Studies on the Metabolism of Gallic Acid by Microorganisms

Part III. On the Intermediary Metabolism of Gallic Acid

by *Aspergillus niger*

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The intermediary metabolism of gallic acid by *Aspergillus niger* under the influence of some added inhibitors has been studied. The decomposition of gallic acid by lyophilized cells under fluoroacetate inhibition allowed *cis*-aconitic acid, α -ketoglutaric acid and citric acid to accumulate. A mechanism of gallic acid decomposition via *cis*-aconitic acid has been inferred.

In the previous paper the author reported the formation and some properties of the gallic acid(GA)-decomposing enzyme of *Aspergillus niger* s-2I.¹⁾ The enzyme could not be extracted in a cell-free state and the lyophilized cells were the most active preparation.

Previously the intermediary metabolism of phenolic compounds by *Aspergillus niger* has been studied by Friedrich.²⁾ Under the arsenite inhibition, levulinic acid was identified in the enzymatic digest of resorcin and hydroquinone, and β -ketoadipic acid was considered to be an intermediate, but no intermediate was identified in the case of GA and pyrogallol.

In this paper, the intermediary metabolism of GA by the lyophilized cells of *Aspergillus niger* s-2I in the presence of enzyme inhibitors was studied and the mechanism of GA decomposition was discussed.

EXPERIMENTALS AND RESULTS

Materials and Methods.

1) **Mycellium:** The lyophilized cells and the

acetone-dried cells were prepared from the cells which had been adapted to the decomposition of GA as described previously.¹⁾

2) **Compounds:** For pure *cis*-aconitic acid could not be obtained from commercial source, it was synthesized from *trans*-aconitic acid by the Malachowski's method³⁾ modified by Tomisawa.⁴⁾ m. p. 127°C. *Trans*-aconitic acid was synthesized from citric acid by the method of Bruce.⁵⁾ m. p. 198°~198.5°C. The other reagents used were of analytical grade.

3) **Incubation for decomposition:** Cell preparations were suspended in 0.1 M or 1% aqueous solution of GA, which was dissolved at pH 5.5 by addition of 2N NaOH, and the reaction mixture was incubated at 40°C with shaking in a Erlenmeyer flask or under aeration in a jar fermenter.

4) **Estimation of GA decomposition:** Decomposition of GA was estimated by the colorimetric determination of remaining GA as described previously.⁶⁾

5) **Ether extraction of the decomposition products:** The incubation mixture was filtered and the cells were washed thoroughly with water of about half the volume of incubation mixture. The filtrate and washings were combined and brought to pH 2.0

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1) A. Watanabe, *J. Agr. Chem. Soc. Japan*, **37**, 469 (1963).

2) H. Friedrich, *Arch. für Microbiol.*, **25**, 297 (1956).

3) R. Malachowski and M. Maslowski, *Ber.*, **61**, 2521 (1928).

4) J. Tomisawa, "Hyojun Seikagaku Jikken" (Standard Experiments of Biochemistry) Kobundo, Tokyo, 1953, p. 568.

5) W. F. Bruce, *Org. Syn.*, **17**, I.

6) A. Watanabe, *J. Agr. Chem. Soc. Japan*, **37**, 464 (1963).

with 4N H_2SO_4 . The decomposition products were then extracted with ether by means of a liquid-liquid extractor for one hundred and twenty hours. The ether extracts of about four times the volume of incubation mixture were dried with sodium sulfate, concentrated to dryness under vacuum, and used for identification of intermediates.

Preliminary Experiments.

Since it was considered that, in the acetone-dried or lyophilized cell, a part of the enzyme system might have been denatured and the permeability of the cell membrane would have increased, the decomposition products of GA by these cell preparations were examined. The reaction mixture was extracted with ether at pH 2.0 for one hundred and twenty hours and the components of the ether extract were examined by paperchromatography. Notable quantities of decomposition products such as phenolic compounds and organic acids were not found. Only in the case of the acetone-dried cell, a trace of acidic substance resulting from the degradation of 5g of GA was found in the ether extract but could not be identified because of its small quantity. Thereupon, effects of inhibitors on the GA-decomposition were studied. Based on the knowledge of some other aromatic nucleus-degrading enzymes such as pyrocatechase⁷⁾ and protocatechuic acid oxidase,⁸⁾ the inhibitors studied included inhibitors for SH-enzymes, some metal-binding agents and others. In a 100-ml Erlenmeyer flask, 0.5 g of lyophilized cells, 5 ml of 0.1 M GA solution and an amount of an inhibitor to give a final concentration of 0.001 M were mixed in a final volume of 50 ml at pH 5.5. After incubation on a reciprocating shaker at 30°C for twenty hours, the inhibition degree for the GA-decomposition was estimated (Table I). Detection of reaction products was performed by paperchromatography with the reaction mixtures incubated with EDTA, sodium azide, fluoroacetate and arsenite, the inhibition degrees of which were comparatively low. Each ether extract from 50 ml of the reaction mixtures was paperchromatographed on Tōyō Roshi No. 50 filter paper with two solvent systems, *n*-butanol-water-formic acid (10:15:2 V/V) and phenol-water-formic acid (75:25:1 V/V). After air-drying the paper, acidic intermediates were detected on the paperchromatogram by spraying 0.04% alcoholic bromphenolblue solution adjusted to pH 4.0. It was found that the acidic intermediates accumulated most evidently in the reaction mixture of fluoroacetate inhibition and to some extent in the reaction mixture of arsenite inhibition.

Effect of variation in concentration of fluoroacetate on its inhibitory action.

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At various concentrations of fluoroacetate the inhibition degree for GA decomposition was estimated. Under the conditions employed the reaction was hardly inhibited by addition of fluoroacetate less than 0.002 M in the final concentration. When 5 g of the fresh

FIG. 1. Relationship between Concentration and Inhibitory Effect of Fluoroacetate on GA Decomposition.

adapted cells, which had about the same activity as 0.5 g of the lyophilized cells, and 0.002 M fluoroacetate were used, GA was degraded completely after fifteen hours and acidic intermediates scarcely accumulated.

Extraction of Intermediates from the Reaction Mixture Inhibited by Fluoroacetate.

Forty grams of the lyophilized adapted cells was added to 3 l of water containing 40 ml of M/3 aqueous solution of fluoroacetate in a 10-l jar and suspended well.

TABLE I. EFFECTS OF INHIBITORS ON GA DECOMPOSITION

Inhibitor (10^{-3}M)	Inhibition degree (%)
<i>p</i> -CMB	75
EDTA	0
$\alpha\alpha'$ -Dipyridyl	45
NaN_3	0
CuSO_4	29
NaHSO_3^*	98
Iodoacetate	13
Arsenite	13
Fluoroacetate	7

* Final conc. M/15.

7) O. Hayaishi, M. Katagiri and S. Rothberg, *J. Biol. Chem.*, **229**, 905 (1957).

8) L. Ottey and E.L. Tatum, *ibid*, **223**, 307 (1956).

FIG. 2. Paperchromatograms of Organic Acids from the Reaction Mixtures Containing the Inhibitors.

I: GA, II: *cis*-aconitic acid, III: *trans*-aconitic acid, IV: α -ketoglutaric acid, V: citric acid, VI: fluoroacetate inhibition, VII: arsenite inhibition.

(Solvent system: (1), *n*-BuOH-Water-Formic acid (10:15:2) V/V.

(2), Phenol-Water-Formic acid (75:25:1) V/V.

After keeping the mixture at 30°C for about thirty minutes, 7.6 g of GA was added as a 50 ml aqueous solution, the pH of which had been adjusted to 5.5 with 2N NaOH. The reaction was allowed to proceed at 30°C under aeration at the rate of 4 l air per minute. During the course of reaction the pH was adjusted to 5.5 by addition of 2N NaOH and GA in the mixture was estimated at time intervals. GA was decomposed completely in thirty-seven hours. The reaction mixture was filtered, the cell paste homogenized in 1 l water with a blender, the homogenate filtered and the residue washed with water. The filtrates and washings were all combined. The combined solution was brought to pH 7.0 with 2N NaOH and concentrated to 1 l under vacuum. After the concentrated solution was brought to pH 2.0 with 4N H₂SO₄ and then saturated with sodium chloride, the decomposition products were extracted with ether for one hundred and twenty hours. The ether extracts were removed from the extractor and the ether container of the extractor was washed with ether thoroughly. Four liters of the combined ether solutions dried with sodium sulfate was concentrated to dryness under vacuum, and 1.95 g of orange-yellow oily residue was obtained. The constituents were examined by paperchromatography and it was con-

firmed that it contained no other acidic constituents than the one which was found in the preliminary experiment, and that no phenolic substances that were positive to the ferric chloride reaction were present. The substances separated on the paperchromatogram with the solvent system of *n*-butanol-water-formic acid were named A, B, C, D and E, respectively, as shown in Fig. 2.

Separation of the Components of the Ether Extracts by Column Chromatography.

After examination of column chromatography with cellulose powder, silica gel⁹⁾ and celite,¹⁰⁾ these acidic substances were found to be best separated by a method which consisted of the primary separation with silica gel and the rechromatography of resulting fractions with cellulose powder.

1) **Silica gel column chromatography:** Thirty-two grams of activated silica gel prepared by the method of Bulen⁹⁾ was mixed thoroughly with 22 ml of 0.5N H₂SO₄ and suspended in 300 ml of chloroform which was equilibrated with 0.5N H₂SO₄. The resulting silica gel was packed in a column

9) W. A. Bulen, J. E. Varner and R. C. Burrell, *Anal. Chem.*, **24**, 187 (1952); J. E. Varner, "Method in Enzymology". Academic Press Inc., New York, 1957, Vol. III, p. 397.

10) H. E. Swin and F. M. Utter, "Method in Enzymology". 1957, Vol. IV, p. 584.

(1.8×24 cm). The solution of 400 mg of the ether extract dissolved in 1 ml of 0.5N H₂SO₄ was adsorbed in 4 g of silica gel uniformly. The loaded silica gel was packed on the top of the silica gel column with about 5 ml of chloroform equilibrated with 0.5N N₂SO₄. The elution was performed at a rate of 80 ml per hour and fractions of 10 ml were collected. The solvent schedule was 400 ml of 5%, 540 ml of 15%, 400 ml of 25%, 1200 ml of 35%, and 600 ml of 50% *n*-butanol in chloroform (V/V) which was equilibrated with 0.5N H₂SO₄ and filtered with dry filter paper. Fractions were examined by paper chromatography for their components and divided into the following groups. Fractions of group 1, 2, 3 and 4 from the ether

Groups	Components
1 (fraction 64~73)	B with a trace of A
2 (fraction 74~79)	A with a trace of B
3 (fraction 85~95)	A and C
4 (fraction 191~204)	D only

extract of 800 mg which had been chromatographed in two portions were combined respectively and concentrated under vacuum. Residues of 250 mg, 270 mg and 25 mg were obtained from group 1, 2 and 3 respectively, and group 4 gave 20 mg of semicrystallized material. E was not found in any fraction.

2) Purification of A and B by column chromatography on cellulose powder: The residues from group 1 and 2 were rechromatographed to remove the contaminating trace of A and B, respectively. Then solution of 200 mg of the residue from group 1 dissolved in 1.5 ml of ethanol was adsorbed in 2 g of cellulose powder (Tōyō Rōshi 100~200 mesh) and mixed thoroughly. After evaporation of ethanol under vacuum, it was packed on the top of a column of cellulose powder (2.2×47 cm, 45 g of cellulose powder). Then the elution was performed with *n*-butanol-water-formic acid (10:15:2 V/V) at a flow rate of 20 ml per hour. Fractions of 5 ml were collected and the components were examined by paperchromatography with the same solvent as in the column chromatography. The solvent of fractions 30~36 was evaporated under vacuum and 85 mg of B was obtained. Similarly, 210 mg of the residue from group 2 was chromatographed on a cellulose column of the same scale, and 120 mg of A was obtained from fractions 24~28 after evaporating the solvent. C which was present in the effluent of group 3 was no longer found in the residue of 25 mg after evaporation of the solvent.

Identification of Intermediates.

Each of the separated substances was examined by paperchromatography as described previously, and upon comparison with authentic substances, it was supposed that A, B and D were *trans*-aconitic acid, α -ketoglutaric acid and citric acid, respectively.

1) Substance A: A was recrystallized from ethyl-acetate-chloroform and 85 mg of small needles was obtained from 110 mg of A, m. p. 194°C. *Anal.* Found: C, 41.65; H, 3.59. *Calcd.* for C₆H₆O₆: C, 41.39; H, 3.47%. The infrared spectra of A and an authentic sample of *trans*-aconitic acid showed no difference in absorption.

2) Substance B: B was recrystallized from acetone-benzene to give 55 mg of crystals from 85 mg of B, m. p. 111°C. To 40 mg of B dissolved in 0.5 ml of water was added 4 ml of 4% solution of 2,4-dinitrophenylhydrazine in methanol saturated with dried HCl gas. After thirty minutes, the hydrazone was precipitated by the addition of 1 ml of water and filtered. The 2,4-dinitrophenylhydrazone was recrystallized from water, m. p. 220°C. *Anal.* Found: C, 40.36; H, 3.15; N, 16.98. *Calcd.* for C₁₁H₁₀O₈N₄: C, 40.50; 3.09; N, 17.18%. Therefore, B was identified as α -ketoglutaric acid.

3) Substance C: Although A was identified as *trans*-aconitic acid, it has been reported that *trans*-aconitic acid does not undergo the enzymatic reaction,¹¹⁾ that in the aqueous solution *cis*-aconitic acid was isomerized completely after boiling for five minutes and mostly warming for ten minutes at 65°C, and that the isomerization was especially rapid in acidic or alkaline solution.^{3, 12)} Therefore, it was considered that *trans*-aconitic acid was produced by isomerization of *cis*-aconitic acid during the separation processes and was not a true intermediate of GA metabolism. In order to examine this possibility the ether extract was examined by paperchromatography, and *R_F* values of the components and authentic *cis*- and *trans*-aconitic acids were compared. It was found that C, which was not separated free from A by the subsequent column chromatography and was considered to be liable to decompose, had the same *R_F* value as *cis*-aconitic acid.

Quantitative Determination of Intermediates.

All the substances found as the intermediates of GA metabolism are members of the tricarboxylic acid

11) C. Martius, *Z. Physiol. Chem.*, **247**, 104 (1937).

12) H. A. Krebs and L. V. Eggelston, *Biochem. J.*, **38**, 426 (1944).

cycle. An ether extract of the reaction mixture of GA under fluoroacetate inhibition was analyzed for the intermediates by the method of Varner.⁹⁾ The reaction mixture of GA under arsenite inhibition and that of citric acid under fluoroacetate inhibition were also analyzed. In the following experiments, all the processes were performed at as low temperature as possible and excessive acidification was avoided to

prevent the isomerization of the *cis*-acid. Incubation mixtures under each inhibition were incubated at 30°C for forty-one hours under shaking and the decomposition products were extracted at pH 2.0 with ether for one hundred and twenty hours. Each about 400 ml of the ether extract was dried with sodium sulfate and the ether was distilled off under vacuum. The residues obtained were used for quantitative

FIG. 3. Quantitative Determination of Organic Acids from the Reaction Mixtures Containing the Inhibitors.

Reaction mixture:	Components contained.
GA-fluoroacetate system:	1 mM GA, 0.1 mM fluoroacetate, 1 g lyophilized cell.
GA-arsenite system:	1 mM GA, 0.1 mM arsenite, 1 g lyophilized cell.
Citrate-fluoroacetate system:	1 mM citrate, 0.1 mM fluoroacetate, 1 g lyophilized cell.

Each reaction mixture in a final volume of 100 ml at pH 5.5 was incubated at 30°C for forty-one hours under shaking. Each ether extract loaded on a silica gel column (1.2×25 cm) was eluted with 200 ml of 5%, 270 ml of 15%, 200 ml of 25%, 600 ml of 50%, and 300 ml of 50% *n*-butanol in chloroform (V/V) saturated with 0.5N H₂SO₄ successively. The flow rate was 80 ml per hour and the each fraction of 3.3 ml was titrated with 0.01N NaOH with phenol red as the indicator under vigorous stirring.

determination. The results are shown in Fig. 3. In the GA-fluoroacetate system, *cis*-aconitic acid, α -ketoglutaric acid and small quantities of citric acid and *trans*-aconitic acid were found. When both aconitic acids were regarded as *cis* form, the molar ratio of *cis*-aconitic acid, α -ketoglutaric acid and citric acid was about 5:5:1. In the GA-arsenite system, α -ketoglutaric acid did not accumulate and, in the citric acid-fluoroacetate system, *cis*-aconitic acid did not accumulate.

DISCUSSION

It was known that tricarboxylic acid cycle is the only metabolic pathway for formation of *cis*-aconitic acid and existence of tricarboxylic acid cycle has been observed in *Aspergillus niger*.¹³⁾ It has been reported that the following reactions, I and II, in tricarboxylic acid cycle proceed by the action of aconitase and at equilibrium the ratio of citric acid, *cis*-aconitic acid and isocitric acid is 89.5:4.3:6.3%.¹⁴⁾

It has also been reported that fluoroacetate,

an inhibitor for aconitase, competitively inhibits reaction I specifically. Therefore, *cis*-aconitic acid cannot be formed from citric acid through reaction I. The present result with the citric acid-fluoroacetate system agrees with this mechanism. In the GA-fluoroacetate system, isocitric acid did not accumulate. Therefore *cis*-aconitic acid can not be the product through the reaction II. From these results, *cis*-aconitic acid found in the present study must have been produced through a pathway other than tricarboxylic acid cycle. Difficulty in securing the decomposing-enzyme system in a cell-free form made the study of its mechanism difficult but it was observed previously that fission of the benzol nucleus of GA proceeds with consumption of atomospheric oxygen.⁶⁾ It is conceivable that GA would be decomposed by the same kind of mechanism as in the case of oxidative decomposition of catechol reported by Hayaishi et al.^{7,15)} Therefore the schema for GA decomposition is proposed in Fig. 4.

FIG. 4. Proposed Mechanism of GA Decomposition.

13) M. N. Mickelson and M. N. Schuler, *J. Bacteriol.*, **65**, 297 (1953).

14) H. A. Krebs and L. V. Eggelston, *Biochem. J.*, **37**, 334 (1943).

15) O. Hayaishi and Z. Hashimoto, *Med. J. Osaka Univ.*, **2**, 33 (1950).

In this schema GA is oxidized to peroxide(a) by an oxygenase with atmospheric oxygen. The peroxide is then decomposed to the tricarboxylic acid(b_1), which is tautomerized to (b_2) form easily, and the two forms are in an equilibrium state. The acid(b_2) is decarboxylated to *cis*-aconitic acid by an oxydative decarboxylase.

Since magnesium ion combines with fluoroacetate to make a complex compound, the accumulation of α -ketoglutaric acid under fluoroacetate inhibition suggests the inhibition of α -ketoglutaric decarboxylase due to the removal of magnesium ion. The inhibitors

for aconitase such as inhibitors for SH enzymes and ferrous ion also appear to inhibit the enzyme which decomposes the benzol nucleus of GA. This would seem to indicate the reason why the fluoroacetate inhibition was the only way to accumulate *cis*-aconitic acid by these cell preparations.

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