Short Communications

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Preparative enzymatic synthesis of the *threo* and *erythro* isomers of γ-hydroxy-L-glutamic acid

γ-Hydroxyglutamate, first isolated from Phlox by VIRTANEN AND HIETALA[1] and first synthesized chemically by BENOITON AND BOUTHILLIER[2], is of metabolic interest as an intermediate in the degradation of hydroxy-L-proline by mammalian enzymes[3–5]. Several methods have previously been described for the preparative synthesis of optically active isomers of γ-hydroxyglutamate. All four isomers are separable from the mixture of racemates obtained by chemical synthesis, utilizing chromatographic separation of the two diastereomeric racemates and enzymatic resolution of each of the latter[6]. This method, however, requires both a commercially unavailable starting compound, ethyl-α-acetoxy-β-chloropropionate, for the initial synthesis, as well as the multiple procedures attendant on enzymatic resolution of each racemate by the deacylase method. The single isomer of primary interest in mammalian metabolism, *erythro*-γ-hydroxy-L-glutamate, can be prepared enzymatically from hydroxy-L-proline *via* the intermediate Δ^1-pyrroline-3-hydroxy-5-carboxylate[3,7]; this method involves an initial step with an unstable enzyme ("proline oxidase"[8]) and a poor yield of the pyrroline product. The most recently described method[9] depends on isolation of *threo*-γ-hydroxy-L-glutamate from the leaves of *Phlox decussata*; facilities for growing the plant in bulk quantities are required and the method yields an isomer which appears not to be that normally produced in the mammalian metabolism of hydroxy-L-proline[3].

We have utilized two other enzymatic reactions that permit the synthesis in good yield of both L-diastereomers of γ-hydroxyglutamate from readily available starting compounds. All reagents are commercial except for a single enzyme preparation that is stable and easily prepared. This method involves two steps: first, the reversible enzymatic condensation of pyruvate and glyoxylate to form DL-α-hydroxy-γ-ketoglutarate*[5,10]. The latter compound is chromatographically separated from the incubation mixture and in a second step is reduced with glutamate dehydrogenase (EC 1.4.1.3), NH_4^+, and DPNH to obtain the *erythro*- and *threo*-diastereomers of γ-hydroxy-L-glutamate. The latter two isomers are isolated separately by chromatography through Dowex-1–acetate as originally described by BENOITON *et al.*[6].

Apart from simplicity and high overall yield, this method has several advantages: it provides approximately equal amounts of both the L-isomers found in nature and active as substrates for transamination by mammalian enzymes[5]; the DL-mixture of the corresponding keto acid is obtained as an intermediate for possible separate use; it permits the preparation of the amino acid or keto acid products

* It has been previously reported that the enzymatic cleavage of α-hydroxy-γ-ketoglutarate is not stereospecific, either antipode being active as a substrate[5].

isotopically labeled in any or all carbon atoms, depending on the choice of specifically labeled glyoxylate and/or pyruvate as starting materials.

The following account of a specific preparation describes the steps in detail: the first incubation mixture contained 9 mmoles of sodium glyoxylate, 27 mmoles of sodium pyruvate, 5 mmoles of potassium phosphate (pH 7.5) and 210 mg of enzyme protein in a total volume of 75 ml. The enzyme was prepared as described earlier, representing the 0.55 ammonium sulfate precipitate* of Fraction III (Table I, see ref. 5). The enzyme is stable and has been found active after 6-month storage at $-15°$.

The components above were incubated at 37° for 12 h at which time 85% of the glyoxylate had been consumed to form α-hydroxy-γ-ketoglutarate, assayed with glutamate dehydrogenase[5]. The reaction was stopped by adding 0.1 vol. of 50% trichloroacetic acid, coagulated protein was removed by centrifugation, and the supernatant solution was adjusted to pH 7.5 with NaOH and placed on a 4 × 36-cm column of Dowex-1–formate. Gradient elution with formic acid was carried out as described earlier[5], collecting 15-ml fractions. α-Hydroxy-γ-ketoglutarate appeared in tubes 160 to 200; the quantity totalled 6.55 mmoles or about 85% of that put on the column. The pooled eluates were concentrated about 10-fold in a flash evaporator, neutralized to pH 7.0 with NaOH, and stored at $-15°$. The preparation was stable for at least 3 months, and served as the source of a number of separate preparations of γ-hydroxyglutamate.

0.95 mmole of α-hydroxy-γ-ketoglutarate were incubated at 37° with 40 mg of crystalline liver glutamate dehydrogenase (Sigma Chemical Company), 1.0 mmole of neutralized DPNH, 0.55 mmole of $Na(NH_4)_2PO_4$, and 5 mmoles of potassium phosphate (pH 7.0), in a total volume of 88 ml. After 5 h all but 0.09 mmole of the keto acid had been consumed, as assayed with excess DPNH and glutamate dehydrogenase. The reaction was stopped by adding 0.1 vol. of 50% trichloroacetic acid, and the supernatant solution obtained after centrifugation was placed on a 2.2 × 32-cm column of Dowex-50 X8 (H^+). The column was washed with 3 column volumes of water and eluted with 0.3 N HCl, collecting 15-ml samples. γ-Hydroxyglutamate was eluted in tubes 25 to 52 and represented 0.77 mmole, or 90% of the α-hydroxy-γ-ketoglutarate consumed. The pooled eluates were repeatedly evaporated to remove most of the HCl, neutralized with NaOH, and placed on a 4.5 × 45-cm column of Dowex-1–acetate; the column was eluted with 0.5 N acetic acid collecting 10-ml fractions. *erythro*-γ-Hydroxy-L-glutamate (0.30 mmole) was eluted in a volume of 400ml, beginning at about 6 column volumes; *threo*-γ-hydroxy-L-glutamate(0.35 mmole) was eluted in 450 ml, beginning 400 ml after the end of the *erythro* peak. In other runs it has been found that up to 0.6 mmole of the mixed diastereomers obtained from a Dowex-50 column can be separated completely by a single passage through a 3.8 × 45-cm column of Dowex-1, with as much as 100 ml of eluate between the peaks.

The γ-hydroxyglutamate so obtained is the L-form, as would be expected from the stereospecificity of liver glutamate dehydrogenase[11]. Thus, polarimetric measure-

* As noted earlier[5], this ammonium sulfate fraction contains the α-hydroxy-γ-ketoglutarate cleavage enzyme and is free of glutamate–aspartate transaminase which aminates the keto acid, while the 0.55–0.7 ammonium sulfate fraction contains transaminase activity but no keto-acid cleavage activity.

ment of the *erythro* peak obtained in the preparation described above gave a molar rotation in 5 N HCl of + 59.6°, on the basis of ninhydrin determination of the concentration, in good agreement with the reported values of + 59.5° (see ref. 3) and +61.6° (see ref. 6) for *erythro*-γ-hydroxy-L-glutamate. The product is homogeneous on paper chromatography and is uncontaminated with residual NH_4^+ or DPN^+, present in the second incubation mixture. Approximately equal amounts of the *erythro* and *threo* forms are recovered, indicating the formation of equal or nearly equal quantities of the two isomers of α-hydroxy-γ-ketoglutarate in the enzymatic condensation reaction, in confirmation of the earlier observation[5] of non-stereospecific enzymatic cleavage of the keto acid. The procedure above has been used repeatedly to obtain both unlabeled and ^{14}C-labeled *erythro*- and *threo*-γ-hydroxy-L-glutamate. The overall yield of both isomers from glyoxylate, as the limiting initial reactant, has been about 40–50%.

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The sulfhydryl content of some lactate dehydrogenases*

Recently evidence has been obtained for the involvement of sulfhydryl groups in the mechanism of action of a number of lactate dehydrogenases (L-lactate: NAD^+ oxidoreductase, EC 1.1.1.27)[1]. Since lactate dehydrogenase now appears to consist of 4 subunits[2,3], it is possible that S–S bridges are involved in holding the subunits together. Therefore, in order to clarify further the role of the SH groups, we thought it of value to determine the cysteine and cystine content of a number of lactate

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