THE ROLE OF $\alpha$-KETOGLUTARATE AND MESACONATE IN GLUTAMATE FERMENTATION BY CLOSTRIDIUM TETANOMORPHUM*

By J. T. WACHSMAN†

(From the Department of Plant Biochemistry, University of California, Berkeley, California)

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The products of L-glutamate fermentation by resting or growing cells of Clostridium tetanomorphum are acetate, butyrate, hydrogen, and approximately stoichiometric amounts of carbon dioxide and ammonia (1-4). Experiments with C$^{14}$-labeled glutamate have shown that acetate, butyrate, and carbon dioxide originate preferentially from glutamate carbons 1 and 2, 3 and 4, and 5, respectively, and that glutamate carbon atom 4 is the precursor of butyrate carbons 1 and 3 (5, 6). These findings are consistent with a path of glutamate fermentation that involves the formation of 2 different C$_2$ units, one active in acetate formation and the other a precursor of butyrate, and exclude the operation of a tricarboxylic acid cycle in the oxidative direction. Experiments on the mechanism of this fermentation carried out with cell-free preparations have furnished evidence for the involvement of both $\alpha$-ketoglutaric and mesaconic acids.

**Methods**

C. tetanomorphum strain H$_1$ was grown in 10 to 20 liter quantities on a medium containing 1.0 per cent yeast extract, 0.3 to 0.5 per cent L-histidine$\cdot$HCl$\cdot$H$_2$O (neutralized) or 0.5 per cent L-glutamic acid (neutralized), 0.05 per cent sodium thioglycolate, 0.05 M potassium phosphate buffer, pH 7.4, and inorganic salts. Cells were harvested and washed as previously described (1), ground with alumina at 0-5°, and extracted with 0.05 M potassium phosphate buffer, pH 7.0, containing 0.05 per cent Na$_2$S$\cdot$9H$_2$O (2 gm. of alumina and 10 ml. of buffer were used per gm. wet weight of cells). The extract which resulted after removal of whole cells and alumina by centrifugation was lyophilized and stored as a powder at $-10°$. For enzymatic studies, the dry powder was dissolved in cold distilled water containing 0.2 per cent Na$_2$S$\cdot$9H$_2$O and adjusted to pH 7 with hydrochloric acid.

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The following technique was used for the chromatography of fixed acids. 2 volumes of acidified reaction mixture, brought to approximately pH 2 with H$_2$SO$_4$, were added to 18 volumes of 95 per cent ethanol and the resulting precipitate was removed by centrifugation. The supernatant fluid was evaporated to dryness at 40° under a stream of air, dissolved in 1 volume of 75 per cent ethanol, and used for chromatography on Whatman No. 1 filter paper. Papers were developed, dried at room temperature, and then analyzed for the presence of ultraviolet-absorbing constituents with the aid of a Mineralight ultraviolet lamp. Papers were then sprayed with bromoresol green for the detection of acids (7). The following developing solvents were employed: n-amyl alcohol-formic acid (8), isoamyl formate-formic acid (9), and n-butanol-acetic acid (10).

For mesaconate analysis, a 1:10 dilution of the reaction mixture in 95 per cent ethanol containing 0.5 N H$_2$SO$_4$ was prepared and centrifuged. The optical density of the supernatant solution at 240 m$\mu$ was then determined by using a similarly treated endogenous control in the reference cell. A solution containing 0.1 $\mu$mole of recrystallized mesaconic acid per ml. of acid alcohol has an optical density of 0.337 at 240 m$\mu$. Mesaconic acid was obtained from the Aldrich Chemical Company.

Manometric experiments were performed at 37° in a nitrogen atmosphere. Anaerobic experiments not involving measurement of gas exchange were carried out in evacuated test tubes.

Fatty acids were estimated by titration after distillation from an acid solution and identified by paper chromatography (11). The procedure of Grant (12) was used to test for the presence of formate.

The reduction of unsaturated acids with palladium and hydrogen was performed essentially according to Davies (13). Keto acids were determined on a 5 per cent trichloroacetic acid extract of the reaction mixture according to Friedemann and Haugen (14). L-Glutamate was estimated manometrically with Clostridium welchii decarboxylase (15).

Optical density measurements in the ultraviolet region were made at room temperature with a model DU Beckman spectrophotometer with silica cells of 1 cm. light path.

We are indebted to Dr. E. Conn for a generous supply of glucose-6-phosphate dehydrogenase prepared according to Kornberg (16).

Results

Glutamate Decomposition—A comparison of the fermentation of L-glutamate by the cell-free extracts and resting cells revealed the following differences. Whereas both acetate and butyrate are formed by resting cells, the extract does not form butyrate. Instead, approximately 2 moles of acetate are formed per mole of glutamate fermented. As a consequence of
the loss in ability to form butyrate, there is an increased production of hydrogen. The molar ratio of hydrogen formed to glutamate used was found to be 0.57 and 0.05 for the alumina extract and for resting cells, respectively.

*Mesaconic Acid Formation and Utilization*—The alumina extract was found to catalyze the decomposition of small quantities of glutamate under aerobic conditions. Paper chromatography of the resulting reac-

**Table I**

Detection of Mesaconate by Paper Chromatography

The reaction mixture contained 300 mg. of a lyophilized alumina extract, 792 μmoles of potassium phosphate buffer, pH 7.0, 2.5 μmoles of Na₂S, and 675 μmoles of potassium L-glutamate in a total volume of 12 ml., and was incubated 6 hours at 37° on a shaker in air. An alcohol extract of the reaction mixture was chromatographed on Whatman No. 1 filter paper, with butanol-acetic acid as the developing solvent (ascending). The ultraviolet-absorbing acidic band was eluted with water and rechromatographed on Whatman No. 1 paper (ascending). Approximately 0.3 μmole of each standard acid was chromatographed simultaneously. Papers were dried for several hours at room temperature, the quenching areas were marked, and acids were detected by spraying with bromocresol green. Chromatography of an endogenous control did not reveal the presence of a quenching acidic component.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Itaconate</th>
<th>Citraconate</th>
<th>Mesaconate</th>
<th>Fumarate</th>
<th>Reaction mixture + mesaconate</th>
<th>cis,cis-Muconate</th>
</tr>
</thead>
<tbody>
<tr>
<td>RF</td>
<td>0.68*</td>
<td>0.63</td>
<td>0.81</td>
<td>0.74</td>
<td>0.81</td>
<td>0.83</td>
</tr>
<tr>
<td>RF</td>
<td>0.56*</td>
<td>0.46</td>
<td>0.75</td>
<td>0.55</td>
<td>0.75</td>
<td>0.74</td>
</tr>
</tbody>
</table>

* The spot formed by itaconate was acidic but non-quenching. All other compounds gave rise to acidic quenching spots.

Chromatography mixtures revealed the presence of an ultraviolet-absorbing ("quenching") acidic constituent. The mobility on paper, rate of potassium permanganate reduction, and quenching properties of this compound were found to be similar to those of fumaric acid in both acidic and basic solvents. However, in all solvents tested, the glutamate-derived constituent had a higher \( R_p \) value than fumarate. These observations led to an investigation of other dicarboxylic unsaturated acids. Authentic mesaconic acid (Table I) forms an ultraviolet-absorbing acidic spot with an \( R_p \) identical with that of the compound formed from glutamate. Cochr-
matography of authentic mesaconate and the enzymatically produced compound resulted in the formation of a single spot with both acidic and ultraviolet-absorbing properties.

The compound formed from glutamate was purified by paper chromatography and catalytically reduced with hydrogen and palladium. The results obtained by paper chromatography (Table II) show that both authentic mesaconate and the enzymatically produced constituent are re-

Table II

Detection of Reduced Mesaconate by Paper Chromatography

An alcohol extract of a reaction mixture similar to the one described in Table I was chromatographed on Whatman No. 3 paper with butanol-acetic acid as the solvent (ascending). The acidic quenching band was eluted with water and subjected to catalytic hydrogenation in a Warburg manometric flask. The reaction mixture contained a sample of eluate, in 2.8 ml. of 1 N H₂SO₄; 0.2 ml. of a 0.5 per cent suspension of 5 per cent palladium on charcoal was added from the side arm after equilibration at 37°. A hydrogen atmosphere was used. A sample containing 4 μmoles of authentic mesaconate was reduced simultaneously. When hydrogen uptake ceased, the reaction mixtures were extracted five times with 5 ml. portions of ethyl ether, evaporated to dryness at 50°, and extracted with 1 ml. of 75 per cent ethanol. An aliquot of the alcohol extract was chromatographed on Whatman No. 1 paper (ascending) and analyzed for acidic and quenching constituents. The chromatogram of a catalytic hydrogenation control failed to show the presence of an acidic component.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>n-Amyl alcohol-formic acid</th>
<th>Isoamyl formate-formic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduced eluate</td>
<td>0.77</td>
<td>0.57</td>
</tr>
<tr>
<td>Mesaconate</td>
<td>0.85*</td>
<td>0.69*</td>
</tr>
<tr>
<td>Reduced mesaconate</td>
<td>0.77</td>
<td>0.55</td>
</tr>
<tr>
<td>Methyl succinate (separate experiment)</td>
<td>0.74</td>
<td></td>
</tr>
</tbody>
</table>

* Acidic quenching spot. All other spots are acidic, but non-quenching.

duced to non-quenching acidic compounds with \( R_F \) values essentially identical with that of methylsuccinic acid. The latter compound is the expected product of mesaconate reduction.

The ability of the alumina extract to produce gas from both mesaconate and glutamate is presented in Fig. 1. It is apparent that gas is evolved at essentially the same rate from both substrates, although the lag observed with mesaconate is considerably shorter than that with glutamate. Since no correction was made for carbon dioxide retention, the rate refers to the time interval between the beginning of the linear portion of the curve and the break in the curve. The resulting fermentation mixtures
were analyzed for fatty acids and shown to contain only acetic acid. The ratio of acetate formed to either mesaconate or glutamate used was 1.91.

In an effort to increase the yield of mesaconate from glutamate, a study of the effect of pH and anaerobiosis was made. Based on the increase in optical density at 240 nm, it was possible to demonstrate a 20 per cent accumulation of mesaconate from glutamate under aerobic conditions at pH 7.0. Under anaerobic conditions at pH 7.0, as much as a 50 per cent con-

![Graph](image-url)

**Fig. 1.** Gas production from mesaconate and glutamate. Each Warburg vessel contained 50 mg of a lyophilized alumina extract, 133 µmoles of potassium phosphate buffer, pH 7.0, 0.41 µmole of Na₂S, and 20 µmoles of potassium salt of substrate in a volume of 2 ml.; nitrogen atmosphere; 37°. Gas production was calculated as CO₂ evolution with no correction made for H₂ production or CO₂ retention.

version was found. However, complete or partial inhibition of mesaconate formation occurred at pH 5.6 or pH 9.2.

In the experiment in Fig. 2, mesaconate formation reaches a maximum at the end of 1 hour and then slowly declines. Coupled with this decline is a large increase in keto acid concentration. Based on α-ketoglutarate as a standard, keto acid accumulation at 6 hours accounts for 108 per cent of the glutamate initially present. The accumulation of keto acid is 10 times greater at pH 8.5 than at pH 7.0.

*α-Ketoglutarate*—Alumina extracts catalyze the reduction of triphospho-
pyridine nucleotide (TPN) in the presence of L-glutamate (Fig. 3). Under the same conditions no reduction of diphosphopyridine nucleotide (DPN) is observed. The rate of the reaction was found to be optimal in the pres-

![Graph](http://www.jbc.org/)  

**Fig. 2.** Mesaconate and keto acid formation from glutamate. The reaction mixture contained 100 mg. of a lyophilized alumina extract, 266 μmoles of potassium phosphate buffer, pH 7.0, 400 μmoles of Tris buffer, pH 9.5, 0.82 μmole of Na₂S, and 40 μmoles of potassium L-glutamate in a volume of 4 ml.; final pH 8.5. Evacuated test tubes; 37°. All the values are corrected for a control without substrate. α-Ketoglutarate was used as a standard for the keto acid determination.

![Graph](http://www.jbc.org/)  

**Fig. 3.** Glutamic dehydrogenase reaction. The cuvette contained 5.0 mg. of a lyophilized alumina extract, 100 μmoles of Tris buffer, pH 8.5, 0.04 μmole of Na₂S, and 4.0 μmoles of potassium L-glutamate in a volume of 1.0 ml. At zero time 0.05 ml. of DPN (0.2 μmole) was added. After 5 minutes, 0.1 ml. of TPN (0.125 μmole) was added. After 30 minutes 0.1 ml. (10 μmoles) of NH₄Cl was added, and to a second identical reaction mixture 0.1 ml. (10 μmoles) of potassium α-ketoglutarate was added. The optical density was read against a cuvette containing all the constituents except glutamate.
ence of tris(hydroxymethyl)aminomethane (Tris) buffer, pH 8.5, and considerably lower in the presence of either phosphate buffer, pH 7.0, or diethanolamine buffer, pH 9.5. The addition of either α-ketoglutarate or ammonia causes a rapid oxidation of the enzymatically reduced TPN (Fig. 3). These findings suggest the presence of a TPN-specific glutamic acid dehydrogenase in extracts of C. tetanomorphum, strain H1.

Further evidence for the presence of this enzyme was obtained by demonstrating the synthesis of L-glutamate, starting with α-ketoglutarate, ammonia, and a system regenerating reduced TPN. The results of the analyses for L-glutamate with C. welchii decarboxylase are presented in Table III. The identity of the compound reacting with glutamate in the C. welchii assay was confirmed by paper chromatography with n-butanol-acetic acid as the developing solvent. It is apparent that the alumina extract catalyzes the formation of glutamate only in the presence of the complete system. There is no detectable glutamate synthesis in the absence of either α-ketoglutarate or ammonia.

Small quantities of α-ketoglutarate are degraded by the alumina extract under aerobic conditions. Paper chromatography of reaction mixtures revealed the presence of an acidic ultraviolet-absorbing constituent, in addition to a large quantity of residual α-ketoglutarate. The $R_F$ value of the ultraviolet-absorbing compound was close to that of authentic mesaconate in four different solvents.

### Table III

**Formation of L-Glutamate from α-Ketoglutarate and Ammonia**

<table>
<thead>
<tr>
<th>Component omitted</th>
<th>L-Glutamate formed, μmoles per ml.</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>14.1</td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>0.0</td>
</tr>
<tr>
<td>Ammonium chloride</td>
<td>0.0</td>
</tr>
</tbody>
</table>

DISCUSSION

Both authentic mesaconate and the compound formed from glutamate are ultraviolet-absorbing acids with essentially identical mobilities in all
the solvents tested. In addition, both compounds are catalytically reduced to non-ultraviolet-absorbing acids with $R_F$ values similar to that of methyl succinate. Mesaconate is metabolized by the alumina preparation, and results in the formation of gas at the same rate and in the formation of acetate in the same molar ratio as is observed with glutamate. These findings support the conclusion that mesaconate is an intermediate in glutamate fermentation.

Based on the increase in optical density at 240 m$\mu$ in the presence of Tris buffer, pH 8.5, a 50 per cent accumulation of mesaconate from glutamate can be demonstrated. The specificity of this method for mesaconate analysis is admittedly poor. However, in each experiment in which there has been an increase in optical density at 240 m$\mu$ in the presence of glutamate, mesaconate was demonstrated chromatographically. The subsequent decrease in mesaconate concentration and the concomitant increase in keto acid concentration indicate that mesaconate is an intermediate between glutamate and keto acid. The identity of the keto acid formed from mesaconate is under investigation.

The demonstration of a glutamic acid dehydrogenase in extracts of $C. tetanomorphum$ strain H$_2$ is in agreement with the work of Fry (17) on another strain of this organism. The enzyme resembles the glutamic dehydrogenase of $Escherichia$ coli (18) and yeast (19) with respect to a specific requirement for TPN, and differs from the DPN-requiring enzymes found in $Clostridium$ sporogenes (20) and $Treponema$ (21). Beef liver glutamic dehydrogenase can function with either DPN or TPN (22).

Although $C. tetanomorphum$ has the enzymatic potential to convert L-glutamate to $\alpha$-ketoglutarate, the intermediary rôle of this compound in glutamate fermentation has not been established. The conversion of $\alpha$-ketoglutarate to mesaconate has been demonstrated, but the possibility that $\alpha$-ketoglutarate is degraded via glutamate cannot yet be excluded.

**SUMMARY**

Mesaconic acid (methylfumaric acid) has been identified as an intermediate in the fermentation of L-glutamate by an alumina extract of $Clostridium$ tetanomorphum, strain H$_2$. The fermentation of either mesaconate or L-glutamate by extracts results in essentially the same rate of gas evolution and the same yield of acetate. The extract was also shown to possess a TPN-specific glutamic dehydrogenase and to catalyze the conversion of $\alpha$-ketoglutarate to mesaconate.

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BIBLIOGRAPHY

THE RÔLE OF α-KETOGLUTARATE AND MESACONATE IN GLUTAMATE FERMENTATION BY CLOSTRIDIUM TETANOMORPHUM

J. T. Wachsman


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