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Acid-Catalyzed Hydrolysis of α-Ketoglutaramic Acid

A Proposed Mechanism Involving Decarboxylation and Amide Hydrolysis of the γ-Lactam, 2-Pyrrolidone-5-hydroxy-5-carboxylic Acid

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The rates of the acid-catalyzed decarboxylation and amide hydrolysis of α-ketoglutaramic acid, the keto analog of glutamine, were investigated and the products of the reactions were characterized. In strong acid at 100°C, amide hydrolysis and decarboxylation occur with about equal facility, yielding α-ketoglutaric acid and 5-hydroxy-2-pyrrolidone, respectively. 5-Hydroxy-2-pyrrolidone undergoes further amide hydrolysis so that the products of complete acid hydrolysis of α-ketoglutaramic acid are ammonia (100%), carbon dioxide (50%), and approximately equal yields (50%) of α-ketoglutaric acid and succinic semialdehyde (β-formylpropionic acid). At increasing pH values, the relative rate of decarboxylation to amide hydrolysis of α-ketoglutaramic acid increases, such that, at pH values of 2 or greater, decarboxylation occurs almost exclusively. The decarboxylation product, 5-hydroxy-2-pyrrolidone, was characterized chromatographically and by its infrared and pmr spectra; the compound may be regarded as the cyclized form of succinamic semialdehyde. A mechanism for the competing amide hydrolysis and decarboxylation reactions is proposed, and the potential biological significance of the decarboxylation pathway is discussed.

INTRODUCTION

Tissue homogenates of liver, kidney, and brain are capable of carrying out the transamination–deamidation of glutamine to yield α-ketoglutaric acid and ammonia, a reaction pathway involving α-ketoglutaramic acid as an intermediate (*1–3*). Recently, two glutamine transaminases (*4, 5*) and the ω-amidase (*6*) that catalyze these reactions have been prepared in highly purified forms and their properties studied in detail. However, the biological significance of the transamination–deamidation pathway in the metabolism of glutamine is still unknown, although the pathway has been suggested

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as a salvage mechanism, reaminating the α-keto acids of certain essential amino acids at the expense of glutamine (*4*). At the present time the only convincingly demonstrated route for the synthesis of α-ketoglutaramic acid in mammals is via transamination of glutamine. Thus, the demonstration that α-ketoglutaramic acid is present in normal rat tissue (*7*) and especially the observation that it accumulates in the cerebrospinal fluid of patients with hepatic coma[4] (*7–9*) indicate that the glutamine transaminases may be of greater biological importance than had previously been recognized.

In our continuing study of the chemical properties of α-ketoglutaramic acid we re-investigated the acid-catalyzed hydrolysis of this compound. Klein *et al.* (*10*) had previously shown that hydrolysis in strong acid at 100°C resulted in the formation of α-ketoglutaric acid and succinic semialdehyde. We confirmed these findings and, in addition, have demonstrated that the precursor of succinic semialdehyde is 5-hydroxy-2-pyrrolidone. The kinetics of the various reactions were examined at different pH values and a mechanism for the competing amide hydrolysis–decarboxylation reactions is proposed.

MATERIALS AND METHODS

2-Pyrrolidone was obtained from Aldrich Chemical Co. L-Glutamine, α-ketoglutaric acid, γ-aminobutyric acid, and 2-pyrrolidone-5-carboxylic acid[5] were obtained from Sigma Chemical Co. 5-Hydroxy-2-pyrrolidone, prepared according to the method of de Mayo and Reid (*11*), was a gift from Dr. de Mayo. The slightly yellow crystals were dissolved in water; the solution was then decolorized with charcoal and lyophilized. The lyophilized powder yielded one spot when subjected to paper chromatography (see below); the pmr spectrum, reproduced in Fig. 3, was consistent with the proposed structure.

The barium salt of α-ketoglutaramic acid was prepared from L-glutamine according to the method of Meister (*2*) except that purified L-amino acid oxidase (25 units, Sigma) was substituted for the crude venom preparation.

Anal. Calc for α-ketoglutaramate (barium), $C_5H_6NO_4Ba_{\frac{1}{2}}$: C, 28.22; H, 2.84; N, 6.58; Ba, 32.37. Found: C, 27.92; H, 3.02; N, 6.38; Ba 32.22.

One-dimensional thin-layer chromatography of the compound was carried out on cellulose using a solvent system consisting of ethanol:ammonium hydroxide:water (8:2:1) (*12*); chromatograms were developed with 0.5% bromcresol green in isopropanol. Only one spot with an R_f value of 0.52 was detected (2-pyrrolidone-5-carboxylic acid and succinamic acid standards yielded R_f values of 0.47 and 0.41, respectively). The preparation of barium α-ketoglutaramate was essentially free of 2-pyrrolidone-5-carboxylic acid (<0.5%) [assessed by measurement of glutamic acid, following hydrolysis of the compound in 1.0 *N* HCl at 100°C (*13*)], glutamine, glutamate, and α-ketoglutarate. In some cases, aqueous solutions of barium α-ketoglutaramate were converted to the corresponding sodium salt by addition of a slight molar excess of 1.0 *M* Na_2SO_4; the precipitated barium sulfate was removed by centrifugation. Solutions of sodium

[4] Glutamine transaminase and ω-amidase have been found to occur in human tissues, including brain (A. J. L. Cooper, unpublished observations).

[5] Pyroglutamic acid, 5-oxo-proline.

α-ketoglutaramate prepared in this fashion were standardized via a two-step reaction involving purified rat liver ω-amidase and glutamic dehydrogenase (*7*).

N-Methyl-α-ketoglutaramic acid (1-methyl-2-pyrrolidone-5-hydroxy-5-carboxylic acid) was synthesized from *N*-methylglutamine by a procedure similar to that described for the preparation of barium α-ketoglutaramate. DL-*N*-Methyl-glutamine was prepared from α-ketoglutaric acid and methylhydrazine according to the method of Kline and Cox (*14*). The amide (4.7 g) was oxidized to *N*-methyl-α-ketoglutaramic acid with purified L-amino acid oxidase (25 units, Sigma) and purified over a column (2.5 × 20 cm) of Dowex 50 (H^+). The pass-through solution was decolorized with charcoal, and the *N*-methyl-α-ketoglutaramic acid was precipitated as the barium salt and reprecipitated twice from water–ethanol (*2*). The pmr spectrum (Fig. 3b) was consistent with the proposed cyclic structure of this compound.

Anal. Calc for *N*-methyl-α-ketoglutaramate (barium), $C_6H_8NO_4Ba_{\frac{1}{2}}$: C, 31.77; H, 3.56; N, 6.18; Ba, 30.28. Found: C, 31.37; H, 3.58; N, 6.18; Ba, 30.31.

Quantification of reaction products. Carbon dioxide, liberated during the hydrolysis of α-ketoglutaramic and *N*-methyl-α-ketoglutaramic acids, was measured according to a procedure modified from that of Pontén and Siesjö (*15*). In a typical experiment, approximately 3 μmoles of barium α-ketoglutaramate were added to 1.0 ml of 1 *N* HCl in Pyrex culture tubes (75 × 100 mm). The tops were fitted with 10-cm lengths of thick-wall Tygon tubing (6-mm internal diameter) and connected to a second set of tubes which contained 0.3 ml of 0.025 *M* $Ba(OH)_2$ and thymolphthalein indicator. The tubes containing α-ketoglutaramic acid were placed in a 99–100°C oil bath for periods of up to 3 hr; the $Ba(OH)_2$-containing tubes were kept at room temperature. Unheated samples, maintained for 3 hr on ice, or acid blanks that did not contain α-ketoglutaramic acid (not different) were taken as zero time. Reactions were terminated at appropriate time intervals by cooling the connected "hydrolysis" and "trap" tubes in an ice bath. At the end of the experiment, all tubes were transferred to an air-tight, Lucite glovebox (Germfree Laboratories, Inc.) and allowed to equilibrate at room temperature for 45 min. During this time CO_2 was removed from the atmosphere within the box by circulating the enclosed air through Ascarite (Arthur H. Thomas Co.) absorption columns. The Tygon connections were then severed, and excess $Ba(OH)_2$ was titrated with 0.02 *N* HCl in the CO_2-free environment. The amounts of carbon dioxide liberated during hydrolysis were calculated from differences in the volume of HCl required to titrate the zero-time (unheated) controls.

α-Ketoglutarate was determined spectrophotometrically essentially according to the procedure of Lowry and Passonneau (*16*). Aliquots of the hydrolysis mixtures were added to a reagent consisting of 100 m*M* Tris–HCl (pH 8.5), 40 m*M* ammonium acetate, 0.1 m*M* ADP, and 0.1 m*M* NADH. The reaction was initiated by the addition of 0.1 mg/ml of liver glutamic dehydrogenase (Boehringer–Mannheim) and followed by the change in absorbance at 340 nm. Ammonia was measured by the method of Folbergrová *et al.* (*17*) in a reagent consisting of 100 m*M* Tris–HCl (pH 8.5), 0.1 m*M* ADP, 0.1 m*M* NADH, 5 m*M* α-ketoglutarate, and 0.15 mg/ml of glutamic dehydrogenase (in glycerol). The reaction was started by the addition of an aliquot of the hydrolysis mixture, and followed spectrophotometrically at 340 nm.

Succinic semialdehyde (β-formylpropionic acid) was assayed with a cell-free preparation from *Pseudomonas fluorescens* (GABase, Worthington Biochemical Corp.) (*18*),

which contained NADP-dependent succinic semialdehyde dehydrogenase activity. The assay mixture contained 100 m*M* Tris–HCl (pH 8.5), 10 m*M* 2-mercaptoethanol, and 1 m*M* NADP. After the addition of sample, the reaction was started with 0.35 mg/ml of GABase and followed by the change in absorption of the nucleotide at 340 nm; oxidation of succinic semialdehyde was completed in 30 min at room temperature. Purified succinic semialdehyde was a gift from Dr. F. N. Pitts.

γ-Aminobutyric acid (GABA), the product of hydrolysis of 2-pyrrolidone, was also determined with GABase by a spectrophotometric technique. Samples of the 2-pyrrolidone hydrolysis mixture were added to a reagent consisting of 100 m*M* potassium pyrophosphate (pH 8.6), 10 m*M* 2-mercaptoethanol, 1 m*M* NADP, and 1 m*M* α-ketoglutarate. The mixtures were read at 340 nm and the reaction initiated by the addition of 0.35 mg/ml of GABase; progress of the transamination–oxidation reaction was followed at 340 nm until completed (ca. 50 min).

Chromatography. Ascending paper chromatography was carried out according to the method of Fink *et al.* (*19*) on Whatman No. 1 paper. The solvent system consisted of *tert*-butyl alcohol:methyl ethyl ketone:formic acid:water (40:30:15:15) (solvent I) and *n*-butyl alcohol:acetic acid:water (50:25:25) (solvent II). γ-Aminobutyric acid was detected with ninhydrin, and compounds containing amide groups were visualized by the modified (*20*) method of Rydon and Smith (*21*), or with iodine vapor. α-Ketoglutaric acid and succinic semialdehyde were chromatographed as their 2,4-dinitrophenylhydrazone derivatives on cellulose thin-layer plates with a solvent system consisting of *n*-butyl alcohol:acetic acid:water (10:2:6) (solvent III) and were found to have R_f values of 0.75 and 0.94, respectively.

Proton magnetic resonance studies. All pmr spectra were recorded in deuterium oxide (99.7%, Wilmad Glass Co.) with a Bruker WH 90 Fourier transform spectrophotometer. Chemical shifts ($\Delta\nu$) were measured as parts per million (ppm) from the methyl resonance of the internal standard, sodium 3-(trimethylsilyl)propane sulfonate (DSS, Wilmad Glass Co.), and then converted to standard τ values. Spectral classification is based on the scheme devised by Pople *et al.* (*22*).

Infrared spectra. Infrared spectra were recorded with a Perkin–Elmer 621 grating infrared spectrometer using either (a) a mull obtained with approximately 2 mg of sample suspended in Nujol between two salt blocks or (b) carbon tetrachloride solvent. The 6.2434-μm peak of styrene was used as an internal reference.

RESULTS

Hydrolysis of α-Ketoglutaramic Acid in 1 N HCl

Solutions containing approximately 3 μmoles of barium α-ketoglutaramate in 1.0 ml of 1 *N* HCl were heated at 100°C in Pyrex tubes that were connected via Tygon tubing to individual CO_2 traps (see methods section). At intervals, the reactions were terminated by cooling the mixtures in ice, and the hydrolysis products were analyzed (Fig. 1). After complete acid hydrolysis, the yield of ammonia (3.05 μmoles) was found to be the same as that obtained by complete enzymatic hydrolysis with ω-amidase (*7*), i.e., 100% of the theoretical value. Whereas enzymatic hydrolysis of α-ketoglutaramate yielded stoichiometric amounts of α-ketoglutarate, complete acid hydrolysis yielded only

about 50% of α-ketoglutarate; succinic semialdehyde (~47%) accounted for most of the remaining products.

In order to characterize further the products of acid hydrolysis of α-ketoglutaramic acid, an aliquot of the hydrolysis mixture was withdrawn at 2 hr (Fig. 1) and treated with saturated 2,4-dinitrophenylhydrazine in 6 *N* HCl (*23*). The precipitated 2,4-dinitrophenylhydrazones were subjected to thin-layer chromatography (solvent III, methods section) and yielded two spots with R_f values identical to those of the 2,4-dinitrophenyl-

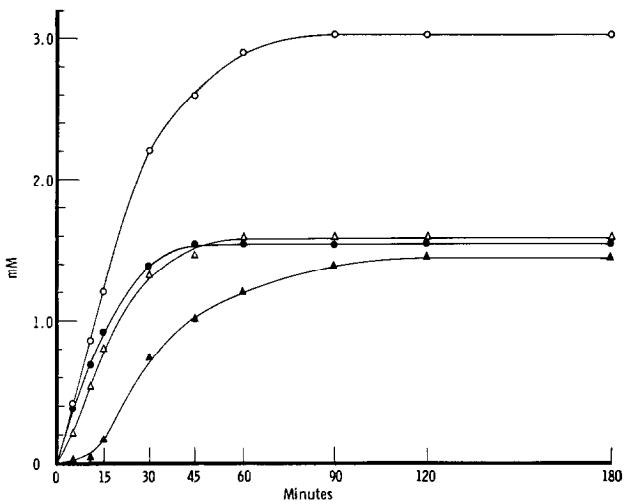

FIG. 1. Hydrolysis of α-ketoglutaramic acid in 1 *N* HCl. Pyrex tubes containing approximately 3.0 μmoles of barium α-ketoglutaramate dissolved in 1.0 *N* HCl were heated at 99–100°C for periods up to 3 hrs, and the reactions were terminated by chilling the mixtures in ice. See the text for the reaction apparatus and methods of quantitation of the products. (o——o), Ammonia; (●——●), α-ketoglutarate; (△——△), carbon dioxide; (▲——▲), succinic semialdehyde.

hydrazones of α-ketoglutaric acid and succinic semialdehyde. Thus, complete hydrolysis of α-ketoglutaramic acid in 1 *N* HCl may be essentially described by the equation:

$$\alpha\text{-Ketoglutaramic acid} \xrightarrow[\Delta]{} NH_3 + 0.5\ \alpha\text{-ketoglutaric acid} + 0.5\ CO_2 + 0.5\ \text{succinic semialdehyde.}$$

Solutions of α-ketoglutaric acid and succinic semialdehyde (3 m*M* in 1 *N* HCl) were completely stable when heated at 100°C for 3 hr. However, in order to eliminate the possibility that succinic semialdehyde arises via catalytic decarboxylation of α-ketoglutaric acid, a mixture containing α-ketoglutaric acid (10 m*M*), $BaCl_2$ (10 m*M*), and NH_4Cl (10 m*M*) in 1 *N* HCl (total volume = 1 ml) was heated at 100°C for 3 hr. α-Ketoglutarate was quantitatively recovered, and no succinic semialdehyde was detected under these conditions.

Formation of α-ketoglutaric acid was rapid and parallel with the rate of ammonia formation for the first 5 min (Fig. 1). The reaction appeared to follow first-order kinetics with a calculated rate constant of 0.062 min^{-1}. Carbon dioxide appearance was slower and showed a brief initial induction period, indicating that CO_2 is not formed directly but via some intermediate compound. Succinic semialdehyde formation also exhibited

sigmoidal kinetics but with a longer latency period. Rate constants for CO_2 and succinic semialdehyde formation, calculated at late times when the reactions were first order, had values of 0.058 and 0.030 min^{-1}, respectively. The rates of α-ketoglutaric acid formation in 0.1, 1, and 2 *N* HCl at 100°C were of the order of 1:10:20.

Hydrolysis of α-Ketoglutaramic Acid at Various pH Values

The rate of CO_2 production from α-ketoglutaramic acid at 100°C was determined at different hydrogen ion concentrations in the hydrolysis mixture (Fig. 2). As the hydrogen ion concentration was decreased from 1.0 to 0.01 *M*, the yield of CO_2 progressively increased from 49.2 to 88.9% of the theoretical maximum. When the reactions were allowed to proceed to completion, it was found that, although α-ketoglutaric acid and

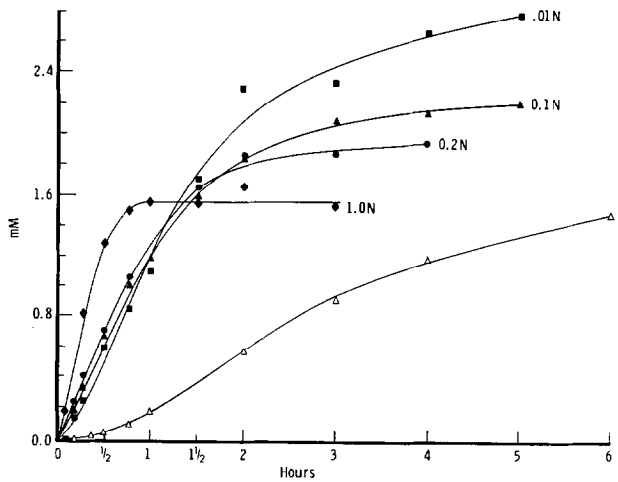

FIG. 2. Carbon dioxide release from α-ketoglutaramic acid at 100°C and at various hydrogen ion concentrations. Conditions as for Fig. 1. Filled symbols denote CO_2 formation; the open triangles denote succinic semialdehyde formation during the hydrolysis in 0.1 *N* HCl. It may be noted that the lag in CO_2 production increases with decreasing hydrogen ion concentration (the slopes of the curves for 0.2, 0.1, and 0.01 *N* HCl are quite similar but are displaced to the right with decreasing $[H^+]$). Hydrolysis of 10 m*M* α-ketoglutaramic acid in 0.01 *N* HCl (not shown) yielded a lag time for CO_2 production identical to that for hydrolysis of 3 m*M* α-ketoglutaramic acid in 0.01 *N* HCl (shown). These findings eliminate the possibility that the lag in CO_2 production might be due to a finite rate of CO_2 distillation from dilute solution.

succinic semialdehyde were the only products formed in appreciable quantities, the ratio of succinic semialdehyde to α-ketoglutaric acid increased from 0.97 in 1 *N* HCl (or 2 *N* HCl, not shown) to 1.59 in 0.2 *N* HCl. Thus, with increasing pH, decarboxylation of α-ketoglutaramic acid was favored over amide hydrolysis. Moreover, it is noteworthy that the rate of succinic semialdehyde formation in 0.1 *N* HCl (Fig. 2) lagged far behind that of CO_2 production, indicating the intermediate formation of a relatively stable decarboxylated precursor of succinic semialdehyde. During the hydrolysis at pH 2 (0.01 *N* HCl), negligible amounts of succinic semialdehyde were formed. This stability of the decarboxylated intermediate probably explains why Klein *et al.* (*10*) were unable

to detect succinic semiadlehyde formation (as its 2,4-dinitrophenylhydrazone) following hydrolysis of α-ketoglutaramic acid at pH 2 for 1 hr (100°C) or at pH 0 for 14 hr (60°C). The precursor of succinic semialdehyde was identified as 5-hydroxy-2-pyrrolidone (see below).

Identification of 5-Hydroxy-2-pyrrolidone as the Precursor of Succinic Semialdehyde

Inspection of Fig. 1 reveals that during the hydrolysis of α-ketoglutaramic acid in 1 *N* HCl the maximal rate of succinic semialdehyde formation occurs at approximately 20 min; therefore, precursor concentration should be at a maximum at this time. Accordingly, we attempted to isolate the precursor in the following way: Barium α-ketoglutaramate (625 mg) was dissolved in 10 ml of 1 *N* HCl, heated for 20 min in an oil bath at 100°C, and then chilled in ice. The mixture was passed over a column (1 × 15 cm) of Dowex 50 (H^+) and the column was washed with 15 ml of water. The pass-

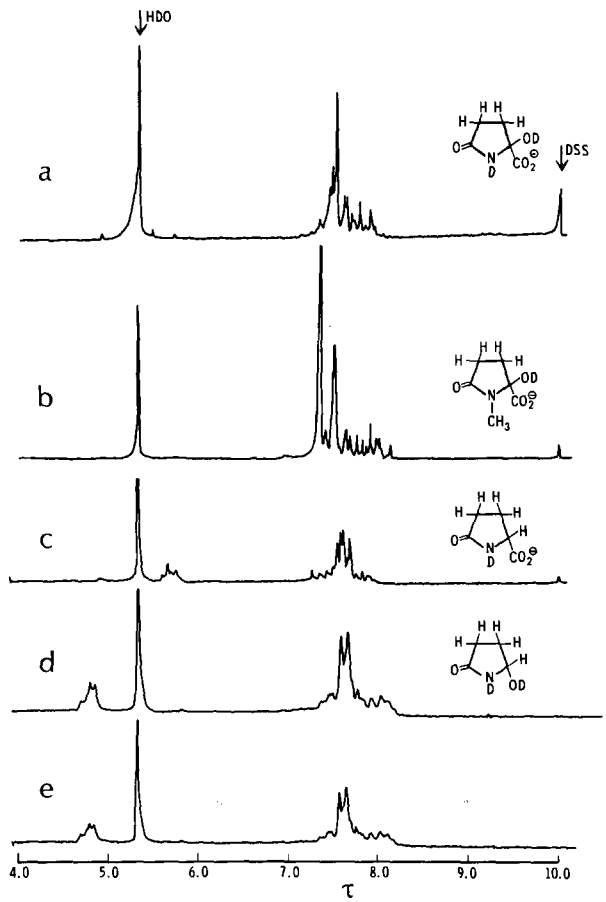

FIG. 3. Proton magnetic resonance spectra of (a) α-ketoglutaramic acid, (b) *N*-methyl-α-ketoglutaramic acid, (c) 2-pyrrolidone-5-carboxylic acid, (d) 5-hydroxy-2-pyrrolidone, and (e) compound X. Each spectrum was obtained on approximately 3 mg of sample disolved in 0.4 ml of D_2O, as described in the methods section. Compound X denotes the precursor of succinic semialdehyde that was isolated from the acid hydrolysis mixture of α-ketoglutaramic acid.

through volume was brought to pH 4.7 with 2 *M* NH_4OH and added to the top of a column (1 × 20 cm) of Dowex 1 (acetate). The column was washed with 50 ml of water and the pass-through solution was decolorized with charcoal and lyophilized to a white powder.

Proton magnetic resonance evidence. A portion of the lyophilized precursor material (compound X) was lyophilized twice from D_2O and suspended in 0.4 ml of D_2O, and the pmr spectrum was determined (Fig. 3e). The spectrum of the purified precursor was identical to that of authentic 5-hydroxy-2-pyrrolidone (Fig. 3d). For comparative purposes, the pmr spectra of α-ketoglutaramic acid,[6] *N*-methyl-α-ketoglutaramic acid, and 2-pyrrolidone-5-carboxylic acid are also recorded in Fig. 3. None of the four known compounds depicted in the figure has a plane of symmetry. Therefore, the spectra resulting from the four methylene protons of each structure are complex and of the ABCD type (cf. Fig. 3 a–d, the region encompassing τ values of 7.3–8.3). In addition, downfield resonance peaks due to the proton at C-5 of 2-pyrrolidone-5-carboxylic acid and the proton at C-5 of 5-hydroxy-2-pyrrolidone can be discerned. The distorted spectra corresponding to these protons derives from the fact that, in both instances, the hydrogens on the adjacent methylene groups are magnetically nonequivalent (i.e., they constitute the "A" part of an ABC-type spectrum). The proton at C-5 of 5-hydroxy-2-pyrrolidone is farther downfield (more deshielded) than the proton at C-5 of 2-pyrrolidone-5-carboxylic acid. This finding is to be expected since the carboxylate anion is

TABLE 1

PAPER CHROMATOGRAPHY OF 5-HYDROXY-2-PYRROLIDONE, COMPOUND X,[a] AND RELATED COMPOUNDS[b]

Compound	R_f value × 100	
	Solvent I	Solvent II
Compound X	94	77
5-Hydroxy-2-pyrrolidone	95	77
Reduced compound X[c]	95 (trace)	78 (trace)
	73	63
	56 (trace)	50 (trace)
Reduced 5-hydroxy-2-pyrrolidone[c]	74	63
	56 (trace)	50 (trace)
2-Pyrrolidone	75	65
γ-Aminobutyric acid	57	49

[a] Compound X denotes the precursor of succinic semialdehyde that was isolated from the acid hydrolysis mixture of α-ketoglutaramic acid.

[b] The chromatographic procedure was as outlined in the methods section. Each spot, except for γ-aminobutyrate, was visualized by the modified (*20*) Rydon and Smith procedure (*21*) or with iodine vapor. γ-Aminobutyrate was visualized with ninhydrin.

[c] Samples were reduced with cyanoborohydride according to the procedure of Cooper and Redfield (*25*).

[6] The pmr spectrum of α-ketoglutaramic acid in D_2O has been published previously (*25*). We have also obtained a spectrum in d_6-DMSO. A singlet, which integrates for one proton and is assigned to the amide hydrogen, was found at 7.8 ppm downfield from the external TMS signal.

electron donating (shielding), whereas the hydroxyl group is electron withdrawing (deshielding) (*24*).

Infrared spectra and chromatographic evidence. 5-Hydroxy-2-pyrrolidone, unlike 2-pyrrolidone, was found to be insoluble in CCl_4, $CHCl_3$, acetonitrile, and ethanol. The infrared spectrum of 5-hydroxy-2-pyrrolidone, obtained from a Nujol film, revealed the presence of a broad absorption region at approximately 3.15 μm, ascribed to O–H and N–H resonances, and a broad carboxyl-absorbing region at about 5.8 μm.

Samples of 5-hydroxy-2-pyrrolidone and the purified precursor compound (compound X) were chromatographed on paper according to the ascending technique (see methods section). Aliquots (3 mg) of both samples were also reduced with cyanoborohydride according to the method of Cooper and Redfield (*25*), purified, and similarly chromatographed (Table 1). Reduction products were found to include 2-pyrrolidone and γ-aminobutyric acid. The infrared spectra and the chromatographic behaviors of compound X and of authentic 5-hydroxy-2-pyrrolidone were indistinguishable.

Kinetic evidence. When 5-hydroxy-2-pyrrolidone was subjected to amide hydrolysis in 1 *N* HCl at 100°C, the products of the reaction were equimolar amounts of ammonia and succinic semialdehyde; the calculated first-order rate constant for this reaction was 0.025 min^{-1}. This value is in good agreement with the constant calculated from the data in Fig. 1 (0.030 min^{-1}).

Equilibria between Open-Chain and γ-Lactam Forms of 2-Pyrrolidone-5-hydroxy-5-carboxylic Acid and 5-Hydroxy-2-pyrrolidone in Strong Acid

5-Hydroxy-2-pyrrolidone may be regarded as the cyclic form of succinamic acid semialdehyde; its structure is therefore analogous to that of 2-pyrrolidone-5-hydroxy-5-carboxylic acid, the γ-lactam form of α-ketoglutaramic acid. In evaluating the mechanism of the acid-catalyzed amide hydrolysis of these compounds, it seemed necessary to determine whether the cyclic or open-chain isomeric forms were the structures actually undergoing hydrolysis. The maximal amounts of the open-chain forms expected to exist in 1.0 *N* HCl were assessed from the relative rates at which α-ketoglutaramic acid and 5-hydroxy-2-pyrrolidone react with semicarbazide and 2,4-dinitrophenylhydrazine (see below).

The pseudo-first-order rate constant for α-ketoglutaramate semicarbazone formation was determined from measurements of the increase in absorbance (248 nm) at 25°C (*26*) in the presence of 1.0 *M* semicarbazide (pH 6.5) and compared with the value obtained for α-ketoglutarate semicarbazone formation. The rate constant for α-ketoglutarate semicarbazone formation was calculated to be 0.48 min^{-1}; the constant obtained for α-ketoglutaramate semicarbazone formation was 1×10^{-4} min^{-1}, a value only 0.02% of that for the analogous open-chain α-keto acid.

The rates of 2,4-dinitrophenylhydrazone formation of α-ketoglutaramic acid and α-ketoglutaric acid were determined by incubating either α-ketoglutaramic acid (varying in concentration from 25 to 100 m*M*) or α-ketoglutaric acid (varying in concentration from 0.1 to 2.0 m*M*) with 2.5 m*M* 2,4-dinitrophenylhydrazine in 1 *N* HCl, at 25°C. At intervals, aliquots (0.1 ml) of the mixtures were added to 1.0 ml of 1 *N* NaOH in order to stop the reaction, and the absorbance at 430 nm was read. Initial rates of

hydrazone formation were directly proportional to α-ketoglutaric acid or α-ketoglutaramic acid concentrations and the pseudo-first-order rate constants were calculated to be 0.51 min^{-1} and 0.50 × 10^{-3} min^{-1}, respectively. Assuming that α-ketoglutaric acid and the open-chain form of α-ketoglutaramic acid react with 2,4-dinitrophenylhydrazine at comparable rates under these conditions, then the maximal amount of the open-chain form of α-ketoglutaramic acid present in 1 *N* HCl probably does not exceed 0.1%.[7] These low estimates of the amount of open-chain α-ketoglutaramic acid that would be expected to exist under acidic conditions are consistent with earlier data (*6*), indicating that at higher pH values (7.1–7.4) the equilibrium between open-chain α-ketoglutaramate and 2-pyrrolidone-5-hydroxy-5-carboxylate was approximately 99.7% in favor of the cyclic isomer.

In parallel experiments with 5-hydroxy-2-pyrrolidone, no semicarbazone formation over the pH range 3.0 to 7.0 could be detected, nor was there any evidence of 2,4-dinitrophenylhydrazone formation in 1 *N* HCl after incubations at 25°C lasting up to 2 hr. These findings indicate that 5-hydroxy-2-pyrrolidone exists overwhelmingly in the cyclic configuration.

Acid Hydrolysis of N-Methyl-α-ketoglutaramic Acid (1-Methyl-2-pyrrolidone-5-hydroxy-5-carboxylic Acid) and Other Amides

In order to investigate further the mechanism of amide hydrolysis and decarboxylation of α-ketoglutaramic acid (2-pyrrolidone-5-hydroxy-5-carboxylic acid), we examined the rates of hydrolysis of other substituted and unsubstituted amides in 1 *N* HCl at 100°C (Fig. 4 and Table 2). Acid-catalyzed amide hydrolysis of *N*-methyl-α-keto-

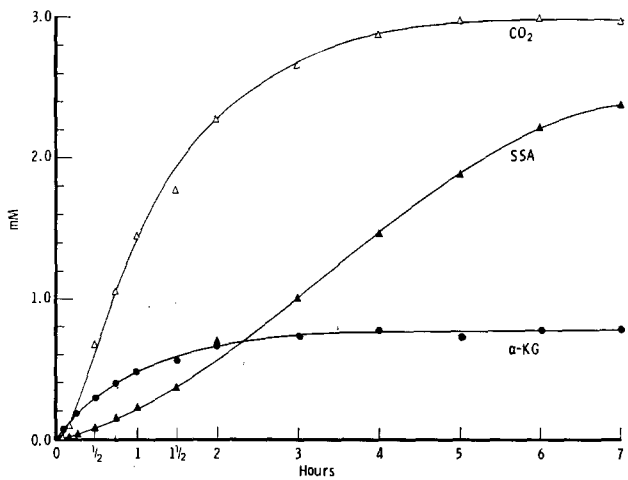

FIG. 4. Acid-catalyzed amide hydrolysis and decarboxylation of *N*-methyl-α-ketoglutaramic acid. Barium *N*-methyl-α-ketoglutaramate (3.75 μmoles) was dissolved in 1.0 ml of 1 *N* HCl and heated at 99–100°C as described in the text and in Fig. 1. SSA, succinic semialdehyde; α-KG, α-ketoglutarate.

[7] In fact, even this estimate may be too high because some hydrolysis of α-ketoglutaramic acid may occur in 1 *N* HCl at 25°C giving rise to α-ketoglutaric acid and succinic semialdehyde; the 2,4-dinitrophenylhydrazones of these products would have been indistinguishable from that of α-ketoglutaramic acid itself in the spectrophotometric assay.

TABLE 2

PSEUDO-FIRST-ORDER RATE CONSTANTS FOR AMIDE HYDROLYSIS AND DECARBOXYLATION OF α-KETOGLUTARAMIC ACID (2-PYRROLIDONE-5-HYDROXY-5-CARBOXYLIC ACID) AND OTHER AMIDES AT 100°C IN 1 *N* HCl[a]

Compound	Rate constant (min^{-1})	
	Amide hydrolysis	Decarboxylation
2-Pyrrolidone-5-hydroxy-5-carboxylic acid	0.062	0.058
2-Pyrrolidone-5-carboxylic acid	0.022	Not detected
5-Hydroxy-2-pyrrolidone	0.030	
2-Pyrrolidone	0.015	
1-Methyl-2-pyrrolidone-5-hydroxy-5-carboxylic acid	0.004	0.017
Asparagine	0.085	

[a] Rate constants were calculated from rates of product formation. The products measured were α-ketoglutaric acid (from 2-pyrrolidone-5-hydroxy-5-carboxylic acid and 1-methyl-2-pyrrolidone-5-hydroxy-5-carboxylic acid), succinic semialdehyde (from 5-hydroxy-2-pyrrolidone), glutamic acid (from 2-pyrrolidone-5-carboxylic acid), γ-aminobutyric acid (from 2-pyrrolidone), and ammonium ion (from asparagine).

glutaramic acid (1-methyl-2-pyrrolidone-5-hydroxy-5-carboxylic acid) was first order with respect to α-ketoglutaric acid formation (Fig. 4); the calculated rate constant for the reaction was 0.004 min^{-1}, a value only 6.4% of that obtained for the amide hydrolysis of α-ketoglutaramic acid. Decarboxylation of *N*-methyl-α-ketoglutaramic acid was slower than that of α-ketoglutaramic acid and showed a distinct initial lag phase. The apparent rate constant, calculated at late times when the reaction was first order, was found to be 0.017 min^{-1}, a value about one-third that for decarboxylation of α-ketoglutaramic acid (Table 2). Whereas introduction of a methyl group on the amide nitrogen slowed the decarboxylation process, the rate of amide hydrolysis was reduced even more so. In fact, α-ketoglutaric acid accounted for only 20% of the products, but the sum of the yields of α-ketoglutaric acid and CO_2 together constituted 97% of the theoretical yield. As was the case for α-ketoglutaramic acid, the hydrolysis of *N*-methyl-α-ketoglutaramic acid gave rise to succinic semialdehyde but more slowly, following a longer latency period (Fig. 4). However, the sum of α-ketoglutaric acid and succinic semialdehyde, when plotted against time over a 4-hr period, yields a straight line. The slope of this plot is virtually identical to the rate of methylamine formation from *N*-methyl-α-ketoglutaramic acid, obtained under similar hydrolytic conditions by Meister (*2*).

Hydrolysis of asparagine, a simple, open-chain amide, occurs at about five times the rate at which 2-pyrrolidone and at more than 20 times the rate at which 1-methyl-2-pyrrolidone-5-hydroxy-5-carboxylic acid are hydrolyzed (Table 2). These findings are consistent with the known resistance of sterically hindered (substituted) amides to acid hydrolysis (*27*, *39*). The greater ease with which 2-pyrrolidone-5-hydroxy-5-carboxylic acid is hydrolyzed as compared to that of 2-pyrrolidone (four-fold) may be due to facilitation by the electron-withdrawing groups at C-5; the presence of either a

carboxyl group (2-pyrrolidone-5-carboxylic acid) or an hydroxyl group (5-hydroxy-2-pyrrolidone) also increases the rate of hydrolysis, and the effect appears to be additive.

DISCUSSION

Proposed Scheme for the Acid-Catalyzed Conversion of α-Ketoglutaramic Acid (2-Pyrrolidone-5-hydroxy-5-carboxylic Acid) to α-Ketoglutaric Acid

The proposed scheme of the acid-catalyzed conversion of α-ketoglutaramic acid to α-ketoglutaric acid is outlined in Fig. 5 (pathway 1). The scheme consists of the amide hydrolysis of the γ-lactam, 2-pyrrolidone-5-hydroxy-5-carboxylic acid, leading to the formation of a carbinolamine intermediate (I). Such carbinolamines are known to be unstable and break down spontaneously to the corresponding α-keto acids with the liberation of ammonia (cf. *28*).

Several features of the reaction pathway are noteworthy. First, the contribution to amide hydrolysis via the open-chain form of α-ketoglutaramic acid is considered to be negligible. Meister (*2*) showed that α-ketoglutaramic acid and the structurally related compounds, asparagine, isoasparagine, isoglutamine, and α-ketosuccinamic acid, are all hydrolyzed at comparable rates, i.e., there was only a 50% difference in the $t_{\frac{1}{2}}$ value for the hydrolysis of α-ketosuccinamic acid (fastest) and α-ketoglutaramic acid (slowest). Our data for the rates of hydrolysis of asparagine and α-ketoglutaramic acid are consistent with these findings (Table 2). However, in 1 *N* HCl less than 1% of α-ketoglutaramic acid exists in the open-chain form; therefore, if only the open-chain form were undergoing hydrolysis, the rate of hydrolysis should have been at least two orders of magnitude slower than, e.g., that of asparagine. Second, the conversion of 2-pyrrolidone-5-hydroxy-5-carboxylic acid to 2-hydroxyglutamic acid (carbinolamine structure I, Fig. 5) is analogous to the conversion of 2-pyrrolidone-5-carboxylic acid to

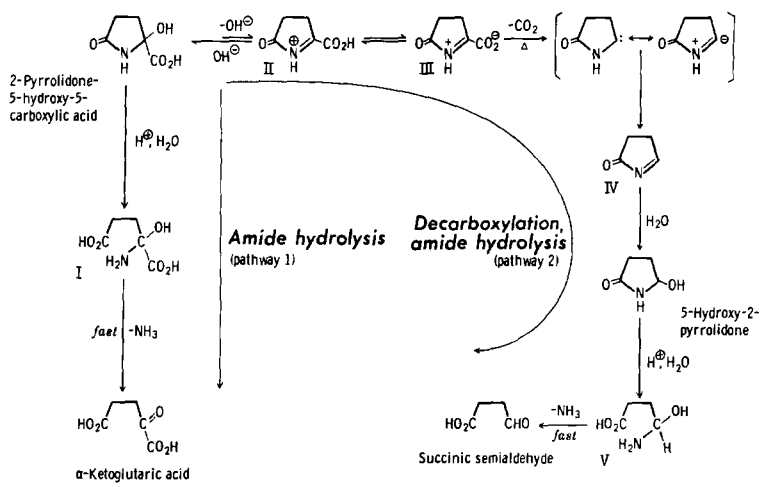

FIG. 5. Proposed mechanism of the amide hydrolysis–decarboxylation of the γ-lactam structure of α-ketoglutaramic acid.

glutamic acid (*13*). Cleavage of the acyl, as opposed to the alkyl, amide bond is consistent with the general observation that hydrolysis of *N*-substituted amides leads to the formation of amines rather than alcohols (*27*). Third, the rate of amide hydrolysis of α-ketoglutaramic acid was found to be proportional to the molar concentration of HCl, but it has not been established whether the reaction is general or specific acid-catalyzed. Since the mechanism for acid-catalyzed hydrolysis of amides is still uncertain (cf. *29*), speculation on a more detailed mechanism for pathway 1 is unwarranted.

Proposed Scheme for the Decarboxylation of 2-Pyrrolidone-5-hydroxy-5-carboxylic Acid to 5-Hydroxy-2-pyrrolidone and Subsequent Formation of Succinic Semialdehyde

Conversion of 2-pyrrolidone-5-hydroxy-5-carboxylic acid to succinic semialdehyde is thought to proceed by pathway 2 (Fig. 5). The scheme involves dehydration to the cyclic dehydropeptide (structure II), followed by decarboxylation of the zwitterion species derived therefrom (structure III). Hydration of the decarboxylated product (IV) gives rise to the relatively stable intermediate, 5-hydroxy-2-pyrrolidone. Succinic semialdehyde arises from acid-catalyzed amide hydrolysis of 5-hydroxy-2-pyrrolidone via the intermediate formation of the unstable carbinolamine (4-amino-4-hydroxybutyric acid, structure V), a sequence analogous to that of pathway 1.

Support for pathway 2 derives from several lines of evidence. Inasmuch as 2-pyrrolidone-5-carboxylic acid does not decarboxylate during acid hydrolysis (*13*), the alternative mechanism that decarboxylation occurs via protonation of the amide oxygen (i.e., *N*-protonated Δ^1-2-hydroxypyrroline-5-hydroxy-5-carboxylate zwitterion) seems unlikely; according to that mechanism, 2-pyrrolidone-5-carboxylate would be expected to decarboxylate as readily as 2-pyrrolidone-5-hydroxy-5-carboxylate. The presence of the hydroxyl group in 2-pyrrolidone-5-hydroxy-5-carboxylic acid, therefore, must somehow facilitate decarboxylation. However, compounds such as malic acid, which contain a hydroxyl group alpha to a carboxyl group, do not readily decarboxylate, presumably because alkoxyl (hydroxyl) groups exert only a very small stabilizing effect on adjacent carbanions (*30*). The small but measurable lag in CO_2 production (Figs. 1 and 2) indicates formation of a precursor prior to decarboxylation. Such a precursor is likely to be the *N*-protonated dehydrated compound (1-pyrrolin-5-one-2-carboxylic acid acylimmonium ion, II; Fig. 5) or the zwitterion (III). Cooper and Redfield (*25*) postulated on the basis of pmr data that 2-pyrrolidone-5-hydroxy-5-carboxylic acid exists in equilibrium with a dehydrated species, with the equilibrium position favoring the hydrated species. Interconversion between the two forms appears to be rapid under basic conditions but slow in acid (*25*). Thus, under the conditions of acid hydrolysis several minutes may be required to achieve optimal concentrations of structure III; this lag would account for the delay in CO_2 formation. Additional support for the formation of the cyclic dehydropeptide include the following: (a) Linear dehydropeptides are well known (*31*); presumably, unlike the cyclic structures, the equilibrium between hydrated and dehydrated forms favors the dehydrated species (*31*). (b) Several *N*- and 4-substituted derivatives of 5-hydroxy-2-pyrrolidone have been prepared (*32*, *33*) and applied to the synthesis of certain alkaloids (*33*). The reactive species in such reactions was suggested to be the cyclic acylimmonium ion, a structure exactly analogous to intermediate II (Fig. 5). (c) 2-Pyrrolidone-5-hydroxy-5-carboxylic acid and 5-hydroxy-2-pyrrolidone are reduced under relatively mild conditions (with cyanoborohydride)

to 2-pyrrolidone-5-carboxylic acid and 2-pyrrolidone, respectively (*25* and Table 1), suggesting the presence of an imine-type intermediate. (d) The lower rate of decarboxylation of *N*-methyl-α-ketoglutaramic acid compared with that of α-ketoglutaramic acid is ascribed to differences in the steady-state concentration of the dehydrated intermediate; formation of the acylimmonium ion is expected to be more difficult with the disubstituted amide.

Whereas the initial rate of formation of α-ketoglutaric acid from amide hydrolysis of ketoglutaramic acid was directly proportional to hydrogen ion concentration, no such correlation was observed for decarboxylation (Fig. 2). This behavior is consistent with the hypothesis that structure III is the precursor for the decarboxylation reaction inasmuch as its concentration will be a complex function of pH. Since the pK_a of a simple aliphatic acid is between 4 and 5 (*32*), increasing pH should favor formation of the carboxylate anion and hence increase the rate of decarboxylation. On the other hand, increasing pH may lead to deprotonation of the amide nitrogen which would tend to decrease the rate of decarboxylation. Assessment of the relative concentration of structure III is further complicated by the fact that the equilibrium between the hydrated and dehydrated forms of 2-pyrrolidone-5-hydroxy-5-carboxylic acid will depend to some extent upon the ionization states of the amide and carboxyl groups.

The decarboxylation mechanism outlined in Fig. 5 is analogous to that proposed by Bender and Breslow (*35*) for the amine-catalyzed decarboxylation of α-keto acids in which the transition state energy is lowered by resonance between a zwitterion and a carbene. Brown lists several examples of decarboxylation reactions thought to involve zwitterions, including α-imino acids of the picolinic and quinaldinic type (*36*). These acids decarboxylate readily under acidic conditions and in support of the zwitterion mechanism, Brown and Hammick (*37*) pointed out that *N*-methylquinaldinic acid (a betaine, which is analogous in structure and charge distribution to the zwitterion) decarboxylates more than 50 times faster than the unsubstituted acid. In contrast to these findings, it has been proposed that 5-azaorotic acid undergoes acid-catalyzed decarboxylation via a mechanism involving the free acid and an unprotonated nitrogen (*38*). We are unable to exclude completely the neutral species (azaorotic acid-type mechanism), as opposed to the zwitterion (III) (picolinic acid-type mechanism), as the decarboxylating species. However, the finding that *N*-methyl-α-ketoglutaramic acid is preferentially decarboxylated in 1 *N* HCl (Fig. 4) argues against the neutral species as the susceptible form in the decarboxylation of this *N,N*-disubstituted amide. (A five-centered mechanism of the azaorotic acid type could only occur in the hydrated form and would involve ring opening with no resonance stabilization of the resultant carbene.)

Possible Biological Significance of the Decarboxylation–Deamidation Pathway

At present, the only known route for the degradation of α-ketoglutaramate *in vivo* is via deamidation by ω-amidase (*2, 6*). However, enzymatic or spontaneous decarboxylation of α-ketoglutaramate and subsequent hydrolysis of the intermediate, 5-hydroxy-2-pyrrolidone, might lead to the *in vivo* formation of succinic semialdehyde. Enzymatic hydrolysis of 2-pyrrolidone-5-carboxylate to glutamate does occur (i.e., the ATP-dependent 5-oxo-prolinase reaction (*40*)), and an analogous cleavage of 5-hydroxy-2-pyrrolidone would give rise to succinic semialdehyde. If this reaction occurs in brain, transamination of succinic semialdehyde with glutamic acid (γ-aminobutyrate trans-

aminase) would yield γ-aminobutyric acid, a known and potentially important neurotransmitter. In this connection it is noteworthy that concentrations of γ-aminobutyrate are increased in the cerebrospinal fluid of patients with hepatic encephalopathy (*41*), a condition that is also associated with elevated levels of α-ketoglutaramate in the cerebrospinal fluid (*8*).

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