10^{-2} mmHg) (2.7 Pa) yielded the phthalimidothiazole (**6**) (68 mg, 67%): mp 146–147 °C (lit.[58] mp 143–146 °C); ^{1}H NMR ($CDCl_3$) δ 2.40 (s, 3 H), 3.20 (t, 2 H), 3.93 (t, 2 H), 7.64–7.97 (m, 4 H), 8.64 (s, 1 H); MS, *m/e* 272 (M^+).

Degradation of 4-Methyl-5-(β-phthalimidoethyl)thiazole (6). C-4,4′ by Kuhn–Roth Oxidation.[59] 4-Methyl-5-(β-phthalimidoethyl)thiazole

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(57 mg) was oxidized to sodium acetate (26 mg, 92%), and a portion of the sodium acetate was converted to *N*-acetyl-α-naphthylamine (**8**) by previously described procedures.[5]

C-4′ by Schmidt Reaction on Acetic Acid.[50] Sodium acetate (18 mg) was dissolved in concentrated sulfuric acid (1 mL). Sodium azide (60 mg) was added and the mixture was heated on a steam bath in a flask attached to a system of three gas traps. The carbon dioxide that evolved was passed by means of a stream of nitrogen into potassium hydroxide solution (15%, w/v). When gas evolution had ceased (approximately 4 h), the potassium hydroxide solutions in the traps were replaced by hydrochloric acid (1 M, 1 mL per trap). The acidic reaction mixture was cooled to near its freezing point in a dry ice/acetone bath, basified (to pH >12) by addition of potassium hydroxide solution (15%, w/v), and then heated 1.5 h on the steam bath while the system was flushed with nitrogen.

***N*-Methylphthalimide (9).**[60] *N*-Carbethoxyphthalimide (32 mg) was added to the hydrochloric acid solutions contained in the first two traps. The mixture was stirred, solid sodium carbonate was added to bring the mixture to about pH 9, and the mixture was stirred at room temperature for 1 h. The precipitate was collected by filtration and then sublimed at 75 °C (2×10^{-2} mmHg) (2.7 Pa). Recrystallization from methanol yielded pure *N*-methylphthalimide (9 mg, 42%). Thin-layer chromatography (silica gel, 1:4 ethyl acetate/cyclohexane, v/v) was used to monitor the presence of unchanged *N*-carbethoxyphthalimide (R_f 0.15) in the *N*-methylphthalimide (R_f 0.27): mp 134–135 °C (lit.[61] mp 134 °C); ^{1}H NMR ($CDCl_3$) δ 3.17 (s, 3 H), 7.61–7.94 (m, 4 H); MS, *m/e* 161 (M^+). Potassium phthalimide and methyl iodide were stirred in dimethylformamide for 3 h at room temperature. *N*-Methylphthalimide so formed was identical with the above sample.

C-5,6,7 by Permanganate Oxidation.[62] 4-Methyl-5-(β-phthalimidoethyl)thiazole (**6**) (21 mg) was suspended in dilute sulfuric acid (0.5 M, 1.5 mL) at room temperature. Potassium permanganate (80 mg) was added in portions over a 1-h period, and the mixture was stirred for an additional 2 h. It was decolorized by adding sodium hydrogen sulfite and extracted with ether (4 × 5 mL). The ether extracts were combined, washed with water (1 × 5 mL), dried over anhydrous magnesium sulfate, and evaporated in vacuo to dryness. The residue was sublimed at 105 °C (2×10^{-2} mmHg) (2.7 Pa) and recrystallized from water to yield pure *N*-phthaloyl-β-alanine (**7**) (9 mg, 53%): mp 150–151 °C (lit.[63] mp 150–151 °C); ^{1}H NMR (CD_3COCD_3) δ 2.74 (t, 2 H), 3.94 (t, 2 H), 7.83 (s, 4 H); MS, *m/e* 219 (M^+). This sample was identical with one obtained[64] by fusion of β-alanine with phthalic anhydride at 170 °C for 15 min.

Degradation of D-[6-^{14}C,6-^{3}H]Glucose (Experiment 18). Carrier D-glucose (500 mg) was added to a small portion (approximately 3 μCi) of the D-[6-^{14}C,6-^{3}H]glucose feeding solution, and this mixture was diluted to 100 mL with water.

β-D-Glucose Pentaacetate.[65] A portion (approximately 10 mL) of the above D-[6-^{14}C,6-^{3}H]glucose solution was evaporated to dryness in vacuo. Anhydrous sodium acetate (47 mg) and acetic anhydride (1 mL) were added to this noncrystalline residue (56 mg). The mixture was heated on a steam bath for 2.5 h, and ice water (5 mL) was added. An oil separated from solution at this point, but when it stood at 4 °C for 2 days, it became crystalline. The crystals were filtered off and sublimed at 120 °C (2×10^{-2} mmHg) (2.7 Pa) to yield pure β-D-glucose pentaacetate (42 mg, 34%), mp 131–132 °C (lit.[65] mp 131–132 °C).

C-6 of D-[6-^{14}C,6-^{3}H]Glucose as Formaldehyde Dimethone.[66] A small amount (approximately 6 mL) of the above D-[6-^{14}C,6-^{3}H]glucose solution was evaporated to dryness in vacuo. The noncrystalline residue (33 mg) was redissolved in water (2 mL), sodium bicarbonate solution (1 M, 2 mL) and sodium metaperiodate (200 mg) in water (2 mL) were added, and the mixture was kept at room temperature for 1 h. 5,5-Dimethylcyclohexane-1,3-dione (dimedone) (100 mg) in 95% ethanol (1 mL) was added, and after 10 min at room temperature, the white product was collected by filtration. Sublimation at 110 °C (2×10^{-2} mmHg) (2.7 Pa) yielded pure formaldehyde dimethone (53 mg, 98%), mp 190–192 °C (lit.[66] mp 189–190 °C).

Radioactivity Measurements. Triplicate samples of each compound were counted by liquid scintillation counting (Mark I liquid scintillation computer, Model 6860, Nuclear Chicago Corp.). Samples were dissolved in either water, aqueous ammonia (1%), *N*,*N*-dimethylformamide, or dimethyl sulfoxide and dispersed in Aquasol (New England Nuclear) (10 mL). The efficiency of counting was determined by external standardization with ^{133}Ba. The confidence limits shown in Tables I–VI are standard deviations from the mean.

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^{1}H NMR Study of Cobalt(II)-Substituted Carboxypeptidase A

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Abstract: The water ^{1}H NMR relaxation rate has been measured for solutions containing cobalt(II)-substituted carboxypeptidase A in the Larmor frequency range 5–60 MHz. The values, corrected for the diamagnetic zinc enzyme, have been interpreted within the frame of the Solomon–Bloembergen theory and provide evidence for water coordinated at the metal. When the inhibitor β-phenylpropionate is added to the solution, the above type of measurements are consistent with the substitution of a water molecule by the inhibitor whereas a second water molecule remains coordinated. The correlation time is consistent with the five-coordination of the chromophore in both cases. The ^{1}H NMR spectra of the histidines bound to the metal have also been recorded. These data have been compared with those of a very refined X-ray structure.

Carboxypeptidase A (CPA hereafter) is a zinc enzyme of molecular weight 34 500 containing one zinc ion per molecule.[1-3] Its biological role is the hydrolysis of the C-terminal amino acid from a polypeptide substrate. X-ray data at 2-Å resolution have

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