L-ASCORBIC ACID DEGRADATION BY BACTERIA

IV. DEGRADATION PRODUCTS OF L-ASCORBIC ACID

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In the present study it was investigated if the products by Fentone oxidation of L-ascorbic acid (L-AsA) which had been found as a model experiment of biological oxidation of L-AsA (1), can be the intermediates in the metabolism of the vitamin by L-AsA-adapted cells, and the metabolic products by bacteria was studied using paper chromatography to clarify the pathway of L-AsA degradation.

EXPERIMENTAL

1. Preparation of Microorganisms

Adapted and unadapted cells for L-AsA were prepared as described in previous paper (2). The cells used for investigating the metabolic products of L-AsA was prepared as follows. A loopful of adapted cells was inoculated and cultured in three conical flasks with 20 ml of peptone liquid medium containing 1% L-AsA. After incubation for 2 days, they were transferred to three plate flasks containing 500 ml of the same medium. After the L-AsA completely consumed, the cells were collected and washed with distilled water, and 0.5 g of the cells (dry weight) was used for the experiment.

2. Manometric Determination of L-AsA Metabolism

The experiment was performed using the conventional Warburg technique as described in the previous paper (3). 15 μ moles each of the substrates were used in this experiment.

3. Degradation of the Products of Fentone Oxidation of L-AsA

It was investigated whether or not L-threonic, glyceric, glycolic and formic acid produced by Fentone oxidation of L-AsA are the intermediates in the L-AsA degradation by L-AsA-adapted bacteria. A loopful of unadapted cells was inoculated in a synthetic liquid medium containing 1% of each substrate, and incubated at 30°. Only when glyceric acid was used as a substrate, the medium became turbid. By determining the O₂ uptake of L-AsA-adapted cells in the presence of each of these substrates, glyceric acid alone was found to be metabolized.

4. Metabolic Products of L-Ascorbic Acid by the Adapted Cells

The cells, described above, was added to a synthetic medium consisted of the following components, and incubated at 30° for 24 hours. After half or more of the L-AsA was degraded, the metabolic products were subjected to paper chromatography.

The components of medium: L-AsA (neutralized with 4% NaOH), 3 g; 1/15 M phosphate buffer (pH 6.0), 150 ml; and sterile distilled water, 150 ml.

Paper Chromatography of Nonvolatile Fatty Acids—As the developing solvent system *n*-butanol acetic acid-water (4:1:1) was used in an ascending method at room temperature. Spots were located with 0.1% brom phenol blue-alcohol solution.

Paper Chromatography of Volatile Fatty Acids—The acids, as ammonium salt, were spotted and developed by an ascending method at room temperature using ethanol containing 3% ammonia as a developing solvent. The spots were located by 0.1% brom phenol blue-alcohol solution containing 200 mg per 100 ml of citric acid.

The separation of the products was carried out as shown in Fig. 1. The

FIG. 1 Separation of the Metabolic Products of L-Ascorbic Acid

culture filtrate was neutralized with sodium hydroxide to pH 7, and concentrated under reduced pressure to separate the distillate from the residual solution. The former was used for neutral volatile components and the latter was treated with Amberlite IR-120 to remove the cations and extracted with ether for 90 hr to separate the soluble fraction from the insoluble one (D). The former was, after removal of ether, was subjected to vapor distillation to separate the distillate (B) and the residue (C). B was tested for volatile acids and C for nonvolatile acids.

5. Investigation of the Products after Degradation of Half of the L-AsA

Neutral Volatile Fraction—Following reaction were all negative: (a) Agulhon's reaction, (b) Davy's reaction, (c) Tollens' reaction, (d) Guyon's reaction. Therefore, alcohols and aldehydes were proved to be absent.

Ether Soluble Volatile Fraction—This fraction had a strong odor of acetic

acid and it made a sublimate solution slightly turbid. Therefore, the presence of formic acid was suggested. After the vapor distillate was neutralized with ammonia water, concentrated under reduced pressure, it was subjected to paper chromatography for volatile acids. As shown in Fig. 2, a single spot, R_F 0.34, was detected which agreed with that of acetic acid developed simultaneously. Further, following qualitative tests were carried out. (a) Heating with arseneous anhydride gave cacodyl oxide with a garlic-like smell. (b) Addition of ferric chloride gave a reddish brown color which disappeared by heating. Thus the spot was concluded as acetic acid, yielding 184 mg.

FIG. 2 Paper Chromatography of Volatile Acids (1)

FIG. 3 Paper Chromatography of Volatile Acids (2)

FIG. 4 Paper Chromatography of Nonvolatile Acids

Ether-Soluble Nonvolatile Fraction—The residual fraction, after removal of volatile acids, was subjected to paper chromatography. As shown in Fig. 3, a single spot, R_F 0.68, was found, which agreed with that of lactic acid. Therefore, it was tested as follows. (a) Uffelmann's reaction: To 10 ml of 1% phenol several drops of dilute ferric chloride solution was added. After mixing with this reagent, the test solution became yellow. (b) Guaiacol reaction: To the test solution, concentrated sulfuric acid was added and heated in a boiling water bath for 2 minutes. After cooling, a few drops of 5% alcoholic guaiacol solution was added. It gave a pink color.

Ether Insoluble Fraction—The ether insoluble fraction was, after concentration under reduced pressure, subjected to paper chromatography. Two spots, R_F 0.31 and 0.55, were detected, which gave a yellow color with bromophenol blue. They were in agreement with those of L-AsA and phosphoric acid, respectively. The spot, R_F 0.31, was detected also by 2,6-dichlorophenol indophenol reagent, showing to be the L-AsA unchanged.

6. The Metabolic Products of L-Ascorbic Acid after Complete Degradation

The bacteria (0.5 g of dry weight) were cultivated secondarily for 48 hours

in the medium of the same composition as described above. After the L-AsA was completely disappeared from the medium, the products were investigated by paper chromatography and qualitative tests as described above. As the result, lactic acid was absent, but acetic acid (203 mg) and a trace of formic acid were detected.

7. Investigation of the Metabolic Products of Dehydroascorbic Acid by L-Ascorbic Acid-Adapted Cells

In the previous paper (4), L-AsA is reported to be metabolized *via* DAsA. Therefore, when DAsA is used as a substrate, the same metabolic products are expected to be produced. In order to test this assumption, the following experiments were carried out. 3 g of DAsA was treated in the same way as described above. It was found that acetic acid and a trace of formic acid (equivalent to 83 mg acetic acid) were present in the medium after half the DAsA was consumed and that acetic acid and a small amount of formic acid (equivalent to 183 mg acetic acid) were present, but no lactic acid was detected.

8. Metabolic Products of Pyruvic Acid and of Lactic Acid by L-Ascorbic Acid-Adapted Cells

500 mg each of pyruvic and lactic acid was dissolved in 20 ml of 1/15 M phosphate buffer (pH 6.0), and after adjusting the pH of the medium to 6, 100 mg (dry weight) of L-AsA-adapted cells was added and the whole was cultured with shaking at 30° for 10 hours. After the substrate was completely degraded, the metabolic products were separated as shown in Fig. 5, and subjected to paper chromatography. Acetic and formic acid were thus identified. The yield, as acetic acid, gave 15 mg from pyruvic acid, and 16.3 mg from lactic acid. Further, the same amount of pyruvic acid was dissolved in phosphate buffer as described above and after adjusting the pH and adding the L-AsA-adapted cells, the whole was placed in a dessicator and evacuated to 10 mm Hg. After static culture for 24 hr the products were tested as shown in Fig. 5. It was found that formic and acetic acid (equivalent to 7.6 mg

FIG. 5 Separation of the Degradation Products of Pyruvic and Lactic Acid by L-Ascorbic Acid-Adapted Cells

FIG. 6 Degradation of Pyruvic and Lactic Acid

●, Pyruvic acid, ▲, Lactic acid, ---, CO₂ output, —, O₂ uptake

using the Warburg manometer. As shown in Fig. 6, the oxygen uptake and CO₂ output were marked in both the substrates, the rate of pyruvic acid degradation being somewhat greater than that of lactic acid.

DISCUSSION

As the Fentone oxidation products of L-AsA, except glyceric acid, failed to be metabolized by the L-AsA adapted cells, the mechanism of L-AsA degradation by the microorganism seems to be different from that of the chemical decomposition of the vitamin. Lactic acid, which was found on the way of L-AsA metabolism by the adapted cells, was not detected in the culture medium in which L-AsA had been completely degraded. When DAsA was used as a substrate, lactic acid was not detected even on the way of L-AsA degradation. From these findings it is suggested that lactic acid was produced only in the medium under anaerobic condition due to the high reduction potential of L-AsA, and it was then metabolized as the medium was gradually changed to aerobic condition following the disappearance of L-AsA. It was further manometrically proved that pyruvic and lactic acid were fairly well metabolized by the L-AsA-adapted cells. Both the substrates were mostly degraded to carbon dioxide and water under aerobic condition, except for the production of small amounts of acetic and formic acid. Pyruvic acid was partly reduced to lactic acid under anaerobic condition. Considering the above findings, L-AsA is considered to be metabolized *via* the pathway given in Fig. 7. The

FIG. 7 A Plausible Pathway of L-Ascorbic Acid Degradation by the Adapted Cells

acetic acid) as volatile acids and lactic and pyruvic acid (remaining unattacked) as nonvolatile acids were present.

9. Manometric Test for Degrading Lactic and Pyruvic Acid

The degradation of lactic and pyruvic acid by L-AsA-adapted cells were tested

pathway from diketo-L-gulonic to pyruvic acid remains to be settled.

SUMMARY

It was clarified that the mechanism of chemical degradation of L-ascorbic acid was different from that by microorganism. The metabolic products were identified by paper chromatography and the mechanism of it was partially elucidated.

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