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Studies with Specific Enzyme Inhibitors

XIV. The Effects of Enzymatically Synthesized (−)-*erythro*-Fluoromalic Acid on Malate Dehydrogenase and on Anion Carriers of Liver Mitochondria

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SUMMARY

Enzymatic synthesis and isolation of fluoromalate with a tentatively assigned (−)-*erythro* configuration is described. Fluoromalate inhibits purified malate dehydrogenase (K_i = 13 or 16 μM) but has no effect on the oxidation of pyruvate and succinate by liver mitochondria, indicating that fluoromalate does not penetrate the inner mitochondrial membrane. Oxidation of externally added L-malate is reversibly inhibited by fluoromalate when the latter is present at higher concentrations than malate. Oxidation of tricarboxylic acids and of α-ketoglutarate is greatly increased under state 3 conditions by fluoromalate. The rate of citrate-isocitrate translocation in mitochondria is activated by fluoromalate to the same extent as the metabolism of tricarboxylic acids. It is concluded that fluoromalate competitively inhibits the malate carrier and activates the carriers of tricarboxylic acids and of α-ketoglutarate.

INTRODUCTION

Based on its high potency and relative specificity, fluoro-oxalacetate appeared to be a suitable tool for the study of the regulatory role of malate dehydrogenase (EC 1.1.1.37) in cellular and subcellular systems (1, 2). Although in short-term (10–15-min) experiments with isolated mitochondria, fluoro-oxalacetate can be successfully applied as an inhibitor of malate dehydrogenase (3), its use is complicated by nonenzymatic decarboxylation to fluoropyruvate (4). According to Gal (5) and Krasna (6, 7), DL-*erythro*-fluoromalic acid inhibits malate dehydrogenase competitively (K_i = 34 μM). Whereas the inhibitory effect of DL-*erythro*-fluoromalate on malate dehydrogenase appears to be about 100 times weaker than that of fluoro-oxalacetate (K_i = 0.1 μM; cf. ref. 1), its greater stability may outweigh this disadvantage and could make it suitable for inhibition of malate dehydrogenase in subcellular multienzyme systems. These considerations led to a reinvestigation of the biochemical properties of fluoromalate.

Previous workers (5–7) employed the racemic mixture of the *erythro* diastereoisomer of fluoromalic acid as enzyme inhibitor. In order to avoid the uncertainty

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introduced by the possible interference of the noninhibitory optical isomer, we prepared (−)-*erythro*-fluoromalic acid enzymatically. The present paper is concerned with (a) the method of enzymatic synthesis of (−)-*erythro*-fluoromalic acid, (b) its effect on purified malate dehydrogenase (cf. ref. 1), and (c) its effect on certain metabolic activities of isolated rat liver mitochondria.

MATERIALS AND METHODS

Crystalline fluoro-oxalacetic acid was prepared and purified as described previously (4). NAD^+, $NADP^+$, commercial yeast alcohol dehydrogenase (EC 1.1.1.1.), malate dehydrogenase, and isocitrate dehydrogenase (EC 1.1.1.42) were purchased from Boehringer Company. Purified ox kidney malate dehydrogenase was isolated by a published method (8). Quantitative analysis for fluoro-oxalacetate was based on the determination of absorbance at 240 nm, indicating the amount of enol-fluoropyruvate derived from fluoro-oxalacetate by decarboxylation with Al^{3+} (4). Details of this assay will be given under RESULTS. Fluoromalate was determined by a colorimetric method for hydroxycarboxylic acids (9) as modified subsequently (10). Spectrophotometric assays were carried out in a Gilford multichannel recording spectrophotometer or in a Unicam SP-825 double-beam instrument. The rate of mitochondrial conversion of citrate to isocitrate was followed in an Aminco-Chance dual-wavelength spectrophotometer equipped with a log converter, by monitoring extramitochondrial NADPH formation in the presence of externally added isocitrate dehydrogenase and $NADP^+$. Wavelength settings were 374 and 340 nm. Details will be given under RESULTS. Oxygen uptake by mitochondria was determined either in a Gilson oxygraph or, in more prolonged experiments, in Gilson respirometers. Rat liver mitochondria were prepared in a sucrose-mannitol–bovine serum albumin medium by differential centrifugation (11). Their functional integrity was controlled by the determination of oxidative phosphorylative efficiency with glutamate as substrate (P:O = 2.8–3.1 was acceptable) and by occasional examination with the electron microscope.[2] Absence of microsomal contamination was tested by glucose 6-phosphatase (EC 3.1.3.10) assays.

Malate dehydrogenase activity was assayed at 25° both by the reduction of oxalacetate by NADH and by the oxidation of L-malate by NAD^+. In the second assay, oxalacetate was removed by coupling malate oxidation to glutamate-oxalacetate transaminase (EC 2.6.1.1.) obtained from Boehringer. It was ascertained in separate experiments that fluoromalate at the concentrations used had no effect on the transaminase activity.

The evolution of $^{14}CO_2$ from labeled substrates was measured in Gilson differential respirometers by absorbing $^{14}CO_2$ in 0.2 ml of Hyamine hydrochloride placed in the center well. The metabolism of mitochondria was stopped by the addition of 0.15 ml of 70% perchloric acid from the side arm, and incubation was continued for 15 min to allow quantitative trapping of $^{14}CO_2$. Radioactivity was determined by scintillation counting in a mixture containing 0.3% 2,5-diphenyloxazole and 0.03% *p*-bis[2-(5-phenyloxazolyl)]benzene in toluene. A Packard Tri-Carb instrument, model 3375, was used for scintillation counting.

RESULTS

Enzymatic Synthesis of (−)-erythro-Fluoromalate

Stereospecific reduction of fluoro-oxalacetate by NADH was carried out by malate dehydrogenase. In this system, NADH was continuously generated by alcohol dehydrogenase plus NAD^+ and ethanol.

Reaction system. Four milliliters of pig heart malate dehydrogenase [Boehringer; 5 mg of protein per milliliter in 3.2 M $(NH_4)_2SO_4$] and 2 ml of yeast alcohol dehydrogenase [Boehringer; 30 mg of protein per milliliter in 3.2 M $(NH_4)_2SO_4$] were dialyzed at 4° against three successive batches of 0.1 M Tris-acetate, pH 7.0 (each batch contained 2 liters), for 4–6 hr in order to remove excess $(NH_4)_2SO_4$.

Fluoro-oxalacetic acid (1.6 g, 8.6 mmoles) and NAD^+ (0.460 g, 0.7 mmole) were dis-

[2] We are indebted to Dr. W. Stoeckenius for electron microscopy.

solved in 200 ml of 0.1 M Tris-acetate buffer and titrated to pH 8.20 with 1 N NaOH. One milliliter of this solution was set aside for determination of the spontaneous rate of decarboxylation of fluoro-oxalacetate (4). This rate served as a control for the determination of the rate of enzymatic reduction of fluoro-oxalacetate to fluoromalate (see Fig. 1). Dialyzed malate dehydrogenase and alcohol dehydrogenase were then added to the reaction mixture (total volume, 210 ml, containing 41 mM fluoro-oxalacetate, 3.3 mM NAD^+ plus NADH, 1.6 M ethanol, 0.095 mg/ml of malate dehydrogenase, and 0.29 mg/ml of alcohol dehydrogenase). The reaction mixture was kept at 20°, and the progress of the reaction was monitored by spectrophotometric analysis of the disappearance of fluoro-oxalacetate as follows. Samples (10 μl) of the reaction mixture were added to 3-ml cuvettes of 1-cm light path, maintained at 40°, and containing 3 ml of 1 N formic acid. The background absorbance at 240 nm was recorded for a few seconds in a Unicam SP-825 spectrophotometer, and 20 μl of 1 M $AlCl_3$ were added. Within 1 min, maximal absorbance was reached at 240 nm, which, as shown previously (4), was due to the enol-fluoropyruvate formed from the Al^{3+}-catalyzed decarboxylation of fluoro-oxalacetate. By extrapolation from the relatively slow rate of enol-keto conversion of fluoropyruvate, the concentration of fluoro-oxalacetate present at zero time could be readily determined (4). Apparent first-order kinetics for the rate of fluoro-oxalacetate consumption was found during the first two half-lives of the reaction, as demonstrated by plotting of the logarithm of absorbance (at 240 nm) against time (Fig. 1). The upper curve illustrates the rate of spontaneous decarboxylation of fluoro-oxalacetate in the absence of malate dehydrogenase and alcohol dehydrogenase, while the lower curve is an indication of enzymatic reduction of fluoro-oxalacetate to fluoromalate.

Isolation of fluoromalic acid. After the reaction had proceeded for about 4 hr, when the estimated consumption of fluoro-oxalacetate reached 90%, it was terminated by passing the reaction mixture through an Amicon membrane filter (No. PM-30) at 4°.

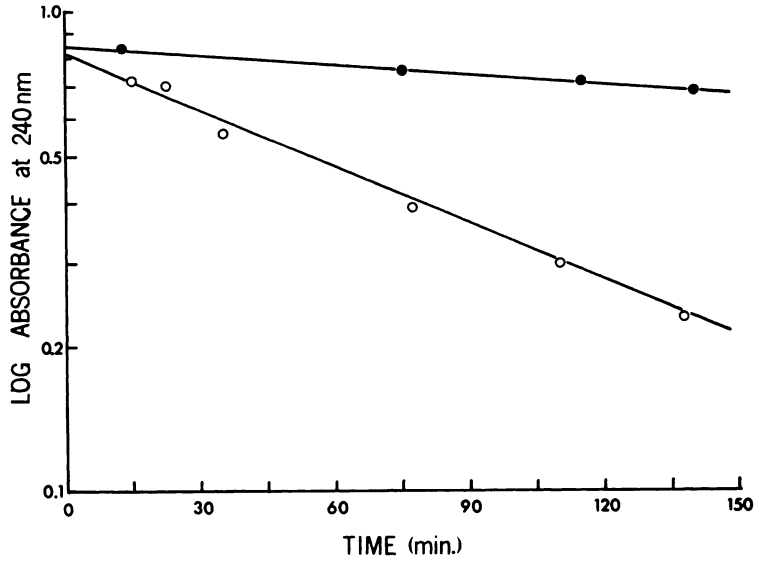


FIG. 1. *Kinetics of fluoro-oxalacetate disappearance during enzymatic synthesis of (−)-erythro-fluoromalate*

The upper curve (●——●) is the control containing no enzymes, and the lower curve (○——○) represents the complete system. For details, see MATERIALS AND METHODS. The ordinate represents the absorbance of the enol-fluoropyruvate–Al^{3+} complex, and the abscissa, time in minutes.

The retained enzyme was recovered and used again for a subsequent batch.

The filtrate (200 ml) was stirred for 45 min with activated charcoal (Norit, 8 g). Charcoal adsorption removed 95% of the material which absorbed at 340 nm. Norit was removed by filtration.

To the clear, colorless filtrate, 43 ml of 1 M lead acetate solution were added, and the pH was adjusted to 6.0 with 1 N NaOH. A white precipitate, consisting of the crude lead salt of fluoromalate, was removed by centrifugation. The removal of fluoromalate as the lead salt was monitored by colorimetric analysis for fluoromalate in the supernatant solution (9, 10). The lead salt precipitate was dissolved by the addition of 14 ml of moist Dowex 50-H^+ (13 g). The slurry (consisting of Dowex 50 resin) was poured on a Dowex 50-H^+ column (50 ml, 44 g) and eluted with distilled water until the pH of the effluent rose to 4.0. The effluent (50 ml) was filtered through Celite and evaporated to a brown syrup under vacuum. An acetone extract (15 ml) of the syrup was filtered and evaporated to dryness, leaving a semicrystalline product (0.403 g).

Purification and characterization of fluoromalic acid. The combined products of five enzymatic syntheses yielded 2.63 g of crude fluoromalic acid. Recrystallization from ethyl acetate (10 ml) by addition of benzene (10 ml) yielded 1.27 g of white, crystalline solid (m.p. 159–159°). Concentration of filtrates gave an additional 0.44 g. Based on the combined weight of starting material (8 g of fluoro-oxalacetic acid), five preparations produced 1.70 g of crystalline fluoromalic acid, or an over-all yield of 21%.

$C_4H_5FO_5$

Calculated: C 31.55, H 3.29, F 12.50

Found: C 31.64, H 3.21, F 12.20

The infrared spectrum measured in KBr discs, showed peaks at 2.9, 3.3, 5.8, 7.0, 7.4, 8.0, 8.4, 9.1, 9.9, 10.5, 11.6, 12.0, 13.0, 13.7, and 15.2 μ. The specific rotation of the product (in 23% ethanol) was $[\alpha]_D^{20}$ −2.3°. The NMR spectrum of (−)-*erythro* fluoromalate is shown in Fig. 2.

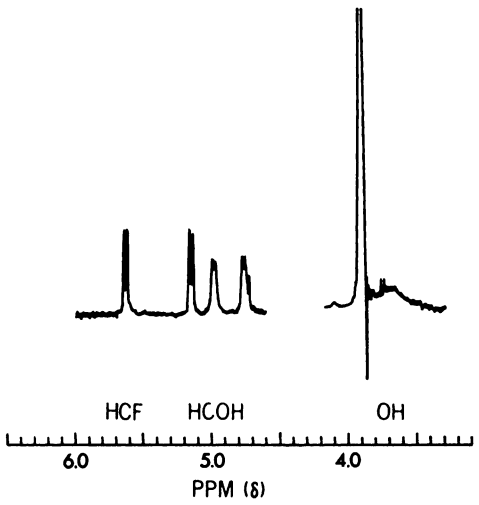
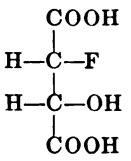


FIG. 2. *Nuclear magnetic resonance spectrum of (−)-erythro-fluoromalic acid in D_2O*

The spectrum was recorded with a 100 MHz Jeol spectrometer. Tetramethylsilane was used as an internal standard.

Configuration of enzymatically synthesized fluoromalic acid. Since the configuration of the hydroxyl center is known to be L from the stereospecificity of malate dehydrogenase, and the *erythro* configuration has been established (7), the most probable structure of enzymatically synthesized (−)-*erythro*-fluoromalic acid is according to the Fischer convention and is



According to the R/S nomenclature, this acid is 2R-3R-2-fluoro-3-hydroxysuccinic acid.[3]

Inhibition of Malate Dehydrogenase by (−)-erythro-Fluromalate

Inhibition of ox kidney cytoplasmic malate dehydrogenase is shown in Fig. 3.

[3] The hydroxyl center of L-malic acid is designated S in the R/S system, but introduction of a fluorine atom on the adjacent center alters the priority sequence of the groups around the hydroxyl center and changes the designation to R.

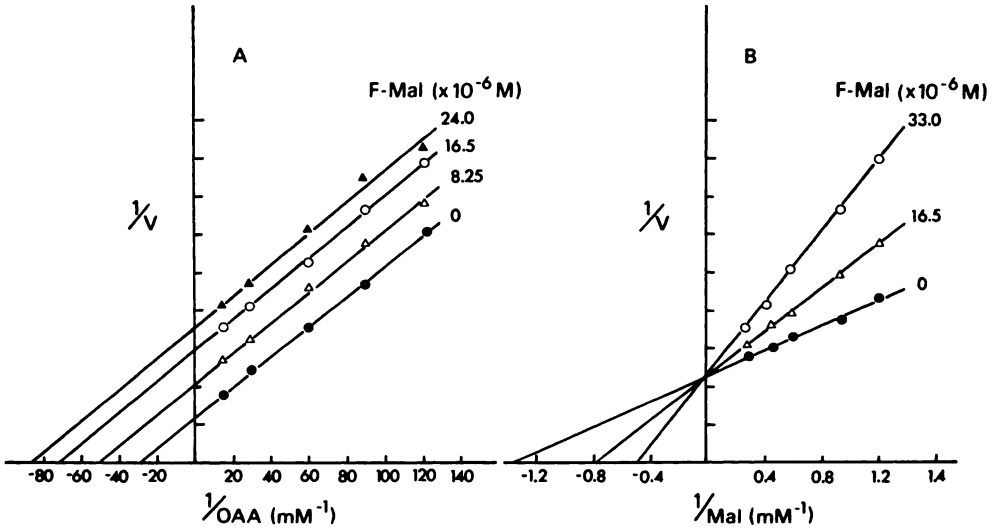


FIG. 3. *Kinetics of inhibition of malate dehydrogenase by (−)-erythro-fluoromalate*

A. Incubation mixtures (3 ml, 25°) contained 0.2 mM NADH, 0–24 μM fluoromalate (F-Mal), and 5 μg of purified cytoplasmic malate dehydrogenase (8) in 0.1 M Tris-HCl buffer, pH 7.4, with oxalacetate (OAA) as the variable substrate. Absorbance changes at 340 nm were recorded at a sensitivity of 0–1.0.

B. Incubation mixtures comprised 0.5 mM NAD^+, 0–33 μM fluoromalate, (F-Mal), 1 mM glutamate, 5 μg of glutamate-oxalacetate transaminase (Boehringer), and 5 μg of purified cytoplasmic malate dehydrogenase (8) in 0.1 M Tris-HCl buffer, pH 7.4, with malate (Mal) as the variable substrate. Absorbance increments at 340 nm were measured at a sensitivity of 0–0.2.

When the reaction was measured from the oxalacetate side (i.e., NADH + oxalacetate), at pH 7.4, an apparent uncompetitive type of inhibition by fluoromalate was obtained, with an apparent K_i of 13 μM (Fig. 3A). On the other hand, when the reaction was measured from the malate side (i.e., malate + NAD^+), also at pH 7.4, competitive inhibition was observed, with an apparent K_i of 16 μM. Detailed two-substrate kinetic analysis with respect to coenzyme and carboxylate substrates was not carried out because the purpose of preliminary kinetic analyses was to compare the effectiveness of fluoro-oxalacetate (1) and fluoromalate. According to these results, fluoromalate is about 150 times less inhibitory than fluoro-oxalacetate under comparable conditions.

Effects of (−)-erythro-Fluoromalate on Liver Mitochondria

Because of its inhibitory effect on malate dehydrogenase, fluoromalate, like fluoro-oxalacetate (3), would be expected to inhibit the metabolism of substrates which are oxidized by the mitochondrial citric acid cycle. Pyruvate oxidation was measured under state 3 conditions in the polarographic system (Fig. 4), and in Warburg respirometers for more prolonged (40-min) periods (Table 1). In the polarographic system 1–5 mM fluoromalate had no measurable effect on O_2 uptake in the presence of 2 mM pyruvate as substrate. The rates of O_2 uptake and $^{14}CO_2$ evolution from 5 mM uniformly ^{14}C-labeled pyruvate, as measured in respirometers for 40 min, were also unaffected by up to 10 mM fluoromalate. When [1,4-^{14}C]succinate (5 mM) and unlabeled pyruvate (2 mM) were oxidized simultaneously in Warburg respirometers by mitochondria, neither the O_2 uptake nor the $^{14}CO_2$ formation rate was significantly inhibited even by 10 mM fluoromalate (Table 2). These results indicate that fluoromalate did not penetrate the inner mitochondrial membrane.

The oxidation of other carboxylic acid substrates was also determined in the presence of fluoromalate under state 3 metabolic conditions. As shown in Table 3,

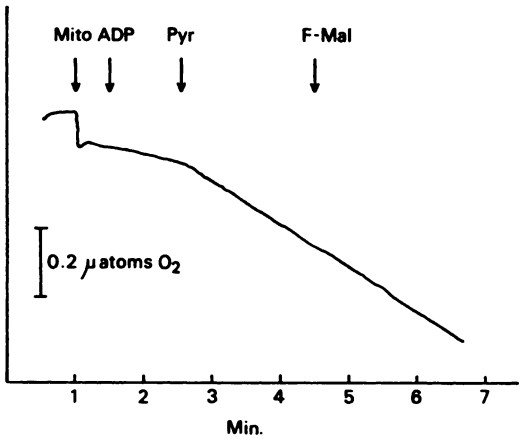


FIG. 4. *Ineffectiveness of (—)-erythro-fluoromalate on pyruvate oxidation*

Oxygen uptake (in microatoms, ordinate) was determined with the aid of a Gilson oxygraph, model K, using a Clark electrode. The reaction mixture (5 ml of 0.15 M KCl plus 0.05 M Tris-HCl and 0.01 M phosphate, pH 7.4) was stirred magnetically and kept at 30° by a circulating water jacket. Components were added at the time intervals shown: 8.4 mg of mitochondrial protein (Mito), 2 mM ADP, 2 mM pyruvate (Pyr), and 1 or 5 mM fluoromalate (F-Mal).

TABLE 1

Effect of (—)-erythro-fluoromalate on $^{14}CO_2$ evolution from oxidation of [^{14}C]pyruvate

Incubation mixtures (3 ml) comprised 5 mM uniformly ^{14}C-labeled pyruvate (0.016 μCi/μmole), 0–10 mM fluoromalate, 11.5 mg of mitochondrial protein, 2 mM ADP, and 10 mM potassium phosphate in 0.05 M Tris–0.15 M KCl buffer, pH 7.4. Incubation time was 40 min at 30° in Warburg respirometers, the CO_2 being trapped in 0.2 ml of Hyamine hydroxide (methylbenzethonium chloride, Rohm and Haas) for subsequent counting as described under MATERIALS AND METHODS.

Fluoromalate concentration	Oxygen uptake[a]	$^{14}CO_2$
M	*μl*	*cpm*
	100	88,591
2×10^{-5}	104	87,441
2×10^{-4}	102	87,599
1×10^{-3}	110	87,114
1×10^{-2}	106	87,285

[a] Values corrected for endogenous respiration.

rates of O_2 uptake with citrate and isocitrate (both 2 mM) were stimulated 4- and 8-fold by 1 mM fluoromalate, and the oxidation of α-ketoglutarate (2 mM) was about doubled under the same experimental conditions.

The time course of the activation of the metabolism of tricarboxylic acids and of α-ketoglutarate is illustrated in Fig. 5. The

TABLE 2

Effect of (—)-erythro-fluoromalate on O_2 uptake and $^{14}CO_2$ evolution from oxidation of [1,4-^{14}C]succinate in the presence of pyruvate

Incubation mixtures (3 ml) comprised 5 mM [1,4-^{14}C]succinate (0.0160 μCi/μmole), 2 mM pyruvate, 0–10 mM fluoromalate, 9.2 mg of mitochondrial protein, 2 mM ADP, and 10 mM potassium phosphate in 0.05 M Tris–0.15 M KCl buffer, pH 7.4. Incubation time was 30 min at 30° in Warburg respirometers, the CO_2 being trapped in 0.2 ml of Hyamine hydroxide for subsequent counting (see MATERIALS AND METHODS).

Fluoromalate concentration	Oxygen uptake[a]	$^{14}CO_2$
M	*μl*	*cpm*
	140	4118
2×10^{-5}	145	4126
2×10^{-4}	146	4196
1×10^{-3}	146	3707
1×10^{-2}	128	3986

[a] Values corrected for endogenous respiration.

TABLE 3

Rates of oxidation of carboxylic acids in the presence of (—)-erythro-fluoromalate

Rates of O_2 uptake were calculated from polarographic experiments as described in the legend to Fig. 4.

Carboxylic acid substrate (2 mM)	Rate of oxidation	
	No fluoromalate	With fluoromalate (1 mM)
	nmoles/min/mg mitochondrial protein	
1 Citrate	3.7	16
2. Isocitrate	3.7	29
3. α-Ketoglutarate	6.8	17
4. Malate	15.0	0[a]
5. Pyruvate	6.7	6.7

[a] Fluoromalate (4 mM) was present.

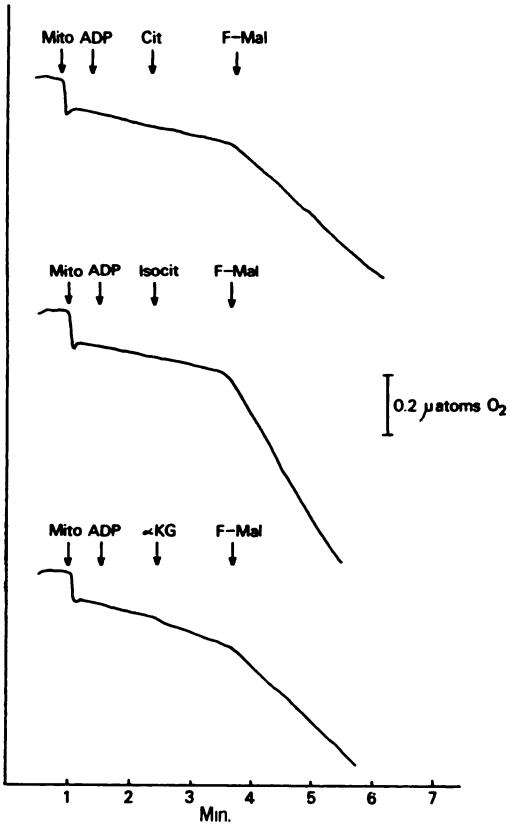


FIG. 5. *Effect of (−)-erythro-fluoromalate on oxidation of tricarboxylic acids*

The additions to the incubation were as follows: 8.3 mg of mitochondrial protein (Mito), 2 mM ADP, 2 mM citrate (Cit) or α-ketoglutarate (αKG), 1 mM isocitrate (Isocit), and 1 mM fluoromalate (F-Mal). Experimental conditions were the same as described in the legend to Fig. 4.

effect of fluoromalate was instantaneous and depended on the presence of both orthophosphate and ADP (not shown).

In marked contrast to other substrates, the metabolism of L-malate was inhibited in the presence of fluoromalate at higher concentrations than malate (experiment 4 in Table 3). The concentration-dependent, reversible inhibition of malate metabolism by fluoromalate is further illustrated in Fig. 6. When malate was added at 1 mM concentration, the subsequent addition of 1 mM fluoromalate had no effect on O_2 uptake, but 4 mM fluoromalate inhibited malate metabolism (Fig. 6A). Furthermore, the inhibitory effect of 4 mM fluoromalate on the oxidation of 1 mM malate was completely abolished when the malate concentration was raised to 4 mM (Fig. 6B). It is unlikely that the mitochondrial system which is responsible for the inhibition of malate metabolism by 4-fold higher concentrations of fluoromalate than malate is mitochondrial malate dehydrogenase, which sould have been inhibited according to the K_i value of 16 μM. The possibility that higher concentrations of fluoromalate could be oxidized to fluorooxalacetate, which might subsequently inhibit malate metabolism, is ruled out by the time course of inhibition. It was found that (−)-*erythro*-fluoromalate was oxidized by NAD^+ in the presence of purified malate dehydrogenase at less than 0.1% of the rate obtained with L-malate as substrate. It would have taken a reaction time of 3–4 hr

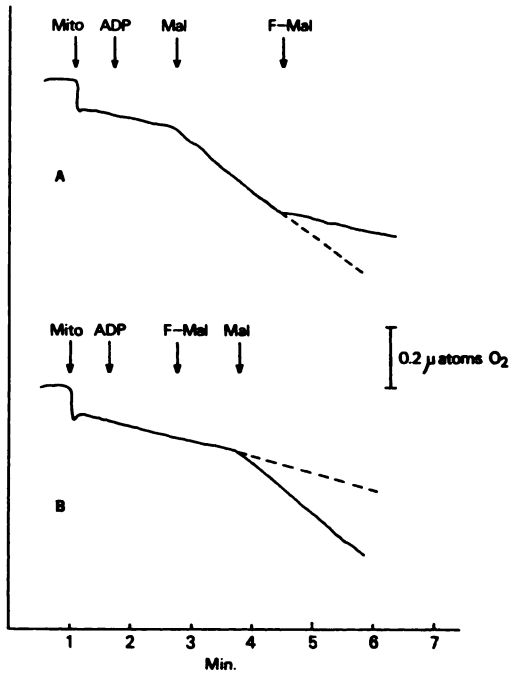


FIG. 6. *Effect of (−)-erythro-fluoromalate on malate oxidation*

Measurements were made in the polarographic system described in the legend to Fig. 4. The additions to the incubation mixture were as follows: 8.3 mg of mitochondrial protein (Mito), 2 mM ADP, and either 1 mM malate (Mal) followed by 1 mM (- - -) or 4 mM (——) fluoromalate (F-Mal) (A) or 4 mM fluoromalate followed by 1 mM (- - -) or 4 mM (——) malate (B).

(at 25°) to produce micromolar concentrations of fluoro-oxalacetate in the presence of a solution of purified malate dehydrogenase and a carbonyl reagent to trap fluoro-oxalacetate (at pH 8.0). Since the inhibitory effect of fluoromalate was instantaneous (Fig. 6), this characteristic property of the inhibition itself excludes the possibility that slow metabolism of fluoromalate could produce inhibitory fluoro-oxalacetate. The ineffectiveness of fluoromalate as an inhibitor of pyruvate and succinate oxidation showed that fluoromalate did not enter the matrix space of mitochondria, where malate dehydrogenase and fumarase are localized (12, 13). These results also exclude the remote possibility that fluoromalate may be metabolized by mitochondria. A comparison of the effects of fluoromalate on malate and pyruvate oxidation is also shown in Table 3 (experiments 4 and 5).

The most unusual activating effect of fluoromalate was observed when tricarboxylic acids were oxidized by mitochondria under state 3 conditions (Table 3 and Fig. 5). Since this experimental model is complicated, involving both anion translocation and metabolism, a simpler system was applied which more directly measures tricarboxylate entry and exit in mitochondria. As shown by Chappell and Robinson (14), in the presence of externally added isocitrate dehydrogenase and $NADP^+$ the rate of isocitrate efflux (as measured by NADPH formation) is a direct measure of citrate translocation through the inner membrane. At a chosen sensitivity of the dual-wavelength spectrophotometer ($A_{340-374} = 0.1$), intramitochondrial pyridine nucleotides do not interfere and the rates of citrate-isocitrate conversion by mitochondria can be directly

TABLE 4

Rates of isocitrate efflux from liver mitochondria with citrate as substrate

The suspending medium in all experiments consisted of 10 mM potassium phosphate, 5 mM $MgCl_2$, 0.3 mM $NADP^+$, 2.2 mg of mitochondrial protein, 0.3 mg of isocitrate dehydrogenase, and 0.05 M Tris-HCl–0.15 M KCl, pH 7.4. The reaction was started by the addition of citrate, followed after 3–4 min by fluoromalate (see Table 5 and Fig. 7). The volume of the reaction mixture was 3 ml.

Additions	Isocitrate efflux
	nmoles/min/mg mitochondrial protein
1. 2 mM citrate (±2 mM ADP)	1.7
2. 2 mM citrate (±2 mM ADP) + 1.0 mM fluoromalate	6.1
3. 10 mM citrate (±2 mM ADP)	1.7
4. 10 mM citrate (±2 mM ADP) + 1.0 mM fluoromalate	6.3
5. 2 mM citrate + 1 μM rotenone	1.6
6. 2 mM citrate + 1 μM rotenone + 2 mM ADP	2.3
7. 2 mM citrate + 1 μM rotenone + 0.1 mM fluoromalate	7.1
8. 2 mM citrate + 1 μM rotenone + 2 mM ADP + 1.0 mM fluoromalate	7.4
9. 10 mM citrate + 1 μM rotenone	1.7
10. 10 mM citrate + 1 μM rotenone + 2 mM ADP	2.9
11. 10 mM citrate + 1 μM rotenone + 0.1 mM fluoromalate	7.4
12. 10 mM citrate + 1 μM rotenone + 2 mM ADP + 1.0 mM fluoromalate	7.4

TABLE 5

Activation of citrate transport by various concentrations of (—)-erythro-fluoromalate

To an incubation mixture (3 ml) comprising 2 mM ADP, 10 mM potassium phosphate, 5 mM $MgCl_2$, 1 μM rotenone, 0.3 mM $NADP^+$, 1.8 mg of mitochondrial protein, and 0.3 mg of isocitrate dehydrogenase (Calbiochem) in 0.05 M Tris–0.15 M KCl buffer, pH 7.4, 1.5 mM citrate was added, followed after several minutes by the addition of fluoromalate in the concentrations indicated below. Incubations were performed at 25° in an Aminco-Chance dual-wavelength spectrophotometer equipped with a log converter at a sensitivity setting of 0–0.1 optical density unit ($A_{340-374}$).

Fluoromalate concentration	Rate of isocitrate exit
M	*nmoles/min/mg mitochondrial protein*
0	2.1
3.3×10^{-6}	3.2
1×10^{-5}	4.7
3.3×10^{-5}	5.4
1×10^{-4}	6.7
5×10^{-4}	7.6
1×10^{-3}	7.6

monitored. Table 4 shows the rates of isocitrate efflux under a variety of experimental conditions. There was significant isocitrate efflux when citrate was added externally, even in the absence of an inhibitor of metabolism (experiments 1–4). The only requirement for maximum citrate translocation and isocitrate efflux was orthophosphate and fluoromalate, while ADP under these conditions had no detectable effect. Variation of the citrate concentration from 2 to 10 mM did not modify the rates of isocitrate efflux, and 1 mM fluoromalate augmented the rate to the same maximum (experiments 1–4). When electron transfer was inhibited by 1 μM rotenone (experiments 5–12), 1 mM fluoromalate increased the efficiency of maximal rates of citrate-isocitrate conversion by about 16% above the rate determined in the absence of rotenone. It is apparent from this reconstructed system that under normal cell physiological circumstances about 84% of cytoplasmic citrate, which enters mitochondria, could reappear in the cytosol as isocitrate and be oxidized to α-ketoglutarate. It is of interest, but as yet unexplained, that in the presence of rotenone ADP alone increased citrate entry by about 40% (experiments 6 and 10) but had no effect in respiring mitochondria.

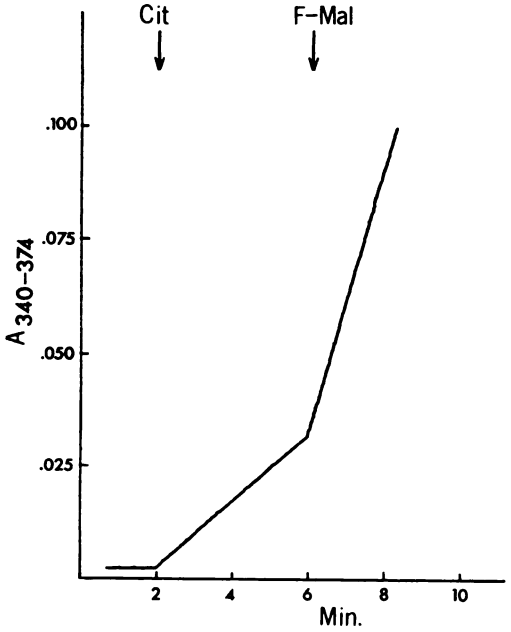


FIG. 7. *Spectrophotometric assay of citrate entry into mitochondria*

Details of the incubation mixture are given in the legend to Table 4. Cit, citrate; F-mal, fluoromalate.

The relationship between the rates of citrate transport and the concentration of fluoromalate is illustrated in Table 5. Above 0.1 mM fluoromalate, citrate transfer was maximal. The kinetics of isocitrate efflux (or citrate entry) is shown in Fig. 7, as recorded in the dual-wavelength spectrophotometer.

DISCUSSION

Enzymatic synthesis of (−)-*erythro*-fluoromalate resulted in a product which was suitable for unambiguous application in enzymatic and metabolic systems. Inhibition of malate dehydrogenase by fluoromalate resembles inhibition of lactate dehydrogenase by L(+)-fluorolactate (15). With both dehydrogenases at fixed concentrations of pyridine nucleotides, the fluorohydroxy acid exhibited competition toward the hydroxy acid substrate, but with respect to the keto acid substrate the apparent inhibition was uncompetitive. As shown in more extensive studies with lactate dehydrogenase (15), K_i can be a function of the concentration of the second substrate if the inhibition is uncompetitive; therefore the same K_i for fluoromalate with respect to both substrates of malate dehydrogenase is probably a fortuitous result and merely reflects experimental values at one given concentration of NADH (Fig. 3A).

The absence of an inhibitory effect of fluoromalate on the oxidation of pyruvate or the pyruvate plus succinate substrate couple demonstrates that fluoromalate cannot penetrate the inner membrane of mitochondria. These results could not have been obtained if fluoromalate had found its way to mitochondrial malate dehydrogenase, which should have been inhibited in accordance with the K_i value of 13–16 μM. Similar conclusions were drawn by Chappell (16), who observed that fluoromalate (which was probably the synthetic, unresolved product) did not inhibit isocitrate oxidation after preliminary incubation of mitochondria with

2,4-dinitrophenol and arsenate. No stimulation of isocitrate oxidation in the presence of fluoromalate was reported under these conditions (16). It seems possible that the absence of stimulation of O_2 uptake by fluoromalate could be due to the prior treatment of mitochondria with 2,4-dinitrophenol and arsenate, reagents known to interfere with various energy-coupled inner membrane functions. It has been reported that tricarboxylate translocation is an energy-coupled process (17). Alternatively, the (+)-isomers of racemic fluoromalate or impurities may have interfered with the activating effect of (−)-*erythro*-fluoromalate, as reported in the present paper.

The two apparently opposite types of effects of fluoromalate on mitochondria—inhibition of malate and activation of tricarboxylate and α-ketoglutarate metabolism—can be explained on the basis of the modification of anion carriers by fluoromalate. Detailed molecular information related to the nature and function of these anion carriers is missing, although their operational significance in metabolic regulation is well established (16, 18–20). According to Chappell (14, 16, 18), the phosphate-requiring dicarboxylate carrier is capable of translocation of both succinate and malate. Our results show that fluoromalate inhibits malate but not succinate metabolism, suggesting the existence of a specific malate carrier. Since none of the anion carrier proteins has so far been isolated, it is impossible to decide whether or not specific succinate and malate carriers exist, or whether the same carrier protein has sites with different affinities toward various dicarboxylate anions. The second effect of fluoromalate—the apparent activation of the metabolism of tricarboxylic acids and of α-ketoglutarate—can also be correlated with the operation of specific anion carriers. Both the tricarboxylate carrier (14, 16, 18, 19) and the α-ketoglutarate carrier (20) are known to be activated by malate; thus it is to be expected that a structural homologue of malate, (−)-*erythro*-fluoromalate, has effects similar to those of malate.

The inaccessibility of mitochondrial malate dehydrogenase to even high concentrations of fluoromalate implies that this fluoro acid could be used as a specific inhibitor of cytoplasmic malate dehydrogenase in a cellular system. Further studies with isolated liver parenchymal cells have indicated that this is the case.[4]

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[4] M. N. Berry and E. Kun, unpublished observations.