Studies on Specific Enzyme Inhibitors

VII. SYNTHESIS AND ENZYME-INHIBITORY PROPERTIES OF α-MONOFUROGLUTARIC ACID*

L. K. GOTTWALD, J. E. AYLING, AND E. KUN†

From the Departments of Pharmacology and Biochemistry, School of Medicine, and the Department of Pharmaceutical Chemistry, School of Pharmacy, the University of California, San Francisco 22, California

(Received for publication, June 13, 1963)

In the course of investigations, aiming at the development of enzyme inhibitors to be used as "enzyme reagents" in complex biochemical systems (1-6), special attention was paid to analogues of physiological enzyme substrates which occupy key positions in branching points of metabolic pathways. The present work deals with the chemical and enzyme-inhibitory properties of a hitherto unknown analogue of glutamic acid, which contains a fluorine atom in place of NH₂ as a substituent on the α carbon.

EXPERIMENTAL PROCEDURE AND RESULTS

Chemical Synthesis of α-Mono fluoroglutaric Acid

Diethyl fluoromalonate (7) (10.74 g, 0.065 mole) was added in one portion to a solution of sodium (0.14 g, 0.006 mole) in absolute ethanol (20 ml). Ethyl acrylate (7.4 g, 0.074 mole), dissolved in ethanol (10 ml), was added dropwise over a period of 15 minutes to the rapidly stirred solution of diethyl sodio-fluoro-malonate. Heat was evolved and the reaction mixture turned brown. The mixture was left at room temperature for 20 hours and then heated under reflux for 30 minutes. After cooling, the mixture was treated with glacial acetic acid to bring the pH to 7.0 and then the solvents were evaporated at 50-60° under vacuum. The residue was taken up in 50 ml of anhydrous ether and filtered in order to remove salts. The solvent was removed under vacuum and the residue distilled, yielding diethyl α-fluoroglutarate (4.3 g, 37%), b.p. 120-128° at 5 to 8 mm. A sample, redistilled for analysis, had a boiling point of 110-112° at 5 mm. Analysis,

\[ \text{C}_6\text{H}_{10}\text{FO}_4 \]

Calculated: C 52.84, H 7.13, F 9.74
Found: C 52.62, H 7.32, F 9.17

A second, higher boiling fraction was identified as triethyl α-fluoro-c-carboxyglutarate (6.1 g, 37%), b.p. 158-160° at 5 to 8 mm. Analysis,

\[ \text{C}_6\text{H}_{12}\text{FO}_4 \]

Calculated: C 51.8, H 6.9, F 6.8
Found: C 51.7, H 6.8, F 6.9

This primary reaction product of condensation of diethyl sodio-fluoromalonate and ethyl acrylate, triethyl α-fluoro-c-carboxyglutarate, can be prepared in 67% yield by repeating the above synthetic procedure without heating under reflux.

α-Fluoroglutaric Diamide—Diethyl α-fluoroglutarate (2.06 g; 0.01 mole) was mixed with ammonium hydroxide (7 ml, 15% w/w) while cooling in an ice bath. On standing over night at 0° white crystals deposited and were filtered, washed with ethanol, and dried, yielding 1.0 g of the diamide, m.p. 165-167°.

A sample recrystallized from ethanol had a melting point of 169-169.5°. Analysis,

\[ \text{C}_3\text{H}_7\text{FN}_2\text{O}_4 \]

Calculated: C 40.54, H 6.52, N 18.91, F 12.83
Found: C 40.25, H 6.30, N 18.71, F 12.69

α-Fluoroglutaric Acid—A mixture of diethyl α-fluoroglutarate (4.3 g, 0.022 mole) and 10% hydrochloric acid (30 ml) was heated under reflux for 10 hours. The solvent was removed under vacuum, leaving 3.4 g of white crystalline product. After one recrystallization from trifluoroacetic acid, colorless crystals were obtained (2.0 g, 61%), m.p. 110-113°. One subsequent recrystallization from trifluoroacetic acid raised the melting point to 113°. Analysis,

\[ \text{C}_3\text{H}_7\text{FO}_4 \]

Calculated: C 40.00, H 4.70
Found: C 40.10, H 4.76

Infrared spectrum of the free acid (0.5% in KBr pellet): λ_{max} = 3.4 (broad), 5.8, 7.0, 7.45, 7.8, 8.15, 9.1, 9.2, 10.8, 11.35, 12.43, 13.5, 14.4, and 15.3 μ. Alternatively, α-fluoroglutaric acid can be prepared from triethyl α-fluoro-c-carboxyglutarate in 40% yield by heating the ester under reflux with 37% HCl for 12 hours. On evaporation of the solvent the crude acid is obtained, which upon recrystallization from trifluoroacetic acid yields the pure product, m.p. 113°.

Enzyme-inhibitory Properties of α-Mono fluoroglutaric Acid

Inhibitory properties were first tested on enzymes which are known to play a major role in the metabolism of glutamate. Glutamic-aspartic aminotransferase of rat tissues was tested spectrophotometrically (2) and by direct analyses of aspartate formation from glutamate in the presence of tissue homogenates. In the latter type of tests quantitative amino acid analyses were performed by the method of Heilmann, Barollier, and Watzke...
(8) after electrophoretic separation of amino acids on filter paper at pH 3.6 (sodium formate buffer). No effect of fluoroglutarate on glutamate-aspartate aminotransferase was observed up to 10^{-2} M. Glutamate-alanine aminotransferase of the soluble fraction of rat liver homogenates was tested according to Segal, Beattie, and Hopper (9), and again no inhibition could be detected by fluoroglutarate at 10^{-2} M. Malate dehydrogenase (NAD^+-linked), isocitrate dehydrogenase (NADP^+-linked), citrate- condensing enzyme, aconitase (tested as previously described (1, 3, 5, 6)), and succinic dehydrogenase (10) of sonically disrupted mitochondria (cf. (5, 6)) were tested in the presence of fluoroglutarate at concentrations exceeding 10-fold of that of each particular substrate, but no inhibition was observable. Glutamine formation, as determined by paper electrophoresis, from glutamate (10^{-3} M) + ATP (3 \times 10^{-3} M), in the presence of rat tissue homogenates (prepared in 0.15 M KCl + 5 \times 10^{-3} M MgCl_2 and 5 \times 10^{-3} M phosphate of pH 7.4), was uninfluenced by 5 \times 10^{-2} M fluoroglutarate.

On the other hand glutamic dehydrogenase of beef liver (Mann Research Chemicals) was inhibited by fluoroglutarate in a purely competitive fashion, with a $K_i$ of 3.3 \times 10^{-4} (Fig. 1). It should be remembered that synthetic $\alpha$-fluoroglutarate, containing 1 asymmetrical carbon atom, is a racemate of two optical isomers only one of which is likely to be the inhibitor. The $K_i$ for the inhibitory isomer is thus more correctly expressed as 1.6 \times 10^{-4}.

Mitochondrial respiration in a recording polarograph (Oxigraph; Gilson Electronics, Madison, Wisconsin) was also measured in the presence of oxaloacetate alone, oxaloacetate plus pyruvate combined, and $\alpha$-ketoglutarate as substrates (each at 2 \times 10^{-3} M concentration) in the presence of ADP and inorganic phosphate as described earlier (6). Fluoroglutarate at 4 \times 10^{-2} M concentrations did not influence the rate of O_2 uptake, indicating that none of the enzymatic components of the citric acid cycle, or of the electron transfer chain to molecular oxygen, were primarily affected by this substance.

On the other hand, respiration of rat kidney, heart, and, to a lesser extent, brain mitochondria with pyruvate (2 \times 10^{-3} M) as the single added substrate, under conditions described earlier (6), was markedly depressed (50 to 60%) by 4 \times 10^{-2} M fluoroglutarate. Preliminary studies concerning the mechanism of this inhibition disclosed that the site of inhibition was not pyruvate dehydrogenase, as measured by the method of Jagannathan and Schweet (11). Another inhibitory effect of fluoroglutarate (4 \times 10^{-4} M) on mitochondrial O_2 uptake was detected when succinate was used as the single added substrate. The degree of inhibition (25 to 30%) was independent of succinate concentration, which was varied between 5 \times 10^{-4} M and 2 \times 10^{-3} M.

Fluoroglutarate had no inhibitory effect on succinic dehydrogenase system with either 2,6-dichlorophenolindophenol and phenazine methosulfate (10) or cytochrome c (in the presence of KCN) as electron acceptors and submitochondrial particles obtained by sonic disruption (4, 6) as the catalytic system. Further work is required for the clarification of this inhibitory effect of fluoroglutarate, which does not, however, directly involve recognizable enzymatic components of the citric acid cycle.

**DISCUSSION**

Among the major enzymes for which glutamate is a recognized substrate, only glutamic dehydrogenase was found to be inhibited by fluoroglutarate. Glutamate-aspartate aminotransferase can be effectively inhibited by a combination of mono- and difluoro-oxaloacetate (1, 2, 5), but none of these acids act on glutamic dehydrogenase. Specificity of these inhibitors was further tested on glutamate-alanine aminotransferase. This enzyme is unaffected by either mono- or difluoro-oxaloacetate, but, in agreement with Hopper and Segal (12), is powerfully inhibited by aminooxyacetic acid. Aminooxyacetic acid, on the other hand, has no inhibitory effect on glutamate dehydrogenase or glutamate-aspartate aminotransferase.

Although the influence of fluoroglutarate on mitochondrial metabolism does not involve a direct effect on enzymes primarily concerned with the enzymatic disposition of glutamate, except glutamate dehydrogenase, its usefulness as a specific enzyme reagent is complicated by its inhibitory action on "pyruvate" and to a lesser extent on "succinate" respiration. With these reservations in mind, it still seems possible to use a combination of the above listed inhibitors for a study of functional participation of enzymes in complex systems (13) acting on glutamate as a common substrate. This work and further studies concerning the mode of action of fluoroglutarate are being continued.

Comparison of $\gamma$-fluoroglutamate (14, 15) and $\alpha$-monofluoroglutarate, with respect to their effect on glutamate dehydro-
genase, showed that, in contrast to fluoroglutarate, the former is a substrate of this enzyme. At a concentration of $10^{-3}$ M the velocity of enzymatic oxidation of $\gamma$-fluoroglutarate by crystalline liver glutamate dehydrogenase is about one-tenth of that of glutamate. Further characterization of this reaction is in progress.

**SUMMARY**

1. Chemical synthesis of $\alpha$-monofluoroglutarate by a Michael type of condensation of diethyl sodio-fluoromalonate with ethyl acrylate is described.

2. Monofluoroglutarate has no inhibitory effect on malate, isocitrate, succinate, and pyruvate dehydrogenases, nor on citrate-condensing enzyme or aconitase. Respiration of mitochondria is unaffected by fluoroglutarate with oxaloacetate, oxaloacetate plus pyruvate, or $\alpha$-ketoglutarate as substrates.

3. Glutamate dehydrogenase is inhibited competitively by fluoroglutarate, whereas transaminases, involving glutamate or alanine, are uninfluenced by this acid.

4. Respiration of mitochondria in the presence of pyruvate as the only added substrate is strongly inhibited by relatively high concentrations of fluoroglutarate. A lesser inhibition of $O_2$ uptake in the presence of succinate was also recorded. The nature of this inhibitory effect is at present unknown.

**REFERENCES**

Studies on Specific Enzyme Inhibitors: VII. SYNTHESIS AND ENZYME-INHIBITORY PROPERTIES OF α-MONOFLUOROGLUTARIC ACID

L. K. Gottwald, J. E. Ayling and E. Kun


Access the most updated version of this article at http://www.jbc.org/content/239/2/435.citation

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/239/2/435.citation.full.html#ref-list-1