dependent on crystallization conditions.

Layered crystals were only obtained from racemic, or close to racemic, solutions. Solutions that contained an appreciable enantiomeric excess, ~ 20 percent, deposited optically pure hexahelicene crystals. This result is due to the well-documented (*15*) solubility difference between pure enantiomers and racemic mixtures of conglomerates, a difference that is of practical use in the preparative resolution of hexahelicene by repeated crystallization after use of TAPA (*6*).

It has usually been taken for granted that crystallization in a chiral space group is sufficient to allow resolution of enantiomers if the crystals are sorted according to a chirality observation such as sign of optical rotation. The hexahelicene case exemplifies a situation that may have been overlooked in other systems as well, where crystals grow as nearly racemic mixtures of enantiomers and are indistinguishable from genuinely chiral material as measured by standard x-ray diffractometry.

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7. Crystals of the same substance often occur in aggregates; when individuals are in a definite geometric relation to each other, such crystals are said to be twinned. The term is used when two or a symmetry-dependent number of individuals are aggregated.
8. We determined the enantiomeric purities of hexahelicene by polarimetry, using known weights, or by high-performance liquid chromatography, using the TAPA columns developed by Gil-Av and his co-workers [F. Mikes, G. Boshart, E. Gil-Av, *J. Chromatogr.* **122**, 205 (1976); Y. H. Kim, A. Tishbee, E. Gil-Av, in preparation].
9. Layers were cleaved under a light microscope with an ordinary surgical blade after partial dissolution of the crystals. The brittleness of hexahelicene made it difficult to cleave many consecutive layers.
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12. In the only case, to the best of our knowledge, of lamellar twinning that has been described in the literature (*10*), a similar rationale can be made on the basis of molecular packing. In the crystal structure of β-phenylglyceric acid (*13*), planes parallel to (0,0,1) separate the molecules into stacks; the "chiral interactions" are only between molecules in the same stack. The twin plane is indeed (0,0,1) (*10*). [Different conventions have been chosen in (*10*) and (*13*) to define *a*- and *c*-axes.]
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14. Recent calculations by S. Ramdas, J. M. Thomas, and C. J. Eckardt (personal communication) on the hexahelicene crystals demonstrate that the interfacial energy associated with twinning across the (1,0,0) plane is much lower than the energy for twinning across the (0,1,0), (0,0,1), (1,1,0), (1,0,1), or (0,1,1) planes.
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Incorporation of 4-Amino-5-Hydroxymethylpyrimidine into Thiamine by Microorganisms

Abstract. *One possible route for the biosynthesis of the (4-amino-2-methyl-5-pyrimidyl)-methyl moiety of thiamine would involve the formation of a methyl group on the demethylated pyrimidine, 4-amino-5-hydroxymethylpyrimidine, before its incorporation into thiamine. This possibility was tested by preparing the 4-amino-5-hydroxymethylpyrimidine and feeding it to* Escherichia coli, Bacillus subtilis, *and* Saccharomyces cerevisiae. *Analysis of the thiamine produced by these organisms showed that 4-amino-5-hydroxymethylpyrimidine was readily incorporated into thiamine without the addition of a methyl group, and no evidence was found for the conversion of this pyrimidine into normal methylated pyrimidine. Substitution of the demethylated thiamine for thiamine had no effect on the growth rate or the yield of* E. coli *cells. Complete substitution of the thiamine with the (4-amino-5-pyrimidyl)-methyl moiety was possible in an* E. coli *pur I mutant. The extent of incorporation of the demethylated pyrimidine decreased in some organisms and increased in others by the addition of adenine to the growth medium; this difference led to a simple test to separate organisms that use 5-aminoimidazole ribonucleotide for the biosynthesis of thiamine pyrimidine from those that do not.*

Early studies on the incorporation of radioactive precursors into the pyrimidine moiety of thiamine showed that this pyrimidine was formed by a pathway different from that of other pyrimidines (*1, 2*). Studies of microbial genetics and of certain types of purine toxicity found in microorganisms have shown a connection between purine metabolism and the biosynthesis of thiamine (*3, 4*).

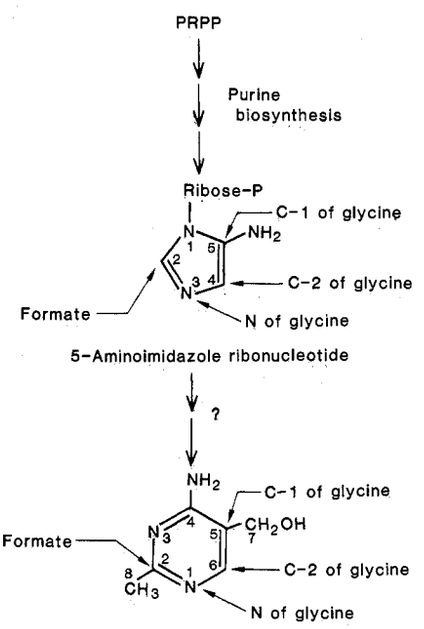

Fig. 1. Biosynthesis of the pyrimidine moiety of thiamine starting from phosphoribosyl pyrophosphate (*PRPP*).

Newell and Tucker (*5*) showed that, in *Salmonella typhimurium*, 5-aminoimidazole ribonucleotide, an intermediate in purine metabolism, is converted into the pyrimidine moiety of thiamine. Laboratory studies with stable isotope–labeled glycine confirmed these results (*6*) and showed how the conversion takes place in enteric bacteria (Fig. 1).

The problem in relation to 4-amino-5-hydroxymethyl-2-methylpyrimidine biosynthesis is to understand how the methyl group is formed at C-2 of the pyrimidine and how the two-carbon unit is inserted into the imidazole ring. One possible pathway is for the imidazole ring to expand to a pyrimidine, with the methyl group introduced as a last step. If this pathway is correct then 4-amino-5-hydroxymethylpyrimidine might function as a precursor; it is clear, however, from other work that if this pathway is correct, the introduced methyl group does not come from methionine (*1, 7, 8*). This hypothesis was tested by growing organisms with 4-amino-5-hydroxydideuteromethylpyrimidine. Conversion into the pyrimidine of thiamine was determined by gas chromatographic–mass spectrometric analysis of deuterium

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incorporation into 4-amino-5-ethylthiomethyl-2-methylpyrimidine, a product of the cleavage of thiamine with ethanethiol (*6*). When this was done for the thiamine produced by the organisms shown in Table 1, no indication of thiamine containing a deuterated 4-amino-2-methyl-5-pyrimidylmethyl moiety was found. However, 4-amino-5-ethylthiomethylpyrimidine was clearly present, indicating that the demethylated pyrimidine was being incorporated directly into the thiamine. That this pyrimidine had been incorporated into the thiamine was proved conclusively by isolating, dephosphorylating, and oxidizing the thiamine to the thiochrome (*9*). The resulting thiochrome and demethylthiochrome were then separated by thin-layer chromatography and quantified by their fluorescence. Reference thiochrome and demethylthiochrome were prepared as in (*10*). The required demethylthiamine was prepared from 4-amino-5-hydroxymethylpyrimidine as described for other thiamine analogs (*11*). The compounds had a relative mobility (R_F) of 0.29 and 0.24, respectively, on silica gel with a mixture of methanol and ethyl acetate (3:1) as solvent. These determinations showed that the demethylated pyrimidine was readily incorporated into thiamine without the methyl group.

That this demethylated thiamine was in fact functioning as a thiamine coenzyme in the cells was confirmed by work on the *Escherichia coli* K 12 pur I mutant. Since this mutant lacks phosphoribosylaminoimidazole synthetase, it requires both the pyrimidine moiety of thiamine and a purine for growth. Only the demethylated thiamine was found in cells grown with 4-amino-5-hydroxymethylpyrimidine (Table 1). Addition of this compound (0.5 to 100 μg/ml) gave log phase growth rates that are the same as those obtained with wild-type *E. coli* K 12 or the mutant grown with thiamine. The demethylated pyrimidine is metabolized in the same manner as the normal pyrimidine, as shown by the incorporation of the 4-amino-5-formylpyrimidine compound (Table 1) into thiamine. 4-Amino-5-formyl-2-methylpyrimidine has been implicated as a possible intermediate in the biosynthesis of the normal pyrimidine moiety of thiamine (*11*).

The data in Table 1 also establish that, at least for *E. coli* and *Bacillus subtilis*, a connection exists between purine metabolism and the biosynthesis of the pyrimidine moiety of thiamine. Addition of increasing amounts of adenine to the medium results in incorporation of increased amounts of 4-amino-5-hydroxymethylpyrimidine into the thiamine. Adenine, as a feedback inhibitor, inhibits the production of 5-aminoimidazole ribonucleotide, which occurs at the branch point for the pyrimidine biosynthesis. The cell simply makes up for the loss in the production of the methylated pyrimidine by using the demethylated pyrimidine. This argument is consistent with the well-established inhibition of purine biosynthesis in these organisms by adenine (*4*, *12*).

Purine biosynthesis in yeasts is also inhibited by adenine (*13*) but, in *Saccharomyces cerevisiae*, does not result in increased incorporation of 4-amino-5-hydroxymethylpyrimidine into thiamine (Table 1). This is consistent with the hypothesis that biosynthesis of the pyrimidine moiety of thiamine in yeast does not originate from an aminoimidazole ribonucleotide and is also consistent with results of incorporation studies, which showed that formate is incorporated into the C-4 of the pyrimidine in yeasts (*14*), whereas it is incorporated at C-2 in *E. coli* (*15*).

Table 1. Incorporation of 4-amino-5-hydroxymethylpyrimidine and 4-amino-5-formylpyrimidine into thiamine by microorganisms. The *E. coli* and *B. subtilis* strains were grown on 200 ml of a defined medium containing 1 g of glucose and 1 g of caseamino acids (*16*). The yeasts were grown on 400 ml of a medium containing glucose (20 g/liter) and caseamino acids (20 g/liter) substituted for the sucrose, thiamine, and Tween 40 of a previously described medium (*17*). The pyrimidines and adenine were added to give the indicated concentrations. 4-Amino-5-formylpyrimidine was prepared as follows: to a stirred solution of 1.2 g of sodium dissolved in 80 ml of methanol was added 5.2 g of formamidine acetate and then 6.1 g of ethoxymethylenemalononitrite. Within 30 seconds, there was a rapid crystallization of 4-amino-5-cyanopyrimidine, which was removed and crystallized from water to 3.3 g of a colorless solid [melting point, 250°C (*18*); M^+, 120 *m/z*]. One gram of this product was dissolved in 10 g of H_20 (D_2O) in which 1.6 g of acetyl chloride had been reacted and hydrogenated with H_2 (D_2) at 10 pounds per square inch with 600 mg of 5 percent palladium on carbon as a catalyst. The yield was 500 mg of 4-amino-5-formylpyrimidine (melting point, 188° to 190°C; M^+, 123 *m/z*). Reduction of 100 mg of this compound in 2 ml of methanol with 30 mg of $NaBH_4$ (D_4) gave 73 mg of 4-amino-5-hydroxymethylpyrimidine (melting point, 167° to 168°C; M^+, 125 *m/z*). The ratio of demethylthiochrome to thiochrome was determined from fluorescence intensity measurements of the separated thiochromes. The excitation maximum was at 372 nm and the emission maximum was at 433 nm for both compounds dissolved in methanol.

Organism/strain	Adenine-fed (μg/ml)	4-Amino-5-formylpyrimidine-fed (ng/ml)	4-Amino-5-hydroxymethylpyrimidine-fed (ng/ml)	Ratio of demethylthiochrome to thiochrome	Total thiamine (μg/g wet weight)
E. coli K 12 pur I	0.1		50	> 55	4.7
E. coli K 12 pur I	0.1	50		> 100	
E. coli K 12 wild type	0.2		50	0.88	2.8
E. coli B	0.0		50	0.14	1.1
E. coli B	0.1		50	0.50	
E. coli B	0.2		50	0.93	
E. coli B	0.4		50	1.42	0.69
E. coli B	0.2	50		0.48	
B. subtilis	0.0		50	2.20	0.80
B. subtilis	0.3		50	4.80	0.53
S. cerevisiae	0.0		50	1.71	0.60
S. cerevisiae	0.3		50	1.49	1.31

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