Fluorocitric Acid: Selective Microbial Degradation of the Inhibitory Isomer

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In Peters's laboratory it has been shown that the toxicity of fluoroacetate for mammals can be attributed to the 'lethal synthesis' of fluorocitrate, a potent inhibitor of the tricarboxylic acid-cycle enzyme aconitase (EC 4.2.1.3) (Peters, Wakelin, Buffa & Thomas, 1953; Morrison & Peters, 1954). The organic synthesis of fluorocitrate yields four diastereoisomers, only one of which has inhibitory activity (Ward & Peters, 1961; Fanshier, Gottwald & Kun, 1964); this activity has been ascribed to an enantiomer of the DL pair tentatively assigned the *erythro* configuration by Fanshier *et al.* (1964). Each enantiomer of the DL pair can be synthesized enzymically by using citrate synthase (EC 4.1.3.7), the inactive one from acetyl-CoA and fluoro-oxaloacetate and the active one from fluoroacetyl-CoA and oxaloacetate (Fanshier, Gottwald & Kun, 1962, 1964; Brady, 1955). The absolute configuration of the individual enantiomers has not been determined because of the small amount of material provided by the enzymic synthesis.

In order to provide enough of the active enantiomer (or its mirror image) for physical-chemical studies we have used the enrichment culture technique to obtain a micro-organism that will selectively degrade one member of the DL-*erythro* pair. The organism isolated, classified as *Pseudomonas* species, is capable of completely degrading the inhibitory isomer, leaving only its inactive enantiomer in the medium. While work was in progress to characterize this isomer, we became aware of the resolution of these isomers as the deoxyephedrine salts by Dummel & Kun (1969). It seems clear that the procedure of these workers should provide enough of the resolved *erythro* enantiomers to determine the absolute stereochemistry of the inhibitory isomer of fluorocitrate. A brief report on our approach to this problem, however, seems appropriate. First, it provides an independent confirmation of the results of Dummel & Kun (1969) and, secondly, it indicates the availability of a pseudomonad capable of living in a concentration of fluorocitrate greater than 1 mM and capable of degrading a compound known to inhibit the metabolism of animals and bacteria (Jayasuriya, 1956; Behrman & Stanier, 1957).

Diethyl monofluorocitrate was prepared by the condensation of diethyl monofluoro-oxaloacetate

with malonic acid, converted into the triethyl ester and purified by distillation (Fanshier *et al.* 1964). Analysis of this material by g.l.c. indicated two peaks present in the ratio approx. 4:1, which presumably represent the *erythro* and *threo* racemates described by Fanshier *et al.* (1964). The triethyl ester was hydrolysed in base and the racemates were separated by ion-exchange chromatography. For a typical preparation 1.7 g of mixed fluorocitrates was added to a column (2 cm × 28 cm) of Dowex 1 (Bio-Rad AG1-X4; 200–400 mesh; formate form), which was then eluted by a linear gradient of formic acid (1.0–5.0 M). Samples of each fraction were assayed colorimetrically (Hartford, 1962). After the elution of several uncharacterized compounds, presumably resulting from the hydrolysis of triethyl ester, the *erythro* racemate of fluorocitric acid was eluted. This was related to the *erythro* racemate described by Fanshier *et al.* (1964) by comparison with the published values of the melting point and i.r. spectrum of the cyclohexylammonium salt (Fanshier *et al.* 1964; Dummel & Kun, 1969). Further support for this assignment came from the n.m.r. spectrum of the free acid in D₂O and the retention time of the trimethyl ester on g.l.c., both of which are identical with the values obtained with material synthesized by the published method (Fanshier *et al.* 1964).

The pseudomonad was isolated from local mud at room temperature on a medium with the following composition (per l): KH₂PO₄, 0.5 g; K₂HPO₄·3H₂O, 1.2 g; NH₄Cl, 10 g; racemic sodium *erythro*-fluorocitrate (2.63 g); 5 ml of salts A and 1.5 ml of salts B (Goldman, Milne & Keister, 1968). At later stages in the isolation of the organism 0.05% or 0.1% of yeast extract was added to the above medium. The organism was maintained on slants of this composition supplemented with 0.05% of yeast extract and 0.85% of Ionagar no. 2 (Consolidated Laboratories, Chicago, Ill., U.S.A.).

To degrade one isomer of the DL-*erythro* pair the organism was allowed to grow on a medium identical with the one described for isolation except that 0.1% of yeast extract was added and the concentration of racemic sodium *erythro*-fluorocitrate was 1.32 g/l. A slant was used to inoculate 100 ml of this medium, and after 24 h on a rotary shaker

Fig. 1. Release of F^- ion from racemic *erythro*-fluorocitric acid. F^- ion was measured by a specific ion electrode (Orion Research, Cambridge, Mass., U.S.A.). ○ and ● refer to different experiments. For details see the text.

at 28–30°C the bacterial suspension was divided and used to inoculate three 2litre flasks, each containing 630ml of the same medium. The progress of the microbial degradation, as indicated by the release of F^- ion from fluorocitrate, is shown in Fig. 1. The F^- ion concentration reached a value of 2.1mm in one experiment and 2.4mm in the other. These concentrations represent essentially one-half the racemic *erythro*-fluorocitric acid (4.8mm) added to the medium. The quantitative appearance of F^- ion in the medium is consistent with the selective microbial degradation of one of the two isomers present.

To recover the remaining isomer, the growth medium was filtered through Celite 545 (Johns Manville Corp., New York, N.Y., U.S.A.) and acidified by the addition of dry Dowex 50 (H^+ form). The fluorocitric acid was extracted continuously with ether for 3 days and further purified on Dowex 1 as described above.

The enantiomer of *erythro*-fluorocitric acid was obtained in 70% yield based on 2g of racemic fluorocitric acid originally added to the growth medium. After three crystallizations the melting point of the cyclohexylammonium salt was 164–172°C and the elemental analysis was in agreement with the theoretical value.

This single isomer isolated from the growth medium had no inhibitory effect on aconitase, purified as far as the first ethanol fractionation step (Morrison, 1954) and assayed and activated by the

method of Peters (1961). This was in contrast with the inhibition observed under these conditions with racemic *erythro*-fluorocitrate.

In order to compare the optical rotation of the non-inhibitory isomer with the published value of the inhibitory isomer, the cyclohexylammonium salt (5.028mg, 10 μ mol) was acidified on a column of Dowex 50 (H^+ form), evaporated to dryness and dissolved in a solution that was half-saturated with ammonium molybdate. This solution gave $[\alpha]_D +315^\circ$, which is consistent with the value of -314° found in Peters's laboratory for the inhibitory biosynthetic isomer (Pattison & Peters, 1966). Our results are also consistent with those of Dummel & Kun (1969), since they found $[\alpha]_D -83.5^\circ$ for the cyclohexylammonium salt of the active isomer in half-saturated ammonium molybdate, and we found $[\alpha]_D \pm 84.7^\circ$ for the inactive isomer under these conditions. The inactive isomer has a positive Cotton effect when examined as the cyclohexylammonium salt (c 0.199 in ethanol).

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