REQUIREMENT OF GTP FOR PTERIDINE SYNTHESIS IN SALMONELLA TYPHIMURIUM AND ITS INHIBITION BY AMP*

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We have been studying a number of mutants of Salmonella typhimurium whose growth is acutely inhibited by adenine. The growth patterns obtained and the types of agents that can relieve the inhibition has suggested that adenine impairs growth by decreasing the availability of single carbon units for various biosynthetic reactions. These considerations have led us into an analysis of folic acid biosynthesis in Salmonella and the possible effect of adenine derivatives as inhibitors. This paper deals with the first enzymes to be studied, those required for the conversion of a guanine derivative to a pteridine (Reynolds and Brown, 1964) and the condensation of reduced hydroxymethylpteridine with pABA to form dihydropteroic acid (Brown, 1959; Shiota, 1959).

The results show that the participating guanine congener is GTP and that its conversion to a pteridine is inhibited by adenine ribonucleotides. Shiota (personal communication) using Lactobacillus and Levenberg (1965) with Pseudomonas also showed that GTP is specifically required for pteridine formation.

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MATERIALS AND METHODS

The preparations and experiments were patterned after the work of Reynolds and Brown (1964). The bacteria used were Salmonella typhimurium, strain LT-2 and some adenine sensitive mutants (ads-) derived from it (obtained from M. Demerec). Cultures were grown in a minimal salts-glucose medium for 18 hours with aeration. The cells were harvested, washed twice with saline, suspended in phosphate buffer (0.03 M at pH 7.2), containing 2-mercaptoethanol (4 mM), ruptured by a MSE ultrasonic disintegrator and centrifuged at 30,000 X g for 1 hour. The clear extract was stirred with 5% acid washed Darco grade 20 x 40 for 20 minutes at 4 C and recentrifuged to remove the charcoal. The charcoal treated extract was then dialyzed twice, overnight at 4 C, each time against fresh tris buffer (0.03 M at pH 8.0) containing 2-mercaptoethanol (4 mM).

Enzyme studies (see Table I) were carried out at 37 C under paraffin oil in 11 x 100 mm test tubes. Folic acid compounds were determined by microbiological assay with Streptococcus faecalis (ATCC 8043) as the test organism and folic acid as the standard. Activity was expressed in terms of mμg of folate equivalents formed.

The folate compound formed was identified by paper ascending chromatography of an aliquot of the reaction mixture on Whatman No.3 MM paper using phosphate buffer (0.1 M at pH 8.0) as developing solvent and then visualized by tetrazolium bioautography (Usdin et al., 1954) with S. faecalis.

Reduced 2-amino-4-hydroxy-6-hydroxymethylpteridine (hydroxymethylpteridine) was prepared chemically from pteroylglutamic acid by the method of Waller et al. (1950) and purified by paper chromatography to remove all traces of pteroylglutamic acid. The hydroxymethylpteridine was reduced just before use with sodium borohydride and acid for 30 minutes and adjusting to pH 8.0.

RESULTS AND DISCUSSION

Synthesis of dihydropteroic acid by extracts of Salmonella showed the same requirements as the Escherichia coli system of Brown et al. (1961), viz, ATP, pABA, $MgCl_2$ and reduced hydroxymethylpteridine. Increase in ATP concentration to four times the optimal level did not inhibit synthesis. Previous growth in the presence of adenine did not repress the activity, nor was there any significant difference between the activities of the wild type strain and the adenine sensitive mutants. Attempts to replace the hydroxymethylpteridine with guanosine or GMP were unsuccessful, but GTP allowed detectable synthesis. The low activity with GTP eventually was found to be due to inhibition by ATP (Table III).

Table I

Two Step Conversion of GTP to Dihydropteroate

System	Additions to reaction mixture in		Total folate equivalents formed (mμg)
	Stage 1	Stage 2	
A	—	ATP + pABA + HMPt*	69.1
B	GTP	ATP + pABA	42.2
C	GTP + ATP	pABA	14.7
D	GTP + ATP + pABA	—	16.6
E	—	GTP + ATP + pABA	5.1
F	—	ATP + pABA	0
G	ATP	pABA	0
H	ATP + pABA	—	0
I	—	ATP + pABA	0

The reaction mixture contained: $MgCl_2$, 2.5 mM; potassium phosphate, 10 mM; $CuCl_2$, 5×10^{-4} mM; sodium ascorbate, 5 mM; and 2-mercaptoethanol, 5 mM in a total volume of 1 ml of 0.1 M tris buffer, pH 8.0. Incubation for the first stage was carried out for 2 hours with 0.2 ml cell-free extract (3.2 mg protein) and the reaction was stopped by heating to 100° for 1 minute. After cooling incubation for the second stage was carried out with 0.1 ml of fresh cell-free extract (1.6 mg protein) for a further 2 hours. GTP, 0.4 mM; reduced hydroxymethylpteridine (HMPt*), 0.4 mM; ATP, 1 mM, and pABA, 4 mM, were added as indicated above. 0.2 m moles of 2-mercaptoethanol was added and the reaction was stopped by immersing the tubes in a boiling water bath for 1 minute.

In order to study the conversion of GTP to hydroxymethylpteridine it was necessary to split the overall reaction into two stages. Preliminary experiments had shown that GTP was not converted to pteroate in the absence of either pABA or ATP. Incubation carried out in the absence of pABA should therefore presumably result only in the conversion of GTP to hydroxymethylpteridine which then could be converted to pteroate by the subsequent addition of pABA, ATP and fresh enzyme. The eight fold higher conversion obtained when GTP is added during the first stage (Table I, B vs E) indicates that GTP is indeed being converted to hydroxymethylpteridine during this stage and that ATP inhibits this conversion (Table I, B vs C).

The initial observation that GTP rather than guanosine, GMP or GDP is the most active precursor of hydroxymethylpteridine, is further borne out by data presented in Table II. Unlike the results obtained by Reynolds and Brown (1964) in E.coli, negligible folate activity is observable when either guanosine or GMP is substituted for GTP, and GDP gives only about 16% of the activity of GTP.

A comparison of ATP, AMP and adenosine as inhibitors is seen in Table III. AMP is the most efficient inhibitor of the three. It is unlikely that AMP acts by depleting GTP via transphosphorylation since chromatographic analysis showed that appreciable

Table II

Ability of Guanine Compounds to Form Hydroxymethylpteridine

Guanine compound	Folate equivalents formed (mμg/mg protein)
None	0
Guanosine	0
GMP	0.36
GDP	2.00
GTP	12.80

The reaction was carried out as in Table I system B. GTP was substituted by 0.4 mM of the guanine compound indicated which were added in Stage 1.

amounts of GTP remained after the reaction. The adenine sensitive mutants are inhibited to a slightly greater extent than the wild type strain LT-2 and the presence of relatively high concentrations (50 μg/ml) of adenine during growth of the mutants does not repress enzyme activity (Table III).

Table III

Inhibition of Formation of Hydroxymethylpteridine from GTP by Adenine Compounds

Adenine compounds added		Folate equivalents formed (mμg/mg protein in stage 1)				
Stage 1	Stage 2	LT-2	ads-2		ads-4	
			(a)	(b)	(a)	(b)
—	ATP	12.4	14.4	15.0	14.6	15.0
ATP	—	6.8	5.2	4.6	8.7	9.1
—	AMP	9.2	9.6	8.8	10.1	12.1
AMP	—	3.8	1.7	2.1	2.4	2.5
—	Adenosine	14.4	13.0	11.0	16.8	16.0
Adenosine	—	13.2	12.7	11.7	14.9	16.0

The reaction mixture was as in Table I. GTP, 0.4 mM was added in stage 1; ATP, 1 mM and pABA, 4 mM in stage 2. The adenine compounds, 1 mM were added as indicated. For adenine sensitive mutants: (a) indicates cells grown without supplementation. (b) indicates cells grown in medium containing adenine (50 μg/ml) and thiamine (2 μg/ml) to permit growth.

Table IV

Inhibition of Formation of Hydroxymethylpteridine from GTP by Different Concentrations of AMP

AMP concentration in stage 1	Folate equivalents formed (mμg/mg protein in stage 1)				
mM	LT-2		ads-4	ads-5	
	(a)	(b)	(a)	(a)	(b)
0	12.1	10.2	13.1	12.4	12.5
0.2	8.6	6.8	6.2	9.9	9.4
0.5	5.1	5.5	3.6	6.6	5.2
1.0	2.8	3.2	1.8	3.4	3.3
2.0	1.6	1.5	0.8	0.9	0.9

The reaction mixture was as in Table III. AMP was added in stage 1 as indicated, and in stage 2 to give a final concentration of 2.0 mM in all cases. For adenine sensitive mutants, (a) and (b) are as in Table III. For LT-2: (a) indicates cells grown without supplementation (b) indicates cells grown in 200 μg/ml adenine.

The inhibition produced by increasing concentrations of AMP can be seen in Table IV. The activity of the adenine sensitive mutant (ads-4) is greater than that of the wild type at almost all AMP concentrations. Though inhibition of growth of the wild type strain at high concentrations of adenine may be due to this type of enzyme inhibition, it is unlikely that the acute sensitivity of the mutants can be explained entirely on this basis. Other reactions of folic acid metabolism are being investigated for this purpose.

REFERENCES

Brown, G.M., Fed.Proc.18,19 (1959).
Brown, G. M., Weisman, R.A. and Molnar, D.A., J.Biol.Chem.236,2534 (1961).
Levenberg, B., Fed.Proc.24,669 (1965).
Reynolds, J.J. and Brown, G. M., J.Biol.Chem.239,317 (1964).
Shiota, T., Arch.Biochem.Biophys.80,155 (1959).
Usdin, E., Shockman, G.D. and Toennies, G., Appl.Microbiol.2,29 (1954).
Waller, C.W., Goldman, A.A., Angier, R.B., Boothe, J.H., Hutchings, B.L., Mowat, J.H. and Semb, J., J.Am.Chem.Soc.72,4630 (1950).