Thiamin Biosynthesis in *Saccharomyces cerevisiae*: Origin of the Pyrimidine Unit

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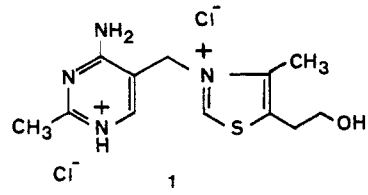
Abstract: Radioactivity from [^{14}C]formate, from D-[1-^{14}C]-, D-[2-^{14}C]-, and D-[6-^{3}H,6-^{14}C]glucose, from D-[1-^{14}C]fructose and from [1,3-^{14}C]- and [2-^{14}C]glycerol is incorporated nonrandomly into the pyrimidine moiety of thiamin in *Saccharomyces cerevisiae*. The observed incorporation pattern, established by a new chemical degradation, leads to the inference that there are two biosynthetic pathways to the pyrimidine moiety of thiamin in yeast. In the major pathway, formate is the precursor of C-4 of the pyrimidine nucleus, while hexose metabolites serve as the source of the remaining five carbon atoms of the pyrimidine unit. In the minor pathway, it is C-2 and not C-4 of the pyrimidine nucleus which is derived from formate, while C-4,-5 originates from carbohydrate. The source of C-2′, C-5′, and C-6 of the pyrimidine unit in this minor pathway remains unknown. Activity from [*ureido*-^{14}C]citrulline and [^{14}C]urea is not incorporated into thiamin.

Present knowledge of the biosynthetic origin of thiamin (vitamin B_1) (**1**) is still fragmentary. It was shown more than 20 years ago that the final step of its biosynthesis is the union of the intact



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thiazole unit with the intact pyrimidine unit. The route to each of these two subunits remains to be clarified, and not even primary precursors have been fully identified.[1,2]

The thiazole unit originates from an amino acid, a five-carbon sugar, and a sulfur source. In yeast, the precursors are glycine and a pentulose phosphate,[3-5] and in bacteria they are tyrosine and a deoxypentulose.[6-9] The sulfur source has not been identified but is presumed to be cysteine. The steps of the pathway from the precursors to the thiazole ring system are hypothetical.[5]

Even less is known of the primary precursors of the pyrimidine unit, beyond the fact that this unit differs in origin in yeasts and other eukaryotes on the one hand and in prokaryotes, such as *Escherichia coli* and *Salmonella typhimurium*, on the other.[10]

One of the reasons for the delay in determining primary precursors of the thiamin subunits was, without question, that mutually contradictory observations of substrate incorporations were made in different microorganisms and that, until recently, the possibility that the pathways to the subunits differed in yeasts and in bacteria, while recognized,[11] was not seriously considered.

The results of early experiments made it unlikely that the pyrimidine unit (**6**) of thiamin originated by the same route as the nucleic acid pyrimidines, which in bacteria and in animal tissues are derived from aspartic acid, via orotic acid.[12] Orotic acid did not serve as a precursor of thiamin, either in yeast[3,13] or in *E. coli*.[14] Reports on the incorporation of aspartic acid are conflicting. In yeast, aspartic acid was not incorporated into thiamin.[3,13] In bacteria, no incorporation was originally reported in *E. coli*.[14] Later, incorporation was reported in *B. subtilis*[15] and in *E. coli*,[16-18] label from [U-^{14}C]aspartate entering C-4 and C-6 of the pyrimidine unit.[18] This distribution of label from aspartate cannot be reconciled with the observation that C-4 and C-6 of the pyrimidine unit of thiamin are derived from the carboxyl and the methylene group of glycine (**3**), respectively, in *E. coli*[19] as well as in *Salmonella typhimurium*[20,21] in which the two C atoms of labeled glycine are incorporated without dilution of radioactivity.[22,23] Since the N atom of glycine serves as the precursor of N-1 of the pyrimidine unit (**6**),[19] it would appear that an intact glycine molecule serves as the source of C-4, C-6, and N-1 of the pyrimidine unit of thiamin in bacteria. In yeast, glycine is not a precursor of the pyrimidine moiety of thiamin.[4]

The role of formate as a precursor of the pyrimidine unit has been the subject of numerous investigations. Incorporation of [^{14}C]formate into the pyrimidine unit of thiamin in *B. subtilis* was originally reported over 25 years ago[15,24] but only recently was the site of labeling determined[10] to be C-2. Formate enters C-2 of the pyrimidine unit of thiamin also in *E. coli*,[25] in *S. typhimurium*,[20] and in two other bacteria.[10,26] Entry of formate into C-2 appears to be general in prokaryotes.

Formate is a precursor of the pyrimidine unit also in eukaryotes,[27-29] but the site of incorporation is not C-2. In *Saccha-*

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(1) Leder, I. G. In "Metabolic Pathways", 3rd ed.; Greenberg, D. M., Ed.; Academic Press: New York, 1975; Vol. 7, pp 57-85.
(2) Brown, G. M.; Williamson, J. M. *Adv. Enzymol.* **1982**, *53*, 345-381.
(3) Linnett, P. E.; Walker, J. *Biochem. J.* **1968**, *109*, 161-168.
(4) White, R. L.; Spenser, I. D. *Biochem. J.* **1979**, *179*, 315-325.
(5) White, R. L.; Spenser, I. D. *J. Am. Chem. Soc.* **1982**, *104*, 4934-4943.
(6) Estramareix, B.; Thérisod, M. *Biochim. Biophys. Acta* **1972**, *273*, 275-282.
(7) Bellion, E.; Kirkley, D. H.; Faust, J. R. *Biochim. Biophys. Acta* **1976**, *437*, 229-237.
(8) White, R. H. *Biochemistry* **1978**, *17*, 3833-3840.
(9) David, S.; Estramareix, B.; Fischer, J.-C.; Thérisod, M. *J. Am. Chem. Soc.* **1981**, *103*, 7341-7342.
(10) Yamada, K.; Morisaki, M.; Kumaoka, H. *Biochim. Biophys. Acta* **1983**, *756*, 41-48.
(11) Brown, G. M. In "Comprehensive Biochemistry"; Florkin, M., Stotz, E. H., Eds.; Elsevier: Amsterdam, 1971; Vol. 21, pp 1-9.
(12) Hartman, S. C. In "Metabolic Pathways", 3rd ed.; Greenberg, D. M., Ed.; Academic Press: New York, 1970; Vol. 4, pp 1-68.
(13) Johnson, D. B.; Howells, D. J.; Goodwin, T. W. *Biochem. J.* **1966**, *98*, 30-37.
(14) Goldstein, G. A.; Brown, G. M. *Arch. Biochem. Biophys.* **1963**, *103*, 449-452.
(15) Tomlinson, R. V.; Kuhlman, D. P.; Torrence, P. F.; Tieckelmann, H. *Biochim. Biophys. Acta* **1967**, *148*, 1-10.
(16) Nakamura, M. *Bitamin* **1965**, *32*, 383-386.
(17) Nakamura, M.; Nakata, T.; Nose, Y. *J. Vitaminol.* **1968**, *14*, 211-218.
(18) Estramareix, B.; Gaudry, D.; Thérisod, M. *Biochimie* **1977**, *59*, 857-859.
(19) White, R. H.; Rudolph, F. B. *Biochemistry* **1979**, *18*, 2632-2636.
(20) Estramareix, B.; Lesieur, M. *Biochim. Biophys. Acta* **1969**, *192*, 375-377.
(21) Estramareix, B. *Biochim. Biophys. Acta* **1970**, *208*, 170-171.
(22) Newell, P. C.; Tucker, R. G. *Nature (London)* **1967**, *215*, 1384-1385.
(23) Newell, P. C.; Tucker, R. G. *Biochem. J.* **1968**, *106*, 271-277.
(24) Pine, M. J.; Guthrie, R. *J. Bacteriol.* **1959**, *78*, 545-549.
(25) Kumaoka, H.; Brown, G. M. *Arch. Biochem. Biophys.* **1967**, *122*, 378-384.
(26) Yamada, K.; Katagi, T.; Kumaoka, H. *Bitamin* **1981**, *55*, 37-42.

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romyces cerevisiae,[30-32] as well as in *Neurospora crassa* and in two other eukaryotes,[10,26] label from [^{14}C]formate is incorporated specifically and exclusively into C-4 of the pyrimidine unit. A contrary report is that of Johnson et al.[13] who recovered [^{14}C]-formate activity entirely within the acetate obtained, by permanganate oxidation, from C-2′,-2 of the pyrimidine unit of thiamin from *S. cerevisiae* and claimed without further experimental support that this activity was present at C-2′. Label was presumably present at C-2.

An important contribution to the understanding of the biosynthesis of the pyrimidine unit of thiamin in bacteria was the discovery by Newell and Tucker[22,23,33] of mutants of *Salmonella typhimurium* with a dual growth requirement for purines as well as for the pyrimidine moiety of thiamin. It was found that the pyrimidine (**6**) was synthesized in large quantity from intermediates of the purine pathway and that the dual growth requirement was satisfied by 5-aminoimidazole ribonucleotide (AIR) (**2**) alone and by compounds that precede AIR on the purine pathway. It was suggested that AIR was the last common intermediate on the routes to the purines and to the pyrimidine unit of thiamin.

Formate is a precursor of C-2 of AIR (**2**). The carboxyl carbon, the methylene carbon, and the nitrogen atom of glycine, respectively, are precursors of C-5, C-4, and N-3 of AIR, in microorganisms and animals.[12] The proposal that AIR is incorporated into the pyrimidine unit of thiamin is consistent with the observed incorporation pattern of label from formate and from glycine into the pyrimidine moiety of thiamin in bacteria. The proposal requires that, in a subsequent step, a C-methyl group is added to C-2 of AIR as a C_1 unit and that a carbon atom of a C_2 unit is inserted between C-4 and C-5 of AIR.[20,21]

Several investigations were carried out to determine the origin of these additional units. Methionine does not serve as the source of the C_1 unit: label from [*methyl*-^{14}C]methionine is not incorporated into thiamin, either in *S. typhimurium*,[23,33] in *E. coli*,[14] or in *B. subtilis*.[15] Nor is methionine incorporated into thiamin in yeast.[4]

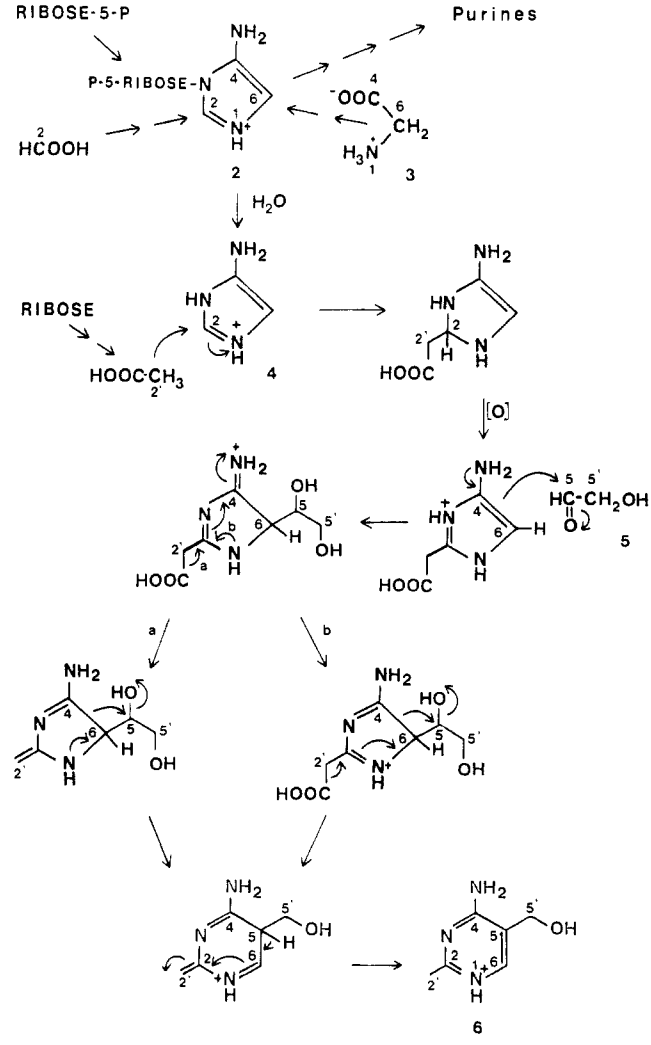
Acetate has been investigated as a potential source of the C_1 unit. Whereas in *E. coli* acetate was discounted as a direct source of carbon for the pyrimidine unit of thiamin,[25] label from [2-^{14}C]-, [2-^{2}H]-, and [2-^{2}H,2-^{14}C]acetate has been reported to enter the pyrimidine unit of thiamin in *B. subtilis*,[15,34] and it was concluded that the methyl group of acetate serves as the donor of the C_1 unit, C-2′. This inference supported an earlier suggestion[27] that the final step in the elaboration of the pyrimidine unit of thiamin was the decarboxylation of a 2-(carboxymethyl)pyrimidine, generated by the attack of acetyl CoA on the corresponding 2-desmethylpyrimidine.

The possibility that the addition of the C_1 unit at C-2 was the final step in the biosynthesis of the pyrimidine unit and that the desmethylpyrimidine unit, 4-amino-5-(hydroxymethyl)pyrimidine, indeed serves as an intermediate on the route from AIR to the pyrimidine unit (**6**) has been tested in *E. coli*, *B. subtilis*, and *S. cerevisiae*.[26,35] 4-Amino-5-(hydroxymethyl)pyrimidine was not converted into the corresponding 2-methyl derivative, and thus does not serve as a precursor of the pyrimidine unit of thiamin.

In view of these results, it must be concluded that entry of the acetate unit whose methyl group supplies the C_1 unit, C-2′, takes place before the ring expansion of the aminoimidazole to the pyrimidine. A chemically rational sequence of steps leading from AIR to the pyrimidine unit is shown in Scheme I.

It has recently been reported[36,37] that, in *E. coli*, label from

Scheme I. Hypothetical Scheme Accounting for the Biogenetic Origin of the Pyrimidine Unit of Thiamin in Bacteria (Numbers Refer to the Numbering System of the Pyrimidine Unit (6) of Thiamin, Throughout)



[6-^{14}C]glucose enters C-5′ of the pyrimidine unit, i.e., the exocyclic C atom of the extra C_2 unit. It was suggested that the C_2 unit originates from C-5,-6 of glucose, via C-4,-5 of ribose. Experimental support for the suggestion that the extra C_2 unit is supplied by ribose, and, more specifically, by the ribose moiety of 5-aminoimidazole ribonucleotide (AIR) (**2**), comes from experiments in *S. typhimurium*.[38] It was observed that label from a sample of ^{14}C-labeled AIR, which contained radioactivity mainly (and uniformly) in the five C atoms of the ribose moiety, entered the two carbon atoms C-5,-5′ of the pyrimidine moiety of thiamin, as well as the C-methyl group, C-2′. It remains to be shown which of the five carbon atoms of ribose serve as the sources of these three carbon atoms of the pyrimidine moiety of thiamin and how the fragmentation of the ribose unit of AIR takes place in order to supply a C_1 unit and a C_2 unit for the construction of the pyrimidine ring by expansion of the aminoimidazole system of AIR.

It would appear that in *S. typhimurium* and presumably also in other bacteria and possibly in prokaryotes in general, 5-aminoimidazole ribonucleotide (AIR), whose C atoms are derived from glycine, formate, and ribose, serves as the sole precursor of the skeleton of the pyrimidine unit of thiamin.

In previous publications we reported that in yeast label from glycine does not enter the pyrimidine unit of thiamin[4] but that

(27) David, S.; Estramareix, B. *Biochim. Biophys. Acta* **1960**, *42*, 562-563.
(28) David, S.; Estramareix, B. *Biochim. Biophys. Acta* **1961**, *49*, 411.
(29) David, S.; Estramareix, B. *Bull. Soc. Chim. Fr.* **1964**, 2023-2024.
(30) David, S.; Estramareix, B.; Hirshfeld, H. *Biochim. Biophys. Acta* **1966**, *127*, 264-265.
(31) David, S.; Estramareix, B.; Hirshfeld, H. *Biochim. Biophys. Acta* **1967**, *148*, 11-21.
(32) Uchida, K.; Yamada, K.; Kuamoka, H. *Bitamin* **1982**, *56*, 235-240.
(33) Newell, P. C.; Tucker, R. G. *Biochem. J.* **1968**, *106*, 279-287.
(34) Tomlinson, R. V. *Biochim. Biophys. Acta* **1966**, *115*, 526-529.
(35) White, R. H. *Science (Washington, D.C.)* **1981**, *214*, 797-798.
(36) Yamada, K.; Kumaoka, H. *Biochem. Int.* **1982**, *5*, 771-776.
(37) Yamada, K.; Kumaoka, H. *J. Nutr. Sci. Vitaminol.* **1983**, *29*, 389-398.
(38) Estramareix, B.; Thérisod, M. *J. Am. Chem. Soc.* **1984**, *106*, 3857-3860.

Table I. Uptake of Labeled Substrates by *Saccharomyces cerevisiae* (ATCC 24903)[a,b]

expt no.	labeled substrate	nominal total radioactivity, μCi	nominal specific radioactivity, mCi/mmol	culture size, mL	radioactivity remaining in medium, % of total
15	D-[1-^{14}C]glucose[c]	500	56	2800	6
16	D-[1-^{14}C]fructose[d]	500	57	2800	9
17	D-[6-^{14}C]glucose[c]	250	53	1400	14
18	D-[6-^{3}H]glucose[c]	5000	33 990	2800	39
	D-[6-^{14}C]glucose[c]	500	53		15
19	D-[2-^{14}C]glucose[c]	500	53	2800	9
20	[1,3-^{14}C]glycerol[c]	500	56	2800	11
21	[2-^{14}C]glycerol[d]	300	25.7	1400	23
22[b]	[^{14}C]formate[c]	340	52	1400	4
23	[^{14}C]formate[d]	1000	58.6	1400	12
24	[*ureido*-^{14}C]citrulline[c]	250	47.9	1400	5
25	[^{14}C]urea[c]	250	57	1400	0.3

[a] Ethanol (0.5% v/v, 0.082 M) as carbon source (in experiments 15–21 and 23–25). [b] Glucose (0.11 M) as carbon source (in experiment 22). [c] New England Nuclear. [d] Amersham/Searle.

radioactivity from hexoses is incorporated.[5] We now report that label from specifically ^{14}C-labeled samples of glucose and fructose, as well as of glycerol, enters the pyrimidine unit in nonrandom fashion. This was shown by a new chemical degradation (Scheme II) which permits assay of radioactivity at each of the six C atoms of the pyrimidine unit.

Further, it is now demonstrated that label from [^{14}C]formate is incorporated not only into C-4 but also into C-2 of the pyrimidine unit. It follows that not one but two biosynthetic pathways lead to the pyrimidine unit of thiamin, in yeast. Neither of these pathways is identical with the route which operates in bacteria.

Results

Radioactive-tracer experiments were carried out with cultures of *Saccharomyces cerevisiae* (ATCC 24903), growing on thiamin-free medium, with either glucose (experiment 22) or ethanol (experiments 15–21 and 23–25) serving as the carbon source (Table I). Radioactive tracer was added at the onset of logarithmic growth, and cells were collected after maximum growth had been attained. Labeled thiamin was isolated from the cells by carrier dilution and degraded by the reactions described below (Scheme II) which permitted assay of radioactivity at each of the six carbon atoms as well as at the hydrogen atoms at C-2′ of the pyrimidine moiety of thiamin.

Chemical Degradation of the Pyrimidine Unit of Thiamin (Scheme II). Thiamin was cleaved with bisulfite to the pyrimidinesulfonic acid **7** and the thiazole derivative **8**. The pyrimidinesulfonic acid **7** was methylated at N-1 with dimethyl sulfate. The (4-amino-1,2-dimethylpyrimidinium-5-yl)methanesulfonate (**9**) so obtained was refluxed in hydrochloric acid to yield (4-hydroxy-1,2-dimethylpyrimidinium-5-yl)methanesulfonate (**10**). The pyrimidine ring was then cleaved by refluxing in sodium hydroxide solution. The products that were obtained were methylamine (*N*-1, N-*Me*), acetate (C-2,-2′), ammonium hydroxide (*N*-3), 3-sulfopropanoate (**11**) (C-4,-5,-5′), and formate (C-6). The three carboxylic acids were isolated by ion-exchange chromatography. Formic and acetic acids were separated from 3-sulfopropanoic acid by distillation and were converted into their 1-naphthylamides, **16** and **15**, which were separated from one another by preparative chromatography. Acetic acid (C-2′,-2) was further degraded by a Schmidt reaction to yield methylamine (C-2′), which was isolated as *N*-methylphthalimide (**17**). A portion of the 3-sulfopropanoic acid was converted into a crystalline anilide salt **12** (C-4,-5,-5′). Another portion was converted into 2-aminoethane-1-sulfonic acid (taurine) (**13**) (C-5,-5′) by a Schmidt reaction. A third portion was pyrolyzed to acrylic acid (**14**). This was oxidized with osmium tetroxide/sodium periodate to give formaldehyde (isolated as its dimethone derivative (**18**) (C-5′), formic acid (C-5), and carbon dioxide (C-4).

That formaldehyde indeed represented C-5′, i.e., the methylene group of acrylic acid, and formic acid represented C-5, i.e., the methine group of acrylic acid, was confirmed by degradation of a sample of 3-sulfo[3-^{14}C]propanoic acid, prepared from [3-^{14}C]propanoic acid (Scheme IV). The formaldehyde which was obtained in the degradation (see Scheme II) contained essentially all the activity of the intact 3-sulfopropanoic acid (96 ± 1%) (Table II).

Table II. Degradation of Synthetic 3-Sulfo[3-^{14}C]propanoic Acid[a]

	SA[b]	RSA[c]
N-phenyl-3-sulfopropanoamide ammonium salt	1.81 ± 0.02	100 ± 1
2-aminoethane-1-sulfonic acid	1.82 ± 0.02	101 ± 1
formaldehyde dimethone	1.74 ± 0.02	96 ± 1

[a] Prepared from sodium [3-^{14}C]propanoate (NEN, see Experimental Section). [b] Specific activity: (dpm per mmol) $\times 10^{-5}$. [c] Relative specific activity (percent) (*N*-phenyl-3-sulfopropanoamide ammonium salt = 100%).

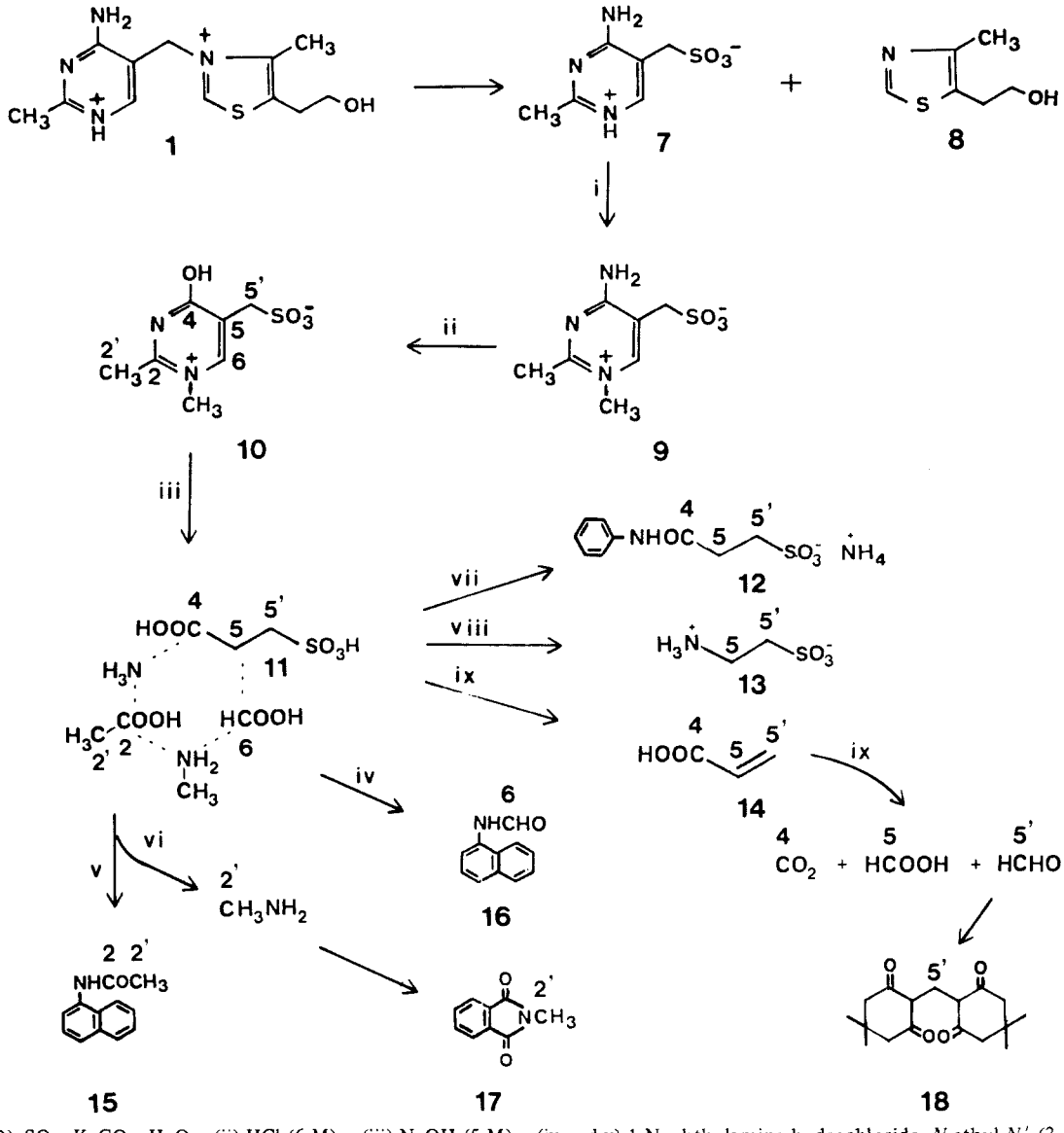
Distribution of ^{14}C and ^{3}H within the Pyrimidine Unit of Thiamin. The samples of pyrimidinesulfonic acid obtained from thiamin, isolated from the experiments with labeled hexoses (experiments 15–19), glycerol (experiments 20 and 21), and formate (experiments 22 and 23) all showed nonrandom distribution of label (Tables III–V). Experiments with [*ureido*-^{14}C]citrulline (experiment 24) and with [^{14}C]urea (experiment 25) did not yield labeled thiamin. The labeling patterns within the pyrimidine moiety of the samples of thiamin isolated from the various tracer experiments are presented in Scheme V.

The distribution of label in the pyrimidine moiety from D-[1-^{14}C]glucose (experiment 15, Scheme V, Figure 1) and D-[1-^{14}C]fructose (experiment 16, Scheme V, Figure 2) was identical. Two-thirds of the activity was located at C-2′, one-fifth at C-5, and the remaining activity was equally distributed between C-2 and C-5′. When D-[2-^{14}C]glucose was the precursor (experiment 19, Scheme V, Figure 3), three-quarters of the activity in the pyrimidine unit was equally distributed between C-2 and C-2′ and the remaining one-quarter of activity was equally distributed between C-4 and C-5. In the pyrimidine samples from D-[6-^{14}C]glucose (experiments 17 and 18, Scheme V, Figure 4), label was distributed among C-2′, C-5, and C-5′ in an approximate ratio of 2:1:6.

In the pyrimidine sample from [1,3-^{14}C]glycerol (experiment 20, Scheme V, Figure 5), an equal amount of activity was found at C-5′ (27%) and C-6 (28%). Twice as much activity was found at C-2′ (20%) than at C-2 (10%), and approximately twice as much activity was found at C-5 (10%) than at C-4 (4%). In the sample from the complementary experiment with [2-^{14}C]glycerol (experiment 21, Scheme V, Figure 6), somewhat more than one-third of the label was located at C-5. Half of the label was distributed between C-2 and C-2′, with slightly more of that activity at C-2′. Activity at C-4 accounted for 12%, and very little activity was found at C-5′ and C-6.

The two experiments with [^{14}C]formate (experiments 22 and 23, Scheme V, Figures 7 and 8) were carried out under different culture conditions. In one experiment (experiment 22), glucose served as the carbon source; in the other (experiment 23), ethanol was used. The distribution of label within the pyrimidine unit was identical in the two experiments: One-quarter of the total

Scheme II. Chemical Degradation of Thiamin To Isolate All Carbon Atoms of the Pyrimidine Unit[a]



[a] (i) $(CH_3O)_2SO_2$, K_2CO_3, H_2O. (ii) HCl (6 M). (iii) NaOH (5 M). (iv and v) 1-Naphthylamine hydrochloride, *N*-ethyl-*N'*-(3-(dimethylamino)propyl)carbodiimide, H_2O. (vi) NaN_3, H_2SO_4. (vii) Aniline hydrochloride, *N*-ethyl-*N'*-(3-(dimethylamino)propyl)-carbodiimide, H_2O. (viii) same as (vi). (ix) 200 °C, followed by OsO_4, $NaIO_4$.

activity of the pyrimidine unit was located at C-2 and three-quarters at C-4.

The pyrimidine degradation was applied also to the sample of intermolecularly doubly labeled pyrimidine obtained from D-[6-^{3}H,6-^{14}C]glucose (experiment 18). There was no change in the $^3H/^{14}C$ ratio in the conversion of (4-amino-2-methyl-5-pyrimidin-5-yl)methanesulfonate (**7**) into the 4-hydroxy derivative **19** (Table IV). Since, under the conditions of this transformation, the protons at C-2′, the C-methyl group, exchange completely, as determined by a control experiment in deuteriated solvent (Scheme III), no label was originally present at C-2′ of the pyrimidine moiety.

In the solvent exchange in 6 M hydrochloric acid, a $^1H/^2H$ isotope effect, K_H/K_D ca. 5, was observed. This corresponds[39] to a $^1H/^3H$ isotope effect, $K_H/K_T = 5^{1.44} = 10.2$, in the degradation of tritium-labeled pyrimidine.

The formic acid, representing C-6 of the pyrimidine moiety, which was obtained by hydrolysis of the product from [6-^{3}H,6-^{14}C]glucose (experiment 18), could not be used to determine the amount of tritium which was originally present at C-6 since, under the degradation conditions employed, the hydrogen at C-6 exchanged completely with solvent protons. This was established by performing the degradation in deuteriated solvent and by determining the ^2H content of the formate derivative obtained in this degradation by mass spectrometry.

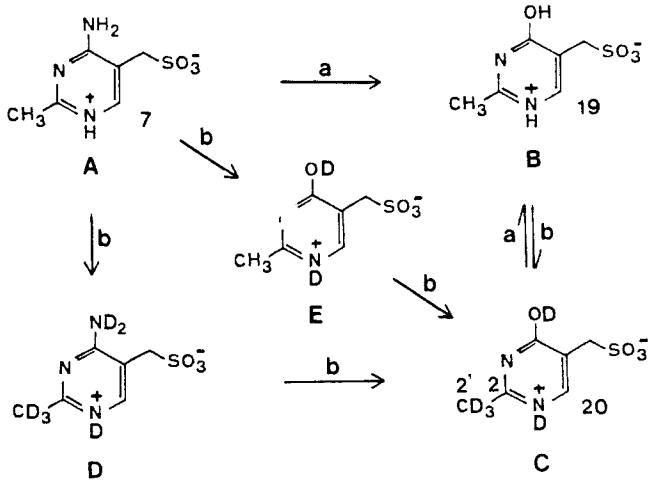
(39) Swain, C. G.; Stivers, E. C.; Reuwer, J. F., Jr.; Schaad, L. J. *J. Am. Chem. Soc.* **1958**, *80*, 5885–5893.

Discussion

The circumstantial evidence which was reviewed in yeast introduction, incomplete though it is, suggests that in bacteria the purine intermediate, 5-aminoimidazole ribonucleotide (AIR) (**2**), whose carbon atoms are derived from glycine, formate, and ribose, serves as the sole precursor of the skeleton of the pyrimidine unit of thiamin. Formate ultimately supplies C-2 and glycine C-4 and C-6 of this pyrimidine. The nitrogen atom of glycine yields N-1. Some of the carbon atoms of ribose supply the remaining carbon atoms, a C_1 unit, C-2′, and a C_2 unit, C-5,5′. C-5′ is supplied by C-6 of glucose, suggesting that C-4 and C-5 of ribose are the source of C-5 and -5′, respectively, of the pyrimidine. The mode of entry of the carbon atoms of ribose remains to be clarified.

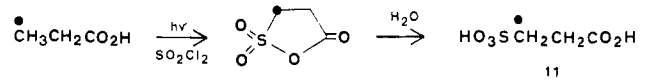
The available evidence suggests further that in yeast the pyrimidine unit of thiamin is derived in an entirely different manner. The only substrate that has been identified with certainty as a precursor of the pyrimidine unit of thiamin in yeast is formate, which is reported to serve as the source of C-4[30-32] but not of C-2. This mode of entry of the precursor is quite distinct from that

Scheme III. Determination of Rate of Exchange of ^1H and ^2H at C-2′ of the Pyrimidine Unit of Thiamin[a]



[a] Reaction conditions: 0.6 M A, B, or C in either (a) 6 M HCl/H_2O or (b) 6 M $^2HCl/^2H_2O$, 100 °C. Conversion of A to B (in HCl/H_2O): $t_{1/2} \sim 150$ min. Proton exchange at the C-methyl group (in $^2HCl/^2H_2O$) ($t_{1/2}$ is defined as the time at which the 2′-methyl group contained an average of 1.5 ^1H, as determined by ^1H NMR): $t_{1/2}$ (B → C) = $t_{1/2}$ (E → C) ~ 30 min, $t_{1/2}$ (A → D) < 30 min. Deuterium exchange at the C-methyl group (in HCl/H_2O) ($t_{1/2}$ defined as above): $t_{1/2}$ (C → B) ~ 150 min.

Scheme IV. Synthesis of 3-Sulfo[3-^{14}C]propanoic Acid



found in bacteria. Nor is glycine a precursor of the pyrimidine ring of thiamin in yeast.[4] Indeed, it has been suggested[35] that the origin of the pyrimidine unit of thiamin in yeast, unlike that in bacteria, is not linked to 5-aminoimidazole ribonucleotide and, thus, not to purine biosynthesis. Nor does it share a common origin with the nucleic acid pyrimidines.[3,13]

Clearly, the time is ripe for a renewed effort to clarify the biosynthetic origin of the pyrimidine moiety of thiamin in yeast.

In an earlier paper[5] we drew attention to the obvious structural analogy between the pyrimidine unit **6** of thiamin and another B vitamin, pyridoxol (**21**).[40] Pyridoxol is derived from carbohydrate precursors. The incorporation patterns of label from D-[1-^{14}C]- and D-[6-^{14}C]glucose[41,42] and from [1,3-^{14}C]- and [2-^{14}C]glycerol[41,43-45] show that, in the major route to pyridoxol in *E. coli*, the vitamin is generated from three triose units. One of these yields a C_3 fragment, C-5′,-5,-6, of pyridoxol which, on the basis of structural analogy, corresponds to the C_3 fragment, C-5′,-5,-6, of the pyrimidine moiety of the thiamin. Another triose unit, after loss of a terminal carbon atom, yields the C_2 fragment, C-2′,-2 of pyridoxol, which corresponds to the C_2 fragment, C-2′,-2, of the pyrimidine. A third triose unit yields the C_3 fragment, C-4′,-4,-3, of pyridoxol. This is the fragment which, in the pyrimidine, is replaced by the fragment, N-3,C-4,NH_2, whose carbon atom, in the thiamin pyrimidine unit from yeast, is derived from formate.[30-32]

Table III. Incorporation of ^{14}C-Labeled Hexoses into the Pyrimidine Moiety of Thiamin

	C atom(s)	specific activity[a] (relative specific activity)[b] 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
(4-amino-2-methylpyridimidin-5-yl)-methanesulfonic acid (**7**)	all	7.97 ± 0.05 (100 ± 1)	7.79 ± 0.05 (100 ± 1)	2.68 ± 0.03 (100 ± 2)	7.64[c] ± 0.04 (100 ± 1)	19.60 ± 0.10 (100 ± 1)
(4-amino-1,2-dimethylpyrimidinium-5-yl)methanesulfonate (**9**)	all	7.83 ± 0.05 (98 ± 1)	7.77 ± 0.07 (100 ± 1)	2.63 ± 0.04 (98 ± 2)		19.38 ± 0.10 (99 ± 1)
N-acetyl-1-naphthylamine (**15**)	2,2′	5.84 ± 0.04 (73 ± 1)	5.66 ± 0.03 (73 ± 1)	0.61 ± 0.02 (23 ± 1)	1.66 ± 0.02 (22 ± 1)	14.66 ± 0.07 (75 ± 1)
N-methylphthalimide (**17**)	2′	5.43 ± 0.04 (68 ± 1)	5.19 ± 0.05 (67 ± 1)		1.55 ± 0.04 (20 ± 1)	7.25 ± 0.05 (37 ± 1)
N-formyl-1-naphthylamine (**16**)	6	0.08 ± 0.02 (1 ± 1)	0.11 ± 0.02 (1 ± 1)	0.23 ± 0.03 (9 ± 2)	0.18 ± 0.02 (2 ± 1)	0.17 ± 0.02 (1 ± 1)
N-phenyl-3-sulfopropanoamide ammonium salt (**12**)	4,5,5′	2.06 ± 0.04 (26 ± 1)	2.23 ± 0.04 (29 ± 1)		5.78 ± 0.03 (76 ± 1)	4.69 ± 0.05 (24 ± 1)
taurine (**13**)	5,5′	1.90 ± 0.02 (24 ± 1)	1.99 ± 0.03 (26 ± 1)	1.99 ± 0.02 (74 ± 1)	5.58 ± 0.02 (73 ± 1)	2.34 ± 0.03 (12 ± 1)
formaldehyde dimethone (**18**)	5′	0.43 ± 0.02 (5 ± 1)	0.43 ± 0.03 (6 ± 1)		4.63 ± 0.05 (61 ± 1)	0.29 ± 0.03 (1 ± 1)

[a] Specific activity: (dpm per mmol) × 10^{-3}. [b] Relative specific activity (percent) [(4-amino-2-methylpyrimidin-5-yl)methanesulfonic acid = 100%]. [c] ^{14}C-activity only.

(40) It is noteworthy that pyridoxol inhibits thiamin biosynthesis in *Neurospora* and that this inhibition has been ascribed to the structural similarity of pyridoxol to the pyrimidine fragment of thiamin (Harris, D. L. *Arch. Biochem. Biophys.* **1956**, *60*, 35-43).

(41) Hill, R. E.; Rowell, F. J.; Gupta, R. N.; Spenser, I. D. *J. Biol. Chem.* **1972**, *247*, 1869-1882.

(42) Hill, R. E.; Spenser, I. D. *Can. J. Biochem.* **1973**, *51*, 1412-1416; **1974**, *52*, 271.

(43) Hill, R. E.; Horsewood, P.; Spenser, I. D.; Tani, Y. *J. Chem. Soc., Perkin Trans. 1* **1975**, 1622-1627.

(44) Hill, R. E.; Miura, I.; Spenser, I. D. *J. Am. Chem. Soc.* **1977**, *99*, 4179-4181.

(45) Vella, G. J.; Hill, R. E.; Spenser, I. D. *J. Biol. Chem.* **1981**, *256*, 10469-10474; **1982**, *257*, 4008.

Table IV. Incorporation of [6-^{3}H,6-^{14}C]Glucose into the Pyrimidine Moiety of Thiamin (Experiment 18)

			specific activity[a] (relative specific activity)[b]		
	H atom(s)	C atom(s)	^{3}H	^{14}C	^{3}H/^{14}C ratio
(4-amino-2-methylpyridimidin-5-yl)-methanesulfonic acid (**7**)	all	all	27.1 ± 0.1 (100 ± 1)	7.64 ± 0.04 (100 ± 1)	3.55 ± 0.02
(4-amino-1,2-dimethylpyrimidinium-5-yl)methanesulfonate (**9**)	at C-5′,C-6	all	26.5 ± 0.2 (98 ± 1)	7.60 ± 0.08 (99 ± 1)	3.49 ± 0.05
(4-hydroxy-2-methylpyrimidin-5-yl)-methanesulfonic acid (**19**)					
(after 24 h reflux in HCl (6 M) at 100 °C)	at C-5′,C-6	all	27.8 ± 0.1 (103 ± 1)	7.86 ± 0.04 (103 ± 1)	3.54 ± 0.02
(after 50 h reflux in HCl (6 M) at 100 °C)		all	26.9 ± 0.1 (99 ± 1)	7.72 ± 0.04 (101 ± 1)	3.48 ± 0.02

[a] Specific activity: (dpm per mmol) × 10^{-3}. [b] Relative specific activity (percent) [(4-amino-2-methylpyrimidin-5-yl)methanesulfonic acid = 100%].

Table V. Incorporation of ^{14}C-Glycerol and ^{14}C-Formate into the Pyrimidine Moiety of Thiamin

		specific activity[a] (relative specific activity)[b]			
	C atom(s)	expt 20 [1,3-^{14}C]glycerol	expt 21 [2-^{14}C]glycerol	expt 22 [^{14}C]formate	expt 23 [^{14}C]formate
(4-amino-2-methylpyridimidin-5-yl)methanesulfonic acid (**7**)	all	12.30 ± 0.08 (100 ± 1)	5.04 ± 0.04 (100 ± 1)	12.03 ± 0.05 (100 ± 1)	15.75 ± 0.07 (100 ± 1)
(4-amino-1,2-dimethylpyrimidinium-5-yl)methanesulfonate (**9**)	all	12.07 ± 0.08 (98 ± 1)		11.91 ± 0.06 (99 ± 1)	15.39 ± 0.10 (98 ± 1)
N-acetyl-1-naphthylamine (**15**)	2,2′	3.68 ± 0.04 (30 ± 1)	2.57 ± 0.02 (51 ± 1)	2.80 ± 0.03 (23 ± 1)	3.40 ± 0.02 (22 ± 1)
N-methylphthalimide (**17**)	2′	2.51 ± 0.02 (20 ± 1)	1.42 ± 0.01 (28 ± 1)	0.22 ± 0.03 (2 ± 1)	0.24 ± 0.07 (2 ± 1)
N-formyl-1-naphthylamine (**16**)	6	3.49 ± 0.03 (28 ± 1)	0.25 ± 0.02 (5 ± 1)	0.12 ± 0.02 (1 ± 1)	0.17 ± 0.03 (1 ± 1)
N-phenyl-3-sulfopropanoamide ammonium salt (**12**)	4,5,5′	5.07 ± 0.05 (41 ± 1)	2.56 ± 0.03 (51 ± 1)	8.89 ± 0.05 (74 ± 1)	11.40 ± 0.06 (72 ± 1)
taurine (**13**)	5,5′	4.56 ± 0.04 (37 ± 1)	1.96 ± 0.02 (39 ± 1)	0.07 ± 0.01 (1 ± 1)	0.24 ± 0.01 (2 ± 1)
formaldehyde dimethone (**18**)	5′	3.34 ± 0.08 (27 ± 1)	0.15 ± 0.03 (3 ± 1)		

[a] Specific activity: (dpm per mmol) × 10^{-3}. [b] Relative specific activity (percent) [(4-amino-2-methylpyrimidin-5-yl)methanesulfonic acid = 100%].

Our observation[5] that the pyrimidine moiety of the samples of thiamin obtained from experiments in which yeast had been incubated with ^{14}C-labeled hexoses (experiments 14–19) and glycerol (experiment 20) contained ca. 50% of the radioactivity of the intact thiamin (ref 5, p 4936, Table III) encouraged us to pursue the working hypothesis that there was analogy in biosynthetic origin between pyridoxol (**21**) and the pyrimidine unit **6** of thiamin. We anticipated that the distribution of label within the pyrimidine samples might correspond to that observed in samples of pyridoxol from correspondingly labeled substrates (Scheme VI). In order to test this supposition, the precise distribution of label within the pyrimidine had to be determined. To do so, a rational chemical degradation of the pyrimidine unit was required which not only permitted assay of radioactivity at every one of its six carbon atoms but which also provided a check of the internal consistency of the data.

The degradation sequences which had been employed in the past were flawed in several ways. Some did not permit radioactive assay of more than one individual carbon atom.[28] Others permitted assay of groups of carbon atoms together but not of individual carbon atoms, or did not account for the total specific activity of the intact pyrimidine in terms of the sum of the specific activities of the degradation products.[13,15,25,29,32] In several degradations, a complete determination of activity at all six carbon atoms was possible, in principle. However, even with these degradation sequences, it appeared to be difficult to account for the total activity of the intact pyrimidine,[20,21,30,31] or else the sequence was likely to be successful only if all activity was restricted to one of the six carbon atoms of the intact pyrimidine.[10,36,37] The degradation sequence which we have devised (Scheme II) accounts for the total activity of the intact pyrimidine unit in terms of three fragments, of unequivocal origin, all of which can be isolated from the reaction mixture: acetic acid (C-2,2′), formic acid (C-6), and 3-sulfopropanoic acid (C-4,5,5′). Separation of C-2′ from C-2 is achieved by a conventional Schmidt reaction. Individual assay of label within C-4,C-5 and C-5′ is accomplished by pyrolysis of 3-sulfopropanoic acid to acrylic acid,[46] followed by controlled oxidation of the latter to formaldehyde (C-5′) and formic acid (C-5) on the one hand and by conversion of 3-sulfopropanoic acid to taurine (C-4, by difference) by means of a Schmidt reaction, on the other.

The results of the degradation sequences performed on the ^{14}C-labeled samples of pyrimidinesulfonic acid obtained from the nine tracer experiments (experiments 15–23) are recorded in Tables III and V. The distribution of activity within these samples, deduced from the degradation results, is summarized in Scheme V. It is evident that in each case incorporation of precursor into the pyrimidine had taken place in nonrandom fashion.

In Scheme VI, the distribution of label within the pyrimidine unit of thiamin, which is predicted (column 3) on the basis of structural and biosynthetic analogy with that observed within pyridoxol (column 2) derived from the same labeled precursor (column 1), is compared with the distribution of label actually established by degradation of the pyrimidine samples (column 4).

In the case of samples derived from [1,3-^{14}C]glycerol and [6-^{14}C]glucose the match between prediction and observation is not unreasonable, with the exception that, in each case, C-2′ carries significantly less than the expected fraction of label and that more label than can be reasonably accounted for on the basis of randomization of background radioactivity is present at C-5 and, in the case of the sample from [1,3-^{14}C]glycerol, also at C-2. However, if these had been the only experiments that had been performed, the temptation would have been overwhelming to regard these results as experimental substantiation of the hypothesis that there exists analogy between pyridoxol and the pyrimidine unit of thiamin, not only in structure but also in biosynthesis.

That such a view would have been entirely erroneous is shown by a comparison of the predicted with the observed distribution of label in the samples of the pyrimidine derived from [2-^{14}C]-glycerol and from [1-^{14}C]glucose. In the case of [2-^{14}C]glycerol, it is predicted that activity is equally distributed between C-2 and C-5 and that other sites would be unlabeled. In the event, while C-2 and C-5 of the isolated sample do indeed carry label (Scheme V, Figure 6), even though not to an equal extent, more than one-quarter of the activity is present at C-2′, and an eighth at C-4, two sites predicted to be unlabeled.

In the case of the sample derived from [1-^{14}C]glucose, it is

Scheme V. Distribution of Radioactivity (Percent of Total Activity of Intact Pyrimidine Unit at Indicated Carbon Atoms) within the Pyrimidine Unit of Thiamin Derived from Hexoses, Glycerol, and Formate[a]

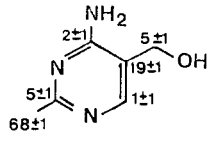


Figure 1
from 1-^{14}C-Glucose
Experiment 15

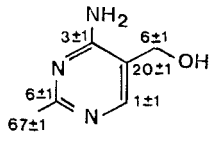


Figure 2
from 1-^{14}C-Fructose
Experiment 16

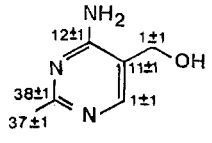


Figure 3
from 2-^{14}C-Glucose
Experiment 19

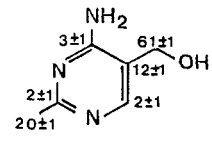


Figure 4
from 6-^{14}C-Glucose
Experiments 17, 18

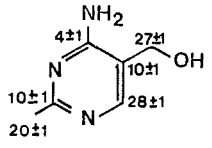


Figure 5
from 1,3-^{14}C-Glycerol
Experiment 20

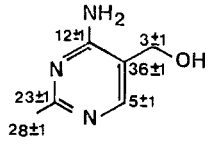


Figure 6
from 2-^{14}C-Glycerol
Experiment 21

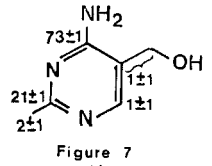


Figure 7
from ^{14}C-Formate
Experiment 22

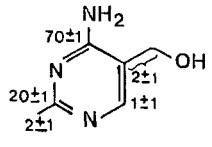


Figure 8
from ^{14}C-Formate
Experiment 23

[a] This distribution was deduced from the results of the controlled chemical degradation of the labeled samples of (4-amino-2-methylpyrimidin-5-yl)methanesulfonic acid (7).

predicted that about two-thirds of the label should reside at C-2′, and this is indeed observed. However, the rest of the activity was predicted to lodge at C-5′ which, in the experimental sample (Scheme V, Figure 1), in fact contains little activity, while C-5, predicted to be unlabeled, in fact carries a fifth of the activity of the molecule. That this observed distribution within the sample from [1-^{14}C]glucose is significant is shown by the fact that the sample, derived from [1-^{14}C]fructose (Scheme V, Figure 2), shows the identical distribution of label, within experimental error. This correspondence in the labeling patterns of these two samples reflects the close metabolic relationship between glucose and fructose. It also inspires further confidence in the reliability of the degradation sequence.

The distribution of label from these substrates, determined by the degradation experiments, clearly shows that there is no analogy in origin between the pyrimidine unit of thiamin in yeast and pyridoxol and that our working hypothesis must be discarded. Another interpretation of the pattern of distribution of label must be sought.

The key to the interpretation of the complex labeling pattern within the pyrimidine samples derived from hexoses and from glycerol (experiments 15–21) was found in the results of two experiments with [^{14}C]formate (experiments 22 and 23). Even though culture conditions in these two experiments differed from one another in that glucose was used as the general carbon source[4] in one case (experiment 22, Table I) while ethanol was employed[5] in the other (experiment 23, Table I), the distribution of label was the same in the two pyrimidine samples. In each case, approximately three-quarters of the activity was located at C-4, while approximately one-quarter of the label was present at C-2. The other four carbon atoms of the pyrimidine moiety were essentially free of activity (Scheme V, Figures 7 and 8).

These results differ markedly from those of earlier experiments in which yeast was incubated with [^{14}C]formate and the distribution of label within the pyrimidine unit of thiamin was determined. The work of three groups must be considered. Firstly, it is reported by Goodwin and his collaborators[13] that permanganate oxidation of a sample of (4-amino-2-methylpyrimidin-5-yl)methanesulfonic acid (**7**), obtained from thiamin derived from [^{14}C]formate in a yeast culture, "yielded acetic acid and, probably, propionic acid as the two volatile products when examined in a gas chromatogram coupled with a radioactive scanner; essentially all the radioactivity was associated with the acetic acid peak." Thus, all label from [^{14}C]formate was present within the acetic acid, presumably derived from the C_2 unit, C-2′,-2, of the pyrimidine unit of the yeast-derived thiamin.

Secondly, and in complete contradiction to the above result, David and his collaborators[31] reported that Kuhn–Roth oxidation of a radioactive sample of (4-hydroxy-2-methylpyrimidin-5-yl)-methanesulfonic acid (**19**), obtained from thiamin derived from [^{14}C]formate in a yeast culture, yielded acetic acid, isolated as the *p*-phenylphenacyl derivative, which was totally nonradioactive. Similarly, degradation of a sample of 4-hydroxy-2,5-dimethylpyrimidine, obtained from [^{14}C]formate-derived thiamin from a similar yeast culture, contained all label within the C_3 unit, C-4,-5,-5′, which was extruded as propionic acid, and none within the C_2 unit, C-2,-2′, which was obtained as acetic acid.[30,31] Thus, none of the activity was present at C-2 but all of it at C-4. Yet, in an earlier paper,[29] the same authors report a different result. Degradation of the pyrimidine unit of a similarly [^{14}C]formate-derived sample of thiamin yielded a fragment, 1,3-diamino-2-methylpropane, representing the four carbon atoms, C-4,-5,-5′,-6 of the pyrimidine unit. This fragment accounted for only 73% of the total activity of the pyrimidine unit. Taken at face value, this result indicates that 27% of the label was present within the C_2 unit, C-2,-2′ of the pyrimidine. Since it was later shown[31] that two of the carbon atoms of the sample of 1,3-diamino-2-methylpropane, representing C-5,-5′, were free of activity, it would appear that this sample of [^{14}C]formate-derived pyrimidine contained 73% of its label at C-4 (assuming C-6 to be unlabeled) and 27% of its label at C-2 (assuming C-2′ to be free of activity).

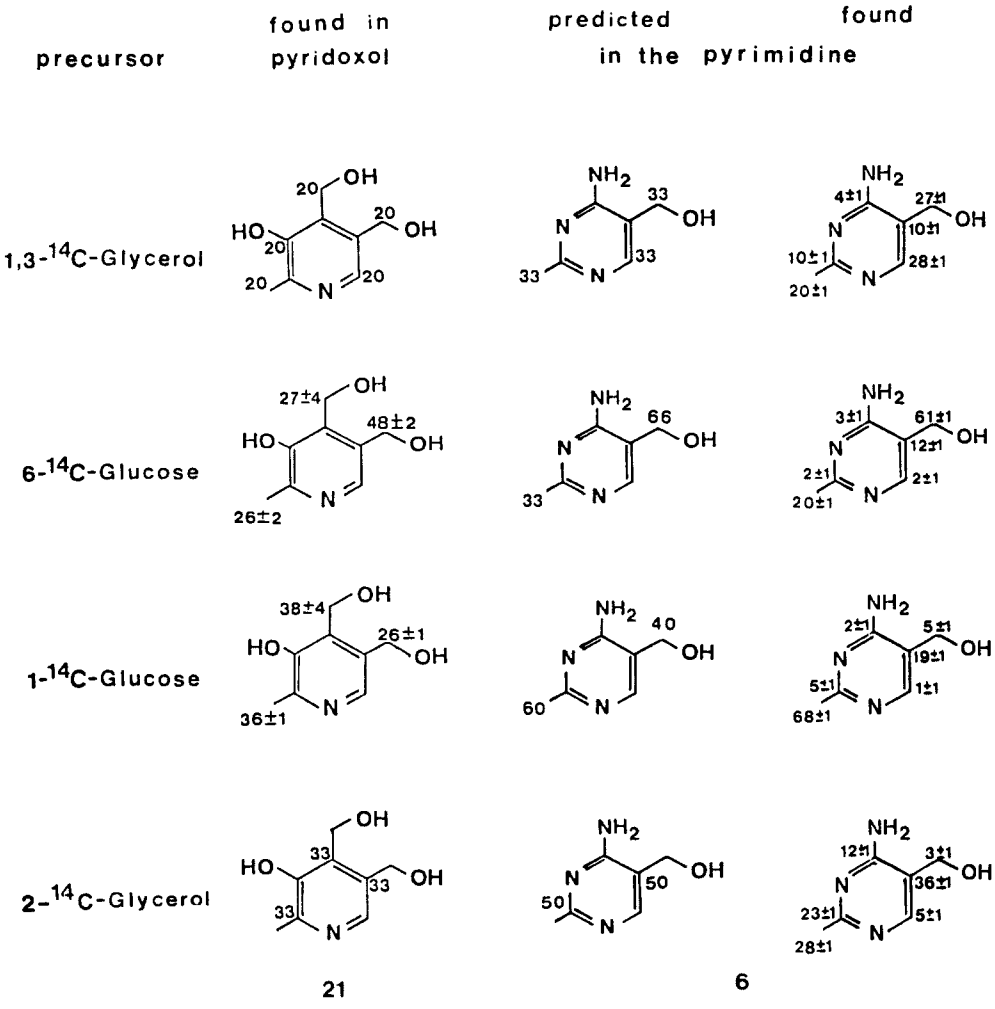
This is a distribution of [^{14}C]formate activity similar to that found in our present work (experiments 22 and 23) and different from that reported subsequently[31] by David et al.

The most recent investigations on the distribution of activity from [^{14}C]formate are those of Kumaoka and his collaborators.[10,26,32] Kuhn–Roth oxidation of samples of pyrimidinesulfonic acid **7**, derived from thiamin obtained from yeast[26,32] and other eukaryotes[10] which had been incubated with [^{14}C]formate, gave acetic acid (i.e., C-2,-2′) that was not radioactive. Most, if not all, activity was present at C-4.

Thus, there are three different, apparently contradictory, sets of results describing the mode of entry of formate into the pyrimidine unit of thiamin in yeast: (i) incorporation into the C_2 unit, C-2′,-2 (presumably into C-2);[13] (ii) incorporation into C-4;[26,31,32] and (iii) incorporation into two sites, C-4 and C-2, with 3 times greater efficiency of entry into C-4 than into C-2, as shown in the present results (experiments 22 and 23) and possibly also in ref 29.

Among possible explanations for the divergent results are that the strains of yeast, employed by the different groups of investigators, differed with respect to thiamin biosynthesis or that thiamin biosynthesis within a given strain of yeast differs under different conditions (e.g., aerobic vs. anaerobic metabolism; time of administration of tracer and duration of the incubation with respect to the growth curve of the culture, from lag phase through

Scheme VI. Distribution of Label from Glucose and Glycerol, Observed in Pyridoxol, Compared with That Predicted and That Actually Observed within the Pyrimidine Unit of Thiamin (Numbers Represent Percent of the Total Activity of the Intact Molecule within the Indicated Carbon Atom)



log phase to stationary phase; photochemical conditions), quite apart from the question of the reproducibility, sensitivity, and reliability of the degradation sequences which were employed.

Our own degradation procedure accounts for the total activity of the intact pyrimidine moiety in terms of the activity of the degradation products in each of the nine experiments which are reported here (experiments 15–23), and we will confine ourselves to a discussion and interpretation of our own results.

Label from formate is incorporated, in two experiments (experiments 22 and 23) (Scheme V, Figures 7 and 8) with different carbon sources, into two sites of the pyrimidine ring of thiamin. In each case C-4 accounts for approximately 70% of the activity of the pyrimidine and C-2 for approximately 20%. In other words, the molar specific activity at C-4 was approximately 3.5 times higher than the molar specific activity at C-2. This means that the administered [^{14}C]formate, en route into C-2, apparently suffered dilution by an endogenous one-carbon donor which was 3.5 times greater than the dilution of the same [^{14}C]formate entering C-4.

It is most unlikely that ethanol and glucose, the substances serving as general carbon sources in the experiments with [^{14}C]formate, would supply, in exactly the same proportions, two different one-carbon intermediates, one of which serving to dilute label from the administered formate en route into C-4, the other serving to dilute, to a much larger extent (3.5 times larger), label from the same formate, by way of a different route, into C-2. Production of two different one-carbon intermediates from the general carbon sources is particularly unlikely in view of the lack of incorporation, into the pyrimidine unit of thiamin in yeast, of such one-carbon donors as [*methyl*-^{14}C]methionine,[4] L-[3-^{14}C]-serine,[5] [2-^{14}C]glycine,[4] and [^{14}C]bicarbonate.[47] Two other potential C_1 donors whose incorporation was tested, [*ureido*-^{14}C]citrulline (experiment 24) and [^{14}C]urea (experiment 25), did not serve as precursors of thiamin.

Nor are the C_1 units entering C-4 and C-2, respectively, supplied by any of the other substrates tested in the present investigation. This is shown by the fact that while the ratio of label within C-4 and C-2, derived from formate, is 3.5/1 (Scheme V, Figures 7 and 8, experiments 22 and 23), the ratio, within these two carbon atoms, of label derived from the other substrates shows a much lower value [0.3, [2-^{14}C]glucose (Scheme V, Figure 3, experiment 19); 0.4, [1-^{14}C]glucose (Scheme V, Figure 1, experiment 15); 0.4, [1,3-^{14}C]glycerol (Scheme V, Figure 5, experiment 20); 0.5, [1-^{14}C]fructose (Scheme V, Figure 2, experiment 16), [2-^{14}C]-glycerol (Scheme V, Figure 6, experiment 21); 1.5, [6-^{14}C]glucose (Scheme V, Figure 4, experiments 17 and 18)]. Entry of formate into C-4 and C-2 of the pyrimidine by way of two different one-carbon relays must be rejected as the explanation for the unequal isotope concentration at the two sites.

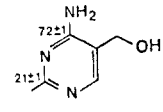
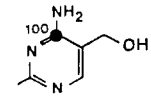
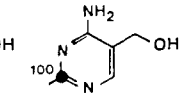
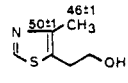
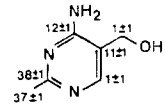
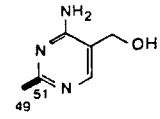
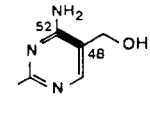
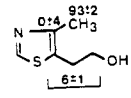
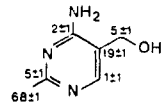
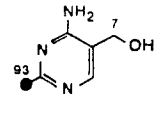
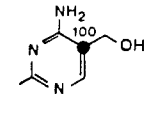
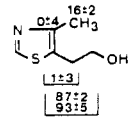
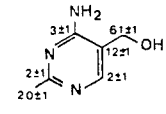
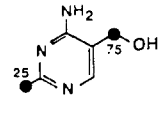
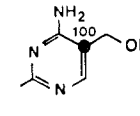
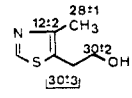
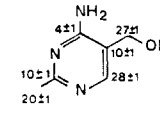
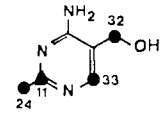
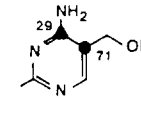
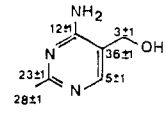
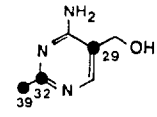
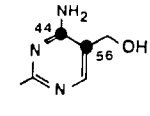
There is an alternative interpretation of the observed distribution of label: The 3.5/1 ratio of activity between C-4 and C-2 of the pyrimidine nucleus is explicable if the ring skeleton of the pyrimidine were formed de novo by two different independent routes, whose overall relative rates of product formation are 3.5 to 1. [^{14}C]Formate enters C-4 by the more efficient route and C-2 by the less efficient route.

The apparently complex pattern of labeling in the pyrimidine moiety of thiamin derived from hexoses and from glycerol is readily

(46) Nagai, T.; Nishitomi, K.; Tokura, N. *Bull. Chem. Soc. Jpn.* **1967**, *40*, 1183–1188.

(47) Yamada, K.; Uchida, K.; Kumaoka, H. *Bitamin* **1981**, *55*, 523–528.

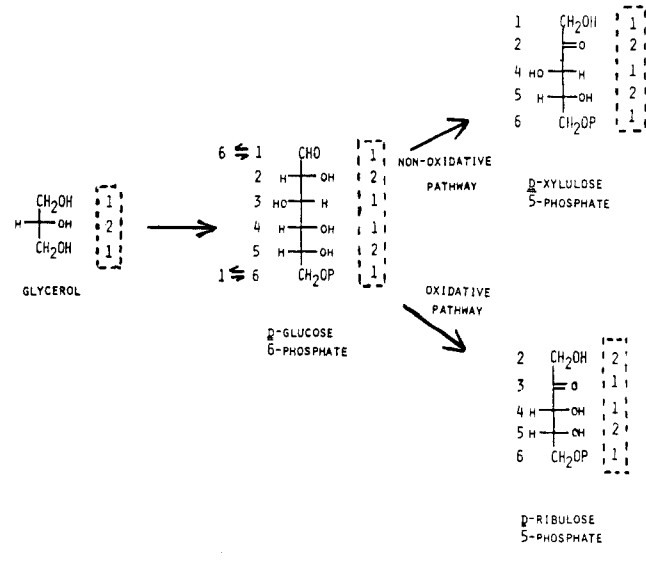
Scheme VII. Interpretation of the Labeling Pattern within Samples of the Pyrimidine Unit of Thiamin, Derived from ^{14}C-Labeled Formate, Hexoses, and Glycerol, based on the Simultaneous Occurrence of Two Independent Pathways

Precursor	Distribution of label found in the thiazole	Distribution of label found in the pyrimidine	Interpretation of the distribution of label in the pyrimidine: MAJOR PATHWAY	Interpretation of the distribution of label in the pyrimidine: MINOR PATHWAY	MAJOR / MINOR
^{14}C-Formate					3.4 ±0.2
2-^{14}C-Glucose					3.4 ±0.3
1-^{14}C-Glucose					3.6 ±0.2
6-^{14}C-Glucose					1.7 ±0.2
1,3-^{14}C-Glycerol					2.0 ±0.1
2-^{14}C-Glycerol					1.9 ±0.2

interpretable on the basis of the premise that two different independent routes are in operation. The interpretation is facilitated by the results of our earlier investigation[5] of the mode of incorporation of label into the thiazole fragment **8** of the thiamin samples whose pyrimidine fragment **6** is now under investigation. The distribution of label from the various precursors within the thiazole unit **8** is shown in Scheme VII, column 2. This distribution has been interpreted in full detail.[5] A brief summary is offered here, since this interpretation is relevant to the distribution of label within the pyrimidine unit.

The C_5 chain, C-4′,-4,-5,-6,-7, of the thiazole unit is derived from the five carbon atoms of a pentulose, related to ribulose or xylulose. Carbon-1 of the pentulose yields C-4′; carbon-5 of the pentulose yields C-7 of the thiazole C_5 unit. The pentulose, in turn, is derived from glucose by two independent routes (Scheme VIII). The first is the nonoxidative pentose phosphate pathway, whose key step, catalyzed by transketolase (EC 2.2.1.1), generates the pentulose from C-1,-2,-4,-5,-6 of glucose. Generation of the thiazole C_5 unit by this route delivers C-1,-2,-4,-5,-6 of glucose into C-4′,-4,-5,-6,-7 of the thiazole unit. The second route is the oxidative pentose phosphate pathway, whose key intermediate, D-gluconic acid 6-phosphate undergoes oxidative decarboxylation catalyzed by 6-phosphogluconate dehydrogenase (EC 1.1.1.44).

Scheme VIII. Predicted Derivation of the Carbon Atoms of a Pentose from the Carbon Atoms of Glucose (Plain Numbers) and from the Carbon Atoms of Glycerol (Ringed Numbers)



Generation of a pentulose by this route delivers C-2,-3,-4,-5,-6 of glucose into C-4′,-4,-5,-6,-7, respectively, of the C_5 unit of the thiazole fragment of thiamin. These relationships are summarized in Scheme VIII. Since approximately one-half of the label from D-[2-^{14}C]glucose is found at each of C-4′ and C-4 of the thiazole unit, it follows that the two pathways from glucose to pentulose make an approximately equal contribution under the conditions of the experiment.

The pyrimidine moiety from thiamin derived from D-[2-^{14}C] glucose shows four major sites of labeling (Scheme VII, column 3): Approximately 75% of its activity is present within the C_2 unit, C-2′,-2, and approximately 23% within the C_2 unit, C-4,-5. It is evident that each of these two pairs of carbon atoms are derived from C-2 of glucose via C-1,C-2 of a pentose.

Since in each of the two C_2 units, C-2′,-2 and C-4,-5, of the pyrimidine moiety one of the carbon atoms, C-2 and C-4, respectively, is fully accounted for by formate, C-4 in the larger fraction of molecules and C-2 in the smaller fraction, it follows that in those molecules in which C-4 is derived from formate, the C_2 unit, C-2′,-2, is derived from C-1,-2 of a pentose (Scheme VII, column 4) and that in those molecules, in which C-2 is derived from formate, the C_2 unit, C-4,-5, is derived from C-1,-2 of a pentose (Scheme VII, column 5).

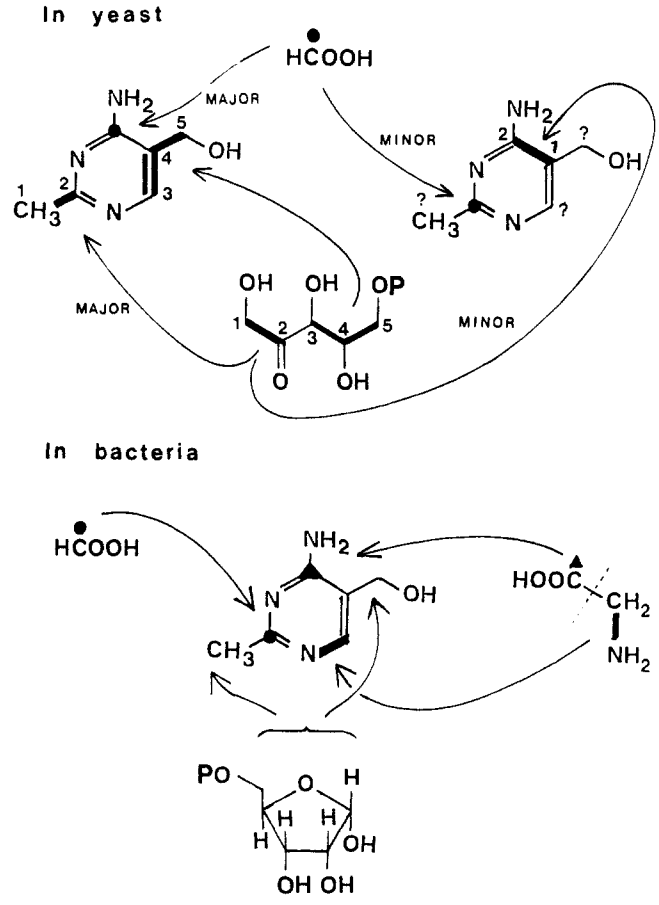
The correspondence of the ratio (37 + 38)/(12 + 11) = 3.3/1, for the activities at C-2′,-2 and at C-4,-5, with the ratio 70/20 = 3.5/1, for the activities due to formate, at C-4 and C-2, respectively, further supports this interpretation.

Which of the two carbon atoms, C-2′ or C-2, and C-4 or C-5, respectively, is derived from C-1, and which from C-2 of the pentose, is shown by the experiment with D-[1-^{14}C]glucose. It is C-2′ and C-5 which account for 87% of the total activity of the pyrimidine sample, in the ratio 68/19 = 3.6/1. In the thiazole moiety, 93% of the label from D-[1-^{14}C]glucose resided at C-4′. The remaining activity was present elsewhere, presumably mainly at C-7, the site derived from carbon-6 of glucose. This scrambling of label is accounted for by glycolytic cleavage into triose units and reutilization of the labeled triose units.

The distribution of label within the samples of the pyrimidine obtained from thiamin derived from D-[6-^{14}C]glucose, [1,3-^{14}C]glycerol, and [2-^{14}C]glycerol is complicated due to increased importance of glycolytic reactions (in the case of D-[6-^{14}C]glucose), and utilization of labeled trioses, but, in general terms, follows the same pattern.

In the sample from D-[6-^{14}C]glucose, the major site of labeling is at C-5′. This site is thus derived from C-5 of a pentose in the major (formate into C-4) pathway. Glycolytic scrambling from C-6 into C-1 of hexose leads to significant labeling at the two sites derived from C-1 of ribose, i.e., at C-2′ and C-5 of the pyrimidine. Under the conditions of this experiment, the ratio of the major (i.e., formate into C-4; C-1 of pentose into C-2′ of pyrimidine) to minor (i.e., formate into C-2; C-1 of pentose into C-5) pathway was 20/12 = 1.7. The two [^{14}C]glycerol experiments lead to similar ratios of major to minor pathways ([1,3-^{14}C]glycerol, 20/10 = 2.0; [2-^{14}C]glycerol 23/12 = 1.9). In the pyrimidine sample from [1,3-^{14}C]glycerol, 55% of the activity is accounted for by C-6 plus C-5′ and is equally divided between these two sites. It is therefore likely that in the major pathway (i.e., formate into C-4) an intact triose unit related to glycerol enters the C_3 fragment, C-3,-4,-5, of pentose directly by the transketolase route and, thence, C-6,-5,-5′ of the pyrimidine. However, this process must be accompanied by indirect entry, via hexose and pentose intermediates: Reversal of glycolysis directs label from [1,3-^{14}C]glycerol into four sites, C-1, C-3, C-4, and C-6, of hexose which, in turn, yields pentose labeled either at C-1, C-3, and C-5 by the transketolase route or at C-2, C-3, and C-5 by the oxidative route. That, in this experiment, the transketolase route from hexose to pentose accounted for approximately twice as much pentose than the oxidative route is shown by the distribution of label within the thiazole unit of thiamin which contained 28% of its activity at C-4′ (C-1 of glycerol into C-1 of hexose and then into C-1 of pentose, by the transketolase route), while C-4 accounted for 12% of the thiazole activity (C-1 of glycerol into C-3 of hexose and then into C-2 of pentose, by the oxidative route) (28/12 = 2.3). The corresponding distribution of label is evident in the pyrimidine unit: The C_2 unit, C-2′,-2, derived from C-1,-2 of pentose in the major (formate into C-4) pathway (C-2′/C-2 = 20/10 = 2.0) and the C_2 unit, C-5,-4, derived from C-1,-2 of ribose in the minor (formate into C-2) pathway (C-5/C-4 = 10/4 = 2.5), each show a corresponding labeling pattern.

Scheme IX. Biosynthesis of the Pyrimidine Unit of Thiamin in Yeast and in Bacteria. Presursor–Product Relationships (Numbers Refer to the Carbon Atoms of a Pentose, Throughout)



Finally, the distribution of label from [2-^{14}C]glycerol is interpretable in corresponding fashion. By reversal of glycolysis, C-2 of glycerol enters C-2 of hexose and thence C-1 of pentose by the oxidative route and C-2 of pentose by the transketolase route. In this experiment the oxidative route predominated slightly. This is shown by the fact that the ratio of activity, C-2′(from C-1 of pentose in the major, formate into C-4, pathway)/C-2(from C-2 of pentose in the major pathway) was 28/23 = 1.2. The pyrimidine carbon atoms derived from C-1 and C-2 of pentose in the minor (i.e., formate into C-2) pathway must show the same ratio. Since C-4 of the pyrimidine, derived from C-2 of pentose, accounts for 12% of the label, it follows that C-5, which in the minor pathway is derived from C-1 of pentose, must account for 12 × 1.2 = 14% of the label. Since C-5, in fact, contains 36% of the label of the intact pyrimidine unit, 36 − 14 = 22% of the label at this site must be due to incorporation in the course of the major pathway. This result complements the conclusion from the experiment with [1,3-^{14}C]glycerol that an intact triose unit, representing C-3,-4,-5 of pentose, accounts for the C_3 moiety, C-6,-5,-5′, of the pyrimidine unit.

The inferences drawn from the nine trace experiments, with formate (experiments 22 and 23), hexoses (experiments 15–19), and glycerol (experiments 20 and 21), on the derivation of the pyrimidine moiety of thiamin in yeast are summarized in Scheme IX. There are two routes to this moiety, one major and the other minor, under the conditions of our experiments. In the major route, formate serves as the source of C-4 of the pyrimidine moiety. The carbon atoms C-2′, C-2, C-6, C-5, and C-5′ are derived,

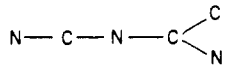
respectively, from the five carbon atoms C-1, C-2, C-3, C-4, and C-5 of a pentose. It is likely that the C_2 fragment, C-1,-2, of the pentose yields the C_2 fragment, C-2′,-2, of the pyrimidine and that the C_3 fragment, C-3,-4,-5, of the pentose yields the C_3 fragment, C-6,-5,-5′, of the pyrimidine, as an intact unit. In the minor route, formate serves as the source of C-2 of the pyrimidine moiety. The C_2 unit, C-5,-4, of the pyrimidine is derived, as an intact unit, from C-1,-2 of a pentose. The origin, in this minor route, of C-2′, C-5′, and C-6 of the pyrimidine unit remains unknown.

It is noteworthy that neither of these pathways is similar to that shown to occur in bacteria, which utilizes glycine as the source of two carbon atoms, C-6 and C-4, of the pyrimidine skeleton.

The biosynthetic anatomy of the two pathways to the pyrimidine unit to thiamin in yeast is very similar even though the incorporation pattern of label from various precursors is markedly different.

In each pathway, a C_2 unit, derived from C-1,-2 of a pentose, is linked, via the carbon atom originating from C-2 of the pentose, to two nitrogen atoms, one of which is attached, in turn, to a carbon atom originating from formate. This carbon atom is joined to yet another nitrogen atom.

It is tempting to speculate that the same unit, of partial structure



participates in the construction of the pyrimidine by both pathways and that this unit originates intact from a precursor which contains it as part of its skeleton. A molecule which fits the bill is 5-aminoimidazole ribonucleotide (AIR) (**2**), the established precursor of the pyrimidine unit of thiamin in bacteria. The structural correspondence of AIR to the pyrimidine unit is shown in Scheme X. The mode of cleavage of AIR which would yield the common C_3N_3 fragment corresponds to that now discarded but originally postulated to rationalize the entry of formate and glycine into the pyrimidine unit of thiamin in *S. typhimurium*.[33]

The origin of AIR in bacteria, from formate and glycine, and its status as a common intermediate in purine biosynthesis and in the biosynthesis of the pyrimidine unit of thiamin are well-established (vide supra). If AIR were to be an intermediate in the same two processes also in yeast, its biosynthesis as well as that of the purine skeleton would have to differ markedly from that established to occur in bacterial systems since glycine cannot be involved as a precursor. While it is generally assumed that purine biosynthesis in yeast follows a pathway similar to that established in bacteria,[48] and glycine appears to be implicated,[49-51] it should be noted that the results of investigations of purine biosynthesis in yeast, which have been reported, are incomplete and therefore not conclusive.

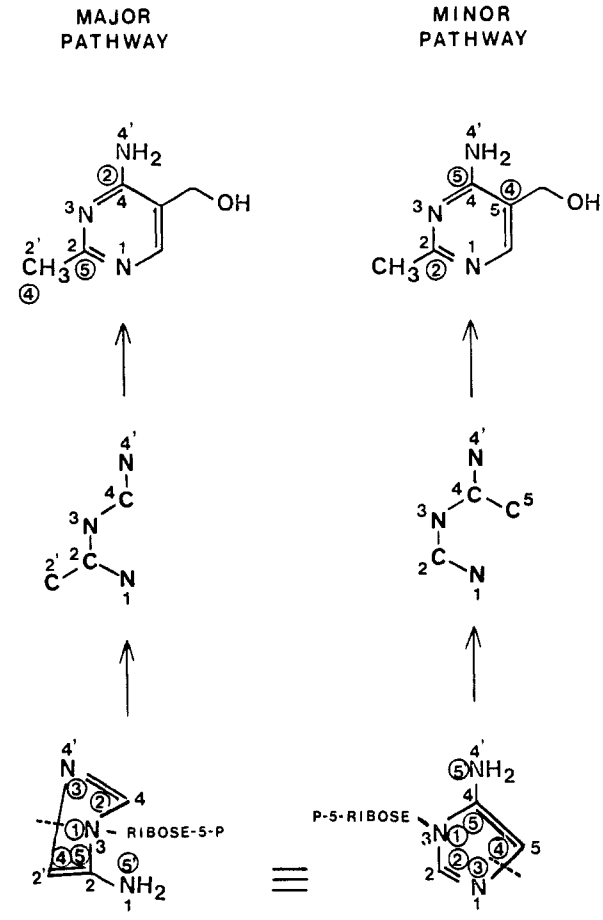
Experimental

Materials. Microorganism. *Saccharomyces cerevisiae* (ATCC 24903) was maintained on malt extract/yeast extract/peptone/glucose slants as described previously.[4]

Media. In one of the experiments with labeled formate (experiment 22) (see Table I), the glucose-based thiamin-free medium, previously described,[4] was used.

In the experiments with labeled glucose (experiments 15 and 17–19), fructose (experiment 16), glycerol (experiments 20 and 21), citrulline (experiment 24), urea (experiment 25), and one of the experiments with labeled formate (experiment 23), a medium was used which contained neither thiamin nor glucose but used ethanol as the general carbon source. Details have previously been reported.[5]

Scheme X. Biogenetic Anatomy of the Pyrimidine Unit of Thiamin[a]



[a] Plain numbers refer to the numbering system of the pyrimidine skeleton; circled numbers refer to the numbering system of the imidazole skeleton. In each case C-circled 2 is derived from formate; C-circled 4, circled 5 is derived from C-1,-2 of glucose, presumably via C-1,-2 of a pentose.

Methods. Procedures for Growth and Administration of Tracer. The procedures employed in the experiments using glucose-based[4] and ethanol-based[5] media have been reported.

Isolation of Thiamin. Details of the methods for centrifugation and extraction of the yeast cells and for the isolation of thiamin have been described previously.[4]

Chemical Degradation of Thiamin (see Scheme II). Separation of the Pyrimidine and Thiazole Moieties by Bisulfite Cleavage. Thiamin was cleaved to (4-amino-2-methylpyrimidin-5-yl)methanesulfonic acid (**7**) and 5-(2-hydroxyethyl)-4-methylthiazole (**8**) by a published procedure.[4]

Degradation of the Pyrimidine Moiety, i.e., of (4-Amino-2-methylpyrimidin-5-yl)methanesulfonic Acid (7). (i) Methylation of (4-Amino-2-methylpyrimidin-5-yl)methanesulfonic Acid (7) with Dimethyl Sulfate (Cf. Reference 52). Potassium carbonate was added to (4-amino-2-methylpyrimidin-5-yl)methanesulfonic acid (**7**) (150 mg) in water (1.0 mL) until a homogeneous solution was obtained at pH ca. 8. Dimethyl sulfate (135 mg) was added, and the two phases were mixed by shaking. After ca. 20 min, a white precipitate formed and the mixture was left for 17 h at 24 °C and 6 h at 5 °C. The wet precipitate obtained from centrifugation was recrystallized from water: yield of (4-amino-1,2-dimethylpyrimidinium-5-yl)methanesulfonate (**9**), 96 mg; ^1H NMR δ (^2H_2O, with 3-(trimethylsilyl)propanoic acid sodium salt (TSPA) as internal reference δ 0.00) 2.67 (s, 3 H), 3.90 (s, 3 H), 4.21 (s, 2 H), 8.22 (s, 1 H). Anal. Calcd for $C_7H_{11}N_3O_3S$: C, 38.70; H, 5.10; N, 19.34; S, 14.76%. Found: C, 38.66, H, 5.13; N, 19.19; S, 14.89.

(ii) (4-Hydroxy-1,2-dimethylpyrimidinium-5-yl)methanesulfonate (10) (Cf. Reference 31). (4-Amino-1,2-dimethylpyrimidinium-5-yl)-methanesulfonate (**9**) (93 mg) was added to the mother liquors from the above recrystallizations and the mixture was evaporated to dryness in vacuo. Hydrochloric acid (6 M, 4 mL) was added, and the mixture was refluxed 24 h. Solvent was removed in vacuo, water (2 mL) was added, and again the solvent was evaporated in vacuo. Crude (4-hydroxy-1,2-dimethylpyrimidinium-5-yl)methanesulfonate (**10**), thus obtained, was

(48) De Robichon-Szulmajster, H.; Surdin-Kerjan, Y. In "The Yeasts"; Rose, A. H., Harrison, J. S., Eds.; Academic Press: New York, 1971; Vol. 2, p 338.
(49) Abrams, R.; Hammarsten, E.; Shemin, D. *J. Biol. Chem.* **1948**, *173*, 429–430.
(50) Abrams, R. *J. Am. Chem. Soc.* **1951**, *73*, 1888–1889.
(51) Edmonds, M.; Delluva, A. M.; Wilson, D. W. *J. Biol. Chem.* **1952**, *197*, 251–259.

used for degradation, without further purification (see (iii), below).

A pure sample of (4-hydroxy-1,2-dimethylpyrimidinium-5-yl)-methanesulfonate (**10**) was obtained by passing a solution (3 mL) of the above crude material through a column of Dowex 50W-X4 (H^+, 6 mL) followed by water (10 mL). The eluate was evaporated to dryness and the solid residue recrystallized from water/methanol: yield ~40 mg; slightly hygroscopic; mp 328–338 °C dec; ^1H NMR δ (^2H_2O/TSPA) 2.66 (s, 3 H), 3.81 (s, 3 H), 4.07 (s, 2 H), 8.07 (s, 1 H). Anal. Calcd for $C_7H_{10}N_2O_4S$: C, 38.53; H, 4.62; N, 12.84; S, 14.69%. Found: C, 38.76; H, 4.61; N, 12.64; S, 14.58.

(iii) Cleavage of (4-Hydroxy-1,2-dimethylpyrimidinium-5-yl)-methanesulfonate (10). The crude (4-hydroxy-1,2-dimethylpyrimidinium-5-yl)methanesulfonate was refluxed 2 h with 5 M sodium hydroxide (2.5 mL). After the solution was cooled to 20 °C water (10 mL) was added and the solution was applied to a column of Dowex 50W-X4 (H^+, 12 mL). The column was further eluted with water (15 mL), and the total eluate was distilled at 1 atm until approximately 5 mL remained in the distillation flask.

The distillate was neutralized with dilute sodium hydroxide to pH 8–9, and the solution was divided into two portions in a ratio 1:2. Each portion was separately evaporated to dryness in vacuo. The former portion was kept for the isolation of formic acid and acetic acid (see (iv), below), and the latter portion was kept for a Schmidt reaction on acetic acid (see (vi), below).

The solution remaining in the distillation flask was evaporated to dryness in vacuo. The residue was dissolved in water (10.0 mL) and divided into three portions in a ratio of 1.5:2.5:6.0 mL. The first portion was neutralized with dilute sodium hydroxide to pH 8–9, evaporated to dryness, and kept for the isolation of *N*-phenyl-3-sulfopropanoamide ammonium salt (**12**) (see (vii), below). The second portion was evaporated to dryness in vacuo and kept for isolation of 2-aminoethane-1-sulfonic acid (**13**) (see (viii), below). The last portion was evaporated to dryness in vacuo and kept for isolation of formaldehyde (see (ix), below).

(iv and v) Isolation of Formic Acid (C-6) and Acetic Acid (C-2′,-2). The portion kept for the isolation of formic acid and acetic acid (see (iii) above) was dissolved in water (1.5 mL), and 1-naphthylamine hydrochloride (75 mg) and *N*-ethyl-*N*′-(3-(dimethylamino)propyl)carbodiimide hydrochloride (130 mg) were added. The mixture was stirred for 15 min and then kept for 2 h at 23 °C. After addition of 1 M HCl (1.0 mL), the mixture was extracted with ether (3 × 2 mL). The ether extracts were dried over anhydrous sodium sulfate and applied to a column of silica gel 40 (70–230 mesh, 15 × 1 cm) and eluted with ether. The eluate was continuously monitored with UV light (254 nm). The fractions containing *N*-formyl-1-naphthylamine (**16**) (fractions 9–12) and *N*-acetyl-1-naphthylamine (**15**) (fractions 15–20) were collected separately and evaporated to dryness in vacuo.

N-Formyl-1-naphthylamine was recrystallized from benzene/petroleum ether (bp 30–60 °C): yield, 10 mg; mp 140 °C [lit.[53] mp 141–142 °C]; MS, *m/e* 171 (100%), 170 (1%), 143 (96%), 115 (81%).

N-Acetyl-1-naphthylamine was recrystallized from benzene/petroleum ether (bp 30–60 °C): yield, 17 mg; mp 160 °C [lit.[54] mp 159–160 °C].

(vi) C-2′ by Schmidt Reaction on Acetic Acid. Concentrated sulfuric acid (2.5 mL) and sodium azide (450 mg) were added to the portion of acetic acid which had been kept for a Schmidt reaction (see (iii), above). The mixture was heated 2 h on a steam bath and then cooled in ice–water. Water (20 mL) was added, followed by solid sodium hydroxide, until the pH was ca. 13. Approximately half of the mixture was distilled into 1 M hydrochloric acid (1 mL). This acidified distillate was evaporated to dryness in vacuo and the residue dissolved in water (1.5 mL). The pH was adjusted to ca. 9 with solid sodium carbonate. *N*-Carbethoxyphthalimide (100 mg) was added, and the mixture was stirred for ca. 20 min. The mixture was centrifuged, and the precipitate was washed with water (0.5 mL) and sublimed at 55 °C/0.025 mmHg. (At 60 °C/0.025 mmHg, the sublimate was found to contain significant amounts of phthalimide, not always removable by recrystallization.) The sublimate of *N*-methylphthalimide (**17**) was recrystallized from methanol/water: yield, 5 mg; mp 133–134 °C [lit.[55] mp 134 °C].

(vii) *N*-Phenyl-3-sulfopropanoamide Ammonium Salt (12) (C-4,-5,-5′). The portion kept for the isolation of *N*-phenyl-3-sulfopropanoamide ammonium salt (see (iii), above) was dissolved in water (0.4 mL). Aniline hydrochloride (30 mg) and *N*-methyl-*N*′-(3′-(dimethylamino)propyl)-carbodiimide hydrochloride (90 mg) were added, and the mixture was kept at 23 °C for 1 h before application to a column of Dowex 50W-X4 (H^+, 5 mL). The column was eluted with water (20 mL) and the eluate was evaporated to dryness in vacuo. The residue was chromatographed on a column of silica gel 40 (70–230 mesh) (25 × 1 cm) with absolute ethanol and continuous monitoring of the effluent at 254 nm. The fractions containing *N*-phenyl-3-sulfopropanoamide were collected and evaporated to dryness in vacuo. The residue was dissolved in water (1 mL) and applied to a column of Dowex 50W-X4 (H^+, 1 mL). The column was eluted with water (5 mL), and the total eluate was evaporated to dryness in vacuo. The residue was dissolved in ethanol (1 mL), and concentrated ammonium hydroxide (0.1 mL) was added. The mixture was centrifuged.

The supernate was evaporated to dryness in a stream of nitrogen, and the residue was recrystallized from ethanol/ethyl acetate: yield, 8 mg; mp 202–203 °C. Anal. Calcd for $C_9H_{14}N_2O_4S$: C, 43.89; H, 5.73; N, 11.37; S, 13.02%. Found: C, 44.02; H, 5.85; N, 11.41; S, 13.20.

(viii) 2-Aminoethane-1-sulfonic Acid (Taurine) (13) (C-5,-5′). The portion kept for the isolation of 2-aminoethanesulfonic acid (see (iii), above) was dissolved in concentrated sulfuric acid (0.6 mL), and sodium azide (170 mg) was added. The mixture was heated on a steam bath for 2.5 h. After the solution was cooled, water (10 mL) was added, and the mixture was applied to a column of Dowex 2×8-100 (CH_3COO^-, 20 mL). The column was eluted with water (40 mL), and the total eluate was collected and evaporated to dryness in vacuo. The residue was dissolved in water (1 mL) and applied to a column of Dowex 50W-X4 (H^+, 5 mL). The column was eluted with water (10 mL) and the total eluate was evaporated to dryness in vacuo. The residue was recrystallized from water/ethanol: yield, 7 mg; mp 325–330 °C dec, [lit.[56] mp 317 °C dec].

(ix) Degradation of 3-Sulfopropanoic Acid to Formaldehyde (C-5′). The portion kept for the isolation of formaldehyde (see (iii), above) was pyrolyzed at 200 °C for 1 h (cf. ref 46). Acrylic acid and water were collected in a trap cooled with ice/water. No other products were observed by ^1H NMR spectroscopy. The acrylic acid/water mixture was diluted with water to 0.5 mL. Osmium tetroxide (2 mg) and sodium periodate (58 mg) were added, and the homogeneous mixture was kept 1 h at 23 °C. The reaction mixture, containing equimolar amounts of formate and formaldehyde (hydrate), as observed by ^1H NMR, was solidified by brief immersion in liquid nitrogen and sublimed into a liquid nitrogen cooled trap at 0.05 mmHg. The sublimate was allowed to warm up to room temperature, and the resulting solution was added to a solution of 5,5-dimethylcyclohexane-1,3-dione (32 mg) in water (7 mL). The pH was adjusted to ca. 5 with sodium hydrogen carbonate. After 1 h, the precipitate was isolated, dissolved in ethanol (1 mL), and filtered through activated charcoal and Celite. The filtrate was evaporated to dryness in vacuo, and formaldehyde dimethone (**18**) was recrystallized from ethanol/water: yield, 5 mg; mp 191–192 °C [lit.[57] mp 189 °C].

Determination of the Rate of Exchange of ^1H and ^2H at C-2′ (Scheme III). Solutions of (4-amino-2-methylpyrimidin-5-yl)methanesulfonic acid (**7**), (4-hydroxy-2-methylpyrimidin-5-yl)methanesulfonic acid (**19**), [^1H NMR δ (^2H_2O/TSPA) 2.69 (s, 3 H), 4.90 (s, 2 H), 8.12 (s, 1 H)], or (2′,2′,2′-$^2H_3$)-(4-hydroxy-2-methylpyrimidin-5-yl)methanesulfonic acid (**20**) (0.6 M in 6 M HCl/H_2O or in 6 M ^{2}HCl/^2H_2O (see Scheme III)) were kept at 100 °C, and the time, $t_{1/2}$, at which equimolar amounts of starting material and product were present was determined by ^1H NMR spectroscopy.

Synthesis of 3-Sulfo[3-^{14}C]propanoic Acid (11) (Cf. Reference 58) (Scheme IV). [3-^{14}C]Propanoic acid sodium salt (New England Nuclear, 6.3 mCi/mmol, 1 μCi) was mixed with propanoic acid (0.74 mL, 10 mmol) and sulfuryl chloride (1.11 mL) in a flask fitted with reflux condenser and thermometer. The mixture was irradiated with a 200-W filament lamp, and the temperature in the reaction mixture was kept at 55 °C. After 7 h, the reaction mixture was allowed to cool to room temperature, benzene/petroleum ether (bp 30–60 °C), 2:8 (3 mL) was added, and the precipitate was washed with the same mixture (2 × 3 mL). Finally the precipitate was washed with benzene (3 mL), dissolved in water, and evaporated in vacuo to give an oily sample of wet 3-sulfo-[3-^{14}C]propanoic acid, which was used in the degradation without further purification (see Table II and (ix), above). ^1H NMR spectroscopy showed only signals due to 3-sulfopropanoic acid.[46]

Cleavage of (4-Hydroxy-1,2-dimethylpyrimidinium-5-yl)methanesulfonate (10) in Deuteriated Solvent. A solution of (4-hydroxy-1,2-dimethylpyrimidinium-5-yl)methanesulfonate (32 mg) and sodium hy-

(52) Zoltewicz, J. A.; Baugh, T. D. *Synthesis* **1980**, 217–218.
(53) Ojima, I.; Inaba, S.; Nagai, Y. *Tetrahedron Lett.* **1973**, 4363–4366.
(54) Leete, E.; Gregory, H.; Gros, E. G. *J. Am. Chem. Soc.* **1965**, *87*, 3475–3479.
(55) Wanag, G.; Veinbergs, A. *Ber. Dtsch. Chem. Ges. B* **1942**, *B75*, 1558–1569.
(56) Heath, R. L.; Piggott, H. A. *J. Chem. Soc.* **1947**, 1481–1485.
(57) Dawson, R. M. C.; Elliot, D. C.; Elliot, W. H.; Jones, K. M. "Data for Biochemical Research", 2nd ed.; Oxford University Press: Oxford, 1969; p 79.
(58) Kharasch, M. S.; Chao, T. H.; Brown, H. C. *J. Am. Chem. Soc.* **1940**, *62*, 2393–2397.

droxide (149 mg) in ^2H_2O (0.75 mL, 99% ^2H) was heated on a steam bath for 2 h. The hydrogen atoms at C-2′ and C-5′ exchange in less than 1 min under these conditions. The mixture was worked up as described above (see (iii) and (iv)), and *N*-formyl-1-naphthylamine (**16**) was isolated: MS, *m/e* 172 (100%), 171 (10%), 144 (91%), 115 (56%). Thus, the sample had ca. 92 atom % ^2H in the formyl position.

Radioactivity Measurements. Radioactivity was assayed by liquid scintillation counting (Beckman LS 9000 Liquid Scintillation System). All samples were recrystallized to constant specific activity, dissolved in water, if necessary, and dispersed in Aquasol (New England Nuclear) and counted in triplicate under comparable conditions of quenching. Confidence limits shown in the tables are standard deviations from the mean.

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Chemoenzymatic Syntheses of Fructose-Modified Sucroses via Multienzyme Systems. Some Topographical Aspects of the Binding of Sucrose to a Sucrose Carrier Protein

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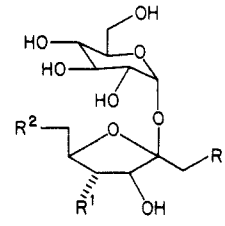
Abstract: 1′-Azido-1′-deoxysucrose (**3**) was synthesized by a sucrose synthetase mediated coupling of 1-azido-1-deoxyfructose with UDP-glucose. 6′-Deoxy- (**7**) and 6′-deoxy-6′-fluorosucrose (**8**) were prepared from the corresponding 6-substituted glucose and UDP-glucose by using an enzyme-couple consisting of glucose isomerase and sucrose synthetase. 4′-Deoxy-4′-fluorosucrose (**13**) was also prepared as above with 4-deoxy-4-fluorofructose (**14**). Fructose **14** was prepared from 4-deoxy-4-fluoroglucose

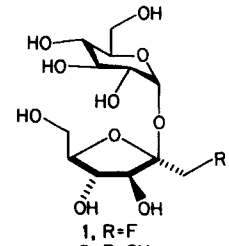


3, R=N_3, R^1=R^2=OH
7, R^2=H, R=R^1=OH
8, R^2=F, R=R^1=OH
13, R^1=F, R=R^2=OH

by conversion to 4-deoxy-4-fluorofructose 1,6-bisphosphate via a three-enzyme-couple consisting of hexokinase, phosphoglucose isomerase, and fructose 6-phosphate kinase. The bisphosphate was hydrolyzed to **14** by use of an alkaline phosphatase. The binding of these sucroses to a sucrose carrier protein is discussed in terms of the topographical surface which sucrose presents to the protein for binding.

Sucrose is the major form of transported carbon in many plant species and is actively transported across cell membranes in several tissue types.[1,2] The carrier protein responsible for this transport is quite specific for sucrose. Using monosaccharides or other disaccharides as competitive substrate inhibitors, workers using several tissue types have found only very weak competition with a very limited number of disaccharides.[3,4] This specificity suggests that either a large portion of the recognition of sucrose by the carrier protein lies with the fructose moiety or that elements of the glucose and fructose rings are involved in binding. To determine the important topographical binding regions of sucrose and the nature of that binding, we have prepared certain modified sucroses and assayed their binding to, and transport by, a sucrose carrier protein. In this paper we report on chemoenzymatic routes to sucroses in which the fructose moiety has been modified.

1′-Deoxy-1′-fluorosucrose (**1**) is an invertase hydrolysis resistant sucrose analogue that we synthesized for use in transport studies were extracellular invertase may exist.[5] While assaying the



1, R=F
2, R=OH
3, R=N_3

transport properties of **1**, we found that **1** was bound by the carrier protein 2 times stronger than natural sucrose (**2**) and that they were binding to the same carrier site in protoplasts derived from

(1) Giaquinta, R. T. "The Biochemistry of Plants"; Preiss, J., Ed.; Academic Press: New York, 1980.
(2) Lichtner, F. T. Spanswick, R. M. *Plant Physiol.* **1981**, *68*, 693.
(3) Giaquinta, R. T. *Plant Physiol.* **1977**, *59*, 750.
(4) Schmitt, M. R.; Hitz, W. D.; Lin, W.; Giaquinta, R. T. *Plant Physiol.* **1984**, *75*, 941.
(5) Card, P. J.; Hitz, W. D. *J. Am. Chem. Soc.* **1984**, *106*, 5348.

0002-7863/86/1508-0158$01.50/0