A Comparison of Hydroxylamine and N-Methylhydroxylamine as Probes for the Mechanism of Action of the Anthranilate Synthetase of Escherichia coli*

MICHAEL J. PABST and RONALD L. SOMERVILLE
From the Department of Biochemistry, Purdue University, West Lafayette, Indiana 47907

SUMMARY
In the presence of hydroxylamine, anthranilate synthetase catalyzes the formation of γ-glutamylhydroxamate. This activity requires enzyme, glutamine, and hydroxylamine and is stimulated by chorismate and inhibited by tryptophan. Measurement of the absorption at 500 nm of the red-violet γ-glutamylhydroxamate-Fea+ chelate provides a convenient assay for the glutaminase activity of this enzyme.

When N-methylhydroxylamine is present in the anthranilate synthetase reaction mixture, N-methylhydroxylamine derivatives of pyruvate are formed. One of these derivatives reacts with Fea+ in acid to form a chromophore which absorbs at 500 nm. The production of these derivatives from pyruvate is nonenzymatic. Their appearance in the anthranilate synthetase reaction mixture is the result of the formation of pyruvate as a product of the enzymatic conversion of chorismate to anthranilate. Anthranilate synthetase is partially inhibited by N-methylhydroxylamine. This inhibition is largely attributable to contaminating Zn2+, a potent inactivator of this enzyme. These results cast doubt on earlier conclusions concerning the mechanism of action of anthranilate synthetase based on studies with N-methylhydroxylamine.

Anthranilate synthetase, the first enzyme specific for tryptophan biosynthesis in Escherichia coli, catalyzes the following reaction: chorismate + glutamine Mg2+ → anthranilate + pyruvate + glutamate. In E. coli, anthranilate synthetase normally occurs aggregated with the next enzyme in the tryptophan pathway, phosphoribosyl transferase. A preliminary report (1) described the inhibition of anthranilate synthetase by hydroxylamine and various methylated hydroxylamines. Inhibition by hydroxylamine and N-methylhydroxylamine was accompanied by the production of substances which complexed with acidic Fea+ to give a red-violet color. These substances were not identified at that time. The requirements for the production of an Fea+-complexing substance in the presence of hydroxylamine were enzyme, glutamine, and chorismate, but not Mg2+; and the reaction was tryptophan-inhibitable. Requirements for the production of such substances in the presence of N-methylhydroxylamine were not determined. Subsequently, Zalkin and Kling (2) found that N-methylhydroxylamine, but not hydroxylamine, inhibited unaggregated anthranilate synthetase isolated from Salmonella typhimurium. (The unaggregated form can use ammonia, but not glutamine, as amino donor.) The production of the Fea+-reactive substance(s) in the presence of N-methylhydroxylamine appeared to require enzyme and chorismate and was not inhibitable by tryptophan.

The product formed when aggregated E. coli anthranilate synthetase is incubated in a reaction mixture containing hydroxylamine was found to be γ-glutamylhydroxamate. This observation was used as the basis of a convenient assay for the glutaminase activity of anthranilate synthetase (3). Tamir and Srinivasan (4) investigated the effects of hydroxylamine and N-methylhydroxylamine on the anthranilate synthetase aggregate from S. typhimurium. Their report is in substantial agreement with the findings presented here with regard to the identity of the products formed, i.e. γ-glutamylhydroxamate and an N-methylhydroxylamine adduct of pyruvate. However, they indicated that production of the N-methylhydroxylamine derivative was enzyme-catalyzed. By contrast, in this paper we demonstrate that the role of anthranilate synthetase is to catalyze the formation of pyruvate, and that the reaction of pyruvate with N-methylhydroxylamine proceeds nonenzymatically. In addition, we show that contamination of commercial N-methylhydroxylamine by Zn2+ may explain the previously reported inhibitory properties of this compound (1, 2).

Hydroxylamine—The identification of the Fea+-chelating substance produced by anthranilate synthetase in the presence of hydroxylamine was hindered by its instability (γ-glutamylhydroxamate readily cyclizes to pyrrolidine carboxylate (5)) and by the appearance of specious acetyl hydroxamate from pyridine-acetate ion exchange buffers. This resulted in our erroneous provisional conclusion that the unknown was not γ-glutamylhydroxamate (1). The unknown was finally identified as follows.
solids were spotted directly on Whatman No. 3MM thick paper. The remaining solids were spotted directly on Whatman No. 3MM thick paper and chromatographed with methanol-water (3:2, run at -15°). Spots were detected by spraying with ninhydrin and FeCl₃ reagent (6).

An anthranilate synthetase reaction mixture which contained hydroxylamine in 0.05 M Tris buffer, pH 7.8, was incubated with 0.35 n mole of enzyme for 1 hour at 25°. The reaction mixture was then filtered through a Diaflo XM-10 membrane to remove protein, and the filtrate was lyophilized. The remaining solids were spotted directly on Whatman No. 3MM thick paper and chromatographed with methanol-water (3:2, run at -15°) and phenol-water (phenol-distilled and saturated with water at 25°, then stored at 5°, whereupon a small amount of a second phase developed; eluent was taken from the major phase and the run made at 25°). Spots were detected by spraying with ninhydrin and FeCl₃ reagent (6).

An anthranilate synthetase reaction mixture which contained hydroxylamine in 0.05 M Tris buffer, pH 7.8, was incubated with 0.35 n mole of enzyme for 1 hour at 25°. The reaction mixture was then filtered through a Diaflo XM-10 membrane to remove protein, and the filtrate was lyophilized. The remaining solids were spotted directly on Whatman No. 3MM thick paper and chromatographed with methanol-water (3:2, run at -15°). Spots were detected by spraying with ninhydrin and FeCl₃ reagent (6).

Enzyme (1.25 μg) was added last to reaction mixtures containing chorismate (0.1 mM), ammonia (100 mM), Mg²⁺ (2 mM), EDTA (0.1 mM), and Tris buffer (0.05 M, pH 7.8), plus either N-methylhydroxylamine or water, as indicated, in a total volume of 1 ml. The formation of anthranilate was followed fluorimetrically (excitation, 310 nm; emission, 400 nm). After approximately 20 min, the synthesis of anthranilate was complete. N-Methylhydroxylamine was then added to the first reaction mixture. Five minutes later, 0.5 ml of FeCl₃ reagent was added to each cuvette. After waiting 20 min for the color to develop, the absorbance of each cuvette was measured. All steps were performed at 25°.

### Table I

<table>
<thead>
<tr>
<th>Chromatography of γ-glutamylhydroxamate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anthranilate synthetase reaction mixture (50 ml), containing 0.1 mM chorismate, 25 mM glutamine, 0.1 mM EDTA, and 200 mM neutralized hydroxylamine in 0.05 M Tris buffer, pH 7.8, was incubated with 0.35 n mole of enzyme for 1 hour at 25°. The reaction mixture was then filtered through a Diaflo XM-10 membrane to remove protein, and the filtrate was lyophilized. The remaining solids were spotted directly on Whatman No. 3MM thick paper and chromatographed with methanol-water (3:2, run at -15°) and phenol-water (phenol-distilled and saturated with water at 25°, then stored at 5°, whereupon a small amount of a second phase developed; eluent was taken from the major phase and the run made at 25°). Spots were detected by spraying with ninhydrin and FeCl₃ reagent (6).</td>
</tr>
</tbody>
</table>

### Table II

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>Anthranilate</th>
<th>Addition</th>
<th>Pyrurate derivative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme + cofactors + water...</td>
<td>100</td>
<td>N-Methylhydroxylamine (200 mM)</td>
<td>0.08</td>
</tr>
<tr>
<td>Enzyme + cofactors + N-methylhydroxylamine (200 mM)...</td>
<td>73</td>
<td>Water</td>
<td>0.077</td>
</tr>
</tbody>
</table>

### Table III

<table>
<thead>
<tr>
<th>Chromatography of pyruvate derivatives</th>
</tr>
</thead>
<tbody>
<tr>
<td>Either chorismate or pyruvate (5 μmoles) was added to 10 ml of a 200 mM neutralized solution (pH 7.8) of N-methylhydroxylamine in water. This mixture was evaporated to dryness under vacuum at 25°, then extracted with chloroform. The chloroform extract was concentrated and spotted on an Eastman silica gel thin layer sheet and developed with either acidic solvent (benzene-acetic acid-water (42:24:1)) or basic solvent (1-butanol-concentrated aqueous ammonia (47:8)). Spots were located with FeCl₃ reagent.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Acidic solvent</th>
<th>Basic solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>RF Color</td>
<td>RF Color</td>
</tr>
<tr>
<td>0.40 Red</td>
<td>0.42 Red</td>
</tr>
<tr>
<td>0.24 Yellow to red</td>
<td>0.24 Yellow to red</td>
</tr>
</tbody>
</table>

Fig. 1. Time course of the production of Fe³⁺-reactive substances from N-methylhydroxylamine and either pyruvate, chorismate, or chorismate plus a complete enzyme system. Pyruvate (1 mM) or chorismate (1 mM) was incubated at 25° for the indicated time with 200 mM N-methylhydroxylamine. The tubes indicated chorismate + enzyme + cofactors contained, in addition to 1 mM chorismate and 200 mM N-methylhydroxylamine, 100 mM ammonia, 0.1 mM EDTA, 2 mM Mg²⁺, and 12.5 pg of enzyme. The purified enzyme aggregate used in this paper had a specific activity of 4100 nmoles of anthranilate produced per min per mg of protein at 25°. N-Methylhydroxylamine was added last in all cases; enzyme was added immediately before the N-methylhydroxylamine. Times of addition of N-methylhydroxylamine were staggered so that all samples came to the end of their incubation simultaneously. FeCl₃ reagent was then added. After 30 min the absorbance at 500 nm was measured.

An anthranilate synthetase reaction mixture which contained hydroxylamine was incubated with enzyme until all chorismate was converted to anthranilate. After removal of protein, the products were subjected to paper chromatography (Table I). A single Fe³⁺-reactive spot (which also reacted positively to ninhydrin) was chromatographed with authentic γ-glutamylhydroxamat (Sigma) in both solvent systems used. Flatbed paper electrophoresis showed that the unknown migrated identically with authentic γ-glutamylhydroxamate at pH 8.2, 7.9, and 3.5. The isoelectric point of the unknown and of γ-glutamylhydroxamate was 7.9 (9).

The identification of γ-glutamylhydroxamate is in accord with the known glutaminase activity of this enzyme. Other glutaminases (7) and a glutamine-dependent amido transferase (8) also catalyze the production of γ-glutamylhydroxamate. Presumably, hydroxylamine acts as a nucleophile competing with water in cleaving a γ-glutamyl-enzyme intermediate (9).

Inhibition by Hydroxylamine—Hydroxylamine (200 mM) inhibits anthranilate synthetase activity less than 30%. The mechanism of this weak inhibition, although not known, may be related to the strong inhibition caused by γ-glutamylhydroxamate. γ-Glutamylhydroxamate (10 mM) produces approximately 50% inhibition of anthranilate synthetase activity when

...
glutamine (10 mM) is the amino donor. With ammonia (100 mM) as amino donor, γ-glutamylhydroxalamine (1 mM) produces 50% inhibition. These inhibition studies were carried out in the presence of 0.2 mM dithiothreitol and 0.1 mM EDTA since our commercial sample γ-glutamylhydroxalamine (Sigma) contained 0.1 μM Cu2+ and 0.05 μM Zn2+ in a 10 mM γ-glutamylhydroxalamine solution.

N-Methylhydroxylamine—The only requirement for the production of Fe3+-reactive material with N-methylhydroxylamine is pyruvate. The amount of pyruvate in an anthranilate reaction mixture depends on the extent of enzymatic or nonenzymatic breakdown of chorismate. Fig. 1 shows the time-dependent appearance of material giving a red color with Fe3+. When chorismate alone is incubated with N-methylhydroxylamine, there is a slow increase in the amount of red material. An equal amount of pyruvate alone gave a high yield of red material within 5 min. This yield remained constant with time. If the chorismate-N-methylhydroxylamine mixture is evaporated to dryness, then redissolved, the reaction goes to completion, producing the same amount of red material as the pyruvate reaction. If enzyme, ammonia, and Mg2+ are added to a chorismate-N-methylhydroxylamine reaction mixture, red material appears faster than with chorismate alone, but more slowly than with pyruvate alone. The simplest interpretation of this experiment is that chorismate breaks down either spontaneously or with the assistance of enzyme to liberate pyruvate, which then reacts nonenzymatically with N-methylhydroxylamine.

The experiment described in Table II demonstrates that Fe3+-reactive material arises from a nonenzymatic reaction between N-methylhydroxylamine and the products of the enzymatic breakdown of chorismate, one of which is pyruvate. Enzyme was added to two complete reaction mixtures, only one of which contained N-methylhydroxylamine. In both cases, the enzyme reaction was allowed to proceed until the formation of anthranilate was completed. N-Methylhydroxylamine was then added to the reaction mixture which lacked it. Five minutes later, both reactions were treated with FeCl3 reagent. Both reactions yielded the same amount of red product, regardless of whether N-methylhydroxylamine was actually present during the conversion of chorismate to anthranilate or not. Since only a catalytic amount of enzyme (ca. 5 pmols) was used, the pyruvate (ca. 100 nmoles) must have dissociated from the enzyme before reacting with N-methylhydroxylamine. The yield of anthranilate was 25% less in the cuvette containing N-methylhydroxylamine from the beginning, suggesting that the N-methylhydroxylamine solution by itself in some way increases the breakdown of chorismate, making it unavailable for conversion to anthranilate.

Chromatography of the products of the reaction of N-methylhydroxylamine with either pyruvate or chorismate yielded identical results (Table III). In the acidic solvent system, one red spot appeared after spraying with FeCl3 reagent. In the basic solvent system, one red and one yellow spot appeared. The yellow spot gradually turned red after several hours. Rechromatography of the material within the yellow spot showed that it was converted spontaneously into the material found in the red spot. The conversion took place in the absence of Fe3+. The proportion of red material is increased on storage in chloroform at −15°, or upon treatment with acid. The shift from yellow to red is probably the same change that is observed in solution when FeCl3 is added, since the red color appears slowly over the course of an hour. (The reaction of FeCl3 with γ-glutamylhydroxalamine, on the other hand, is virtually instantaneous.) Since the pyruvate derivatives were produced nonenzymatically, and could, therefore, shed no light on the mechanism of action of the enzyme, we did not attempt to determine their structures. The chemical nature of a pyruvate-N-methylhydroxylamine adduct has been investigated by Tamir and Srinivasan (4).

Inhibition by N-Methylhydroxylamine—Anthranilate synthetase was completely inactivated by incubation with 200 mM N-methylhydroxylamine in 0.1 mM Tris buffer, pH 7.8, for 20 min at 25°. Since the substances producing a red color with Fe3+ were apparently formed nonenzymatically, and, therefore, probably were not related to the inactivation, we sought another explanation for the loss of activity. Inactivation by N-methylhydroxylamine could be partially prevented by EDTA (0.1 mM) and by substituting phosphate buffer for Tris. On the basis of these clues, the N-methylhydroxylamine hydrochloride (supplied by Aldrich and twice recrystallized by us) was analyzed by atomic absorption and found to contain 1 μM Zn2+ in a 200 mM N-methylhydroxylamine solution. The brand of N-methylhydroxylamine used by Tamir and Srinivasan (4) (K and K Laboratories, Plainview, New York) contained 9 μM Zn2+ in a 200 mM solution. Anthranilate synthetase in 0.1 mM Tris buffer, pH 7.8, can be completely inactivated in 20 min at 25° by 1 μM Zn2+. All activities of the anthranilate synthetase aggregate—anthranilate synthetase using glutamine or ammonia, glutaminase, and phosphoribosyl transferase—are inactivated by Zn2+.

Our evidence proves that the production of pyruvate-N-methylhydroxylamine adducts is not directly catalyzed by anthranilate synthetase. Moreover, the inhibitory properties of N-methylhydroxylamine are probably due to the formation of this reagent by Zn2+. Thus, conclusions about the mechanism of action of anthranilate synthetase based on studies with N-methylhydroxylamine (2,4) are likely to be of limited value, since a nonenzymatic reaction between N-methylhydroxylamine and pyruvate cannot provide information about either the binding of chorismate or the mechanism of its conversion to anthranilate. At present there is no compelling evidence in favor of a covalent intermediate involving anthranilate synthetase and chorismate or any product derived from chorismate (4).

REFERENCES
7. Meister, A., Levintow, L., Greenfield, R. E., and Abend-}
A Comparison of Hydroxylamine and N-Methylhydroxylamine as Probes for the Mechanism of Action of the Anthranilate Synthetase of *Escherichia coli*  
Michael J. Pabst and Ronald L. Somerville  


Access the most updated version of this article at http://www.jbc.org/content/246/23/7214

Alerts:  
- When this article is cited  
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/246/23/7214.full.html#ref-list-1