2-FLUOROUROCANIC ACID, A POTENT REVERSIBLE INHIBITOR OF UROCANASE

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Summary: The K_i for the interaction of 2-fluorourocanic acid with urocanase (from Pseudomonas fluorescens) is 1000 times as great as K_m for the natural substrate, urocanic acid, whereas enzymatic hydration of the fluoro analog occurs ca. 100 times more slowly. Inhibition is competive and is eventually overcome by utilization of the analog. By contrast, 4-fluoro- and 2-amino-urocanic acid are neither significant inhibitors nor substrates for the enzyme. 2-Fluorourocanic acid may prove a useful tool for blocking the utilization of histidine as a one-carbon source in metabolism.

The catabolism of L-histidine, via urocanic acid, 4-imidazolone-5-propionic acid, and N-formimino-L-glutamic acid (1), provides one of the sources of the one-carbon unit necessary for purine biosynthesis and for other metabolic and regulatory processes; although serine is generally considered the principal donor, there presently exists no conclusive evidence as to whether histidine is an essential, alternative, or minor source (2,3). Equally puzzling is the fact that, in certain human genetic diseases in which any one of the steps between histidine and 5-formiminotetrahydrofolate is blocked or reduced in activity, there appears wide variation in symptoms and in the degree of harmfulness of the enzyme defect (4-8). Regardless of the amino acid source of the one-carbon unit, its transfer requires the cofactor, tetrahydrofolic acid, and one effective approach to leukemia chemotherapy utilizes inhibitory analogs of this cofactor (9).

In the course of studies on the catalytic mechanism of histidine ammonia-lyase (10), we found that 2-fluorohistidine is slowly converted by

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Abbreviations: 2-FUr,2-fluorourocanic acid; 4-FUr, 4-fluorourocanic acid: 2-AUr, 2-aminourocanic acid.

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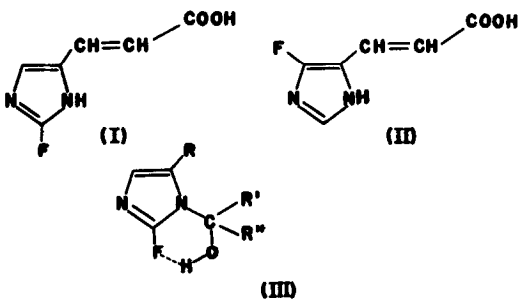
the enzyme to 2-fluorourocanic acid (I), and that the latter species is a potent, reversible inhibitor of urocanase, the second enzyme in the pathway for histidine catabolism. This observation suggested the possibility that either 2-fluorohistidine or 2-fluorourocanic acid may be effective in retarding the formation of formiminoglutamic acid and, thus, in inhibiting purine biosynthesis at an earlier block than that at which tetrahydrofolate antagonists operate. Clearly, such a tool might also help determine the significance of histidine as a one-carbon source. Exploratory studies (11,12) show that 2-fluorohistidine does, in fact, cause a significant depression of murine leukocyte levels and prolongs the survival of leukemia-infected mice. Since elucidation of the mechanisms of the overall inhibitions may prove complex and time-consuming, we present here the data already acquired on the interaction of 2-fluorourocanic acid with urocanase. A general review of fluoroimidazole biochemistry has been prepared by Kirk and Cohen (13).



MATERIALS AND METHODS:

Urocanase from Pseudomonas fluorescens, ATCC 11299b, was prepared by a modification of the method of George and Phillips (14). The material obtained by elution from a DEAE-cellulose column (900 A_{280} units after pooling, with a specific activity of 0.4 enzyme units per A_{280} unit) was subjected to chromatography on an hydroxylapatite column (2.5 x 25 cm), which had been equilibrated with 0.02 M phosphate buffer (pH 7.5) containing 0.2 M NaCl. The elution was performed with a linear gradient composed of 500 ml of the above buffer and 500 ml of 0.2 M phosphate buffer (pH 7.5) containing 0.2 M NaCl. The activity peak from this column was purified further on a DEAE-Sephadex A-50 column (2.5 x 18 cm), which had been equilibrated with 0.015 M phosphate buffer (pH 7.5) containing 0.05 M NaCl. The enzyme fraction was eluted with a linear gradient between 0.05 and 0.5 M NaCl (500 ml of each solution). The specific activity of the combined peak fractions was 1.8 enzyme units per A_{280} unit.

The enzyme was assayed at 25° by the method of Tabor and Mehler (15) in 0.05 M phosphate buffer (pH 7.4), using a urocanate concentration of 0.1 mM. Activity measurements were taken after a 5 min incubation, because of a reproducible lag period which probably involved photoactivation of the enzyme (16). The disappearances of urocanate, 2-FUr, 4-FUr, and 2-amino-

Table 1. Kinetic Constants for Degradation of Urocanic Acid and Its Derivatives by Urocanase.[a]

Compound	$K_m \times 10^4$ M	$K_i \times 10^4$ M	V_{max} [b]
Urocanic acid	0.4 - 1.0		1250
2-FUr	0.01	0.001	8 - 10
4-FUr	nm[c]	nm	nm
2-AUr	nd[d]	ca. 1	nd

[a] Assay conditions are described in legends to Figures 1 and 4

[b] V_{max} is expressed in nmoles of substrate degraded/min/ml of enzyme

[c] Below the level of measurement

[d] Not determined

urocanate were followed at 277, 277, 286, and 289 nm, respectively, these wavelengths corresponding to the absorption maxima of the compounds. One unit of enzyme catalyzes the degradation of one μmole of urocanic acid per min. The kinetic constants were obtained by use of an interactive curve-fitting program, MLAB, and a PDP-10 digital computer (17).

The fluorourocanic acids used in this work were prepared from the corresponding L-histidine derivatives (18,19) by elimination of ammonia with histidine ammonia-lyase (10). The incubation mixture was lyophilized, and the residue was dissolved in a minimum quantity of water. The solution was filtered, adjusted to pH 3, and the precipitate of fluorourocanic acid was collected by filtration. After the compounds had been recrystallized from water, homogeneity and purity were confirmed by elemental analysis (C,H,N) and by mass spectral data. 2-Aminourocanic acid was obtained by total synthesis from urocanic acid (20).

RESULTS:

The kinetic data presented in Table 1 show that 2-FUr has a very strong affinity for urocanase, with a Ki 1000 times as great as Km for urocanic acid itself. Despite this high affinity, the analog is degraded nearly 150-fold more slowly than the natural substrate. Binding of 2-FUr to the enzyme is competitive with that of the natural substrate, showing an apparent K_i of $1 \pm 0.2 \times 10^{-7}$ M (Fig. 1). The high affinity and low reactivity of 2-FUr render this compound an effective reversible inhibitor of urocanic acid breakdown. Thus, addition of 2-FUr to a standard incubation mixture resulted in the immediate reduction of the reaction rate (Fig. 2, arrow). When both sub-

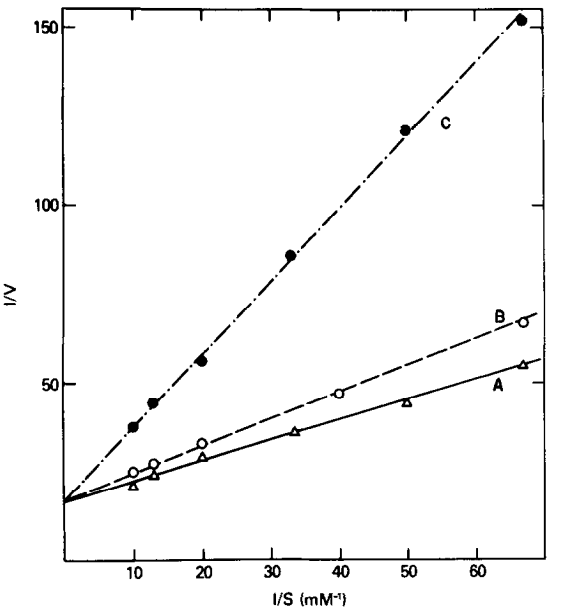


Figure 1. Competitive inhibition of urocanase reaction by 2-fluorourocanate. The incubation mixtures are described in Methods; the enzyme concentration was 0.003 units/ml. V is expressed as Δ_{277} nm/min. —A—, no additions; —B—, 0.5 x 10^{-7} M 2-fluorourocanate added; —C—, 2 x 10^{-7} M 2-fluorourocanate added. Corrections for spectral absorption or degradation of the inhibitor are negligible at these concentrations.

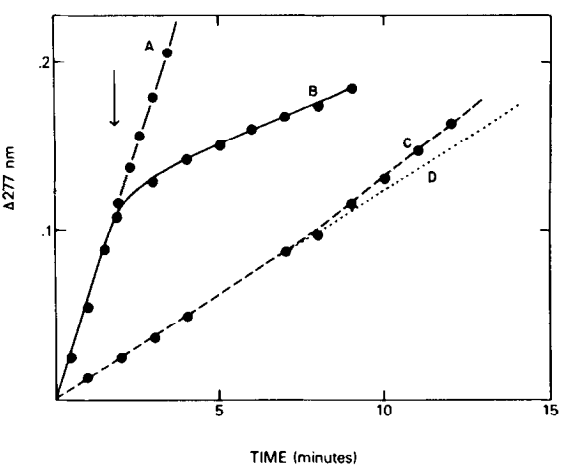
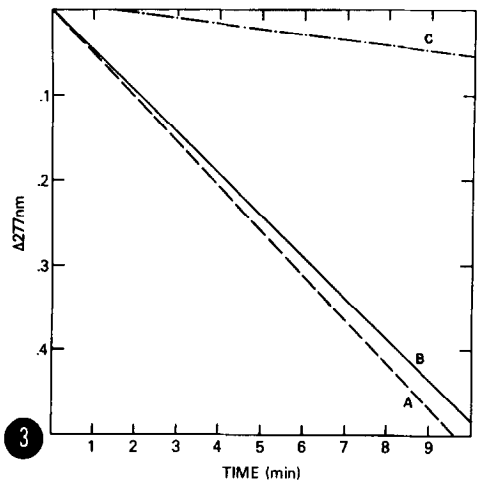


Figure 2. Time course of urocanase reaction in the presence and absence of 2-fluorourocanate. The conditions of the reaction are described in Methods. The concentration of enzyme was 0.004 units/ml. —A—, no additions; —B—, at the arrow, 1.4 mM 2-fluorourocanate was added to one cuvette; —C—, urocanate and 2-fluorourocanate were added at zero time, with an enzyme concentration of 0.0016 units/ml; the extrapolated line (—D—) continues the initial rate.



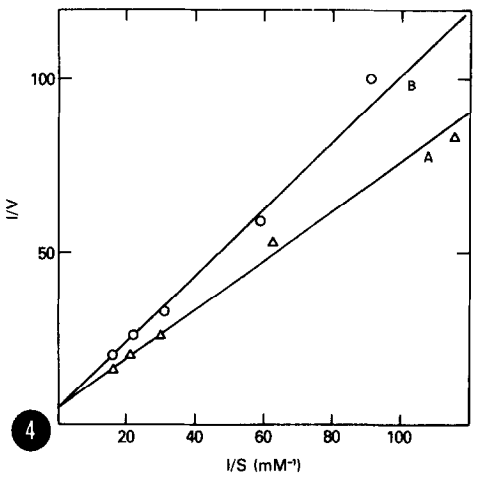


Figure 3. Effect of preincubation of urocanase with 2-fluorourocanate on urocanate degradation. The time course of degradation of urocanate under standard assay conditions was followed in the absence (—A—) and in the presence of 10^{-8} M (—A—) and 10^{-6} M (—C—) 2-fluorourocanate. The enzyme was also preincubated (25°, 30 min) in the presence of 3 x 10^{-6} M 2-fluorourocanate, an aliquot of this mixture was then diluted 150-fold into the assay mixture, and the reaction was followed with time (—B—).

Figure 4. Competitive inhibition of urocanase reaction with 2-aminourocanate. The conditions of the reaction were as described in legend to Figure 1. The enzyme concentration was 0.01 units/ml. —A—, no additions; —B—, 0.026 M 2-aminourocanate added.

strate and analog were added to the incubation mixture at zero time, the reaction proceeded initially at the inhibited rate (Fig. 2, lower line); gradually, the rate of disappearance of urocanate increased, reflecting the slow enzymatic destruction of 2-FUr. The contributions of 2-FUr to optical density were negligible at these concentrations, and required no correction. Further evidence for the reversibility of inhibition is shown in Fig. 3: at a concentration of 3 x 10^{-6} M, 2-FUr gives 90% inhibition of urocanate breakdown (upper line); if the enzyme is preincubated with 2-FUr (25°, 30 min) and an aliquot of this solution is added to the assay mixture to give a final inhibitor concentration of ca. 2 x 10^{-8} M, no significant retardation of reaction is observed (Fig. 3, solid line), even initially.

In contrast to its structural isomer, 4-FUr (II) showed no detectable capacity to inhibit or react with urocanase, while 2-AUr is a relatively poor inhibitor (Fig. 4), and is degraded (at 0.1 mM concentration) ca. 1000-fold more slowly than urocanic acid itself.

DISCUSSION:

These results demonstrate that 2-FUr is a potent, competititve inhibitor of urocanase, that binding to the enzyme is readily and totally reversible, and that urocanic acid does not protect the enzyme against the inhibitor. The difference in binding capacity for the isomeric fluoro analogs is quite remarkable, when one considers that the fluorine atom is small enough to present little steric interference with the active site, and that the geometry of the acrylic acid side chain should be unaffected by the introduction of a fluorine atom or by its ring position. It is noteworthy that corresponding differences between 2-fluoro- and 4-fluorohistidine have been observed for other enzyme systems (10,13). Since 2-fluoroimidazole is 4.5 pK units less basic than imidazole (21), and since a roughly parallel difference in basicity would be anticipated for the urocanic acids, it is clear that ring basicity cannot be critical for binding. Furthermore, 2-aminourocanic acid, whose basicity is probably somewhat higher than that of urocanic acid, is bound very poorly. On the other hand, 2-FUr shows a pK_2 value of 8.4 (ca. 4.5 units less than that expected for urocanic acid), and it is conceivable that the imidazole anion of 2-FUr is actually involved in binding to the enzyme. This possibility is strengthened by the fact that neither 4-FUr (pK_2, ca. 11) nor 2-AUr (pk_2 >12) show significant binding capacity.

On the basis of tentative identification of α-ketobutyrate as the prosthetic group for the enzyme (14), George and Phillips suggested that the carbonyl function may interact covalently with an imidazole ring nitrogen atom. Since there appears to be no direct correlation of amine basicity with the stability of the corresponding Schiff bases or tetrahedral adducts (22), it may be hazardous to place much weight on pK data at the present time. Furthermore, interaction of pyruvate with imidazole could not be detected spectroscopically (22), although the test-tube criterion may be a poor model for the enzyme. If the assumption that covalent interaction between urocanate nitrogen and the enzyme were to prove correct, stabilizaiton of the 2-FUr adduct via an internal hydrogen bond (III) might be offered as another explanation for the difference in binding capacity between 2-FUr and 4-FUr. It would be premature even to speculate on the behavior toward urocanase of other 2-substituted urocanic acids; the poor, but measureable binding and reactivity of 2-AUr may result from a complex combination of steric, conformational (cf. III), and electronic factors. In the course of enzymatic degradation of urocanic acid, removal of a proton from C-4 of the imidazole ring is considered an essential step (23); this process is impossible for 4-FUr, and may account for its failure to show any detectable reactivity with urocanase—although the lack of significant binding may make this explanation superfluous.

Competitive, reversible inhibition of urocanase has been observed with high concentrations of imidazolepropionic acid and with fumaric acid (14). Irreversible inhibition has also been achieved through affinity labeling of the active site with 4-bromocrotonic acid (24). Although the halogen atom in 2-fluoroimidazoles has been found capable of displacement by good nucleophiles (19), the total reversibility of inhibition by 2-FUr shows that it is not acting as an affinity label for urocanase. Indeed, the compound is a remarkably effective reversible inhibitor, and should prove a valuable tool for interference with the catabolism of histidine, and for investigation of its role as a one-carbon donor (25). The biological and pharmacological consequences of *in vivo* production of 2-FUr are under active investigation.

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