# Substrates for Fumarate Hydratase

**Wesley G. Nigh and John H. Richards**

*Contribution No. 3795 from the Gates and Crellin Laboratories of Chemistry, California Institute of Technology, Pasadena, California 91109.*
*Received January 10, 1969*

**Abstract:** Fluorofumaric acid and difluorofumaric acid are found to be substrates for fumarate hydratase. The direction of addition of water to fluorofumarate and, more importantly, the relative reactivities of fluorofumarate and difluorofumarate support a mechanism for the hydration reaction that involves a carbonium ion type intermediate. Neither 2-(*S*)-2-methoxysuccinic nor 2-(*S*)-2-acetoxysuccinic acid nor mono- or difluoromaleic acid are substrates for fumarate hydratase.

The interconversion of fumaric and malic acids is one of the important steps in the Krebs tricarboxylic acid cycle[1] and is catalyzed by the enzyme fumarate hydratase.[2] Until recently fumaric and malic acids were the only two known substrates for fumarate hydratase (EC 4.2.1.2). During the course of the present investigation, reports appeared that L-(−)-tartaric acid, which had been known as a competitive inhibitor,[3,4] was also a substrate[5,6] though less reactive by a factor of around 2000 than the natural substrates.[5] Fluorofumaric acid was also reported to be a substrate.[7] In both cases the product was oxaloacetic acid.

In the present work both mono- and difluorofumaric acids were found to be substrates for fumarate hydratase while 2-(*S*)-2-methoxysuccinic or 2-(*S*)-2-acetoxysuccinic acids were neither substrates nor measurably effective competitive inhibitors. The relative $V_{max}$ for fluorofumaric and difluorofumaric acids are in accord with a rate-determining step which involves carbonium ion character.

## Results

The preparations of monofluorofumaric and difluorofumaric acids have been reported.[8]

On treatment of fluorofumaric acid with fumarate hydratase in buffered solution (10 m*M* phosphate, pH 7.3) at 25° the absorption in the ultraviolet is found to decrease with time below 247 mμ but to increase above 247 mμ (isosbestic point) due to the formation of enolized oxaloacetate. In the case of difluorofumarate, there is no increase in absorption between 250 and 300 mμ, which does not, however, preclude the formation of fluorooxaloacetate as this substance does not enolize to any appreciable extent but exists mainly as the hydrate in solution.[9,10]

Treatment of the reaction products from either fluorofumarate or difluorofumarate with 2,4-dinitrophenylhydrazine by the procedure of Nakamura and Ogata[6] gave a mixture of two 2,4-dinitrophenylhydrazones in each case. Chromatography on silica gel with *n*-butyl alcohol–ethyl alcohol–water (8:2:5) yielded from fluorofumarate the 2,4-dinitrophenylhydrazones of oxaloacetic acid and of pyruvic acid and from difluorofumarate, the 2,4-dinitrophenylhydrazone derivatives of fluorooxalic acid and fluoropyruvic acid.

Aliquots of the enzyme solution were partially denatured by heating and shown to exhibit the same reactivity ratios toward fumarate, fluorofumarate, and difluorofumarate.

By following the rate of hydration spectrophotometrically, the constants in Table I were determined.

**Table I**

| | $K_m$, m*M* | ($V_{max}$ μmol/ml) (min/mg) |
|---|---|---|
| Fumaric acid | 0.33 | 104 |
| Fluorofumaric acid | 2.2 | 410 |
| Difluorofumaric acid | 0.34 | 86 |

Neither fluoro- nor difluoromaleic acids were found to be substrates for the enzyme; 2-(*S*)-2-methoxysuccinic and 2-(*S*)-2-acetoxysuccinic acids were neither substrates nor did they act as competitive inhibitors at concentrations ten times that of substrate.

## Discussion

Fumarate hydratase has long been recognized as an enzyme with exceedingly severe substrate requirements; until recently only *l*-malic acid and fumaric acid would undergo catalytic interaction with this enzyme. Fluorine and hydrogen have similar steric requirements (van der Waals radii: fluorine 1.35 Å, hydrogen 1.2 Å)[11] and might be anticipated, therefore, both to fit at the same site of an enzyme. The close similarity in the values for $K_m$ of fumaric and difluorofumaric acids substantiate this concept.

The addition of water to fluorofumaric acid in the Markovnikov sense is in accord with a carbonium ion type process.[12,13]

The role of polarity in substrate molecules may account for the differences in kinetic values between mono-

(1) H. A. Krebs and W. A. Johnson, *Enzymologia*, 4, 148 (1937).
(2) R. A. Alberty, *Enzymes*, 5, 531 (1961).
(3) V. Massey, *Biochem. J.*, 55, 172 (1953).
(4) P. W. Wigler and R. A. Alberty, *J. Am. Chem. Soc.*, 82, 5482 (1960).
(5) S. Nakamura and H. Ogata, *J. Biol. Chem.*, 243, 528 (1968).
(6) S. Nakamura and H. Ogata, *ibid.*, 243, 533 (1968).
(7) D. D. Clarke, W. J. Nicklas, and J. Palumbo, *Arch. Biochem. Biophys.*, 123, 205 (1968).
(8) M. S. Raasch, R. E. Miegel, and J. E. Castle, *J. Am. Chem. Soc.*, 81, 2678 (1959).
(9) E. Kun, D. R. Grasetti, D. W. Faushier, and R. M. Featherstone, *Biochem. Pharmacol.*, 1, 207 (1958).
(10) W. Kumler, E. Kun, and J. N. Shoolery, *J. Org. Chem.*, 27, 1165 (1962).
(11) L. Pauling, "The Nature of the Chemical Bond," 3rd ed, Cornell University Press, Ithaca, N. Y., p 260.
(12) R. A. Alberty, W. G. Miller, and H. F. Fisher, *J. Am. Chem. Soc.*, 79, 3973 (1957).
(13) D. E. Schmidt, Jr., W. G. Nigh, C. Tanzer, and J. H. Richards, *ibid.*, 91, 5849 (1969).

and difluorofumaric acids. Thus, fluorofumaric acid is a highly polar molecule which may explain its being bound to the active site less effectively than fumarate or difluorofumarate (in which case the symmetry of the molecule destroys any over-all dipole moment). For example, the highly polarized fluorofumarate is either relatively less stable within the less polar environment of the enzyme or induces polarization with the enzyme which lessens the strength of binding interactions. A reaction initiated by addition of a proton may account for the greater reactivity of fluorofumaric acid relative to fumaric acid because of the availability of p electrons on the fluorine to stabilize an adjacent positive charge. However, the replacement of hydrogen by fluorine on the carbon $\beta$ to the positive carbon would be expected to destabilize a carbonium ion because of the inductive effect of the electronegative fluorine. This would lower $V_{max}$ significantly for difluorofumaric acid relative to fluorofumaric acid, as is observed. If, on the other hand, the reaction involved a carbanion intermediate formed by addition of hydroxide ion to the double bond during the hydration reaction or by removal of a proton from C-3 during the dehydration, the reactivity of difluorofumaric acid should exceed or at least be very close to that of fluorofumaric acid. The results with the two fluorinated analogs, therefore, support a mechanism in which the substrate molecule acquires considerable carbonium ion character during the rate-determining step.

The finding that neither 2-(*S*)-2-methoxysuccinic acid nor 2-(*S*)-2-acetoxysuccinic acid bind effectively to the active site may be due either to the requirement for a group no larger than hydroxyl or the necessity of having a group capable of donating a hydrogen for hydrogen bond formation at that site on the enzyme where the hydroxyl group is bound during interaction of *l*-malic acid with the enzyme.

## Experimental Section

**Reagents.** Oxalacetic, fumaric, and *l*-malic acids were obtained from Calbiochem Corp. (A grade) and used without further purification.

**2-*S*-2-Acetoxysuccinic Acid.** Acetyl chloride (22 g, 0.28 mmol) was added to *l*-malic acid (6.7 g, 0.05 mmol) and the mixture heated for 2 hr. Excess acetyl chloride was removed by heating under reduced pressure. Water (1.8 g, 0.1 mmol) was added to hydrolyze the anhydride formed. Upon standing a white solid separated from an oily residue. The solid was filtered, washed with a little cold ether, and recrystallized from toluene (mp 134–135°, lit. 134°). The ir and nmr spectra were also consistent for the expected acetate.

**2-*S*-2-Methoxysuccinic Acid.** The substance was prepared by the published procedure.[14]

**Fluorofumaric acid and difluorofumaric acid** were prepared by the published procedure.[8]

**Fumarate hydratase** was obtained from Nutritional Biochemicals Corp. (Control No. 4728) and purified from ammonium sulfate by centrifugation, removal of the supernatant, and dissolution of the protein in 10 m*M* sodium phosphate buffer. The pH was adjusted to 7.3 by addition of 0.1 *N* sodium hydroxide.

**Kinetic Method.**[15,16] The rate of disappearance of unsaturated acid was followed spectrophotometrically on a Cary M-14 equipped with a thermostated cell compartment. An aliquot of buffered (10 m*M* sodium phosphate, pH adjusted to 7.3) solution of the substrate was placed in the absorption cell and allowed to reach thermal equilibrium with the cell compartment. An aliquot of a solution of fumarate hydratase (100 $\mu$l of about $3.6 \times 10^{-7}$ *M* enzyme in 10 m*M* sodium phosphate buffer at pH 7.3) was added and the cell stoppered and shaken several times. The cell was returned to the spectrophotometer and the decrease in absorbance between 236 and 260 m$\mu$ was followed. From three to six kinetic runs were carried out at five different concentrations of substrate (0.1 to 1.6 m*M*) for fumaric, fluorofumaric, and difluorofumaric acids. The method of Lineweaver and Burk[17] was used to calculate $K_m$ and $V_{max}$.

Buffered (10 m*M* phosphate, pH 7.3) solutions of fluoromaleic, difluoromaleic, 2-(*S*)-2-acetoxysuccinic, and 2-(*S*)-2-methoxysuccinic acids were allowed to interact with buffered solutions of fumarate hydratase. The ultraviolet spectra of these solutions showed no change over a period of 2 days; under similar conditions solutions of *l*-malic acid show a marked increase in absorbance at 230 m$\mu$ within 1 sec.

In addition, concentrations of 2-(*S*)-2-acetoxysuccinic acid or 2-(*S*)-2-methoxysuccinic acid ten times that of *l*-malic acid had no observable effect on the rate of dehydration of *l*-malic acid by fumarate hydratase under conditions identical with those described above.

**Denatured Enzyme.** A solution of fumarate hydratase was partially denatured by heating at 50° for 10 min. This solution showed the following activity (expressed as per cent activity remaining relative to unheated enzyme solutions): toward fumaric acid 48%, toward fluorofumaric acid 49%, and toward difluorofumaric acid 46%.

**Product Analysis.** Treatment of the reaction products from either fluorofumaric or difluorofumaric acids with 2,4-dinitrophenylhydrazine by the procedure of Nakamura and Ogata[6] gave in each case a mixture of diphenylhydrazones. Chromatography on silica gel with *n*-butyl alcohol–ethyl alcohol–water (8:2:5) yielded, from fluorofumaric acid, the 2,4-dinitrophenylhydrazones of oxaloacetic acid and pyruvic acid and from difluorofumaric acid, the 2,4-dinitrophenylhydrazones of 3-fluorooxaloacetic acid (an authentic sample of which was prepared by the method of Kun, *et al.*)[9] and of fluoropyruvic acid.

**Acknowledgment.** We thank the National Institutes of Health of the U.S. Public Health Service (Grant GM-10218) for support of this research.

(14) T. Purdie and G. B. Neave, *J. Chem. Soc.*, **97**, 1517 (1910).
(15) R. M. Bock and R. A. Alberty, *J. Am. Chem. Soc.*, **75**, 1921 (1953).
(16) R. A. Alberty, V. Massey, C. Frieden, and A. R. Fuhlbrigge, *ibid.*, **76**, 2485 (1954).
(17) H. Lineweaver and D. Burk, *ibid.*, **56**, 658 (1934).