ENZYMATIC STUDY ON FOLIC ACID BIOSYNTHESIS BY *MYCOBACTERIUM AVIUM*

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The relationship between purine biosynthesis and the action mechanism of *p*-aminosalicylic acid (PAS), an antimetabolite of tubercle bacilli, has hitherto been studied by the authors and the following findings have been obtained:

(a) 4-amino-5-imidazole carboxamide (AICA) ribotide, an intermediary metabolite of purine biosynthesis, and glycine-amide ribotide, a precursor of AICA ribotide, accumulate in the culture media of *Mycobacterium avium*, partially inhibited by PAS (1, 2). (b) A bacteriostatic action of PAS is weakened by purine derivatives, nucleic acids, and derivatives or components of cotransformylase, *i.e.*, leucovorin, folic acid, *p*-aminobenzoylgutamate (PAG), and *p*-aminobenzoic acid (PABA) (1). (c) The accumulation of AICA derivatives fall by the above substances in spite of the existence of PAS, showing that PAS inhibits the biosynthesis of cotransformylase (3). (d) The biosynthetic pathway to cotransformylase is divided into two parts, *i.e.*, that from folic acid to leucovorin and that up to folic acid. The former pathway was tested with the cell-free extract of this bacterium under the same condition as was performed by Doctor (4) and Nichol (5) and it was found that leucovorin was synthesized from folic acid in the yield of 20 per cent by this bacterium and the pathway was not inhibited at all by PAS (6). (e) The pathway up to folic acid has been studied by Wright and Welch (7), Forest-Walker (8) and Tsesche *et al.* It is now being clarified enzymatically by the authors that the folic acid biosynthesis starts with the synthesis of PAG, followed by the combination with pteridine. In the synthesis of PAG, *i.e.*, peptide biosynthesis, a new finding was obtained concerning the activation of PABA by ATP and a mode of action by CoA. This mode of action is considered to be fairly universal in the systems in which CoA participates. It may also be considered to be a model experiment of protein synthesis. The mechanism of coupling with pteridine remains much to be settled.

Based upon these findings, the direct action point of PAS and sulfa drugs, as well as the mechanism of bacterial resistance, is now being studied by the authors.

EXPERIMENTAL

1. *Extraction of Enzymes*

Mycobacterium avium (Jucho strain) was inoculated in a medium consisting 2/3 volume of glycerol broth and 1/3 volume of Sauton medium and cultured for 3–4 days. The cells harvested were washed with Tris-HCl buffer and ground with an electric roll-mill for 3–4 hours at 0°, keeping the pH at about 7.2 by adding Tris-HCl buffer (pH 8.5). After centrifugation at 3000–4000 rpm for 5 minutes the supernatant is further centrifuged at 16000 rpm for 30 minutes with a refrigerated high-speed centrifuge. The supernatant thus obtained was lyophilized. The lyophilized powder is called cell-free enzyme. This powder was dissolved in Tris-buffer. It was adjusted to pH 8.0 and a 1/5 volume of Amberlite IRA-400 (Cl-form, 200–400 mesh) was added. The mixture is stirred for 3–5 minutes at below 0° and is immediately centrifuged. The supernatant is treated once more with the resin and then lyophilized. The powder thus obtained is called resin-treated enzyme I. By this procedure, apoenzymes largely freed from cofactors are obtained, whereby the majority of folic acid and PABA pre-existed in the cells are adsorbed and removed, the determination becoming thus very easy. The loss of protein was found to be about 20–30 per cent. The fractionation with acetone or ammonium sulfate proved to be unsuccessful due to denaturation of the enzyme.

2. *Conditions for Reactions*

p-Aminobenzoylglutamate Biosynthesis — 1.05 mM of magnesium chloride, 2.4 mM of ATP-Na-salt, 1.2 mM of PABA 2.4 mM of L-glutamate and 20 mg/ml of enzyme powder were added to Tris-buffer (pH 7.2), and the mixture was allowed to react for 3– hours at 35°.

Folic Acid Biosynthesis — 0.58 mM of PAG (or PABA) and 0.0558 mM of xanthopterin were added to Mg⁺⁺-containing Tris-buffer. When PABA was used, 1.16 mM of L-glutamate was added in addition. After reaction for 3 hours, the mixture was heated for 5 minutes at 100°. The supernatant was adjusted to ca. pH 8.5, an aliquot (1 ml) was passed through a resin column and determined fractionally.

p-Aminobenzoyl Hydroxamate Formation — 28 per cent solution of hydroxylamine-HCl salt was neutralized with 14 per cent NaOH solution up to pH 7.2, and the whole was added to Mg⁺⁺-containing Tris-buffer (pH 7.2) in a volume of 1/5. ATP and PABA were used. The reaction was continued for 30–90 minutes, followed by interruption with HCl or trichloroacetic acid (TCA). The hydroxamate was determined according to the Lipmann method (12).

3. *Determination Method*

PAG — The product, PAG, can be separated from the substrate, PABA, by paper chromatography, and be determined by Diazo reaction. The deproteinized solution is lyophilized, dissolved in a little amount of water (0.5 ml) and is developed with 5 per cent citrate-isoamyl alcohol according to Carter (9) or with acetic acid-butyl alcohol-water (1 : 4 : 2). *R_F* of PAG, and PABA are 0.80 and 0.68, respectively, with the former solvent, whereas 0.67 and 0.82

with the latter one.

After color reaction with Diazo reagent using *N*-ethyleneamine, PAG was eluted with 10 per cent TCA-acetone (25 : 75) and was determined photometrically at 540 m μ .

Folic Acid — Deproteinized solution was adjusted to pH 8.5 and was passed through an Amberlite IR-4B (Cl-form, 200–300 mesh) column. Xanthopterin was eluted with 0.25 *M* NaCl solution. After 30 ml being eluted it was made up to 0.70 *M*, whereby citrovorum factor (CF) was eluted. The elution of CF was completed after about 30 ml has been passed through. Then, it was made up to 1.0 *M*, whereby folic acid was eluted. Each fraction was made 5 ml and an adequate flow rate was found to be about 1 ml per 10 minutes. 0.1–0.2 ml from each fraction was mixed with 4 ml of assay medium (11). Using *Leuconostic citrovorum* and *Streptococcus faecalis*, the vitamin was estimated nephelometrically.

Hydroxamic Acid — Lipmann-Tuttle's method (12) was used.

Results

The pathway of folic acid biosynthesis was divided into two steps, *i.e.*, coupling of glutamate with PABA and folic acid synthesis from PAG and xanthopterin.

1. Biosynthesis of PAG

Little PAG was formed when the resin-treated enzyme was incubated with PABA, L-glutamate, ATP and Mg⁺⁺ but the formation increased to four times, when CoA was further added (Fig. 1). When boiled extract was used, the yield increased to two times, far behind that with CoA. The maximum yield was about 25 per cent of the PABA added. This peptide linkage formation, therefore, takes place in the existence of ATP, CoA and Mg⁺⁺. This reaction was strongly inhibited by pyrophosphoric acid as shown in the following experiment.

In the system shown in Table I, 1.2 mM of PABA, 2.4 mM of glutamate and

TABLE I
Biosynthesis of PAG

Reaction system consisting of
Enzyme : resin-treated 25 mg/ml.
Substrate : PABA, 730 μ M; L-glutamate 1460 μ M.
Cofactors : CoA (75% purity), 500 γ /ml; ATP, 4.2 mM.

Cofactor	Formed PAG
	μ M
—	52
Boiled extract	130
CoA	190

cell-free enzyme 1 were used and in addition, 0.6 mM of pyrophosphoric acid was added to a main test and an equimolecular concentration of *o*-phosphoric acid was added to a control test. After reaction, the formation of PAG in a main

FIG. 1 Formation of *p*-Aminobenzoyl CoA PABA, 2.5 and 0 mg/ml; ATP, 4.2 mM; CoA, 60 I.U./ml; IRA-400 resin-treated enzyme powder, 15 mg/ml in final concentration. After reaction hydroxylamine was added, whereby the active intermediate was converted to hydroxamate, which was photometrically determined after adding FeCl_3 .

test was 0.06 mM, whereas that in a control was 0.18 mM. It is suggested, therefore, that pyrophosphoric acid is liberated from ATP, whereby *p*-aminobenzoyl CoA is an active intermediate.

In the experiment shown in Fig. 1, sufficient amounts of CoA and ATP were added to the resin-treated enzyme and varying amounts of PABA were further added but without glutamate. After reaction, the active intermediate formed was determined as hydroxamate according to Lipmann, the amount of the active intermediate formed was found to be directly proportional to the amount of PABA added. It was presumed, therefore, that the hydroxamate formed was *p*-aminobenzoyl hydroxamate (PABH). Furthermore, judging from the reaction condition, it was con-

sidered to be derived from *p*-aminobenzoyl CoA. Next, 5.6 per cent solution of hydroxylamine was added to the reaction system, and a similar experiment was conducted and the hydroxamate which was formed in the reaction was acidified with HCl and TCA and the colored solution after addition of ferric chloride was determined photometrically. In this case the amount of hydroxamate was proportionally increased to that of PABA. Of course, remarkably more hydroxamate formation was observed when hydroxylamine was added in the reaction system.

In a system of PABA, hydroxylamine and ATP, the PABH formation was strongly inhibited by pyrophosphoric acid, as shown in Table II. As stated

TABLE II

Inhibition of PABH Formation by Pyrophosphoric Acid and AMP

PABA, 7.3 mM; ATP, 7.3 mM; hydroxylamine, 5.6 per cent; enzyme, (cell-free enzyme), 10.9 mg/ml; after reaction for 1.5 hr, acidified, FeCl_3 was added, and determined photometrically.

Pyrophosphate	AMP	Hydroxamate
—	—	0.055
+	—	0.000
—	+	0.040

above, the biosynthesis of PAG was strongly inhibited by pyrophosphoric

acid, and the affecting point of the inhibition is assumed from this experiment to be the formation of the active intermediate of PABH. A weak inhibition by AMP was also observed, suggesting that pyrophosphoric acid and AMP were liberated prior to PABH formation.

Then, the pyrophosphoric acid liberated was determined according to Kornberg (13) and the AMP formed was determined by the optical density at 255 m μ according to Umbreit (14), when PABH was formed in the reaction series which consists of PABA, ATP and hydroxylamine. As presented in Table III, the amounts increased of pyrophosphoric acid and AMP by the

TABLE III
Liberation of Pyrophosphate and AMP in PABH Formation
PABA, 7.4 mM; ATP-Na₂, 4 mg/ml. Pyrophosphate was removed by preliminary treatment with MnCl₂.
Enzyme, (cell-free enzyme) 17 mg/ml. Reaction for 1 hr.

PABA	Pyrophosphate	AMP	PABH (optical density)
	mM	mM	
+	0.6	1.172	0.30
—	0.3	0.884	0.07
Difference	0.3	0.288	

addition of PABA were 0.3 and 0.288 mM, respectively, indicating equimolecular liberation.

No pure synthetic PABH preparation being available in this experiment, PABH was expressed in the Table as optical densities estimated with Beckman Spectrophotometer.

From the above experiments, equimolecular amounts of pyrophosphoric acid and AMP were found to be liberated in the *p*-aminobenzoyl CoA formation, possibly in the following reactions.

When the enzyme is treated with resin, the majority of CoA is removed by adsorption, as can be expected from literature (15), and the production of PAG is reduced to 1/4 as mentioned above. Therefore, resin-treated enzyme was expected to produce less PABH in a system consisting of PABA, ATP and hydroxylamine than the enzyme rich in CoA. The result was however opposite, as shown in Table IV, *viz.*, the activity was increased rather than decreased. Further, the addition of ATP and PABA is indispensable and both pyrophosphoric acid and AMP are liberated almost equimolecularly at PABH formation. The active intermediate derived from PABA which is capable of combining with hydroxylamine is therefore considered to be *p*-aminobenzoyl-AMP.

Assuming that *p*-aminobenzoyl-AMP is produced, as shown in Fig. 2,

TABLE IV
PABH Formation with the Resin-Treated Enzyme
 PABA, 4 mg/ml; ATP- Na_2 , 2.5 mg/ml; hydroxylamine, 5.6 per cent.
 After reaction for 2 hr, acidified with HCl and TCA. Ferric chloride
 was added, and the color solution was determined photometrically.

Enzyme	PABA	ATP	PABH/enzyme (mg/ml)
Resin-treated enzyme 1	+	+	0.150
	—	+	0.080
	—	—	0.005
	+	—	0.085
Non-treated enzyme 1	+	+	0.130
	—	+	0.100
	—	—	0.010
	+	—	0.115

PABA is transferred from this intermediate, as well as from *p*-aminobenzoyl-CoA, to hydroxylamine, leading to PABH formation. The reason why PABH formation is not decreased even after removing CoA can thus be explained rationally. But the direct donor of active PABA, which forms PAG by transferring PABA to glutamate, must be *p*-aminobenzoyl-CoA, as clearly be seen from experimental data.

FIG. 2 Mechanism of PABH Formation

Therefore, the improved mechanism of this peptide formation, as presented in Fig. 3, is considered to be rational.

That the hydroxamate thus obtained is PABH, is clear from experimental results. The chemically synthesized PABH by treating PABA-methyl ester with hydroxylamine showed the same R_F with the enzymatic reaction product in paper chromatogram. The identification of the enzymatic reaction product with *p*-aminobenzoyl-AMP and further enzymatic tests of this intermediate are now in progress.

2. Folic Acid Biosynthesis

For folic acid microbioassay *Streptococcus faecalis* has hitherto been used but several folic acid derivatives occurring naturally have folic acid activity to the bacterium. The values determined with this bacterium are therefore

FIG. 3 Mechanism of PAG Formation

not the real values of folic acid alone. Xanthopterin has an activity of as low as about 10^{-4} of folic acid to *Streptococcus faecalis*. It was fractionated in the following way. The reaction solution was adsorbed on Amberlite IR-4B and eluted with NaCl solution as described in experimental part. The fractionally eluted xanthopterin, CF and folic acid were estimated with *Leuconostoc citrovorum* or fluorometrically or with *Streptococcus faecalis* fairly satisfactorily.

The result as given in Fig. 4 shows that the addition of xanthopterin alone to enzyme was almost the same as enzyme alone, but a remarkable folic acid synthesis took place in the system added with both xanthopterin and PAG. It is therefore clear that folic acid is biosynthesized from PAG and xanthopterin. But a fraction active for *Streptococcus faecalis*, differing from both folic acid and CF, was also synthesized as shown in the Figure.

When the resin-treated enzyme was used, scarcely any folic acid was synthesized from PABA, L-glutamate and xanthopterin, but the synthetic activity was completely recovered after the addition of boiled bacterial extract to the enzyme. Thus the separation of active coenzymes from apoenzymes was accomplished by this method, as summarized in Table V.

The optimum pH for the system, resin-treated enzyme plus boiled extract lies sharply at 7.2, whereas an unsharp maximum lies at about pH 6.5 for resting cells.

When the relation of folic acid formed to enzyme concentration in the system of PABA, glutamate, and xanthopterin was compared with that in the system of PAG and xanthopterin, the yield of folic acid was by far higher in the case of PAG, as shown in Fig. 5, and was proportional to enzyme concentration, whereas in the case of the system of PABA and glutamate the

FIG. 4 Fractional Determination with Resin of Folic Acid Biosynthesized

TABLE V
Folic Acid Biosynthesis with Resin-Treated Enzyme
 PABA, 0.29 mM; L-glutamate, 0.58 mM; xanthopterin, 0.056 mM;
 ATP, 0.84 mM; resin-treated enzyme powder, 10 mg/ml.

Substrate	Cofactor	Amount of folic acid formed mg/ml
+	—	3.6
+	Boiled extract	84.9
—	Boiled extract	17.3

FIG. 5 Relation between Enzyme Amount and the Amount of Folic Acid Formed from PABA and PAG

PABA, 0.58 mM; glutamate, 1.16 mM; xanthopterin, 0.056 mM.

PAG, 0.58 mM; xanthopterin, 0.056 mM.

yield was lower and was not proportional to enzyme concentration. Therefore, it was presumed that the pathway from PABA was more complicated than that from PAG, *viz.*, the following pathway was assumed to exist from the experiments hitherto performed by the authors:

PABA + glutamate $\longrightarrow$ PAG $\longrightarrow$ folic acid

The coupling reaction of PAG with xanthopterin remains unsettled, and no analogous coupling reaction of the ring is known. It is therefore a problem of great interest.

As for folic acid synthesis, the pathway, in which the PAG moiety is linked simultaneously with the closure of pteridine ring is studied

by Forest *et al.* and Tsesche *et al.*, whereas the possibility that PAG is formed from PABA plus glutamate and that folic acid is synthesized from PAG plus xanthopterin, was clarified by the authors enzymatically. Furthermore, in the former case the coupling of PABA with glutamate is an indispensable pathway, which has thus far been never analyzed.

Cohen *et al.* (7) proved the necessity of CoA for coupling of benzoic acid with glycine in hippuric acid synthesis, but no further analysis using hydroxamate fixation was made.

SUMMARY

1. The pathway of folic acid biosynthesis by the enzyme extracted from *Mycobacterium avium* is found to be the following: PABA and L-glutamate couple together to form *p*-aminobenzoylglutamate, which is then coupled with xanthopterin to form folic acid.

2. From the experiments using the resin-treated enzyme which is almost

free from coenzymes, the coupling reaction of PABA and L-glutamate, a kind of peptide formation, requires ATP, CoA and Mg^{++} as cofactors, whereby pyrophosphoric acid and AMP are liberated equimolecularly.

3. The amount of *p*-aminobenzoyl hydroxamate formed in this reaction is not reduced even when CoA is removed by resin treatment. It was presumed from this and other reasons that *p*-aminobenzoyl-AMP and *p*-aminobenzoyl-CoA are the active intermediates of PABA.

4. A new method was devised for estimating folic acid alone applying the microbioassay using *Streptococcus faecalis* to a fraction of Amberlite IR-4B. Using this method, folic acid was found to be synthesized from *p*-aminobenzoyl glutamate and xanthopterin.

5. The synthesizing enzyme of this step was found to be able to be divided into active apo-enzymes and co-enzymes by treatment with Amberlite IRA-400.

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