THE MICRODETERMINATION OF FORMATE PRODUCED FROM PYRUVATE BY CELL-FREE EXTRACTS OF ESCHERICHIA COLI*

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Previous studies had demonstrated that Furacin (5-nitro-2-furaldehyde semicarbazone) inhibited the dismutation of pyruvate as well as its conversion to acetylmethylcarbinol by cell-free extracts of both avian (1) and bacterial (2) cells. It was therefore thought desirable to investigate the effect of this compound on the so called phosphoroclastic dissimilation of pyruvate to acetyl phosphate and formate by cell-free extracts of Escherichia coli (3).

Obviously the most satisfactory method for the study of this reaction would be one by which the amount of formate produced could be determined conveniently and accurately. However, all of the methods described previously for formate analysis are cumbersome and ill-adapted for biochemical studies of this sort, which involve the assay of small amounts of formate, contained in a mixture of other interfering substances. The enzymatic method described here appears to be a highly accurate, relatively simple one for the microdetermination of formate produced from pyruvate by extracts of E. coli, and it should prove useful for the determination of formate in other biochemical preparations.

Previous studies by Gale (4) had shown that formate oxidation by cell-free extracts of E. coli was associated with a cytochrome-containing particulate fraction, and later investigations of Sato and Egami (5) had demonstrated that nitrate reduction by whole cells of E. coli was linked to cytochrome b1, which is contained in the particulate fraction (6). In an atmosphere of N2, this particulate fraction is capable of mediating the oxidation of formate with the reduction of an equivalent amount of nitrate to nitrite and the production of an equivalent amount of CO2 as shown in the following equation:

\[
\begin{align*}
O & \quad \text{particulate enzyme system} \\
H-C-OH + KNO_2 & \rightarrow CO_2 + H_2O + KNO_2
\end{align*}
\]

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It has therefore proved possible to assay formate in terms of the CO₂ produced by this reaction in a Barcroft-Warburg micro respirometer. The particulate fraction used can mediate oxidation of a few other substances in the presence of nitrate; *viz.*, succinate, lactate, α-glycerophosphate, molecular hydrogen, and reduced diphosphopyridine nucleotide. However, none of these oxidations involves the production of CO₂, and it appears fairly certain that the particulate fraction can produce CO₂ only in the presence of formate.

**Materials and Methods**

*Preparation of Enzyme System Used for Formate Analysis*—12 hour cultures of *E. coli*, strain ECFS, were grown on the surface of solid media in Roux bottles. The medium consisted of 1.0 per cent Bacto-tryptone, 5.0 per cent Bacto-yeast extract, 0.2 per cent K₂HPO₄, and 2.5 per cent agar, to which 0.5 per cent glucose was added after autoclaving. The cells were washed off the agar with distilled water (25 ml. per Roux bottle), sedimented by centrifugation, and resuspended in M/30 phosphate buffer, pH 7.2, so that the final cell concentration was 20 to 25 mg., dry weight, per ml. The resuspended cells were subjected for 40 minutes to sonic vibrations in a 9 kc. Raytheon magnetostriction oscillator at 5-6°, and the sonically treated material was centrifuged at 10,000 r.p.m. (7000 × g) for 45 minutes in a Spinco refrigerated (3-5°) ultracentrifuge Model L. The supernatant fluid was then further centrifuged in the Spinco ultracentrifuge at 40,000 r.p.m. (102,000 × g) for 40 minutes. The supernatant liquid was removed and the pellet of sedimented particles was broken up and resuspended in a volume of M/15 phosphate buffer, pH 7.2, equal to the original volume of cell-free extract. The suspension was recentrifuged in the Spinco ultracentrifuge at 40,000 r.p.m. for 40 minutes, the particles were resuspended in half the original volume, and this particulate suspension was used for the experiments described here. Particles prepared in an identical manner from *E. coli* cells grown in 6 liter amounts of liquid medium as previously described (7) proved equally satisfactory for formate analysis.

*Analytical Procedure*—The analyses were carried out in a Barcroft-Warburg micro respirometer under an atmosphere of N₂ at 37°. Each Warburg flask contained 1.0 ml. of the formate-containing solution, 1.0 ml. of M/15 phosphate buffer, pH 7.2, 1.0 ml. of a 0.1 M solution of KNO₃, and 1.7 ml. of H₂O in the main chamber. 1.0 ml. of the particulate enzyme system (0.38 mg. of protein N) was contained in a side arm, and 0.3 ml. of 6 N H₂SO₄, saturated with sulfanilic acid, was contained in a second side arm. The reaction was started by adding the enzyme system from...

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the side arm. Manometric readings were taken at half-hourly intervals for 2 hours, after which the acid was added from the second side arm, and a final manometric reading taken 10 minutes later. A correction always had to be made for the CO₂ contained as HCO₃⁻ or CO₃⁻ in the reaction mixture before the start of the enzymatic reaction. This correction was made by adding acid at the start of the reaction to a Warburg flask containing the complete system, estimating the non-enzymatic CO₂ released thereby, and subtracting this amount from the total CO₂ produced in the systems used for formate analysis.

The reaction must be carried out in an atmosphere free of O₂, since the cytochrome-containing enzyme system involved utilizes O₂, where present, in preference to nitrate as a hydrogen acceptor. When the sulfuric acid is added to the reaction mixture, the nitrite present is converted to nitrous acid, which slowly decomposes. This can cause a significant (5 to 10 per cent) analytical error. Saturation of the sulfuric acid with sulfanilic acid effectively eliminates this source of error in analyses involving no more than 20 μmoles of formate. It is recommended that no more than 20 μmoles of formate be contained in the sample for assay, since this amount is the maximum that can be conveniently analyzed with a standard Barcroft Warburg manometer (Table I).

**Table I**

*Results of Typical Set of Analyses for Known Amounts of Formate*

<table>
<thead>
<tr>
<th>Formate in sample analysed (μmoles)</th>
<th>CO₂ produced by enzymatic oxidation of sample (μmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>20.02</td>
</tr>
<tr>
<td>10</td>
<td>10.3</td>
</tr>
<tr>
<td>5</td>
<td>5.2</td>
</tr>
<tr>
<td>2.5</td>
<td>2.6</td>
</tr>
</tbody>
</table>

Stability of Enzyme System—As can be seen in Fig. 1, the enzyme system is not stable, and we were not successful in keeping it active for more than 24 hours. It is to be hoped that some better method of preserving it may be found, possibly by an improved lyophilization technique.

The phosphoroclastic enzyme remained in the supernatant fluid after removal of the particulate fraction by centrifugation at 40,000 r.p.m., as described above. This supernatant liquid was used without further treatment to demonstrate phosphoroclastic activity.

Phosphoroclastic Reaction System—For the data in Fig. 2, the reaction mixture consisted of 1.2 ml. of enzyme, 30 Lipmann units of coenzyme A, 300 γ of cocarboxylase, 20 μmoles of MgCl₂, 200 μmoles of phosphate buffer,
pH 7.2, and 100 μmoles of sodium pyruvate in a final volume of 3.7 ml. Reactions were carried out in Thunberg tubes at 37° under an atmosphere of N₂. They were started by adding the pyruvate in 0.5 ml. of solution from a side arm and were stopped at the designated time intervals by heating in a boiling bath for 10 minutes. After removal of heat-coagulated protein by centrifugation, the material was analyzed for formate by the method described above.

![Graph showing CO₂ production over time](http://www.jbc.org)

**Fig. 1.** Reaction systems were set up as described under "Analytical procedure" with 100 μmoles of formate as the oxidizable substrate. To obtain the rate of reaction of a given enzyme preparation, reactions were started simultaneously in a set of Warburg vessels and then successively terminated by addition of the sulfuric acid from a side arm at the varying time intervals indicated. ○, fresh preparation of enzyme system; X, preparation held 24 hours at 3-5°C; Δ, preparation held 48 hours at 3-5°C; ●, preparation lyophilized and held for 1 week under N₂ at -20°C.

For the analytical data presented in Table II, a proportionately identical reaction mixture forty times larger in final volume was set up under N₂ in a 250 ml. bottle. The reaction was started by adding pyruvate from a separatory funnel inserted in the stopper of the bottle. It was stopped after 60 minutes by heating and the heat-coagulated protein was removed as described above. The pH of the reaction mixture was adjusted to 7.6, and sufficient water was distilled off to reduce its volume to 35 ml. The concentrate was acidified to pH 2.8 with 10 N H₂SO₄ and subjected to steam distillation until 600 ml. of steam distillate had been collected.
The pH of the steam distillate was adjusted to 7.6 with 1.0 N NaOH, and sufficient water was distilled off to reduce its volume to 120 ml. The concentrated steam distillate was then analyzed for formate as in Table II.

**TABLE II**

*Comparison of Results from Chemical and Enzymatic Methods for Measurement of Formate*

<table>
<thead>
<tr>
<th>Material analyzed*</th>
<th>Analytical method</th>
<th>Estimated total formate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat-inactivated reaction mixture</td>
<td>Enzymatic</td>
<td>1225 µmoles</td>
</tr>
<tr>
<td>Concentrated steam distillate</td>
<td>&quot;</td>
<td>1102 µmoles</td>
</tr>
<tr>
<td>&quot; &quot; distillation</td>
<td>Metallic Mg reduction method†</td>
<td>1140 µmoles</td>
</tr>
<tr>
<td>Residue after &quot; distillation</td>
<td>Enzymatic</td>
<td>12 µmoles</td>
</tr>
</tbody>
</table>

* The materials analyzed were prepared as described under the "Phosphoroclastic reaction system."
† See Grant (8).
Chemical analysis for formate in the steam distillate was made by the metallic Mg reduction method as described by Grant (8).

Results

Analytical data such as those in Table I have been reproduced many times, and they indicate that this method is a highly accurate one for the microdetermination of formate in pure solution. The presence of as much as 100 µmoles of pyruvate, lactate, succinate, malate, acetate, oxalate, malonate, or ethyl alcohol, either singly or in combination, had no significant effect on the accuracy of the analysis for formate. The validity of the method for the determination of formate produced from pyruvate by cell-free extracts of *E. coli* is indicated by the data in Table II. Fig. 2 shows a readily reproducible rate curve for this activity. Further studies on the phosphoroclastic system involved are now in progress.

One possible objection to the method might be based on the observation of Gest (9) that, unless the particulate fraction is obtained from cells grown under strictly aerobic conditions, it may contain some formic hydrogenlyase activity. Such activity would lead to erroneous results by mediating the following reaction in which H₂ is produced:

\[ \text{formic hydrogenlyase system} \quad \text{H-O-C-OH} \quad \rightarrow \quad \text{H}_2 + \text{CO}_2 \]

Fortunately, as Gest has also observed (9), nitrate strongly inhibits this reaction. Thus with the amount of nitrate routinely used in our analyses, no detectable hydrogen was ever evolved even when, in the absence of nitrate, the particulate fraction used could be found to have some formic hydrogenlyase activity.

SUMMARY

1. A highly accurate and apparently specific enzymatic method for the microdetermination of formate has been described.

2. The enzyme system used is contained in a cytochrome-containing particulate fraction of extracts of *Escherichia coli*.

3. This enzyme system mediates the coupled oxidation of formate and reduction of nitrate with the formation of an equivalent amount of CO₂.

4. The method has been found to be satisfactory for the determination of formate produced from pyruvate by cell-free extracts of *E. coli*.

BIBLIOGRAPHY

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