The Enzymatic Synthesis of 5-Hydroxy-N-methylpyroglutamic Acid

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SUMMARY

Cell-free extracts of Pseudomonas M.A., a nonphotosynthetic bacterium which grows on methylamine as its sole source of carbon, nitrogen, and energy, have been shown to catalyze a reaction between methylamine and \( \alpha \)-ketoglutaric acid. The product of this reaction has been identified as the cyclic amide, 5-hydroxy-\( N \)-methylpyroglutamic acid. This reaction is catalyzed by an enzyme which differs from the one catalyzing the formation of \( N \)-methylglutamic acid, and it appears to be specific for \( \alpha \)-ketoglutarate. The possibility of a two-step reaction is discussed.

Shaw, Tsai, and Stadtman (1) have described a nonphotosynthetic bacterium, Pseudomonas M.A., which can utilize methylamine as its sole source of carbon, nitrogen, and energy. This organism was shown to possess an inducible enzyme system capable of incorporating methylamine into a new biological compound, \( N \)-methylglutamic acid (Reaction 1). In the course of investigating the chemical nature of this reaction, it was found that Pseudomonas M.A. could also catalyze a reaction between methylamine and \( \alpha \)-ketoglutaric acid (1).

We wish to report the identification of the product of this second reaction (Reaction 2) as 5-hydroxy-\( N \)-methylpyroglutamic acid and to present evidence that this compound is formed by an enzyme which differs from the one catalyzing the formation of \( N \)-methylglutamic acid.

\[
\begin{align*}
\text{COOH} & \quad \text{COOH} \\
\text{CH}_2 & \quad \text{CH}_2 \\
\text{CH}_2\text{NH}_2 + \text{CH}_2 & \rightarrow \text{CH}_2 + \text{NH}_2 \quad (1) \\
\text{CH} & \rightarrow \text{CH}_2 \quad \text{CH} \quad \text{NH} \quad \text{CH}_2 \\
\text{COOH} & \quad \text{COOH} \\
\text{COOH} & \quad \text{COOH} \\
\text{CH}_2\text{NH}_2 + \text{CH}_2 & \rightarrow \text{HO} \quad \text{C} \quad \text{C} = \text{O} + \text{H}_2\text{O} \quad (2) \\
\text{COOH} & \quad \text{COOH} \\
\text{CH}_2 & \quad \text{CH}_2
\end{align*}
\]

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MATERIALS AND METHODS

Preparation of Cell Extracts—Pseudomonas M.A. was grown and harvested as described by Shaw et al. (1). Cell-free extracts were prepared as described (1) with the modification that 20 mM Tricine buffer, pH 8.0, was substituted for 20 mM Tris-hydrochloride buffer, pH 8.5. The enzymatic synthesis of 5-hydroxy-N-methylpyroglutamic acid and of \( N \)-methylglutamic acid was measured as described by Shaw et al. (1) with Dowex 50 chromatography. Unless indicated, reactions mixtures contained 0.1 M Tricine buffer, pH 8.0; 0.005 M 2-mercaptoethanol; 0.10 M \( 14 \)C-methylamine hydrochloride, specific activity 4 \( \times \) \( 10^5 \) cpm per mmole; \( \alpha \)-ketoglutarate or glutamate; and enzyme in a final volume of 0.6 ml. The enzymatic reaction was allowed to proceed for 30 min at 30\( ^\circ \), after which time the reaction was terminated by the addition of 20% trichloracetic acid to a final concentration of 3.3%. The pH of the reaction mixture was 7.9 rather than 8.0 because of the effect of the salt concentration on the buffer. However, the pH remained constant during the course of the reaction. After removal of protein by centrifugation, 0.3 ml of the reaction mixture was chromatographed on a Dowex 50 column prepared in a Pasteur pipette. Radioactive HMPG was eluted from the column by washing with 2 to 3 ml of water; then radioactive \( N \)-methylglutamic acid was eluted from the column with 2 to 3 ml of 2 \( \times \) \( 10^6 \) M \( \text{NH}_4 \). Aliquots of 0.1 to 0.5 ml of column elute were added to 15 ml of Bray's solution and counted in a Nuclear-Chicago Mark I liquid scintillation counter.

Synthesis of 5-Hydroxy-N-methylpyroglutamic Acid—\( \text{DL} \)-\( N \)-Methylglutamine was synthesized from \( \alpha \)-ketoglutaric acid and methylhydrazine according to the procedure of Kline and Cox (2). The amide (2 g) was oxidized with L-amino acid oxidase according to the procedure of Meister (3) with the following modifications. At the end of the reaction, 20% trichloracetic acid was added to a final concentration of 3.3%. The acidified solution (90 ml) was passed over a Dowex 50 column (2 \( \times \) 16 cm), and the product was eluted with water. The product was evaporated to dryness on a rotary evaporator at 50\( ^\circ \) and crystallized from acetone. The material was reprecipitated from ethyl acetate. The melting point was 110-114\( ^\circ \) (uncorrected).

Preparation of Specifically Labeled Radioactive \( \alpha \)-Ketoglutaric Acid—To prepare radioactive \( \alpha \)-ketoglutaric acid labeled in a specific carbon atom, the corresponding \( 14 \)C-labeled glutamic acid was oxidized with glutamic acid dehydrogenase. A typical reaction protocol involved the use of L-glutamic acid dehydrogenase and \( 14 \)C-labeled glutamic acid. The resulting radioactive \( \alpha \)-ketoglutarate was purified by Dowex 50 chromatography.

1 The abbreviations used are: Tricine, tris(hydroxymethyl)methylglycine; HMPG, 5 hydroxy \( N \) methylpyroglutamate.
4678 5-Hydroxy-N-methylpyroglutamate Synthetase

that amount required to form 1 pmole of HMPG per hour. Spec-

Table I
Partial purification of 5-hydroxy-N-methylpyroglutamic acid synthetase

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume</th>
<th>Total protein</th>
<th>Total units</th>
<th>Specific activity</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extraction</td>
<td>81</td>
<td>3550</td>
<td>1300</td>
<td>0.36</td>
<td>%</td>
</tr>
<tr>
<td>Heat step</td>
<td>71</td>
<td>1010</td>
<td>1090</td>
<td>0.72</td>
<td>84</td>
</tr>
<tr>
<td>Ammonium sulfate fraction (50–95% saturated)</td>
<td>21.8</td>
<td>540</td>
<td>525</td>
<td>0.98</td>
<td>40</td>
</tr>
</tbody>
</table>

* Protein was determined by the biuret method (5).
* Enzyme activity was determined as described under "Materials and Methods" using α-ketoglutarate at a final concentration of 0.1 M. One unit of enzyme activity is defined as that amount required to form 1 μ mole of HMPG per hour. Specific activity is defined as units of enzyme activity per mg of protein.

Table II
Demonstration of reaction between α-ketoglutarate and 4C-methylamine

Reactions mixtures contained 0.1 M Tris hydrochloride buffer, pH 8.0; 5 mM 2-mercaptoethanol; 0.1 mM 4C-methylamine hydrochloride, specific activity 9 x 10^6 cpm per μ mole; 25 μ mole of dialyzed extract; and α-ketoglutarate or glutamic acid as indicated in a final volume of 1.0 ml. Control reaction mixtures 6 and 7 were identical with reaction mixture 4. After incubation for 1 hour at 30°, the reaction was terminated by the addition of 0.2 ml of 20% trichloracetic acid. Unreacted glutamic acid was available, the corresponding α-ketoglutarate was labeled in both carbon atoms.

Partial Purification of 5-Hydroxy-N-methylpyroglutamic Acid Synthetase—Cell-free extracts of Pseudomonas M.A., containing 44 μg of protein per ml, were incubated in 15-ml aliquots with 2 ml of 1.0 M α-ketoglutarate, pH 7 to 8, for 15 min at 45°. After chiling in ice, the precipitated protein was removed by centrifugation and discarded. The supernatant solution was then treated with three successive portions of solid ammonium sulfate to give three protein fractions precipitating between 0 and 30%, 30 and 50%, and 50 and 95% saturation with ammonium sulfate. The first two fractions did not contain significant amounts of enzymatic activity and were discarded. The third protein fraction, precipitating between 50 and 95% saturation with ammonium sulfate, was further purified by converting the precipitated enzyme to the free base with 1 M NaOH and diffusing the volatile amine into an acid well containing an excess of 0.2 M sulfuric acid. An aliquot of this 4C-methylamine-sulfate was redissolved in 0.05 M Tris buffer, pH 8.0, and was found to contain the synthetase. A summary of this purification procedure is shown in Table I.

α-C-Methylamine hydrochloride was obtained from New England Nuclear. It was further purified by converting the hydrochloride to the free base with 10 NaOH and diffusing the volatile amine into an acid well containing an excess of 0.2 N sulfuric acid. An aliquot of this 4C-methylamine-sulfate was redissolved in 0.05 M Tris buffer, pH 8.0, and was found to contain the synthetase. A summary of this purification procedure is shown in Table I.

A partially purified preparation of a DPNH flavoprotein dehydrogenase from Clostridium sp. grown on ethylammonium (4) was kindly furnished by Dr. B. Babior.

-action mixture contained 0.1 mmole of Tricine buffer, pH 8.0; 0.6 μ mole of DPN; 0.12 μ mole of menadione; 0.16 μ mole of 14C-glutamic acid, specific activity 4 x 10^6 cpm per μ mole; 0.8 mg of crystalline glutamic acid dehydrogenase; and an excess of a flavoprotein-DPNH dehydrogenase; 2 all in a final volume of 1.0 ml. The reaction was initiated by the addition of glutamic acid dehydrogenase and allowed to proceed for 15 min at room temperature, after which time the reaction was terminated by the addition of 0.2 ml of 20% trichloracetic acid. Unreacted glutamic acid was separated from α-ketoglutarate by chromatography on a Dowex 50-H column (1.9 x 3 cm). α-Ketoglutaric acid was eluted from the column with water while glutamic acid was retained on the column. Chromatography of the product on Whatman No. 1 filter paper with t-butyl alcohol-methyl ethyl ketone-ammonia-water (40:30:10:20), (v/v) (Solvent I) as the developing solvent revealed only one radioactive spot which corresponded to authentic α-ketoglutaric acid. The yields of α-ketoglutaric acids labeled with 14C in carbon atoms 1, 2, or 3 ranged from 16 to 40%. Since only 3,4-14C-glutaric acid was available, the corresponding α-ketoglutarate was labeled in both carbon atoms.

Determination of Incorporation of Specific Carbon Atoms from α-Ketoglutaric Acid into 5-Hydroxy-N-methylpyroglutamic Acid—The specifically 14C-labeled α-ketoglutarates, prepared as described above, and also 5-14C-α-ketoglutarate, obtained commercially, were diluted with unlabeled α-ketoglutarate to give a final specific activity of 3.4 x 10^6 cpm per μ mole. Each radioactive α-ketoglutarate was incubated in a reaction mixture containing 0.2 μ mole of methylamine hydrochloride; 0.23 μ mole of α-ketoglutarate; 0.1 μ mole of Tricene buffer, pH 8.0; 0.005 M 2-mercaptoethanol; and 10 μg of dialyzed extract, all in a final volume of 1 ml. After incubation for 8 hours at 30°, the reaction was terminated by the addition of 0.25 ml of 20% trichloracetic acid. Denatured protein was removed by centrifugation, and the reaction mixtures were chromatographed on Dowex 50-H columns. The radioactive products eluted from the column were then chromatographed on Whatman No. 1 filter paper with Solvent System I described above. The variously labeled 5-hydroxy-N-methylpyroglutamic acids and residual α-ketoglutarate were detected by radioautography and were identified by co-chromatography with the authentic compounds.

Partial Purification of 5-Hydroxy-N-methylpyroglutamic Acid Synthetase—Cell-free extracts of Pseudomonas M.A., containing 44 μg of protein per ml, were incubated in 15-ml aliquots with 2 ml of 1.0 M α-ketoglutarate, pH 7 to 8, for 15 min at 45°. After cooling in ice, the precipitated protein was removed by centrifugation and discarded. The supernatant solution was then treated with three successive portions of solid ammonium sulfate to give three protein fractions precipitating between 0 and 30%, 30 and 50%, and 50 and 95% saturation with ammonium sulfate. The first two fractions did not contain significant amounts of enzymatic activity and were discarded. The third protein fraction, precipitating between 50 and 95% saturation with ammonium sulfate, was further purified by converting the precipitated enzyme to the free base with 10 M NaOH and diffusing the volatile amine into an acid well containing an excess of 0.2 N sulfuric acid. An aliquot of this 4C-methylamine-sulfate was redissolved in 0.05 M Tris buffer, pH 8.0, and was found to contain the synthetase. A summary of this purification procedure is shown in Table I.

14C-Methylamine hydrochloride was obtained from New England Nuclear. It was further purified by converting the hydrochloride to the free base with 10 NaOH and diffusing the volatile amine into an acid well containing an excess of 0.2 N sulfuric acid. An aliquot of this 4C-methylamine-sulfate was redissolved with unlabeled methylamine hydrochloride to obtain the desired specific activity. Chemical analyses were performed by Huffman Laboratories, Inc., Wheatridge, Colorado. Infrared spectra were obtained with a Beckman infrared spectrophotomter. Nuclear magnetic resonance spectra were measured on a Varian A-60 spectrophotometer. Thin layer chromatography was performed with Brinkman Silica Gel F 254 thin layer plates. Crystalline glutamic acid dehydrogenase was obtained from Boehringer. 5-14C-α-Ketoglutarate was obtained from New England Nuclear. All other chemicals were obtained commercially and were used without further purification.

RESULTS

Demonstration of Reaction between α-Ketoglutarate and Methylamine—Table II shows that cell-free extracts of Pseudomonas M.A. catalyze a reaction between methylamine and α-ketoglutarate.
tartrate in which the incorporation of the methyl carbon moiety of methylamine is dependent upon the amount of \( \alpha \)-ketoglutarate added to the reaction mixture. The product from this reaction is not retained on Dowex 50-H and thus differs from \( N \)-methylglutamate, which is retained on the resin (1). The radioactivity incorporated in the presence of glutamic acid can be attributed to the generation of \( \alpha \)-ketoglutarate by glutamic dehydrogenase, which is present in these extracts (1).

**Nature of Reaction**—In order to establish the number of products formed from the reaction of \( \alpha \)-ketoglutarate with methylamine, \( \textsuperscript{14} \)C-labeled methylamine was incubated with \( \alpha \)-ketoglutarate and enzyme for varying lengths of time. Aliquots were taken from the reaction mixture, chromatographed on Dowex 50-H columns, and then on thin layer plates with \( t \)-butyl alcohol-methyl ethyl ketone-ammonia-water (Solvent I) as the developing solvent. At the time periods tested (20, 120, and 1320 min) only a single radioactive spot, \( R_f \) 0.94, was observed on the chromatograms. Chromatography of the unknown compound in two other solvent systems revealed only a single radioactive product in each case: \( t \)-butyl alcohol-methyl ester formic acid-water (40:30:15:15, v/v), \( R_f \) 0.17 (Solvent II), and ethyl acetate-formic acid-water (70:20:10, v/v), \( R_f \) 0.44 (Solvent III). It should be noted that chromatography of the unknown in the solvent system 1-butanol-acetic acid-water (50:25:25, v/v) occasionally leads to the appearance of three or more radioactive spots, but these were shown to be artifacts of the solvent system.

To determine whether the entire carbon skeleton of \( \alpha \)-ketoglutarate is incorporated into the reaction product or only a fragment thereof, specifically labeled \( \textsuperscript{14} \)C-\( \alpha \)-ketoglutarates were incubated with enzyme and methylamine (see "Materials and Methods"). The reaction mixtures were chromatographed on Dowex 50-H and then on Whatman No. 1 filter paper with Solvent I as the developing solvent. In each case, the particular carbon atom of \( \alpha \)-ketoglutarate was found incorporated into the reaction product. Thus the unknown compound contains carbon atoms 1, 2, 3 (and/or 4), and 5 of \( \alpha \)-ketoglutaric acid. (Since only \( \alpha \)-ketoglutarate labeled in both positions 3 and 4 was available, it was not determined whether one or both of these carbon atoms were incorporated into the product.)

**Identification of Unknown Product as 5-Hydroxy-N-methylpyrroglutamic Acid**—In order to isolate the unknown compound, a large scale incubation mixture was prepared containing 5 mmoles of Tricine buffer, pH 8.0; 10 mmoles of \( \textsuperscript{14} \)C-methylamine hydrochloride; specific activity 8.4 \( \times \) 10\(^4\) cpm per mmol; 14 mmoles of \( \alpha \)-ketoglutarate (pH 7); and 1.2 g of dialyzed extract, all in a final volume of 50 ml. The reactions mixture was incubated for 12 hours at 30°, after which time the reaction was terminated by the addition of 20% trichloracetic acid to a final concentration of 3.3%. The precipitated protein was removed by centrifugation, and the solution was chromatographed on a Dowex 50-H column (4.5 \( \times \) 20 cm). The unknown compound was washed through the column with distilled water and concentrated on a rotary evaporator at 50° to a yellowish syrup. (Based on the amount of radioactivity eluted from the column, it could be calculated that 87% of the methylamine was converted to the reaction product.)

The syrup was taken up in acetone, and, as the acetone was evaporated by gently blowing helium over the solution, crystals were formed. After standing overnight at 3°, the crystals were collected and washed with cold acetone. The compound was recrystallized to a constant specific activity from acetone with a final yield of 353 mg, which corresponds to 22% of the theoretical yield.

The elemental analysis of this compound (Table III) indicates a molecular formula of \( \text{C}_8 \text{H}_7 \text{NO}_4 \), which suggests that the compound is derived from the combination of 1 mole each of methylamine and \( \alpha \)-ketoglutarate with the elimination of 1 mole of water. Hence, Structures I to IV have to be taken into consideration.

![Diagram](attachment:structure_diagram.png)

The following chemical and spectroscopic properties of this compound indicate that the correct structure is Structure I.

(a) Treatment of the compound with excess diazomethane in ether furnished a methyl ester, m.p. 81°-82°.

\[
\text{CH}_3\text{NO}_4
\]

Calculated: C 48.54, H 6.40, N 8.09

Found: C 48.81, H 6.38, N 8.09

Infrared IR (CCl\(_4\)): 1718, 1740, 3150, 3250 cm\(^{-1}\)

Nuclear magnetic resonance (CDC\(_3\)): 2.20-2.60 ppm (multiplet, 4H)

The fact that a monomethyl ester was obtained eliminates both Structures III and IV as possible structures. The peaks in the nuclear magnetic resonance spectrum can be assigned as follows:

2.20 to 2.60 to \(-\text{CH}_2\text{CH}_2\text{CO}-\)

2.80 to \(-\text{NCH}_3\)

3.88 to \(-\text{COOCH}_3\)

3.30 ppm (singlet, 3H)

3.20 to \(-\text{OH} \) (vanished upon exchange with D\(_2\)O)

The infrared spectrum of this methyl ester serves to distinguish between Structures I and II. The bands at 3350 and 3150 cm\(^{-1}\) are clearly those of free and bonded OH stretching frequencies and not those of NH which mostly appears at about 3400 cm\(^{-1}\). The band at 1740 cm\(^{-1}\) is best assigned to the ester C=O and that at 1718 cm\(^{-1}\) to the amide C=O: the lack of the amide II band at about 1550 cm\(^{-1}\) suggests a tertiary amide, and the frequency of this band indicates it to be a 5-ring lactam. Structure II, being a lactone, should show a C=O stretching frequency at about 1770 cm\(^{-1}\). Thus these spectral properties are in best agreement for the methyl ester of Structure I.

(b) Treatment of the compound with anhydrous methanolic hydrogen chloride at room temperature for 24 hours gave an ester, m.p. 48°-50°, different from the one described above.
TABLE III

<table>
<thead>
<tr>
<th>Chemical or physical test</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Melting point*</td>
<td>110-113</td>
</tr>
<tr>
<td>2. Chemical analysis</td>
<td></td>
</tr>
<tr>
<td>Carbon</td>
<td>45.26</td>
</tr>
<tr>
<td>Hydrogen</td>
<td>5.83</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>8.78</td>
</tr>
<tr>
<td>Oxygen</td>
<td>40.25</td>
</tr>
<tr>
<td>3. Equivalent weight</td>
<td>158</td>
</tr>
<tr>
<td>4. Keto group determination by reaction with 2,4-dinitrophenylhydrazine (6)*</td>
<td>Negative</td>
</tr>
<tr>
<td>5. Secondary amine test by reaction with nitroprusside (7)*</td>
<td>Negative</td>
</tr>
<tr>
<td>6. Primary amine test by reaction with ninhydrin (8)*</td>
<td>Negative</td>
</tr>
<tr>
<td>7. Amide bond determination by reaction with hydroxylamine (9)*</td>
<td>Positive</td>
</tr>
<tr>
<td>8. α-Hydroxy acid determinations by reaction with molybdate (10)*</td>
<td>Positive</td>
</tr>
</tbody>
</table>

* Mised melting point, 110–113°.

** These tests were performed by allowing the crystalline compounds to react under the conditions of the test.

† These tests were performed by first chromatographing the unknown on thin layer plates and then spraying the chromatograms with the appropriate reagent.

‡ Commercial ninhydrin spray obtained from Mann Research Laboratories was used.

\[
\text{CaH}_{13}\text{N}_2\text{O}_4
\]

Calculated: C 51.32, H 7.00, N 7.48

Found: C 51.46, H 6.83, N 7.47

Infrared (CHCl₃): 1665 and 1750 cm⁻¹

Nuclear magnetic resonance (CDCl₃): 2.75–2.80 ppm (multiplet, 4H)

3.24 ppm (singlet, 3H)

3.81 ppm (singlet, 3H)

Examination of the spectral data reveals the only difference between this ester and that in (a) to be the disappearance of the OH bands in the infrared spectrum and the appearance of the OCH₃ signal at 3.24 ppm in the nuclear magnetic resonance spectrum. This transformation can only be rationalized on the basis of Structure I and not II. Thus the reactions can be summarized as follows.

![Diagram](https://via.placeholder.com/150)

![Diagram](https://via.placeholder.com/150)

Fig. 1. Comparison of the infrared spectrum of HMPG (upper curve) to the Pseudomonas product (lower curve). Spectra were determined in a KBr pellet.

Compound I (5-hydroxy-N-methylpyroglutamic acid) was prepared for comparison with the enzymatic product by the method of Meister (9). The compound is obtained by the oxidation of the N-methylamide of glutamic acid with L-amino acid oxidase from snake venom. The product was purified as the free acid rather than the barium salt. Table III gives a comparison of the properties of the compound isolated from the reaction of methylamine with α-ketoglutaric acid and authentic 5-hydroxy-N-methylpyroglutamic acid. As can be seen, both compounds behave identically and their chemical behavior is consistent with the proposed structure. Fig. 1 shows that both of these compounds have the same infrared spectrum, and Fig. 2 shows that both compounds react at identical rates in the test for an α-hydroxy acid.

Properties of Enzymatic Reaction—Fig. 3 shows that the α-ketoglutarate-dependent incorporation of methylamine into 5-hydroxy-N-methylpyroglutamic acid is proportional to the amount of dialyzed extract added. The \( K_m \) for methylamine is \( 4.6 \times 10^{-2} \) M in the presence of \( 1.0 \times 10^{-2} \) M α-ketoglutarate, and the \( K_m \) for α-ketoglutarate is \( 3.8 \times 10^{-2} \) M in the presence of \( 1.0 \times 10^{-2} \) M methylamine (11). The \( V_{max} \) of the reaction is 3.2 μmoles of methylamine incorporated per min per mg of protein. These kinetic constants were determined under conditions identical with those in Fig. 3 except that Tricine buffer (0.1 M, pH 8.0) was substituted for Tris-hydrochloride buffer. The reaction is linear for several hours under the assay conditions employed.

The enzymatic reaction exhibits a pH maximum of about 8 but is inhibited by Tris-hydrochloride buffer. Triethanolamine, 2-methylimidazole, pyrophosphate, and Tricine buffers had no inhibitory effect on the reaction.

No cofactor requirements have been detected for the reaction. The catalytic activity of dialyzed extracts is unaffected by passage over charcoal or Dowex 50-Na⁺ columns. Also, the 3-fold purified enzyme preparations exhibit no cofactor requirements.
Specificity of Reaction—A series of $\alpha$-ketoglutarate analogues were tested for their ability to act as substrates for the partially purified HMPG synthetase. At concentrations of 0.1 M, no reaction was observed with $\beta$-ketoadipic acid, levulinic acid, succinic acid, $\alpha$-hydroxyglutaric acid, $\beta$-ketoglutaric acid, $\alpha$-ketovaleric acid, or $\alpha$-ketoisovaleric acid.

Nonidentity of 5-Hydroxy-$N$-methylpyroglutamic Acid Synthetase and $N$-Methylglutamic Acid Synthetase—Since it was previously demonstrated that extracts of Pseudomonas MA cata-lyze the incorporation of methylamine into $N$-methylglutamic acid (1), it was of interest to determine whether or not this activity was associated with the 5-hydroxy-$N$-methylpyroglutamic acid synthetase. Table IV shows that there is no inhibition by glutamic acid of the formation of 5-hydroxy-$N$-methylglutamic acid in the presence of saturating levels of substrates. If these two reactions were catalyzed by the same enzyme, one would have expected glutamic acid to inhibit HMPG formation. The observed partial inhibition by $\alpha$-ketoglutarate of the formation of $N$-methylglutamic acid was not investigated and may be due to nonspecific effects.

Further evidence suggesting that these two reactions are catalyzed by separate enzymes can be seen in the heat inactivation
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Fig. 4. Heat inactivation of HMPG synthetase and N-methylglutamic acid synthetase. Two-milliliter aliquots of dialyzed extract (50 mg per ml) were incubated at 45° (---) or 50° (— —). At the time periods indicated an aliquot was withdrawn and assayed for enzyme activity as described under “Materials and Methods.” △, ○, N-methylglutamic acid synthetase activity; △, O, HMPG synthetase activity.

**TABLE V**

**Effect of glutamic acid and α-ketoglutarate on heat stability of HMPG synthetase and N-methylglutamic acid synthetase**

Two-milliliter aliquots of dialyzed extracts were heated at 50° for 12 min in the presence or absence of substrates as indicated. At the end of the incubation period, the samples were chilled in ice and then were assayed for enzyme activities. Control samples were kept in ice until assayed. The levels of glutamic acid (0.03 M) and α-ketoglutarate (0.1 M) used were 3 and 2.5 times their respective K<sub>M</sub> values.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Activity remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HMPG synthetase</td>
</tr>
<tr>
<td></td>
<td>%</td>
</tr>
<tr>
<td>1. Untreated enzyme</td>
<td>(100)</td>
</tr>
<tr>
<td>2. Enzyme heated at 50°</td>
<td>12</td>
</tr>
<tr>
<td>3. Enzyme heated at 50° in presence of 0.1 M α-ketoglutarate</td>
<td>85</td>
</tr>
<tr>
<td>4. Enzyme heated at 50° in presence of 0.03 M glutamic acid</td>
<td>14</td>
</tr>
<tr>
<td>5. Enzyme plus 0.1 M α-ketoglutarate, unheated</td>
<td>102</td>
</tr>
<tr>
<td>6. Enzyme plus 0.03 M glutamic acid, unheated</td>
<td>90</td>
</tr>
<tr>
<td>7. Enzyme heated at 50° then α-ketoglutarate added to 0.1 M</td>
<td>15</td>
</tr>
<tr>
<td>8. Enzyme heated at 50° then glutamate added to 0.03 M</td>
<td>14</td>
</tr>
</tbody>
</table>

curves shown in Fig. 4. At 45°, the 5-hydroxy-N-methylpyroglutamate synthetase is more stable than is the N-methylglutamic acid synthetase although both activities are rapidly inactivated when the preparation is heated to 50°. Table V shows that there is almost complete protection of the 5-hydroxy-N-methylglutamic acid synthetase when α-ketoglutarate is present during the heat treatment. In contrast, the N-methylglutamic acid synthetase is not protected by α-ketoglutarate. Glutamate at saturating levels has no effect on the heat stability of either activity at 50°.

**DISCUSSION**

The demonstration of the formation of 5-hydroxy-N-methylpyroglutamic acid by cell-free extracts of *Pseudomonas M.A.* appears to represent the first demonstration of this compound in a biological system.

Since no cofactor requirement could be demonstrated for the enzymatic reaction, one might speculate that the reaction occurs in two steps (Reactions 3 and 4), where cyclization provides the driving force for the reaction.

\[
\text{CH}_3\text{NH}_2 + \text{COOH} \rightarrow \overset{\text{H}_2\text{O}}{\text{CH}_3\text{N}-\text{CH}_2-\text{COOH}}
\]

The formation of substituted pyroglutamic acids by the action of L-amino acid oxidase on the amide and N-methylamide of glutamic acid (Reactions 5 and 6) (3, 12) provides examples of Reaction 4.

\[
\overset{\text{H}_2\text{O}}{\text{CH}_3\text{NH}-\text{CH}_2-\text{COOH}} \rightarrow \overset{\text{H}_2\text{O}}{\text{CH}_3\text{C}-\text{CH}_2-\text{COOH}}
\]

Since the product of the reaction of L-amino acid oxidase on amino acids is the corresponding keto acid, formation of the substituted pyroglutamic acids must occur by cyclization of the keto amide (12). Cyclization of the proposed imine intermediate in the oxidase reaction (13–17) would not yield the correct product.

It has not been unequivocally established whether HMPG is a true product of the enzymatic reaction or is derived nonenzymatically from an unstable enzyme-produced intermediate, such as that illustrated by Reaction 3. However, it has been shown that extracts of *Pseudomonas M.A.* do catalyze the conversion of
HMPG to free methylamine and α-ketoglutarate (3). This finding suggests that this compound can serve as a substrate for the enzyme in the reverse reaction or that there are other enzymes that can act upon HMPG.

Although studies indicate that HMPG is synthesized by whole suspensions of *Pseudomonas M.A.*, the physiological significance of this compound in the metabolism of this organism is still obscure.

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