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THE BIOSYNTHESIS OF THIAMINE

A YEAST ENZYME SYSTEM WHICH CONVERTS 2-METHYL-4-AMINO-5-AMINOMETHYLPYRIMIDINE TO 2-METHYL-4-AMINO-5-HYDROXYMETHYLPYRIMIDINE*

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SUMMARY

1. An enzyme system obtained from baker's yeast converted 2-methyl-4 amino-5-aminomethylpyrimidine to 2-methyl-4-amino-5-hydroxymethylpyrimidine, a known intermediate in thiamine biosynthesis. The pH optimum of the system was 7.8, and the apparent K_m value for the substrate was $5 \cdot 10^{-5}$ M. The enzyme system was inhibited by 2-mercaptoethanol, pyridoxal phosphate, the amine oxidase inhibitors pargyline and tranylcypromine, and by various carbonyl reagents and chelating agents.

2. Enzyme systems catalyzing a similar reaction were found to be present in *Neurospora crassa* extracts and in a crude preparation of *Bacillus thiaminolyticus* thiaminase.

The biosynthetic pathway for the pyrimidine moiety of thiamine remains relatively obscure. Recent investigations have shown that aminoimidazoleriboside[1,2] and formate[3–5] are incorporated into the molecule. DIORIO AND LEWIN[6,7] demonstrated that 2-methyl-4-amino-5-aminomethylpyrimidine (henceforth called "aminomethylpyrimidine") and 2-methyl-4-amino-5-formylpyrimidine (henceforth called "formylpyrimidine") are formed by thiamine-requiring mutant strains of *Neurospora crassa*. They postulated that these might be intermediates in the formation of the 5-hydroxymethyl derivative, (henceforth referred to as "hydroxymethylpyrimidine"), a known intermediate in thiamine biosynthesis[6,7]. WEI AND LEWIN[8] demonstrated that cell-free preparations of yeast contain an enzyme system which converted formylpyrimidine to hydroxymethylpyrimidine. CAMIENER AND BROWN[9] incubated various substituted pyrimidines, including aminomethylpyrimidine, with cell-free extracts of baker's yeast and found a compound whose R_F value upon paper chromatography was that of hydroxymethylpyrimidine.

In this paper the properties of a cell-free enzyme system of yeast, which converts aminomethylpyrimidine to hydroxymethylpyrimidine, is described and discussed.

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The yeast cell-free extracts were prepared using a French Pressure cell (American Instrument Co., Silver Spring, Md., U.S.A.) as previously described[8], or by toluene autolysis and ammonium sulfate fractionation using the method of CAMIENER AND BROWN[9].

Typical incubation mixtures contained 50 μg of aminomethylpyrimidine, 0.2 ml of enzyme preparation, adjusted to a final volume of 1.0 ml with 0.1 M Tris (hydroxymethylaminomethane) buffer (pH 7.4). Incubation was carried out for 3 h at 37°. The reaction was stopped either by immersing the incubation tubes in boiling water for 5–10 min or by addition of 0.1 ml of trichloroacetic acid (14%). Protein was removed by centrifugation and the supernatant was used either for bioautographic analysis or quantitative microbiological assay for hydroxymethylpyrimidine, as previously described[8, 10].

In initial experiments, extracts of Fleischmann's Baker's yeast, *N. crassa* strain thi2, or a crude preparation of *Bacillus thiaminolyticus* thiaminase I (kindly provided by Dr. R. L. Airth, University of Texas) were incubated with aminomethylpyrimidine for 3 h at 37°, and deproteinized aliquots were bioautographed using the test organisms, *Escherichia coli* strain M70-17 (kindly provided by Dr. J. Gots, University of Pennsylvania) and *Enterobacter aerogenes* strain PD-1 (a gift of Dr. B. Magasanik, Massachusetts Institute of Technology). All three preparations converted aminomethylpyrimidine to a compound whose R_F values by paper chromatography in 4 different solvent systems were identical to hydroxymethylpyrimidine and which satisfied the nutritional requirements of these two test organisms for the pyrimidine moiety of thiamine. Fleischmann's baker's yeast was used as the enzyme source in the remainder of this investigation because of its ready availability.

Further evidence that the product was hydroxymethylpyrimidine was obtained by using ultramicroqualitative analysis techniques in conjunction with bioautography[7]. The product demonstrated the positive Schotten–Baumann reaction and negative Hinsberg test, similar to hydroxymethylpyrimidine. The ultraviolet spectra of the product under both acidic and basic conditions corresponded with those of authentic hydroxymethylpyrimidine. On the basis of this chromatographic, spectrometric and nutritional information, it was concluded that hydroxymethylpyrimidine was synthesized from aminomethylpyrimidine by the yeast enzyme preparation.

The enzymatic nature of the conversion was established by demonstrating that a non-dialyzable, heat-labile (100° for 5–10 min) factor was required for the conversion.

Preliminary experiments established that cell-free yeast preparations, either derived from toluene autolysis or use of the French Pressure Cell, were both enzymatically active. Upon ammonium sulfate fractionation of the yeast autolyzate the activity was greatest in the fraction which precipitated between 50 and 60% saturation with $(NH_4)_2SO_4$. Upon chromatography with Sephadex gel columns (grades G-50, G-75, or G100) the enzyme activity was eluted at about the void volume, indicating a molecular weight greater than 200000. Sephadex filtration removed low-molecular weight materials related to thiamine, which supported growth of the test organisms. These compounds could also be removed from the enzyme preparation with charcoal.

Investigations were conducted to determine the optimum conditions for enzymatic production of hydroxymethylpyrimidine from aminomethylpyrimidine. The

system was active in a pH range of 7.0–8.5 with a pH optimum at 7.8 (see Fig. 1).

The amount of product formed was linear with time, under conditions of the assay, for at least 3 h and was also linear with respect to enzyme concentration with fixed substrate concentrations. The apparent K_m value of $5 \cdot 10^{-5}$ M for aminomethylpyrimidine was obtained (Fig. 2).

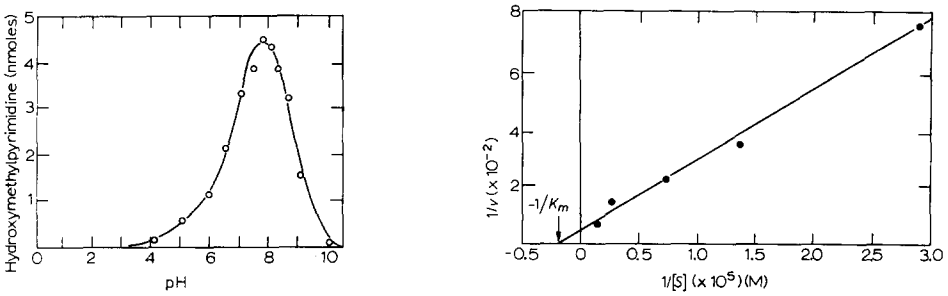

Fig. 1. Production of hydroxymethylpyrimidine from aminomethylpyrimidine as a function of pH. Incubation mixtures contained 50 μg of aminomethylpyrimidine, 0.2 ml of dialyzed enzyme preparation, and 0.1 M buffer (acetate buffers were used for pH values of 4.2–5.9, Tris buffers for pH values 6.5–8.5, and glycine buffers for pH 9–10) in a final volume of 1.0 ml. Following 3-h incubation at 37°, aliquots (50 μl) were spotted on Whatman No. 1 paper and chromatographed ascending in the solvent system *n*-butanol–acetic acid (glacial)–ethanol–water (200:24:10:50, by vol.). Areas corresponding to hydroxymethylpyrimidine were eluted, and the eluates assayed using *E. aerogenes* strain PD1.

Fig. 2. Lineweaver–Burk plot of conversion of aminomethylpyrimidine to hydroxymethylpyrimidine for determination of apparent K_m value for aminomethylpyrimidine. Incubation mixtures containing 0.2 ml of dialyzed enzyme, the above-indicated amounts of aminomethylpyrimidine ($7.2 \cdot 10^{-7}$–$7.2 \cdot 10^{-5}$ M) and 0.1 M Tris buffer (pH 7.4) in a final volume of 1.0 ml, were incubated for 3 h at 37° and chromatographed and assayed as described in Fig. 1, except that *E. coli* strain M70-17 was used as the test organism.

To investigate possible cofactor requirements, enzyme preparations were extensively dialyzed before incubation with or without addition of possible cofactors (Table I). None of the coenzymes which were tested appreciably increased the reaction rate, indicating that none of them were limiting factors in the reaction rate of the unsupplemented system, either because they were not required or because bound cofactors were not lost during dialysis or Sephadex treatment. Pyridoxal phosphate inhibited the reaction significantly (Table I).

Experiments using other classes of inhibitors (Table II) demonstrated significant effects by β-mercaptoethanol but not by cysteine at the concentrations used. *p*-Chloromercuribenzoate was also a relatively ineffective inhibitor. The amine oxidase inhibitors, pargyline and tranylcypromine, were effective inhibitors, as were carbonyl reagents and chelating agents (Table II). These findings are consistent with the proposal that an amine oxidase type of reaction is involved in the conversion of aminomethylpyrimidine to hydroxymethylpyrimidine[7]. Since formylpyrimidine is a postulated intermediate in this scheme, attempts were made to isolate it from the reaction mixtures and to identify it bioautographically. Experiments were conducted using sodium bisulfite as a trapping agent in incubation mixtures. Although sodium bisulfite was shown bioautographically to be an effective inhibitor of hydroxymethylpyrimidine production, there was no convincing evidence of the presence of the formylpyrimidine addition product in bioautograms of the incubation mixtures. If formylpyrimi-

TABLE I

THE EFFECT OF ADDITION OF VARIOUS COFACTORS TO DIALYZED ENZYME PREPARATIONS ON THE PRODUCTION OF HYDROXYMETHYLPYRIMIDINE

Possible cofactors, as shown, were added to reaction mixtures containing 50 μg aminomethylpyrimidine, 0.2 ml of dialyzed enzyme, 0.1 M Tris buffer (pH 7.4) in a final volume of 1.0 ml. Incubation was for 3 h at 37°. Aliquots (50 μl) of the reaction mixtures were chromatographed on Whatman No. 1 paper. Areas corresponding to hydroxymethylpyrimidine were cut out, eluted, and assayed using *E. coli* M70-17.

Expt.	*Addition*	*Amount* (*μmoles*)	*Incubation mixture* (*nmoles of product/ml*)
1	Control	—	3.9
	NAD^+	1.5	5.0
	NADH	1.5	4.0
	$NADP^+$	1.5	4.0
	FAD	0.3	3.4
	FMN	0.3	4.0
2	Control	—	3.9
	Mg^{2+}	3.0	3.7
	Cu^{2+}	3.0	3.9
3	Control	—	2.62
	Pyridoxal phosphate	25	2.44
	Pyridoxal phosphate	50	1.58
	Pyridoxal phosphate	100	0.36

TABLE II

THE EFFECTS OF VARIOUS SUBSTANCES AS INHIBITORS OF THE ENZYMATIC CONVERSION OF AMINOMETHYLPYRIMIDINE TO HYDROXYMETHYLPYRIMIDINE

The test compounds were added to incubation mixtures which were treated as described in Table I.

Expt.	*Inhibitor*	*Inhibitor concn.* (*M*)	*Hydroxymethylpyrimidine synthesized* (*ng/ml*)	*Inhibition* (%)
1	Isoniazide	$1 \cdot 10^{-4}$	333	87
	Tranylcypromide	$1 \cdot 10^{-4}$	560	78
	Pargyline	$1 \cdot 10^{-4}$	1210	53
	Sodium azide	$5 \cdot 10^{-4}$	1268	51
	8-Hydroxyquinoline	$1 \cdot 10^{-4}$	1295	50
	8-Hydroxyquinoline sulfate	$1 \cdot 10^{-4}$	1332	49
	Control	—	2600	0
2	Potassium cyanide	$1 \cdot 10^{-3}$	1268	36
	Semicarbazide	$1 \cdot 10^{-5}$	1069	44
	Hydroxylamine	$1 \cdot 10^{-4}$	826	57
	Phenylhydrazine	$1 \cdot 10^{-4}$	534	73
	Control	—	1974	0
3	Cysteine	$2 \cdot 10^{-3}$	600	0
	Control	—	560	—
4	β-Mercaptoethanol	$2 \cdot 10^{-2}$	44	94
	p-Chloromercuribenzoate	$2 \cdot 10^{-2}$	480	31
	Control	—	700	—

dine is an intermediate, it may have escaped detection either because it was present in very minute amounts or it may have been bound firmly to the enzyme.

The fact that enzyme systems converting aminomethylpyrimidine to hydroxymethylpyrimidine were found in cell-free preparations of baker's yeast, *N. crassa*, and a crude preparation of thiaminase I from *B. thiaminolyticus* lends support to the hypothesis that aminomethylpyrimidine might be an intermediate in the hydroxymethylpyrimidine biosynthesis. The apparent K_m value of $5 \cdot 10^{-5}$ M was sufficiently low to indicate that this reaction might be physiologically significant. The parthway proposed by DIORIO AND LEWIN[7] postulated an oxidation of aminomethylpyrimidine to formylpyrimidine, catalyzed by an amine oxidase type of reaction, followed by reduction of the formyl group to produce hydroxymethylpyrimidine. Our data indicate that the conversion of aminomethylpyrimidine to hydroxymethylpyrimidine is inhibited by amine oxidase inhibitors, including pargyline, tranylcypromine, carbonyl reagents, and chelating agents. Previous data from this laboratory has demonstrated that similar yeast cell-free systems are capable of reducing formylpyrimidine to hydroxymethylpyrimidine[8].

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