L-ASCORBIC ACID DEGRADATION BY BACTERIA
VIII. ALL THE PATHWAY IN THE L-ASCORBIC ACID
METABOLISM

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Two pathways have been shown by the authors to exist in the L-ascorbic acid
(AsA) metabolism by AsA-degrading bacterium, i.e., one through dehydroas-
corbic acid (DAsA), and the other through D-araboascorbic acid (Arabo-AsA).
Both pathways have further been confirmed by detecting the intermediate pro-
ducts, and by testing the effects of various inhibitors.

EXPERIMENTAL

1. Methods

AsA-adapted cells were prepared by the procedure as described in the
previous paper (1), and the determination was made manometrically. The
substrates used were the same as described earlier (1).

2. Detection of Intermediate Products

The medium used contained 3 g of substrate, 150 ml of 1/15 M phosphate
buffer and 150 ml of sterile distilled water. 0.5 g (dry weight) of the adapted
cells was added to the medium and the whole was incubated at 30° for 20
hours. For detecting the intermediate products, paper chromatography was
chiefly used. For developing the nonvolatile acids, n-butanol-acetic acid-water
(4:1:1) was used and the spots located with 0.1% bromphenol blue in ethanol.
In the case of volatile acids, they were converted to the ammonium salts and
developed with 3% ammonia in ethanol. For locating 0.1% bromphenol blue
in ethanol containing 0.2% citric acid was used.

3. Effects of Various Inhibitors on the Metabolism of Glucose, D-Gluconic Acid,
D-Ribose and Arabo-AsA

Using 15 μM each of glucose, D-gluconic acid, D-ribose and arabo-AsA, the
effects of various inhibitors on the metabolism by AsA-adapted cells were
investigated and the result was compared with that of AsA metabolism. As
inhibitors, iodoacetate (10⁻⁴ M), sodium fluoride (10⁻¹ to 10⁻² M), potassium
cyanide (10⁻³ M), arsenous oxide (10⁻³ M), and sodium azide (10⁻³ M) were
used. As the results shown in Figs. 1 to 4, the metabolism of each substrate
was inhibited by all the inhibitors.
FIG. 1 Effect of Various Inhibitors on Glucose Metabolism
I, glucose; II, $10^{-2} M$ NaF; III, $10^{-3} M$ ICH$_2$COOH; IV, $10^{-1} M$ NaF; V, $10^{-3} M$ KCN

FIG. 2 Effect of Various Inhibitors on D-Gluconic Acid Metabolism
I, D-gluconic acid; II, $10^{-3} M$ As$_2$O$_3$; III, $10^{-3} M$ NaN$_3$; IV, $10^{-3} M$ NaN$_3$; V, $10^{-3} M$ As$_2$O$_3$; VI, $10^{-3} M$ ICH$_2$COOH

FIG. 3 Effects of Various Inhibitors on D-Ribose Metabolism
I, D-ribose; II, $10^{-3} M$ KCN; III, $10^{-3} M$ NaN$_3$; IV, $10^{-3} M$ NaN$_3$; V, $10^{-3} M$ KCN; VI, $10^{-3} M$ ICH$_2$COOH

FIG. 4 Effects of Various Inhibitors on Arabo-AsA Metabolism
I, D-arabo-AsA; II, $10^{-3} M$ KCN; III, $10^{-3} M$ ICH$_2$COOH; IV, $10^{-1} M$ NaF

4. Metabolic Products of Glucose
Detection of D-Gluconic Acid
When AsA-adapted cells were incubated in a glucose-peptone medium without calcium carbonate, the growth of the cells stopped in a short time. It is due to the marked fall of pH, suggesting the production of organic acids. For testing the organic acids the following methods were adopted. After innoculating the cells in 20 conical flasks containing 20 ml of a 1% glucose-peptone medium, they were incubated at 30°C for 24 hours. Then the cells were removed after centrifugation and the supernatant was passed through the column of Amberlite IRA-400, followed by
elution with 4% sodium hydroxide. It was further treated with Amberlite IR-120 to remove sodium ions. The effluent was concentrated under reduced pressure and subjected to paper chromatography to identify the organic acids. The result given in Fig. 5 shows a single spot, $R_f$ 0.15, which agreed well with that of D-gluconic acid developed simultaneously. It is therefore evident that the decrease of pH of the medium was caused by gluconic acid.

Metabolic Products of Glucose after Adding CaCO₃—0.5 g of AsA-adapted cells were added to the medium containing 3 g of glucose, calcium carbonate, 150 ml of 1/15 M phosphate buffer and 150 ml of water, and the whole was incubated at 30° for 20 hours. After removing the cells by centrifugation, the supernatant was passed through the column of Amberlite IR-120. The eluate was subjected to vapor distillation to separate the volatile acid. The residual solution was treated similarly to separate the nonvolatile acids. As shown in Fig. 5 b and c, lactic acid ($R_f$ 0.65) was detected, which was positive to both the Uffelman and guaiacol reaction. As the volatile acids, two spots, $R_f$ 0.40 and 0.34, were detected, agreeing with butyric and acetic acid respectively. Further, the volatile fraction was found to reduce mercuric chloride by heating, indicating the presence of formic acid.

5. Metabolic Products of D-Gluconic Acid, D-Ribose and Arabo-AsA

3 g each of D-gluconic acid, D-ribose and arabo-AsA was treated similarly to investigate the volatile and nonvolatile acids. As shown in Fig. 6 a and b, both lactic and acetic acid were detected from each substrate by paper chromatography and formic acid was proved qualitatively by reduction of mercuric chloride.

6. Metabolic Products of DAsA

The adapted cells were cultured anaerobically for 24 hours in phosphate
buffer containing 3g of DAsA, as described above, in a desiccator under reduced pressure. After centrifugation and the supernatant was passed through the column of Amberlite IR-120, followed by extraction with ether. The ether-soluble substances were tested similarly to detect organic acids. As shown in Fig. 7 a and b, lactic and acetic acid were found and formic acid was also detected qualitatively. The ether-insoluble substances were tested after concentration for sugars. As given in Fig. 8, three spots (Rf 0.32, 0.24, 0.13) were detected by the color reaction with ammonia, silver nitrate and aniline hydrogenphthalate, agreeing respectively with d-ribose (Rf 0.32), DAsA (Rf 0.24) and d-xylosone or d-arabinosone (Rf 0.13) developed simultaneously. The part, Rf 0.32, was cut off, and extracted with hot water. After concentration, it was subjected to paper chromatography using water-saturated colloidine. As shown in Fig. 8 b, a spot, Rf 0.65, agreeing with d-ribose was found. Moreover, it was found positive to the pentose reaction with orcinol or phloroglucinol.

7. Lactic Dehydrogenase Activity of AsA-Adapted Cells

D-Lactic acid was found temporarily as a metabolic product of AsA and other substrates on the way of degradation, but it disappeared in a short time. It was proved manometrically in the previous study (2) that the cells were capable of metabolizing lactic acid. In the present experiment, the existence

<table>
<thead>
<tr>
<th>Reaction system</th>
<th>Time for decoloration</th>
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</thead>
<tbody>
<tr>
<td>Lactic acid + cells + methylene blue</td>
<td>60</td>
</tr>
<tr>
<td>Cells + methylene blue</td>
<td>180</td>
</tr>
</tbody>
</table>
of lactic dehydrogenase in the cells was tested as follows. A large Thunberg tube containing 150 \( \mu M \) lactic acid, 20 mg (dry weight) of the cells, 1 ml of methylene blue (1:110 \( \times 10^3 \)), and 4 ml of 1/15M phosphate buffer, was incubated at 30\( ^\circ \) and 10 mm Hg. The time for decoloration of the pigment was measured. As given in Table I, the presence of lactic dehydrogenase in the cells was proved.

8. Optical Rotation of Lactic Acid

The optical rotation of the lactic acid, which was formed on the way of the degradation of AsA and other substances, was investigated. The oxygen consumption of the adapted cells in 15 \( \mu M \) D-, L- and DL-lactic acid respectively was measured manometrically. As shown in Fig. 9, only the D-form of the acid was metabolized. From the fact that the lactic acid, which appeared on the way of AsA degradation, was soon disappeared, it is obvious that the acid was in the D-form.

DISCUSSION

In the previous paper (3) it was reported by the author that the degradation of glucose ceased owing to the decrease of pH, when the adapted cells were grown in a medium containing glucose, whereas glucose was readily degraded without significant change in pH, when the cells were cultured in a medium with calcium carbonate or ascorbic acid. Furthermore, it was assumed that it was due to the degradation of glucose under an anaerobic condition of the medium caused by the high reduction potential of AsA, and that the mechanism was different from the case in the presence of glucose alone. The acid substance which caused the pH to decrease was found to be gluconic acid. This bacterium is therefore considered to be a facultative anaerobic; glucose metabolism passes through Embden-Meyerhof’s pathway under an anaerobic condition, and partially through Warburg-Dickens-Cohen’s pathway under an aerobic condition. From the findings that the metabolisms of glucose, D-gluconic acid, arabo-AsA and D-ribose by AsA-adapted cells were inhibited to the same extent by various inhibitors, e.g., potassium cyanide, sodium azide, iodoacetate, sodium fluoride and arsenous oxide, that carbon dioxide, acetic and lactic acid were always detectable as the metabolic products of D-gluconic acid, arabo-AsA and D-ribose, and that D-lactic acid was formed on the way of the metabolism, but it was further degraded by lactic dehydrogenase, the substrates given above are known to pass the common pathway, i.e., the de-
Fig. 10 Metabolic Pathway of L-Ascorbic Acid by Bacterium
gradation of AsA is considered to pass through Warburg-Dickens-Cohen's pathway via arabo-AsA.

Further, it was shown in the previous paper (4) that a pentose, probably D-ribose, was formed from D-arabinosone in the course of AsA degradation through DAsA. The pentose was identified in the present paper to be really D-ribose. That D-lactic, acetic and formic acid were detected as the metabolic products of DAsA and D-ribose suggest that D-ribose in an intermediate in the degradation of DAsA after having joined Warburg-Dickens-Cohen's pathway. Thus, it might be summarized that the degradation of AsA by the bacterium takes place through both Arabo-AsA and DAsA, joining in the end Warburg-Dickens-Cohen's pathway present in the cells as illustrated in Fig. 10. A part of AsA is isomerized by epimerization of the C5-OH group to arabo-AsA, which in turn partially converted to 3-keto-D-gluconic acid to an equilibrium. After being phosphorylated to 3-keto-D-gluconic acid-6-phosphate, it enters Warburg-Dickens' pathway: It is decarboxylated to ribulose-5-phosphate, which is in equilibrium with D-ribose-5-phosphate. On the other hand, a part of AsA is dehydrogenated to DAsA, which is further converted to diketo-gulonic acid. The latter acid is decarboxylated to L-xylosone, followed by epimerization of the C4-OH group to D-arabinosone. Further, it is reduced to D-ribose, and converted to D-ribose-5-phosphate by phosphorylation, which in turn joins the pathway described above. Further metabolism following D-ribose-5-phosphate are assumed to pass the pathways, which have been reported by many authors (6); namely it is cleaved to active glycolaldehyde and 3-phosphoglyceric acid by aldolase, and the former is converted to acetic acid, whereas the latter to pyruvic acid. The latter acid is, as described in the previous paper (1), partially changed to lactic and formic acid, but is mostly metabolized to carbon dioxide and water through tricarboxylic acid cycle. The end products of AsA metabolism have hitherto been studied mostly in animals.

Thus, Burn, Dayton and Schulenberg (7) found, after intramuscular injection of C14-AsA to a guinea pig, that 30 per cent of the radioactivity was excreted in the expired CO2, and 10 per cent in the urine and concluded that the main pathway of AsA metabolism was CO2 formation by complete oxidation of AsA. Hellman and Burns (8) investigated the AsA metabolism in man and detected diketogulonic and oxalic acid in the urine besides the undegraded AsA. Burns and Kamfer (9) found in the kidney homogenate of a rat that the degradation of AsA took place to CO2 through DAsA and diketogulonic acid. On the other hand, Chan and Becker (10) detected CO2, oxalic acid, L-xylose and glucose as the degradation products of AsA after administering the C14-labeled vitamin to a guinea pigs. Thus the findings of the metabolism of AsA in animals are fragmentary. When these results are compared with the mechanism of AsA degradation clarified by the authors, it is supposed that a part resembles the chemical decomposition, but the most part passes the same route as found in the bacterium.
SUMMARY

1. It is presumed that glucose is metabolized by a AsA-degrading bacterium through Embden and Meyerhof's pathway anaerobically and through Warburg and Dickens' pathway aerobically.

2. The metabolic products of araboacorbic and D-gluconic acid and D-ribose were proved to be lactic, acetic and formic acid by paper chromatography.

3. D-Ribose was found to be a metabolic product of dehydroascorbic acid.

4. The lactic acid, which was found on the way of the degradation of ascorbic acid or other substrates, was proved to be a D-form.

5. The total pathway of ascorbic acid degradation was clarified.

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REFERENCES