The Inhibition of Fumarase and Malic Dehydrogenase by DL-β-Fluoromalic Acid*

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The competitive inhibition of malic dehydrogenase by β-fluoro-oxaloacetate has been reported by Kun et al. (1). In addition to its inhibitory effect, β-fluoro-oxaloacetate also oxidized reduced diphosphopyridine nucleotide enzymatically, but at a rate only 0.02% that of oxaloacetate. It was also shown (2) that β-fluoro-oxaloacetate was a competitive inhibitor of glutamic-aspartic transaminase, and when tested as a substrate, it was transaminated at one-thirtieth the rate of oxaloacetate.

Taylor and Kent (3) prepared disodium β-fluoromalate by the alkaline hydrolysis of dimethyl β-fluoromalate. This product inhibited the malic enzyme and di- and triphosphopyridine nucleotide-dependent malic dehydrogenase, but did not inhibit erythroline fumarase (4). Callely and Dagley (5) found that in the presence of β-fluoromalate, a vibrio accumulated pyruvic acid when grown on malate. The oxidations of pyruvate, malate, fumarate, and succinate were inhibited in the presence of β-fluoromalate.

This paper describes the synthesis in pure form of two isomers of DL-β-fluoromalate and the inhibition of malic dehydrogenase and fumarase by one of these isomers.

EXPERIMENTAL PROCEDURE

Synthesis of DL-β-Fluoromalic Acid

Diethyl β-fluoromalate was prepared from ethyl fluoroacetate (Columbia Organic Chemicals Company, Inc., Columbia, South Carolina) and diethyl oxalate, as described by Blank, Mager, and Bergmann (6). Dimethyl β-fluoromalate was prepared from diethyl β-fluoro-oxaloacetate by reduction with sodium borohydride in methanol, essentially according to the method of Taylor and Kent (3). The fraction boiling at 125-135° at 18 mm was collected. Elementary analysis for dimethyl β-fluoromalate, C₆H₆FO₂,

C₆H₆FO₂
Calculated: C 40.0, H 5.0
Found: C 39.8, H 5.0

The dimethyl β-fluoromalate was hydrolyzed by dissolving 4.8 g of the ester in a mixture of 67 ml of concentrated hydrochloric acid and 133 ml of concentrated glacial acetic acid (7). After standing for 3 days at room temperature, the solution was taken to dryness in a vacuum and placed in a vacuum desiccator over NaOH and P₂O₅ for 3 days. All attempts to crystallize this material were unsuccessful. The material was then chromatographed on a silica gel column, 1.7 by 35 cm. The preparation of the column and the separation of the acids were similar to the procedure of Bulen et al. (8). The column was eluted first with 15% butanol in chloroform and then with 35% butanol in chloroform. The elution chromatogram is shown in Fig. 1. The water layer from each fraction (separated from the CHCl₃) was decolorized with Norit to remove the phenol red indicator, concentrated to a small volume in a vacuum, acidified with H₂SO₄, and extracted with ether. After drying over Na₂SO₄, each fraction was taken to dryness, and the residues were crystallized from ethyl acetate-ligroin (66-75°). Only Fractions A (0.097 g) and B (1.31 g) yielded crystalline solids; the other fractions were oils and were discarded. The analysis of Fractions A and B is shown in Table I. The infrared spectra of Fractions A and B are shown in Fig. 2. The spectra of the two isomers are different, but a definite stereochemical assignment cannot be made on this basis. The OH stretching frequencies from 2.8 to 3.5 μ are essentially identical in both compounds. The C=O frequency is 5.75 μ for A, and in B this absorption is split into two peaks at 5.64 and 5.81 μ. The two compounds differ considerably in the 8.5 to 10.0 μ range which is due to the C=O and C—F absorptions. Compound A absorbs at 8.8 and 9.23 μ, whereas B shows peaks at 8.56, 9.0, 9.16, and 9.89 μ.

When the distillation of the dimethyl β-fluoromalate was continued after the main fraction had been recovered, a higher boiling fraction, boiling point, 146-178° at 18 mm, was obtained. When this fraction (4.61 g) was hydrolyzed and chromatographed as above, β-fluoromalic acids A (0.047 g) and B (0.375 g) were obtained.

To prepare the anhydrous disodium salt of β-fluoromalic acid for use in the enzyme experiments, the acid was neutralized with sodium hydroxide and the solution was lyophilized to dryness and dried in a vacuum desiccator over H₂SO₄ and P₂O₅.

Enzyme Assays

Fumarase—Cell-free extracts of Proteus vulgaris prepared as described previously (9) were used as the source of fumarase. To test the DL-β-fluoromalic acid as a substrate for the enzyme, the disodium salt was dissolved in 0.07 M phosphate buffer pH 7.0, so that the solution after the addition of the enzyme was 1.36 M in DL-β-fluoromalate. As a check on the activity of the enzyme, a similar reaction was carried out with 0.05 M L-malate. The reaction was followed spectrophotometrically at 37° from 220 to 310 μ. Although the reaction with L-malate was completed within an hour, all reactions were continued for 48 hours so that even a small rate of reaction could be determined. With DL-β-fluoromalate B as the substrate there was no change in the
Inhibition of Fumarase and Malic Dehydrogenase

Absorption spectrum on reaction with the enzyme, whereas with L-malate as the substrate the absorbancy increased greatly.

L-Malate and dl-β-fluoromalate gave very similar absorption spectra from 220 to 310 mμ. They both showed only weak end absorption. Fumarate, on the other hand, showed very strong end absorption in this region. After this work was completed, a sample of fluoro-fumaric acid was made available by Dr. Ernst Bergmann and its spectrum was very similar to that of fumaric acid.

To study the inhibitory effect of dl-β-fluoromalate on fumarase, the fluoromalate concentration was kept constant while the concentrations of either L-malate or fumarate were varied. Both reactions were carried out in 0.033 M phosphate buffer pH 7.0 at 25°. With L-malate as substrate the increase in optical density at 240 mμ was measured, whereas with fumarate as substrate the decrease in optical density at 260 mμ was measured (10).

Malic Dehydrogenase—The enzyme preparation from pig heart was purchased from the Worthington Biochemical Corporation. To test the dl-β-fluoromalate as a substrate for the enzyme, the disodium salt at a final concentration of 0.02 M in 0.1 M glycine buffer pH 8.8 was treated with a large amount of the enzyme in the presence of 3.5 × 10⁻⁴ M DPN at 25°. The reaction was followed for a few hours at 340 mμ. Under these conditions with disodium L-malate as substrate, the reaction was completed in a few minutes.

To study the inhibitory effect of dl-β-fluoromalate on the malate → oxaloacetate reaction, the concentration of dl-β-fluoromalate was kept constant while the L-malate concentration was varied. The DPN concentration was 3.5 × 10⁻⁴ M, and the reaction was carried out in 0.1 M glycine buffer pH 8.8 at 25°, and was followed by measuring the increase in optical density at 340 mμ (11). The inhibition of the oxaloacetate → malate reaction was studied by varying the β-fluoromalate concentration at constant DPNH (1 × 10⁻⁴ M) and oxaloacetate (1.3 × 10⁻⁴ M) concentrations in 0.1 M phosphate buffer pH 7.4 at 25° (12). The reaction was followed by measuring the decrease in optical density at 340 mμ.

RESULTS AND DISCUSSION

Synthesis of β-Fluoromalic Acid—The hydrolysis of dimethyl β-fluoromalate by the ester interchange procedure proved to be a very satisfactory method for the preparation of the free acid. This method has also been used for the preparation of β-fluoroxaloacetic acid (1) from its ester. It is superior to alkaline hydrolysis, which can cause the loss of hydrogen fluoride. As can be seen from Fig. 1, hydrolysis of the ester produces a variety of acids, a fact which accounts for the lack of success of all at-

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**Table I**

<table>
<thead>
<tr>
<th>Analysis of β-fluoromalic acid fractions</th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melting point (uncorrected)</td>
<td>192-193°</td>
<td>172-174°</td>
</tr>
<tr>
<td>Equivalent weight</td>
<td>76.2</td>
<td>77.6</td>
</tr>
<tr>
<td>pK₁ (25°)</td>
<td>2.57</td>
<td>2.73</td>
</tr>
<tr>
<td>pK₂ (25°)</td>
<td>3.55</td>
<td>4.25</td>
</tr>
<tr>
<td>% Carbon</td>
<td>31.57</td>
<td>31.68</td>
</tr>
<tr>
<td>% Hydrogen</td>
<td>3.91</td>
<td>3.43</td>
</tr>
<tr>
<td>% Fluorine</td>
<td>12.80</td>
<td>12.45</td>
</tr>
</tbody>
</table>

* Melting point of mixture of Fractions A and B, 160-167°.

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**Fig. 1.** Silica gel chromatography of acid hydrolysate of dimethyl β-fluoromalate. The chromatogram was obtained from the hydrolysis products of 4.8 g (26.6 mmoles) of dimethyl β-fluoromalate with hydrochloric and acetic acids (1:2). The column (1.7 by 35 cm) contained 25 g of silica and 15 ml of 0.5 N H₂SO₄. The column was washed with chloroform before elution with 15% butanol was started. Samples (10 ml) were collected and titrated with 0.1 N NaOH with phenol red as indicator. The acids coming off the column required 37.26 m.eq. of alkali corresponding to 70% of the starting ester. Fraction A contained 3.72 m.eq. of acid and fraction B, 21.6 m.eq.

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**Fig. 2.** Infrared spectra of β-fluoromalic acids A and B. The spectra were recorded with a Perkin-Elmer spectrophotometer model 21. The samples were prepared as KBr pellets in 0.3% concentration.
tempts at crystallization before chromatography. On chromatography, acids equivalent to 70% of the starting ester were obtained. The P-fluoromaleric acids A and B were obtained in 7% and 40% yields, respectively. Elemental analysis and equivalent weights show that both Fractions A and B are DL-P-fluoromaleric acid. The relative position in the chromatogram of B indicates that it is probably more hydrophilic than A.

The two fractions, A and B, are undoubtedly the separated three and erythro isomers of DL-P-fluoromaleric acid. It is not possible at this time to make a definite assignment of configuration to the two isomers. Work is presently in progress to assign these configurations unequivocally. It must be borne in mind that the two separated isomers A and B are not optically pure but are DL mixtures.

In the reduction of diethyl P-fluoro-oxaloacetate with sodium borohydride, Fraction B was formed in 6 times greater quantity than Fraction A. In an effort to prepare larger quantities of A, the reduction of the keto ester with molecular hydrogen was attempted. Diethyl P-fluoro-oxaloacetate (60 m.eq.) in ethyl acetate was reduced in the presence of platinum oxide with 60 m.eq. of hydrogen gas. After removal of the ethyl acetate, the residue was hydrolyzed and chromatographed as described in "Experimental Procedure." The two major fractions obtained were succinic acid (18.6 m.eq.) and oxalic acid (15.6 m.eq.). The formation of 18.6 m.eq. of succinic acid from dimethyl P-fluoro-oxaloacetate would require 56 m.eq. of hydrogen, and 60 m.eq. were actually taken up. The loss of fluorine on reduction is not unexpected, but reduction to succinic acid is. If the fluorine is first removed to form oxaloacetic ester, further reduction would have been expected to form malate, not succinate. Oxalic acid probably was formed by the decomposition of P-fluoro-oxaloacetate during the hydrolysis or chromatography. It has been shown (6) that acid hydrolysis of diethyl P-fluoro-oxaloacetate yields oxalic acid. No P-fluoromaleric acid was formed by the catalytic reduction.

**Inhibition of Fumarase by P-Fluoromalate**—Since the P-fluoromaleric acid A was produced in such small quantities by the synthetic method employed, not enough material was available for studying its substrate and inhibitory properties in the fumarase and malic dehydrogenase enzyme systems. The enzyme experiments to be described were therefore performed only with the DL-P-fluoromaleric acid B.

The fact that the P-fluoromaleric acid B was not optically pure imposes certain limitations on the results to be described. One of the optical isomers may have a much greater affinity for the enzyme than the other and the inhibition constant may apply to only one of the optical isomers so that the actual dissociation constant may be half the apparent value. It is also possible that the inhibition constant is a composite constant for two distinct optical species, one of which might be an inhibitor and the other a weak substrate. However, it is rather unlikely that this would completely mask weak substrate activity. Since the optically pure compounds were not available, the inhibition constants reported below were calculated for the DL acid assuming for the calculation that both optical isomers are equally inhibitory.

The DL-P-fluoromalate B was not a substrate for fumarase. Even when tested at very high concentrations in the presence of large amounts of the enzyme for periods as long as 48 hours, there was no evidence for any reaction. Under identical conditions, L-malate reacted rapidly and reached equilibrium in a short time. The fluorine analogue cannot be completely excluded as a sub-

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**Fig. 3.** Inhibition of fumarase-catalyzed malate → fumarate reaction by DL-P-fluromalate B. The inhibitor concentration was kept constant while the substrate concentration was varied. The reaction was carried out in 0.033 M phosphate buffer pH 7.0 at 25°, as described in the text. The substrate concentration, S, is expressed in moles per liter, and v is the rate of fumarate formation in μmoles per minute.

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strate, but if it is, it reacts at a rate one-hundredth or less of that of L-malate.

Since DL-P-fluromalate B was not a substrate for fumarase, it was of interest to determine whether it inhibited the malate → fumarate reaction catalyzed by the enzyme. The results of such an experiment are shown in Fig. 3, in which the reciprocal of the substrate concentration is plotted against the reciprocal of the velocity of the reaction in the absence of and in the presence of two different concentrations of DL-P-fluromalate B. The curves suggest that the fluorine analogue is a competitive inhibitor of fumarase, which is to be expected in view of the similarity between the substrate and inhibitor. From these data the Michaelis constant K<sub>m</sub> of the fumarase of *Proteus vulgaris* for malate was calculated to be 3.5 × 10<sup>-5</sup>. The apparent K<sub>i</sub> at 0.033 M concentration of inhibitor was 2.8 × 10<sup>-4</sup>, and at double the inhibitor concentration was 2.7 × 10<sup>-4</sup>.

The effect of DL-P-fluromalate B on the reverse reaction, fumarate → malate, catalyzed by fumarase is shown in Fig. 4. The results suggest that P-fluoromalate B is also a competitive inhibitor of this reaction. From the results shown in Fig. 4, the K<sub>m</sub> of the fumarase of *Proteus vulgaris* for fumarate was calculated to be 1.4 × 10<sup>-2</sup>. The apparent K<sub>i</sub> at 0.025 M inhibitor concentration was 3.6 × 10<sup>-3</sup>, and at 0.03 M concentration was 3.0 × 10<sup>-4</sup>.

In Table II are summarized the K<sub>m</sub>, K<sub>i</sub>, and V<sub>max</sub> values for the inhibition of fumarase by DL-P-fluromalate B with both malate and fumarate as substrates. The apparent K<sub>i</sub> values should be the same at different inhibitor concentrations and should be unaffected by the choice of substrate. Therefore, the variations of the K<sub>i</sub> values in Table II are not significant but are merely experimental deviations.

The equilibrium constant for the fumarate = malate reaction was calculated to be 4.06 from the K<sub>m</sub> values and the maximal
velocities (corrected to the same enzyme concentration) with the use of the relationship:

$$K_{eq} = \frac{K_{ma}V_{fumarate}}{K_{fumarate}V_{malate}}$$

Alberty et al. (13) in a study of pig heart fumarase in 0.033 M phosphate buffer pH 7.0, found the $K_m$ for malate to be $1.59 \times 10^{-3}$ and for fumarate $0.87 \times 10^{-3}$. The equilibrium constant obtained from these values and from the maximal velocities was 4.2.

In view of the inhibition of fumarase by DL-$\beta$-fluoromalate B, it is surprising that Gal (4) reported that disodium fluoromalate did not inhibit crystalline fumarase. The fumarase preparation used in the present work was a preparation from Proteus vulgaris, whereas Gal used a crystalline fumarase from pig heart. In addition the disodium fluoromalate used by Gal was prepared by the alkaline hydrolysis of the dimethyl ester and was not further purified.

Inhibition of Malic Dehydrogenase by $\beta$-Fluoromalate—When tested as a substrate for malic dehydrogenase under conditions in which L-malate reacted rapidly, DL-$\beta$-fluoromalate B did not react. Even in the presence of large amounts of enzyme and during extended periods of time, it failed to reduce DPN. As stated above, it is difficult to exclude it completely as a substrate, but even if it is, it reacts at one-hundredth or less the rate of malate. It has been shown by Kun et al. (1) that $\beta$-fluoro-oxaloacetate oxidizes DPNH in the presence of malic dehydrogenase at a rate 0.02% that of oxaloacetate.

The inhibitory effect of DL-$\beta$-fluoromalate B on the malate $\rightarrow$ oxaloacetate reaction catalyzed by malic dehydrogenase is shown in Fig. 5. It can be seen from the figure that the inhibition is of the competitive type. The $K_m$ for malate was calculated to be $1.1 \times 10^{-3}$. The apparent $K_i$ at $5 \times 10^{-4}$ M inhibitor concentration was $1.6 \times 10^{-3}$, and at double the inhibitor concentration was $1.7 \times 10^{-4}$.

The inhibitory effect of DL-$\beta$-fluoromalate B on the reverse reaction, oxaloacetate $\rightarrow$ malate, was studied at pH 7.4 by varying the inhibitor concentration at a fixed concentration of oxaloacetate. When the data were plotted according to Dixon and Webb (14), the value of $K_i$ was estimated at $3.9 \times 10^{-4}$ with the use of the $K_m$ value determined ($3.4 \times 10^{-3}$). These values are quite similar to those reported by Gal (4). This $K_i$ value cannot be compared to the $K_i$ value determined with L-malate as substrate, since the pH was different in both reactions.

**Table II**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Inhibitor concentration</th>
<th>$V_{max}$</th>
<th>$K_m$</th>
<th>$K_i$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malate*</td>
<td>0.033</td>
<td>0.0307</td>
<td>3.5 $\times$ $10^{-3}$</td>
<td>2.8 $\times$ $10^{-3}$</td>
</tr>
<tr>
<td></td>
<td>0.066</td>
<td>0.0290</td>
<td>2.7 $\times$ $10^{-3}$</td>
<td>3.6 $\times$ $10^{-3}$</td>
</tr>
<tr>
<td>Fumarate†</td>
<td>0.025</td>
<td>0.190</td>
<td>1.4 $\times$ $10^{-3}$</td>
<td>3.0 $\times$ $10^{-3}$</td>
</tr>
<tr>
<td></td>
<td>0.030</td>
<td>0.190</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* With malate as substrate, 0.001 ml of the fumarase preparation was used.
† With fumarate as substrate, 0.0033 ml of the fumarase preparation was used.

**Summary**

The erythro and threo isomers of DL-$\beta$-fluoromallic acid have been synthesized, and the effect of one of these isomers on fumarase: vol. 236, no. 3.

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Under the conditions of Fig. 5 in the presence of 0.02 M L-malate, DL-$\beta$-fluoromalate B at concentration of $1.7 \times 10^{-3}$ M inhibited the reaction 78%. DL-$\beta$-fluoromalate A at this concentration inhibited the reaction 50%.
marase and malic dehydrogenase has been studied. It is not a substrate for either enzyme but acts as a competitive inhibitor.

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REFERENCES
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