

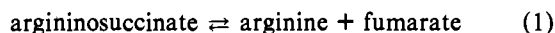
# Substrate-Induced Inactivation of Argininosuccinate Lyase by Monofluorofumarate and Difluorofumarate<sup>†</sup>

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**ABSTRACT:** Monofluorofumarate and difluorofumarate were tested as alternate substrates and inhibitors of the reverse reaction of bovine liver argininosuccinate lyase.  $K_m$  and  $V_{max}$  values relative to fumarate at pH 7.5, 25 °C, and 10 mM arginine are (monofluorofumarate) 1.4 mM and 5% and (difluorofumarate) 46  $\mu$ M and 0.5%. As inhibitors, both of these compounds were shown to inactivate the enzyme activity in a pseudo-first-order process that is dependent on the presence of arginine. The rate of inactivation at saturating monofluorofumarate and difluorofumarate is 13 and 1.3 min<sup>-1</sup>,

respectively. After removal of excess inhibitor, the inactivated enzyme can be restored to greater than 75% of its original activity with half-lives of 6 and 24 min for the monofluorofumarate- and difluorofumarate-inhibited enzyme. Evidence is presented to suggest that the time-dependent inactivation is caused by covalent addition of an enzyme nucleophile with an electrophilic reaction intermediate. In the inhibition by monofluorofumarate, the postulated intermediate is proposed to occur by the spontaneous loss of HF from 2-fluoro-argininosuccinate.

**A**rgininosuccinate lyase catalyzes the reaction:



The enzyme has been purified to homogeneity and extensively studied by Ratner and her colleagues over the past 30 years (Ratner, 1973). Like most of the other enzymes that catalyze  $\alpha,\beta$ -eliminations, the stereochemistry of proton removal and arginine loss from argininosuccinate was found to be trans (Hoberman et al., 1964). However, the chemical mechanism the enzyme uses to facilitate the cleavage of argininosuccinate to arginine and fumarate has remained obscure, although Hansen & Havir (1973) have proposed that the reaction mechanism is concerted.

The question of whether the overall reaction mechanism is concerted or stepwise may be answered by substitution of more electronegative groups on those carbons of the substrate that can develop substantial charge in the transition state. Because of its small size and large electronegativity, the substitution of fluorine for hydrogen has been very successful in the development of alternate substrates for enzymatic reactions. For example, fumarase has been shown to add a water molecule to the double bond of monofluorofumarate and difluorofumarate (Teipel et al., 1968; Nigh & Richards, 1969). Monofluorofumarate was utilized by the enzyme at a rate 4 times faster than that of fumarate and was shown to be hydrated almost exclusively at the carbon bonded to the fluorine. The  $\alpha$ -fluoromalate that was initially produced rapidly lost HF to form oxalacetate in a nonenzymatic step (Teipel et al., 1968; Marletta et al., 1982). Difluorofumarate was found to be hydrated at a rate equal with that of fumarate and eventually formed  $\beta$ -fluorooxalacetate after loss of HF (Nigh & Richards, 1969).

On the basis of the relative rates of hydration for mono- and difluorofumarate by fumarase, Nigh & Richards (1969) have argued that the substrate molecule acquires considerable carbonium ion character in the rate-determining step. However, Blanchard & Cleland (1980) have recently proposed that the reaction occurs via a carbanion intermediate, and Jones et al. (1980) have suggested a concerted pathway.

In this paper, mono- and difluorofumarate were tested as substrates for the reverse reaction of argininosuccinate lyase. Unlike the relatively high reactivity with fumarase, these compounds appeared to be very poor substrates for argininosuccinate lyase. In addition, these compounds were also found to induce a very rapid time-dependent inactivation of argininosuccinate lyase at a very low inhibitor concentrations. The significance and possible mechanisms for the inactivation process are discussed.

## Materials and Methods

Argininosuccinate lyase was isolated from beef liver according to the method of Havir et al. (1965) and Schulze et al. (1970). Mono- and difluorofumarate were synthesized according to the procedure of Raasch et al. (1959), starting from 1,1,2-trichloro-2,3,3-trifluorocyclobutane (PCR Research Chemicals). Acetylenedicarboxylic acid was obtained from Aldrich. [2,3-*guanidino*-<sup>15</sup>N<sub>2</sub>]Arginine was purchased from KOR Isotopes. All other biochemicals were obtained from Sigma.

**Nuclear Magnetic Resonance Measurements.** <sup>19</sup>F NMR<sup>1</sup> spectra were acquired on a JEOL PS-100 spectrometer operating at 94 MHz. Typical acquisition parameters used to obtain the spectra were 90° flip angle, 3-s repetition time, 4000–15 000-Hz sweep width, 1-Hz line broadening, and 16K data points. <sup>15</sup>N NMR spectra were obtained on a Varian XL-200 spectrometer operating at a frequency of 20 MHz. Fully proton-decoupled spectra with a full nuclear Overhauser enhancement were obtained with a pulse width of 60°, 8000-Hz sweep width, 10-s delay time between pulses, 2-Hz line broadening, and 8K data points. Proton-decoupled spectra with suppressed NOE were obtained in an identical manner except that the decoupler was gated off during the 10-s delay time. Some <sup>15</sup>N spectra were also obtained on a Varian FT80 spectrometer at a frequency of 8 MHz. Natural abundance <sup>13</sup>C spectra were obtained on a Varian XL-200 NMR spectrometer at a frequency of 50 MHz. Proton noise decoupled spectra were obtained with a sweep width of 11 000 Hz, 0.7-s acquisition time, 2.3-s delay between pulses, 30° flip angle, 1-Hz line broadening, and 16K data points. The attached

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<sup>1</sup> Abbreviations: NOE, nuclear Overhauser enhancement; Hepes, N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid; NMR, nuclear magnetic resonance.

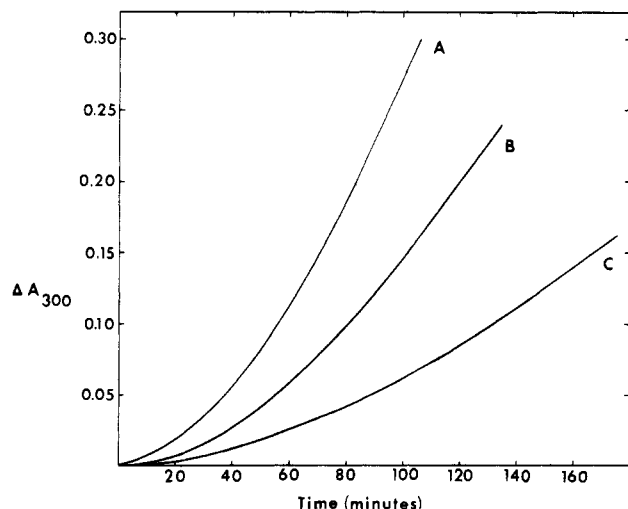


FIGURE 1: Progress curves after addition of argininosuccinate lyase to a solution of monofluorofumarate and arginine at 25 °C. Each 3-mL cuvette contained 10 mM arginine, 50 mM Hepes, pH 7.5, 100 mM KCl, 3.33 mM monofluorofumarate, and various levels of argininosuccinate lyase: (A) 0.072 unit; (B) 0.036 unit; (C) 0.018 unit.

proton test (APT) was used in some instances to determine the number of hydrogens bonded to particular carbon atoms (Le Cocq &ALLEMAND, 1981).

**Enzyme Assays and Inhibition Experiments.** Enzyme assays and absorbance measurements were made with a Gilford 260 UV-vis spectrophotometer and a Linear 255 recorder. A unit of argininosuccinate lyase activity is defined as the amount of enzyme needed to catalyze the formation of 1  $\mu$ mol of fumarate/min at 25 °C and pH 7.5 at saturating argininosuccinate.

## Results

**Monofluorofumarate Substrate Activity.** Catalytic activity of argininosuccinate lyase with fumarate as a substrate is routinely assayed by following the loss of absorbance at 240 nm ( $\epsilon = 2440$ ). In contrast, the addition of enzyme to a solution of arginine and monofluorofumarate at pH 7.5 resulted in a relatively slow increase in absorbance at all wavelengths tested (230–300 nm). The relative increase in absorbance was the greatest at 270 nm when the initial velocity was measured. However, when the rate was followed at wavelengths greater than 290 nm, we noticed a very slow initial rate that gradually increased by an apparent first-order process to a much faster steady-state rate. Shown in Figure 1 are time courses for this increase in absorbance with three levels of enzyme. The first-order rate constants for this lag phase are 0.025, 0.020, and 0.016  $\text{min}^{-1}$  for 0.072, 0.036, and 0.018 unit of added argininosuccinate lyase, respectively.

The product of the reaction between monofluorofumarate and arginine catalyzed by argininosuccinate lyase was isolated by incubating 0.40 unit of argininosuccinate lyase, 33 mM monofluorofumarate, 33 mM arginine, and 200 mM  $\text{P}_i$ , pH 7.5, at room temperature for 3 days. The reaction was complete as determined by  $^{19}\text{F}$  NMR spectroscopy. The product was removed from protein by passage through a YM10 ultrafiltration membrane. The UV spectrum of the product is shown in Figure 2. An absorption maximum at pH 7.5 is seen at 299 nm. At low pH, this maximum is shifted to 279 nm. The  $\text{pK}$  for the transition is 4.75.

The Michaelis constant and relative  $V_{\text{max}}$  for monofluorofumarate were determined for comparison with those of fumarate as a substrate for argininosuccinate lyase. At pH 7.5 and an arginine concentration of 10 mM, the Michaelis con-

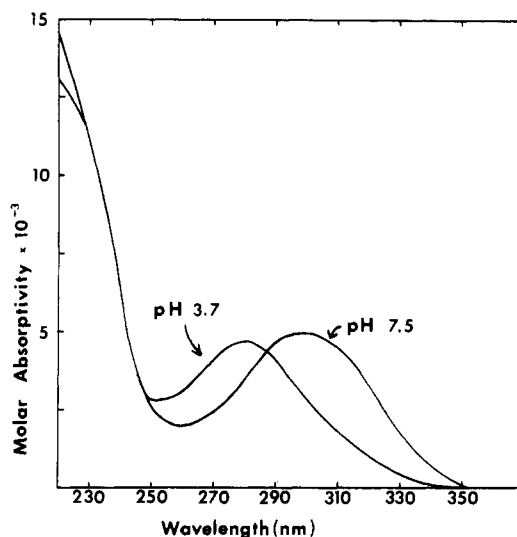


FIGURE 2: UV spectra of product formed by action of argininosuccinate lyase on arginine and monofluorofumarate. Additional details are given in the text.

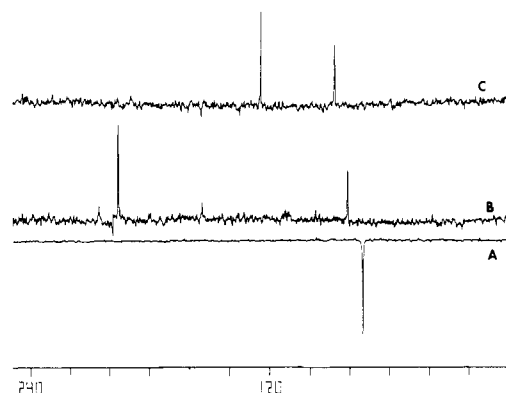


FIGURE 3:  $^{15}\text{N}$  NMR spectra of [2,3-guanidino- $^{15}\text{N}_2$ ]arginine and product formed by action of argininosuccinate lyase on arginine and monofluorofumarate. (A) 33 mM 95%-enriched [2,3-guanidino- $^{15}\text{N}_2$ ]arginine in 200 mM  $\text{P}_i$ , pH 7.5, and 20%  $\text{D}_2\text{O}$  (1800 scans). (B) Product formed by action of argininosuccinate lyase on 33 mM [2,3-guanidino- $^{15}\text{N}_2$ ]arginine and 33 mM monofluorofumarate. Proton-decoupled spectrum with suppressed NOE (20000 scans). (C) Same as in (B) except that the pH was adjusted from 7.5 to 2.75 with HCl (7500 scans). Additional details are given in the text.

stants for fumarate and monofluorofumarate were  $67 \pm 15 \mu\text{M}$  and  $1.4 \pm 0.1 \text{ mM}$ , respectively. The  $V_{\text{max}}$  with monofluorofumarate was 5% as fast as with fumarate.

The products of the reaction were subjected to  $^{19}\text{F}$ ,  $^{15}\text{N}$ , and  $^{13}\text{C}$  NMR spectroscopic analysis. The  $^{19}\text{F}$  spectrum of monofluorofumarate consists of a doublet ( $^3J_{\text{HF}} = 30 \text{ Hz}$ ) at 117 ppm upfield from  $\text{CFCl}_3$ . After incubation with arginine and argininosuccinate lyase, this doublet completely disappears and is replaced by a singlet at 125 ppm. This new peak was determined to be due to  $\text{F}^-$  by addition of NaF to the NMR tube. No significant other resonances were observed at intermediate reaction times.

The fate of the two guanidino amino nitrogens of arginine was determined by incubating [2,3-guanidino- $^{15}\text{N}_2$ ]arginine with monofluorofumarate and argininosuccinate lyase. The proton-decoupled  $^{15}\text{N}$  spectrum of [2,3-guanidino- $^{15}\text{N}_2$ ]arginine at a frequency of 20 MHz is shown in Figure 3A. A single inverted resonance is observed 75 ppm downfield from  $\text{NH}_3$ . After incubation with monofluorofumarate and argininosuccinate lyase, this peak completely disappears and is replaced by two resonances separated by 6 Hz at 82 ppm when a proton-decoupled spectrum with full NOE is obtained at pH

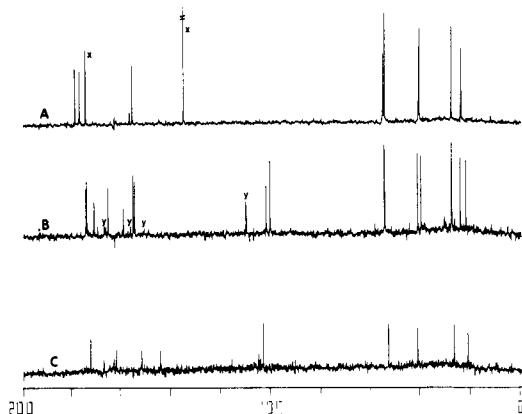


FIGURE 4: Natural abundance  $^{13}\text{C}$  spectra of argininosuccinate and of product formed by action of argininosuccinate lyase on arginine and monofluorofumarate. (A) Argininosuccinate formed by action of argininosuccinate lyase on 33 mM arginine and 67 mM fumarate. The peaks labeled with an "x" are due to unreacted fumarate (20 000 scans). (B) Product formed by action of argininosuccinate lyase on 33 mM arginine and 45 mM monofluorofumarate. The peaks labeled with a "y" are those of unreacted monofluorofumarate (26 000 scans). (C) Same as in (B) except that the pH was adjusted from 7.5 to 2.75 with HCl (22 000 scans). Additional details are given in the text.

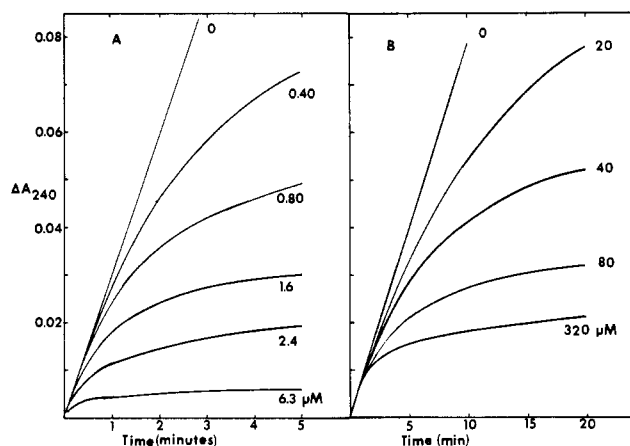


FIGURE 5: Progress curves of argininosuccinate lyase reaction with fumarate as a substrate in the presence of micromolar concentrations of mono- or difluorofumarate. (A) Each 3-mL cuvette contained 0.50 mM fumarate, 10 mM arginine, 50 mM Hepes, pH 7.5, 100 mM KCl, and the indicated concentrations of monofluorofumarate. The reaction was initiated by the addition of argininosuccinate lyase. (B) Same as in (A) except that difluorofumarate was used as the inhibitor at the indicated concentrations.

7.5 (data not shown). With gated proton decoupling (proton decoupling, no NOE), an additional two resonances are observed at 196 ppm separated by 6 Hz (Figure 3B). The separation of 6 Hz is also observed at a frequency of 8 MHz, indicating that the splitting is due to  $^{15}\text{N}$ - $^{15}\text{N}$  coupling. At pH 2.75, the resonance at 196 ppm is shifted to 125 ppm, and the  $^{15}\text{N}$ - $^{15}\text{N}$  coupling is lost (Figure 3C).

The natural abundance  $^{13}\text{C}$  spectra of the product formed from monofluorofumarate and arginine are shown in spectra B (pH 7.5) and C (pH 2.75) of Figure 4. For comparison the  $^{13}\text{C}$  spectrum of argininosuccinate is shown in Figure 4A.

**Monofluorofumarate Inhibition Studies.** Low concentrations of monofluorofumarate were found to significantly inhibit the progress of the reverse reaction of argininosuccinate lyase. Shown in Figure 5A are the time courses of the back-reaction when micromolar levels of monofluorofumarate are included in assay mixtures containing 0.50 mM fumarate and 10 mM arginine. The loss of activity is pseudo first order and dependent on the concentration of monofluorofumarate (Figure 6A). The final steady-state rate at concentrations of mono-

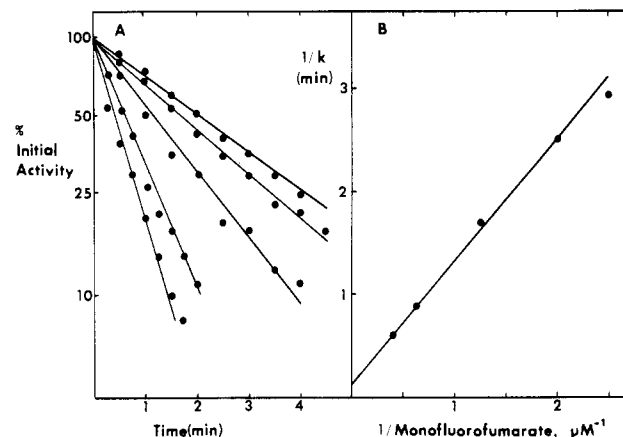


FIGURE 6: Effect of monofluorofumarate on inactivation rate. (A) The percent of initial enzyme activity was determined from the slope of the progress curves (Figure 5A) at various times. (B) A replot of the data shown in (A) and of similar data. The reciprocal of the first-order rate constant is plotted vs.  $[\text{inhibitor}]^{-1}$ .

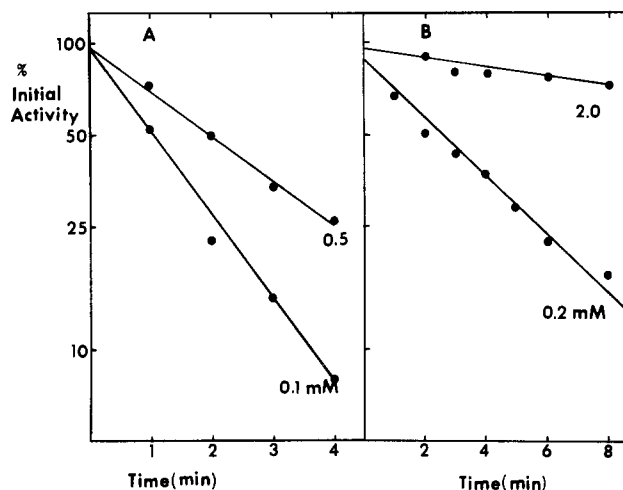
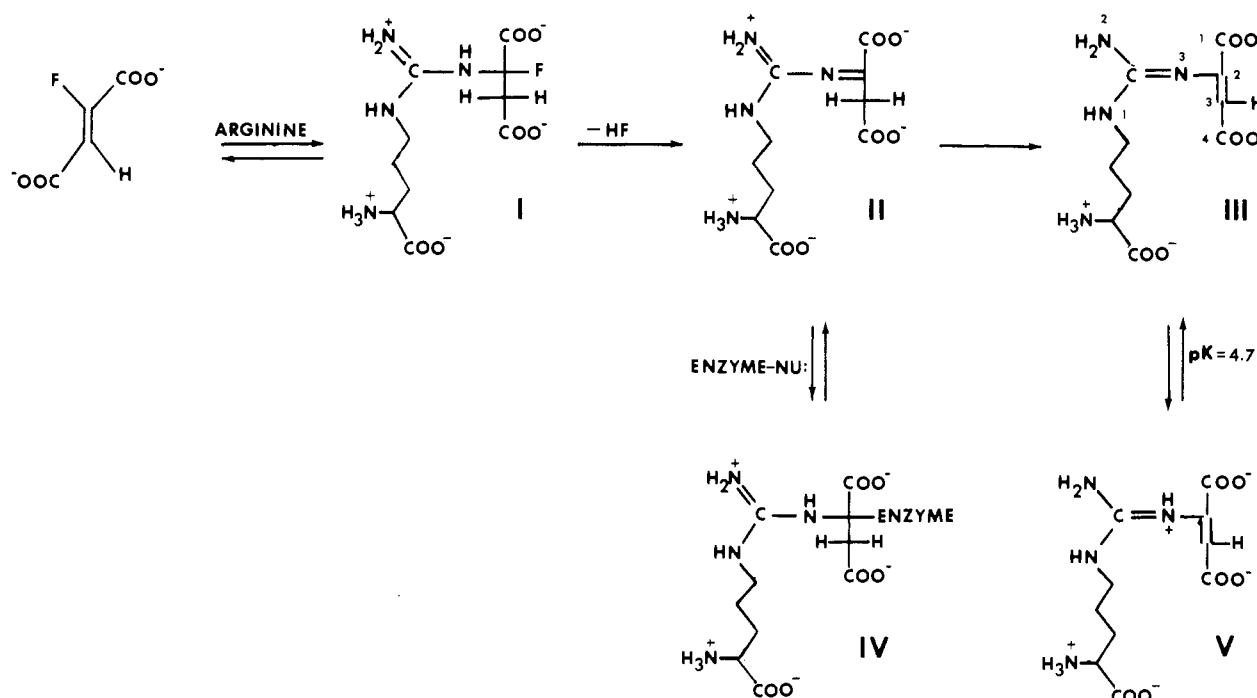


FIGURE 7: Effect of fumarate concentration on inactivation rate. (A) Effect of fumarate (0.1 and 0.5 mM) on inactivation induced by 0.40  $\mu\text{M}$  monofluorofumarate. Other assay conditions the same as in Figures 5A and 6. (B) Effect of fumarate (0.2 and 2.0 mM) on inactivation induced by 20  $\mu\text{M}$  difluorofumarate. Other assay conditions the same as in Figures 5B and 8.

fluorofumarate greater than 3  $\mu\text{M}$  is  $<2\%$  of the uninhibited rate. The inactivation rate constants were fit to the equation  $k = k_{\text{inact}}[I]/(K_{1/2} + [I])$  by the least-squares method (Cleland, 1967). The maximal rate of inhibition ( $k_{\text{inact}}$ ) is  $13 \pm 6 \text{ min}^{-1}$ , and the concentration of monofluorofumarate giving a half-maximal rate of inhibition ( $K_{1/2}$ ) is  $17 \pm 9 \mu\text{M}$  (Figure 6B). The standard errors in the determination of these kinetic constants are large because the rate of inactivation at concentrations of monofluorofumarate greater than 2.4  $\mu\text{M}$  was too fast to accurately measure. The rate of inactivation was dependent on the concentration of arginine (data not shown) and was significantly increased at lower concentrations of fumarate (Figure 7A).

The inhibition induced by monofluorofumarate was found to be reversible. To demonstrate this, argininosuccinate lyase (0.10 unit), 10 mM arginine, 0.1 mM monofluorofumarate, 50 mM Hepes, and 100 mM KCl were incubated in a volume of 0.2 mL for 5 min. A 0.005-mL aliquot was removed and added to a 3.0-mL solution containing 1.5 mM argininosuccinate, 50 mM Hepes, pH 7.5, and 100 mM KCl, and the change in absorbance was monitored at 240 nm. The enzyme gradually regained greater than 75% of its original activity in an apparent first-order fashion.<sup>2</sup> The  $t_{1/2}$  for the reacti-

Scheme I



vation of enzyme activity was 5.9 min. Full activity could be regained by slow passage (~1 h) of the inhibited enzyme through a Sephadex G-25 column.

**Difluorofumarate Substrate Activity.** In contrast with the results obtained with monofluorofumarate, incubation of difluorofumarate and arginine with argininosuccinate lyase produced a slow decrease in absorbance at 240 nm. Assuming that the extinction coefficient of the product at this wavelength is insignificant (see below) compared with that of difluorofumarate, the  $K_m$  and  $V_{max}$  were determined to be  $46 \pm 6 \mu\text{M}$  and 0.5% that seen for fumarate at 10 mM arginine, respectively.

The products of the reaction were subjected to  $^{19}\text{F}$  and  $^{13}\text{C}$  NMR spectroscopic analysis. The  $^{19}\text{F}$  spectrum of difluorofumarate consists of a singlet at 149 ppm upfield from  $\text{CFCl}_3$ . After addition of enzyme to a solution of 33 mM difluorofumarate and 33 mM arginine at pH 7.5, the signal due to difluorofumarate gradually disappears and is replaced by two new resonances. F<sup>-</sup> is observed at 125 ppm and a doublet ( $^2J_{\text{HF}} = 49 \text{ Hz}$ ) is observed at 197 ppm. The doublet at 197 ppm is identical in chemical shift and coupling constant with that of 3-fluoropyruvate. At longer incubation times this doublet gradually disappears and is replaced by a triplet ( $^2J_{\text{HF}} = 47 \text{ Hz}$ ) at 234 ppm. This resonance is coincident with authentic fluoropyruvate (Sigma). The  $^{13}\text{C}$  spectrum confirms the identification of fluoropyruvate as the sole product. The  $-\text{CH}_2\text{F}$  group is observed at 85 ppm as a doublet ( $^1J_{\text{CF}} = 172 \text{ Hz}$ ), and the hydrated carbonyl is observed as a doublet ( $^2J_{\text{CF}} = 22 \text{ Hz}$ ) at 93 ppm from  $\text{Me}_4\text{Si}$ . The carboxylate is observed at 175 ppm and  $\text{HCO}_3^-$  at 160 ppm. Essentially identical chemical shifts and coupling constants were observed with the authentic sample of fluoropyruvate. The spectrum of arginine remained unchanged.

**Difluorofumarate Inhibition Studies.** Difluorofumarate also caused a time-dependent inactivation of argininosuccinate lyase (Figure 5B) that was pseudo first order and dependent on the concentration of difluorofumarate (Figure 8). The final

steady-state rate at concentrations of difluorofumarate greater than 0.2 mM is less than 3% of the uninhibited rate. The  $k_{\text{inact}}$  is  $1.3 \pm 0.1 \text{ min}^{-1}$  and  $K_{1/2}$  is  $320 \pm 40 \mu\text{M}$ . The rate of inactivation is decreased at higher concentrations of fumarate (Figure 7B).

The inactivation process was also found to be reversible. Argininosuccinate lyase (0.10 unit) was incubated with 100  $\mu\text{M}$  difluorofumarate, 10 mM arginine, 50 mM Hepes, and 100 mM KCl at pH 7.5 in a volume of 0.1 mL for 5 min. A 0.015-mL aliquot was removed and assayed in a 3.0-mL volume containing 1.0 mM argininosuccinate, 50 mM Hepes, and 100 mM KCl. The change in absorbance was monitored at 240 nm. The enzyme regained activity in an apparent first-order process with a  $t_{1/2}$  of 24 min.

**Acetylenedicarboxylate.** Acetylenedicarboxylate inhibited the enzyme in a time-dependent process. The  $k_{\text{inact}}$  is  $0.58 \pm 0.05 \text{ min}^{-1}$ , and the apparent  $K_{1/2}$  is  $1.1 \pm 0.25 \text{ mM}$ . The  $t_{1/2}$  for the reactivation is 6.9 min.

## Discussion

The substrate and inhibitory activity of monofluorofumarate with argininosuccinate lyase has been interpreted and summarized according to the reaction sequence depicted in Scheme I. The rationale for this sequence is as follows. Assuming that argininosuccinate lyase catalyzes the addition of arginine across the double bond of fluorofumarate with the same stereochemistry (anti addition) as that with fumarate, then only two initial reaction products are possible. If the nitrogen adds to the carbon bonded to the hydrogen, then the resulting product (3-fluoroargininosuccinate) should be a relatively stable compound since both *erythro*- and *threo*-3-fluoroaspartic acid can be synthesized (Kollonitsch et al., 1979; Wanner et al., 1980). However, if the nitrogen adds to the other carbon, then HF can be liberated spontaneously from 2-fluoroargininosuccinate (I) as observed. This process is analogous to the formation of  $\alpha$ -fluoromalate from fluorofumarate catalyzed by fumarase.

We cannot unequivocally rule out the formation of 3-fluoroargininosuccinate as a minor reaction component. This compound may be formed in low yield in equilibrium with the

<sup>2</sup> Full activity was not regained because of the monofluorofumarate that still remained with the enzyme upon dilution into the assay mixture.

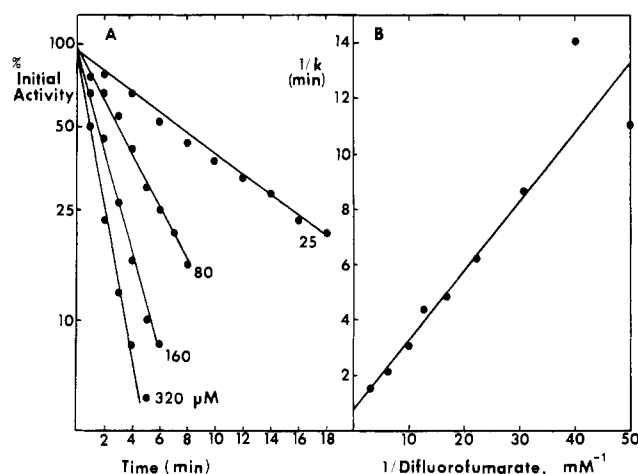


FIGURE 8: Effect of difluorofumarate on inactivation rate of argininosuccinate lyase. (A) The percent of initial enzyme activity was determined from the slope of the progress curves (Figure 5B) at various times. (B) A replot of the data shown in (A) and of similar data. The reciprocal of the first-order rate constant is plotted vs.  $[\text{inhibitor}]^{-1}$ .

starting material, but the course of the reaction is eventually diverted totally through 2-fluoroargininosuccinate because of the irreversible loss of HF. However, we have observed no resonances in the  $^{19}\text{F}$  NMR spectra of the reaction mixture that can be assigned to 3-fluoroargininosuccinate even at intermediate reaction times. This would indicate that 2-fluoroargininosuccinate is the predominant initial product formed.

Depending on which proton is lost, the removal of HF from 2-fluoroargininosuccinate (I) can be envisioned to form either II or III directly. Marletta et al. (1982) have demonstrated that  $\alpha$ -fluoromalate decomposes directly to the keto form of oxalacetate and not to the enol tautomer. Therefore, the loss of HF from I is depicted as directly forming II before tautomerizing to III.

The stable configuration of the product produced by the action of argininosuccinate lyase on fluorofumarate and arginine is consistent with structure III. The  $^{15}\text{N}$  NMR spectrum (Figure 3B) of the selectively  $^{15}\text{N}$ -enriched product indicates two significantly different nitrogen environments. The resonance at 196 ppm is indicative of an imine-type nitrogen (N3) with no attached protons (Levy & Lichter, 1979). This assignment is confirmed by the loss of signal when the spectrum is obtained with nuclear Overhauser enhancement and the observed 71 ppm upfield shift upon proton at pH 2.75 (V). The other  $^{15}\text{N}$  resonance (N2) appears at 82 ppm and is consistent with an amino group of a substituted guanidine (Levy & Lichter, 1979).

The  $^{13}\text{C}$  NMR spectrum of the final product confirms the formation of structure III. The  $^{13}\text{C}$  spectrum at pH 7.5 is complicated because at least 17 different resonances are observed even though there are only 10 carbon atoms in the molecule. The most significant two peaks are those at 100 and 101 ppm. These resonances are consistent with  $\text{sp}^2$ -hybridized carbons that are  $\beta$  to a heteroatom (Levy et al., 1980). The attached proton test (APT) indicated that these carbons were bonded to an odd number of hydrogens. They must therefore be bonded to only one hydrogen because methyl groups at this chemical shift are quite unlikely. The multiplicity of peaks is presumed to arise because of isomer formation about the  $\text{C}=\text{N}$  and  $\text{C}=\text{C}$  double bonds with relatively slow interconversion. When the pH is lowered to 2.75, only a single resonance is observed for each carbon (Figure 4C). This would indicate that at this pH only a single isomer

is present or that there is now rapid acid-catalyzed isomerization.<sup>3</sup>

The extended conjugation of structure III also accounts for the absorption maximum at 300 nm. The lag phase in the enzyme assay with monofluorofumarate at 300 nm most likely represents the slow tautomerization of  $\text{II} \rightarrow \text{III}$ . Since the apparent first-order rate constant for the approach to the steady state is larger at higher enzyme concentrations, this step may be enzyme assisted. The equilibration of the proposed isomers of III may be an alternative explanation for the slow increase in absorption at 300 nm.

The inactivation of argininosuccinate lyase can best be explained by the action of intermediate II. This unstable compound should be susceptible to nucleophilic attack at C-2, which would result in a covalent enzyme-product adduct (IV). Efforts to isolate this intermediate failed because this type of addition reaction should be readily reversible. Alternatively, the inactivation of enzyme activity may be explained by postulating either II or III as transition-state analogues (Wolfenden, 1972; Lienhard, 1973). Many of the known transition-state inhibitors have been found to inhibit activity in a slow, tight-binding, reversible process [Schloss & Cleland (1982) and references cited therein]. The inhibition is not due directly to the formation of  $\text{F}^-$  because fluoride ion does not inhibit the reaction up to a concentration of at least 1 mM.

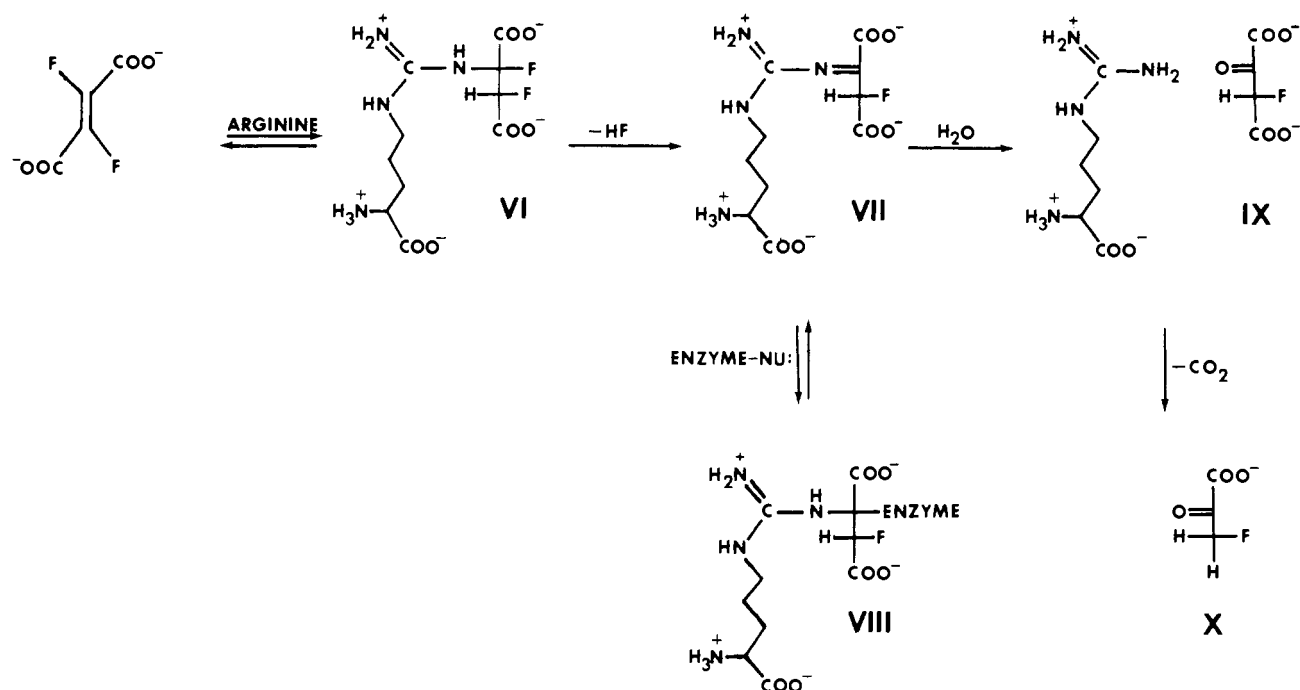
The substrate and inactivation activity induced by difluorofumarate can be rationalized in an analogous manner as outlined in Scheme II. The addition of arginine to difluorofumarate can only produce 2,3-difluoroargininosuccinate (VI). Loss of HF from this compound would then yield intermediate VII. Like the analogous intermediate in the monofluorofumarate reaction, this compound is susceptible to nucleophilic attack by an enzyme nucleophile, which would result in enzyme inactivation. Since the only products that can be identified by  $^{19}\text{F}$  and  $^{13}\text{C}$  NMR spectroscopy are fluorooxalacetate (IX) and fluoropyruvate (X), the attack by water at C-2 of VII must be effectively competing with the tautomerization reaction.

If structures such as II and III are postulated as potent inhibitors for argininosuccinate lyase then acetylenedicarboxylate can be predicted to be an effective inhibitor. Scheme III shows that the addition of arginine to the triple bond of acetylenedicarboxylate will produce these same intermediates. It can also be predicted that the rate constant for the reactivation of enzyme activity will be the same for both the monofluorofumarate-inhibited and acetylenedicarboxylate-inhibited enzyme. As expected, the enzyme is inhibited in a time-dependent process by acetylenedicarboxylate, and the half-life for reactivation ( $t_{1/2} = 6\text{--}7$  min) is the same as that with the fluorofumarate-inhibited enzyme. The maximal rate of inactivation ( $k_{\text{inact}}$ ) with acetylenedicarboxylate is about 20-fold slower than that with monofluorofumarate. This suggests that the rate-limiting step for inactivation by acetylenedicarboxylate is not the formation of the covalent substrate-enzyme adduct but some step that precedes it such as the actual addition of arginine to the triple bond or the tautomerization step.

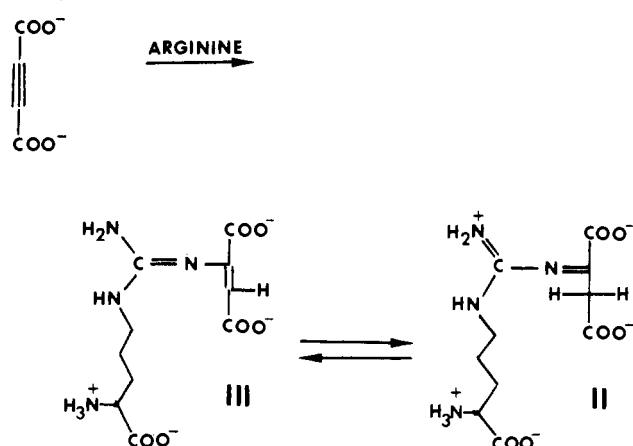
Mono- and difluorofumarate appear to be poor substrates for argininosuccinate lyase only because these compounds can

<sup>3</sup> The compound indicated by structure III was found to be inert to reduction by  $\text{NaBH}_4$  and  $\text{NaBH}_3\text{CN}$  at pH 7.5 and 3.1, respectively. However, we have been able to reduce this compound at pH 3.0 in low yield to argininosuccinate by  $\text{H}_2$  at 1 atm of pressure using Pd on charcoal as a catalyst. The argininosuccinate was determined by enzymatic assay with argininosuccinate lyase.

Scheme II



Scheme III



induce the formation of a highly reactive or tight-binding intermediate that almost totally inactivates the enzyme during catalysis. In the inactivation process, monofluorofumarate is more effective than difluorofumarate because  $k_{\text{inact}}$  is larger and  $K_{1/2}$  is smaller under identical conditions. In fact,  $k_{\text{inact}}/K_{1/2}$  (apparent second-order rate constant is low inhibitor concentrations) favors monofluorofumarate by a factor of almost 200. The reason for this difference in reactivity may be difficult to identify because of the many variables that enter into the inactivation of the enzyme. The actual rate of inhibition or inactivation will depend to a significant extent on the partitioning of the various postulated intermediates and on the individual rate constants for the loss of HF from intermediates I and VI and on the rate of nucleophilic addition of enzyme or H<sub>2</sub>O to intermediates II and VII. Stopped-flow or rapid-quench techniques may be useful in the measurement of the various rate constants.

The inhibition by these fluorine derivatives of fumarate is very much like the mechanism-based (suicide) inactivators previously described in detail by Abeles & Maycock (1976), Walsh (1977), and Rando (1977). The enzyme has taken a relatively unreactive compound and transformed it into a highly reactive electrophile or a tight-binding transition-state

inhibitor. The biggest difference between these compounds and most of the other suicide substrates that have been previously described is that the inactive enzyme can be readily regenerated under the appropriate conditions and the enzyme is never completely "dead".

These compounds may be useful in inhibiting the activity of argininosuccinate lyase in vivo for metabolic studies of urea or arginine biosynthesis. To counteract the utilization of these compounds in the cell by fumarase, 3(*R*)-fluoroargininosuccinate may be needed to deliver fluorofumarate to the active site. The liberated fluorofumarate from the cleavage of 3-(*R*)-fluoroargininosuccinate would be readily available for the synthesis of 2-fluoroargininosuccinate and eventual enzyme inactivation. We are currently testing mono- and difluorofumarate as substrates and inhibitors of aspartase and adenylosuccinate lyase. These are the other two enzymes that add nitrogen across the double bond of fumarate.

**Registry No.** Argininosuccinate lyase, 9027-34-3; monofluorofumarate, 672-18-4; difluorofumarate, 2714-32-1; acetylenedicarboxylate, 142-45-0.

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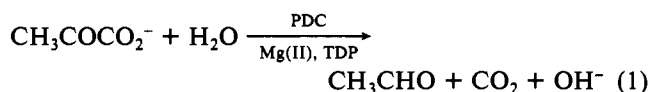
## Active Site Directed Irreversible Inactivation of Brewers' Yeast Pyruvate Decarboxylase by the Conjugated Substrate Analogue (*E*)-4-(4-Chlorophenyl)-2-oxo-3-butenic Acid: Development of a Suicide Substrate<sup>†</sup>

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**ABSTRACT:** (*E*)-4-(4-Chlorophenyl)-2-oxo-3-butenic acid (CPB) was found to irreversibly inactivate brewers' yeast pyruvate decarboxylase (PDC, EC 4.1.1.1) in a biphasic, sigmoidal manner, as is found for the kinetic behavior of substrate. An expression was derived for two-site irreversible inhibition of allosteric enzymes, and the kinetic behavior of CPB fit the expression for two-site binding. The calculated *K*'s of 0.7 mM and 0.3 mM for CPB were assigned to the catalytic site and the regulatory site, respectively. The presence of pyruvic acid at high concentrations protected PDC from

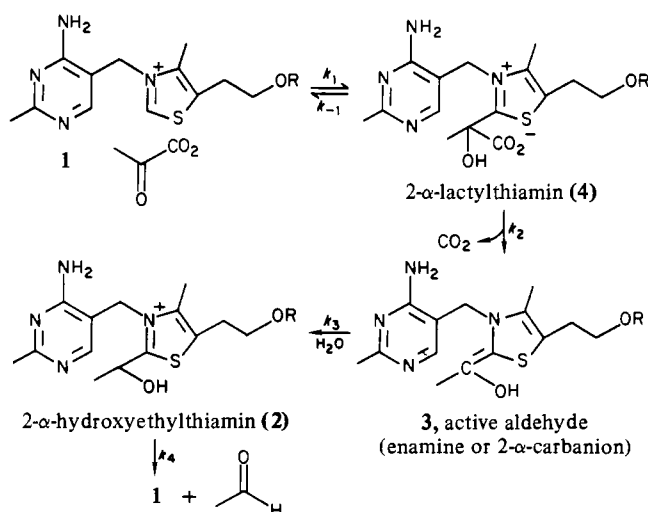
inactivation, whereas low concentrations of pyruvic acid accelerated inactivation by CPB. Pyruvamide, a known allosteric activator of PDC, was found to enhance inactivation by CPB. The results can be explained if pyruvamide binds only to a regulatory site, but CPB and pyruvic acid compete for both the regulatory and the catalytic centers. [<sup>14</sup>C]CPB was found to lose <sup>14</sup>CO<sub>2</sub> concurrently with the inactivation of the enzyme. Therefore, CPB was being turned over by PDC, in addition to inactivating it. CPB can be labeled a suicide-type inactivator for PDC.

**T**hiamin diphosphate (TDP, **1**) was first demonstrated to be an essential cofactor for the nonoxidative decarboxylation of pyruvate by pyruvate decarboxylase (PDC, EC 4.1.1.1; Lohmann & Schuster, 1937). The role of the coenzyme in a variety of other reactions including oxidative decarboxylation of α-keto acids was summarized (Krampitz, 1969; Sable & Gubler, 1982). PDC catalyzes the irreversible decarboxylation of pyruvate, employing tightly bound Mg(II) and TDP as cofactors (Schellenberger, 1967):



Breslow's model studies demonstrated the importance of the C2 atom of the thiazolium ring in TDP-requiring reactions (Breslow, 1957, 1958). Those model studies were confirmed

Scheme I



with the isolation of 2-(1-hydroxyethyl)thiamin diphosphate (HETDP, **2**) from the enzymatic reaction mixtures (Carlson & Brown, 1960; Holzer & Beaucamp, 1961; Krampitz et al., 1961). Scheme I presents a mechanism consistent with the

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