Stereochemical Outcome of Processing of Fluorinated Substrates by ATP Citrate Lyase and Malate Synthase[†]
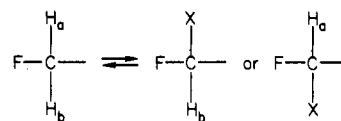
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ABSTRACT: The (–)-*erythro*- and (+)-*erythro*-2-fluorocitrate isomers are substrates for the cytoplasmic ATP citrate lyase from rat liver with V_{max} values 0.13% and 2.3%, respectively, that of citrate when the keto acid products are measured by in situ reduction by NADH and malate dehydrogenase. Anticipated regiospecificity of cleavage by ATP citrate lyase is production of 2-fluoroacetyl-CoA and oxalacetate from a (2*R*,3*R*)-2-fluorocitrate isomer and production of acetyl-CoA and (3*S*)-fluorooxalacetate from cleavage of the other erythro diastereomer, the (2*S*,3*S*)-2-fluorocitrate. The product combinations were validated by in situ reduction with MDH and (4*R*)-[³H]NADH to yield L-(2*S*)-[2-³H]malate from (–)-*erythro*-2-fluorocitrate and to yield L-(2*R*,3*S*)-3-fluoro[2-³H]malate only from the (+)-*erythro*-2-fluorocitrate. The data allow for the first time unambiguous assignment of absolute stereochemistry to the (+)-*erythro*-2-fluorocitrate (2*S*,3*S*) and thereby necessarily to the (–)-*erythro*-2-fluorocitrate as 2*R*,3*R*.

This latter isomer is, of course, the toxic species generated by the "lethal synthesis" from fluoroacetyl-CoA and oxalacetate effected by citrate synthase. Assuming inversion in the citrate synthase reaction, one can now state that it processes 2-fluoroacetyl-CoA chirally with specific *pro-S* hydrogen abstraction in the condensation. Cleavage of (2*R*,3*R*)-2-fluorocitrate by ATP citrate lyase in ³H₂O should yield a product that is chiral, (2*S*)-fluoro[2-³H]acetyl-CoA. Attempts to analyze this product involved in situ coupling with [¹⁴C]-glyoxalate and yeast malate synthase. Surprisingly, both *erythro*- and *threo*-3-fluoromalates formed in about equal amounts, and each had both ³H and ¹⁴C radioactivity. These results suggest that malate synthase, in contrast to citrate synthase, processes one of its two substrates achirally at a prochiral center, either C_2 of fluoroacetyl-CoA or, less likely, the trigonal aldehyde carbon of glyoxalate during fluoromalate biosynthesis.

Substrate analogues with specific replacement of H by F have proved useful in enzymology since enzymes often process such molecules catalytically and thereby reveal mechanistic facets of their action. The small size of fluorine and the short C–F bond are a close steric mimic to the C–H bond, but the high electronegativity of fluorine makes the polarization of the C–F bond more akin to that of a C–OH bond. There are enzymatic precedents for apparent recognition of C–F as either a C–H or C–OH substituent (Filler, 1976). One recent use of fluorine replacement for hydrogen has been to probe enzymatic generation of carbanionic intermediates at an adjacent site by detection of net H–F elimination sequence (Silverman & Abeles, 1976, 1977; Wang & Walsh, 1978; Kollonitsch et al., 1978; Seiler et al., 1978). With several PLP enzymes, such fluorinated substituted molecules behave as suicide substrates when the subsequent enamine intermediate is captured by an enzymatic nucleophile. On the other hand, fluorine substitution in 5-FdUMP[1] (Hartmann & Heidelberger, 1961) generates mechanism-based inactivation of thymidylate synthetase precisely because the enzyme cannot break the C_5–F bond by F⁺ abstraction (Santi et al., 1976).

We have been investigating the consequences of enzymatic processing of fluorinated analogues at the C–F locus in reactions where the C–F bond is itself not broken.

In reversible interconversions of fluoromethyl and fluoromethylene groups, the stereochemical outcome of the reaction reflects chirality of enzymatic recognition. For example, fluoropyruvate is carboxylated to fluorooxalacetate by transcarboxylase with stereospecific removal of only one enantiotopic hydrogen, but fluoropyruvate is enolized achirally by pyurvate kinase (Goldstein et al., 1978).

The simplest stable fluorinated carboxylate is the naturally occurring fluoroacetate (Peters, 1972), which is toxic by virtue of its chiral recognition (as fluoroacetyl-CoA) and condensation with oxalacetate to a single 2-fluorocitrate[2] diastereomer (Fanshier et al., 1964) [(–)-erythro], catalyzed by citrate synthase, the celebrated "lethal synthesis" reaction (Peters, 1972).

In the experiments noted here, we report an apparent achiral processing of fluoroacetyl-CoA by malate synthase. The fluoroacetyl-CoA was generated in situ from cleavage of (–)-*erythro*-fluorocitrate by rat liver ATP citrate lyase (citrate

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[1] Abbreviations used: 5-FdUMP, 5-fluorouridine 5′-monophosphate; CCE, citrate cleavage enzyme (ATP citrate lyase); MDH, malate dehydrogenase; NADH, reduced nicotinamide adenine dinucleotide; ATP, adenosine 5′-triphosphate; CoA, coenzyme A; HPLC, high-performance liquid chromatography; Tris, tris(hydroxymethyl)aminomethane; *t*-Fm, *threo*-3-fluoromalate; *e*-Fm, *erythro*-3-fluoromalate; LDH, lactate dehydrogenase; DTT, dithiothreitol; PLP, pyridoxal phosphate.

[2] The numbering system for the fluorocitrates used in this paper is that which has developed historically and is the most widely quoted; however, IUPAC nomenclature states that the portion of the citrate backbone derived from acetate be numbered 4 and 5. Therefore, under this system, the (–)-erythro isomer would be 4-fluorocitrate and the (+)-erythro would be a 2-fluorocitrate. Another numbering system is that designated as parent numbering (pn) where the *pro-R* branch has lower numbering than the *pro-S* branch [(1979) *Bioorg. Chem. 2*, 301–310].

cleavage enzyme), and studies on the regio- and stereospecificity of (+)- and (−)-*erythro*-2-fluorocitrate cleavage by this latter enzyme have now provided assignment of absolute stereochemistry to these fluorotricarboxylate isomers.

Materials and Methods

The (+)- and (−)-*erythro*-2-fluorocitrate enantiomers were synthesized and resolved according to procedures in the literature (Dummel & Kun, 1969). The yields obtained in the various synthetic and resolution steps were comparable to those reported. The optical rotations attained were also equivalent to those reported by Dummel & Kun (1969). The individual isomers were crystallized as the tricyclohexylammonium salts and used in this form in the enzyme incubations. Fluoroacetyl-CoA was generated enzymatically as discussed below. The diastereomeric fluoromalate standards (erythro, $2R,3R$ and $2S,3S$; threo, $2R,3S$ and $2S,3R$) were synthesized and separated by methods we reported earlier (Goldstein et al., 1978).

The (+)-deoxyephedrine [(+)-methylamphetamine] hydrochloride salt was purchased from Sigma. To obtain the free base, the hydrochloride was dissolved in water, titrated with NaOH to pH 14, and extracted with ether, the ether solution was dried over sodium sulfate, and the solvent was removed under reduced pressure. The (−)-deoxyephedrine hydrochloride was synthesized from (−)-pseudoephedrine (Sigma) by treating the pseudoephedrine (25 g, 0.151 mol) dissolved in 200 mL of dry $CHCl_3$ with a solution of 37.5 mL of $SOCl_2$ in 90 mL of dry $CHCl_3$. The addition of the $SOCl_2$ solution was carried out at a rate which maintained a gentle reflux while the reaction mixture was cooled in an ice bath. Initially, pseudoephedrine hydrochloride precipitated, but with constant addition and stirring, the salts redissolved, which resulted in a red-brown colored solution. The reaction mixture was then refluxed for about 1 h during which time some precipitate appeared. The solution was cooled and then diluted with dry Et_2O (500 mL), whereupon the HCl salt of β-chloromethylamphetamine precipitated. The light yellow solid was filtered, washed with ether, and recrystallized from CH_3CN, affording a light yellow solid: mp 197–199 °C (28 g, 0.134 mol); 89% yield. A portion of this product (25 g, 0.117 mol), anhydrous sodium acetate (10 g, 0.133 mol), and 10% Pd on charcoal (1.25 g) were added to 150 mL of distilled water. The mixture was hydrogenated under 50 psi of H_2 for 1.5 h. The catalyst was removed by filtration and washed with H_2O. The filtrate was brought to pH 14 with NaOH, extracted with Et_2O (4 × 50 mL), and dried over Na_2SO_4. HCl gas was added to the ether solution which had been cooled to ice-bath temperature; a white solid precipitated. The solid material was filtered and recrystallized from acetonitrile, yielding 20 g of (−)-deoxyephedrine hydrochloride (0.116 mol, 87%).

ATP citrate lyase (CCE) was purified to homogeneity from rat liver as described by Srere and colleagues (Singh et al., 1976), and the assay has also been reported (Cottam & Srere, 1969). Malate synthase (yeast) was the most generous gift of Dr. Ronald Woodard and Professor Heinz Floss and was assayed according to Powell & Beevers (1968). MDH was purchased from Sigma.

$(4R)$-[^3H]NADH was synthesized enzymatically according to Oppenheimer et al. (1971) to a specific activity of 3.7 μCi/μmol. Sodium [^{14}C]glyoxalate was purchased from Amersham (5.5 mCi/mmol). ^3H_2O (1 Ci/mL) was obtained from New England Nuclear. Cyclohexylamine was distilled prior to use, and it and all other chemicals used were of the best quality available. Analytical methods for the separation of the diastereomeric fluoromalates and malate were carried

Table I: Cleavage of the 2-Fluorocitrates by ATP Citrate Lyase[a]

substrate	rel velocity (%)	sp act.[b]
citrate	100	2150
(+)-*erythro*-2-fluorocitrate	2.3	50
(−)-*erythro*-2-fluorocitrate	0.13	2.7

[a] The conditions for the incubation were as follows: Tris-HCl (pH 8.0), 200 μmol; ATP, 5 μmol; CoASH, 0.2 μmol; DTT, 10 μmol; $MgCl_2$, 10 μmol; NADH, 0.2 μmol; MDH, 25 units; substrate, 20 μmol. The reaction was initiated with ATP citrate lyase, 40 mg/mL (1.0 μL in the case of citrate and 12 μL with both fluorocitrates). The reaction volume was 1.0 mL, and the decrease in A_{340} was observed. [b] Specific activity is expressed as nmol min^{-1} mg^{-1}.

out as described earlier (Goldstein et al., 1978), and the incubation conditions for each enzyme experiment are given with the respective table or figure.

Results and Discussion

(+)- and (−)-erythro-2-Fluorocitrates. The synthetic routes described by Kun and colleagues worked well in our hands both for the separation of the diastereomeric erythro from the threo compounds and also for the separation of (+)-*erythro*- from the (−)-*erythro*-2-fluorocitrates by repeated fractional crystallization to equal and opposite optical rotation in accord with literature values (Dummel & Kun, 1969). Subsequent enzymatic experiments required this high degree of purity of (+)- and (−)-erythro forms. (*Caution*: the (−)-erythro isomer, but not the (+)-erythro, is a highly poisonous neurotoxin and should be handled with extreme care.)

Activity of ATP Citrate Lyase with (−)-erythro- and (+)-erythro-2-Fluorocitrate. To test if one of the above 2-fluorocitrate isomers could serve as a source of fluoroacetyl-CoA, of eventual known chirality in ^3H_2O, we determined substrate activity with homogeneous ATP citrate lyase from rat liver which catalyzes citrate cleavage in mammalian cytoplasm to provide acetyl-CoA groups for fatty acid biosynthesis (eq 1). A convenient continuous assay of enzyme
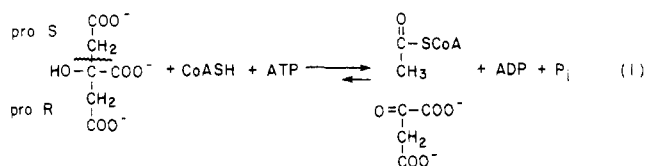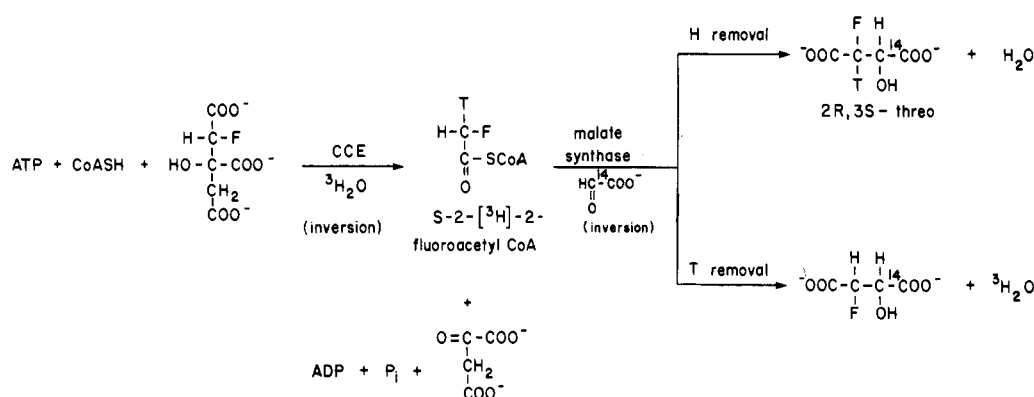

activity is the in situ reductive trapping of α-keto acid with malate dehydrogenase and NADH. We have previously determined that (R)- and (S)-3-fluorooxalacetate is a reducible substrate for MDH to yield $(2R,3R)$-erythro- or $(2R,3S)$-threo-fluoromalates, respectively, so this assay was suitable for monitoring 2-fluorocitrate processing since both possible keto acid products can be detected (Goldstein et al., 1978).

The data of Table I show a comparison of the V_{max} rates for citrate (100%), (+)-erythro (2.3%), and (−)-erythro (0.12%) at 20 mM concentrations of the substrates. Due to the low rates, K_m values have not yet been determined for fluorocitrates, but the K_i values are 0.192 mM and 0.073 mM for (−)-erythro and (+)-erythro forms, respectively. This MDH/NADH reduction assay does not per se distinguish between oxalacetate or 3-fluorooxalacetate (and, correspondingly, 2-fluoroacetyl-CoA or acetyl-CoA) as cleavage products, but it does show that aldol cleavage is taking place. The regospecificity issue is solved below.
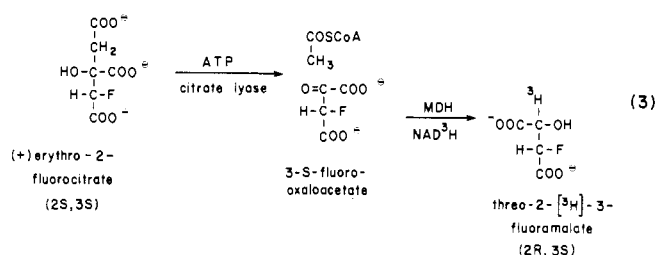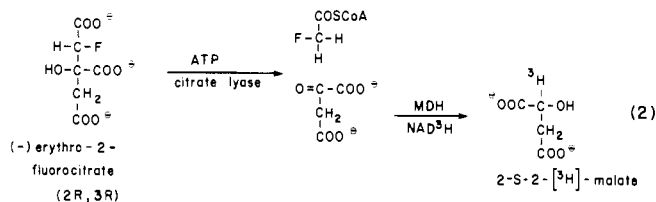
The very slow rate of the (−)-erythro isomer was worrisome, albeit qualitatively in the right direction if it had as its absolute configuration the $2R,3R$ geometry and the cleavage involved

Scheme I

$$\text{ATP + CoASH + } \begin{array}{c} COO^- \\ | \\ H-C-F \\ | \\ HO-C-COO^- \\ | \\ CH_2 \\ | \\ COO^- \end{array} \xrightarrow[^3H_2O \ (inversion)]{CCE} \begin{array}{c} T \\ | \\ H-C-F \\ | \\ C-SCoA \\ \| \\ O \end{array}$$

S-2-[^{3}H]-2-fluoroacetyl CoA

+

$$\begin{array}{c} O=C-COO^- \\ | \\ CH_2 \\ | \\ COO^- \end{array}$$

ADP + P$_i$ +

malate synthase
$$\begin{array}{c} HC-COO^- \\ \| \\ O \end{array}$$
(inversion)

H removal → $\begin{array}{c} F \ H \\ | \ | \\ ^-OOC-C-C-COO^- \\ | \ | \\ T \ OH \end{array}$ + H$_2$O

2R,3S- threo

T removal → $\begin{array}{c} H \ H \\ | \ | \\ ^-OOC-C-C-COO^- \\ | \ | \\ F \ OH \end{array}$ + ^{3}H$_2$O

a carbanion equivalent at the fluorinated carbon. The (+)-erythro form then should be cleaved at the CH$_2$ carbon. The obvious problem was that the (−)-erythro form might actually be inactive and be contaminated with 3% of the (+)-erythro isomer, thus yielding the observed data. Such contamination is not the case. As shown below, the (−)-erythro isomer is indeed cleaved at $^1/_{20}$ the V_{max} rate of (+)-erythro, as determined by product analysis, revealing unique products from each fluorocitrate isomer.

Product Analysis of Keto Acids from ATP Citrate Lyase Action on (−)- and (+)-erythro-2-Fluorocitrate. The regiospecificity for ATP citrate lyase cleavage on prochiral citrate in the physiological reaction is uniquely at the *pro-S* arm, as indicated in eq 1. We anticipated this regiospecificity might persist in 2-fluorocitrate processing. Earlier X-ray analysis of the (±)-erythro racemate suggested a 2R,3R and 2S,3S mixture, and it was guessed but not proven that the (−)-erythro form has the 2R,3R configuration (Carrel et al., 1970). This hypothesis is, in fact, correct according to a recent X-ray analysis carried out by Glusker and co-workers on the (+)-erythro-2-fluorocitrate, performed after this work was completed (Stallings et al., 1980). The first half of eq 2 and 3 shows the anticipated products from expected regiospecific

$$\begin{array}{c} COO^= \\ | \\ H-C-F \\ | \\ HO-C-COO^= \\ | \\ CH_2 \\ | \\ COO^= \end{array} \xrightarrow[\text{citrate lyase}]{ATP} \begin{array}{c} COSCoA \\ | \\ F-C-H \\ | \\ H \end{array} + \begin{array}{c} O=C-COO^= \\ | \\ CH_2 \\ | \\ COO^\circleddash \end{array} \xrightarrow[NAD^3H]{MDH} \begin{array}{c} ^3H \\ | \\ ^-OOC-C-OH \\ | \\ CH_2 \\ | \\ COO^\circleddash \end{array} \quad (2)$$

(−) erythro - 2 - fluorocitrate (2R,3R)

2-S-2-[^{3}H]-malate

$$\begin{array}{c} COO^\circleddash \\ | \\ CH_2 \\ | \\ HO-C-COO^\circleddash \\ | \\ H-C-F \\ | \\ COO^- \end{array} \xrightarrow[\text{citrate lyase}]{ATP} \begin{array}{c} COSCoA \\ | \\ CH_3 \end{array} + \begin{array}{c} O=C-COO^\circleddash \\ | \\ H-C-F \\ | \\ COO^- \end{array} \xrightarrow[NAD^3H]{MDH} \begin{array}{c} ^3H \\ | \\ ^-OOC-C-OH \\ | \\ H-C-F \\ | \\ COO^\circleddash \end{array} \quad (3)$$

(+) erythro - 2 - fluorocitrate (2S,3S)

3-S-fluorooxaloacetate

threo-2-[^{3}H]-3-fluoromalate (2R,3S)

cleavage of (−)-(2R,3R)-erythro- and (+)-(2S,3S)-erythro-2-fluorocitrate by ATP citrate lyase. Oxalacetate should be produced from (−)-erythro- and (3S)-fluorooxalacetate from (+)-erythro-fluorocitrate.

In situ reductive trapping with malate dehydrogenase and (4R)-[^{3}H]NADH provided three useful features: (a) it prevented racemization and loss of chiral integrity at C$_3$ of the α-keto acids; (b) it introduced a tritium label only into the C$_4$-hydroxydicarboxylates to be analyzed; (c) by chiral introduction of the second asymmetric center, the C$_2$-OH as 2R,[3]

the MDH reduction allowed physical separation of the diastereomeric products chiral at C$_3$ by virtue of fluorine substitution (eq 2 and 3). We have previously reported the column separation of (2R,3R)-erythro- and (2R,3S)-threo-3-fluoromalates (Goldstein et al., 1978).

The data of Figure 1 corroborate the above expectations fully and suggest several points. Since (−)-erythro-2-fluorocitrate yields only [2-^{3}H]malate and (+)-erythro-2-fluorocitrate only threo-fluoro[2-^{3}H]malate (2R,3S), the isomers are pure, and each fluorocitrate is indeed a substrate for ATP citrate lyase. The result also confirms the anticipated regiospecificity of cleavage, suggesting fluorine substitution causes no gross alterations in enzyme–substrate orientation.

The (+)-erythro-2-fluorocitrate enzymatic cleavage data are particularly revealing. Since a (3S)-fluorooxalacetate is the initial product, the CHF carbon is not the carbon site involved in C–C cleavage, and its stereochemical integrity is unaltered and must be the same in the starting fluorocitrate, i.e., 2S. This 2S configuration fixes the absolute stereochemistry of (+)-erythro-2-fluorocitrate since the (±)-erythro pair is a (2S,3S),(2R,3S) mixture. The (+)-erythro must be (2S,3S)- and the (−)-erythro (2R,3R)-2-fluorocitrate by these results, a fact now independently verified by Glusker's recent X-ray result (Stallings et al., 1980). Assigning the 2R,3R configuration to (−)-erythro-2-fluorocitrate and assuming the normal inversion result seen with citrate synthase and its physiological substrates (Eggerer et al., 1970), one can then say that the synthase is acting chirally to remove only the *pro-S* hydrogen from C$_2$ of fluoroacetyl-CoA during condensation in the "lethal synthesis" reaction. This stereochemical outcome was suggested by Klinman & Rose (1971).

The absolute stereochemical assignment and the enzymatic cleavage product data allow one to suggest that the 20-fold higher V_{max} rate of (+)-erythro-2-fluorocitrate with ATP citrate lyase may reflect fluorine-linked electronic effects at the reacting CHF site in the (−)-erythro isomer. It is not yet known what step is rate determining in catalysis with the (−)- or (+)-erythro-2-fluorocitrates by ATP citrate lyase.

Lastly, these results should permit use of ATP citrate lyase as a reagent for generation of tritiated fluoroacetyl-CoA of known absolute chirality. For example, cleavage of (2R,3R)-2-fluorocitrate in ^{3}H$_2$O should yield, if it proceeds with normal inversion stereochemistry (Eggerer et al., 1970), (S)-2-fluoro[2-^{3}H]acetyl-CoA.

Worthy of mention are the data obtained on the inhibition and substrate reactivity of the 2-hydroxycitrates with ATP

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[3] L-Malate is (2S)-malate, but L-fluoromalate diastereomers are 2R chiral species. The S center becomes R because of the fluorine substituent at C-3 which reverses the usual atomic number priority of α-hydroxy acids.
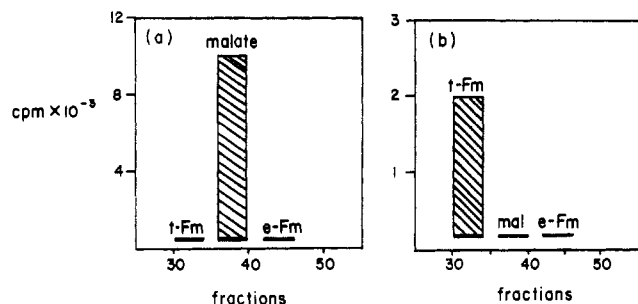
FIGURE 1: (a) Histogram of chromatographic results from the fluorocitrate incubations with ATP citrate lyase. The α-ketodicarboxylic acids produced were trapped with MDH and (4R)-[³H]NADH. (a) In 1 mL, (-)-*erythro*-2-fluorocitrate, 20 μmol; Tris-HCl buffer (pH 7.5), 200 μmol; ATP, 5 μmol; CoA, 0.2 μmol; MgCl₂, 10 μmol; (4R)-[³H]NADH, 0.2 μmol; MDH, 25 units. The reaction was initiated with 25 μL of ATP citrate lyase (40 mg/mL) and allowed to proceed for 1.5 h at 23 °C. Removal of protein and NADH was accomplished as reported earlier (Goldstein et al., 1978). The added standards *threo*-fluoromalate, malate, and *erythro*-fluoromalate, are *t*-Fm, malate, and *e*-Fm, respectively; relative chromatographic elution profile is indicated on the figure. (b) (+)-*erythro*-2-Fluorocitrate, 20 μmol; incubation time 50 min; all other conditions the same as in (a).

citrate lyase (Sullivan et al., 1977). They investigated all four isomers (i.e., (+)- and (-)-erythro and (+)- and (-)-threo); however, the ones corresponding to the *erythro*-fluorocitrates, (-)-allohydroxycitrate (2R,3S), and (+)-allohydroxycitrate (2S,3R) are related to the (-)- and (+)-*erythro*-fluorocitrates and therefore can be directly compared.

The (-)-allohydroxycitrate K_I is 0.003 mM whereas the (+)-allohydroxy isomer is 0.013 mM. The opposite trend is seen with the fluorocitrates with the (+) isomer having a 2-fold lower K_I than the (-). As substrates, the hydroxycitrates again

(-) allohydroxycitrate   (+)allohydroxycitrate
     (2R,3S)   (2S,3R)
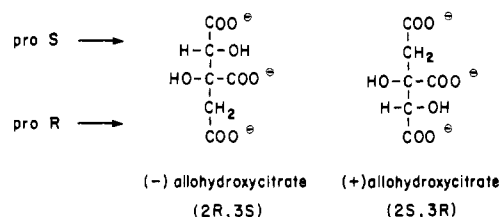
show an opposite trend when compared to the fluorocitrates, with the (-)-hydroxy isomer being about 14 times more reactive than the (+) isomer. Whether the differences between hydroxyl and fluorine analogues of citrate reflect steric and/or electronic distinctions and whether rate-determining steps are changed remain to be determined. Also, unambiguous product identification from hydroxycitrate isomer processing has not yet been reported.

Action of Malate Synthase of Fluoroacetyl-CoA. As a preliminary test of the projected chiral outcome from inversion at C₂ with the (-)-*erythro*-fluorocitrate and ATP citrate lyase, we coupled this reaction run to ³H₂O to yeast malate synthase to trap the fluoro[³H]acetyl-CoA with glyoxalate and make a 3-fluoromalate that should be either erythro (2R,3R) or threo (2R,3S) (Scheme I). If malate synthase were to remove only one of the C₂-hydrogen species (¹H or ³H) from fluoroacetyl-CoA chirally, a single fluoromalate product diastereomer should result (either tritiated or not). If both prochiral hydrogens were removed achirally, then both erythro- and threo-fluoromalate diastereomers should form, but again only one should retain the tritium label. For detection of the presence of both isomers, [¹⁴C]glyoxalate was used in the malate synthase incubation. The HPLC system used separates malate and both fluoromalate diastereomers (Goldstein et al., 1978).
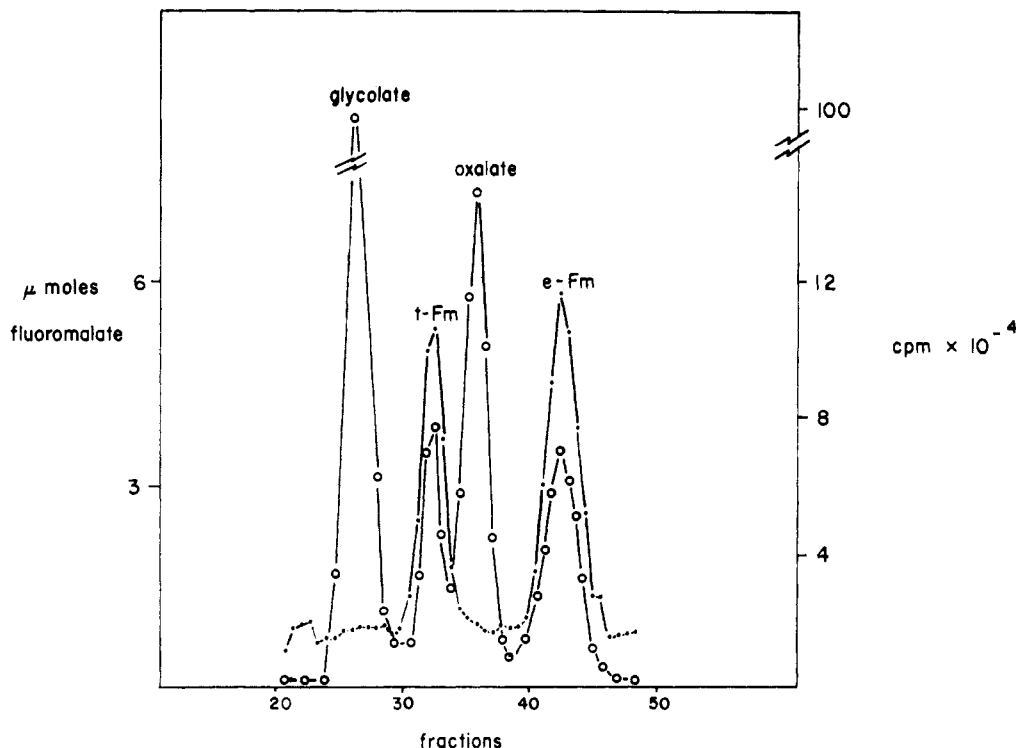

FIGURE 2: Chromatogram of the malate synthase reaction with [¹⁴C]glyoxalate and fluoroacetyl-CoA. The fluoroacetyl-CoA was generated in situ from (-)-*erythro*-2-fluorocitrate by ATP citrate lyase. The incubations conditions were the following: in 1 mL, (-)-*erythro*-2-fluorocitrate, 20 μmol; Tris-HCl (pH 7.5), 100 μmol; ATP, 5 μmol; MgCl₂, 10 μmol; CoA, 0.2 μmol; ATP citrate lyase (40 mg/mL), 100 μL; malate synthase, 50 μL (125 nmol of malate/min). The reaction was initiated with [¹⁴C]glyoxalate (1 μmol), allowed to proceed for 2 h at 23 °C, and quenched with the addition of NADH (1 μmol) and LDH (5 units). Protein and NADH were removed as previously reported (Goldstein et al., 1978). Solid circles indicate authentic *erythro*-3-fluoromalate (*e*-Fm) and *threo*-3-fluoromalate (*t*-Fm) standards added to the sample prior to the chromatographic analysis. The open circles indicate ¹⁴C radioactivity. The [¹⁴C]oxalate peak is a contaminant of the commercial [¹⁴C]glyoxalate. Later experiments utilized purified [¹⁴C]glyoxalate (Warren, 1970).

The results of such a coupled two-enzyme experiment unexpectedly showed that both fluoromalate product diastereomers were formed and both were tritiated. The $^3H/^{14}C$ ratios for the *erythro*- and *threo*-fluoromalates were 0.76 and 0.94, respectively. Conditions for the experiments were the same as stated with Figure 2 except that ^3H_2O (0.5 Ci) was substituted for unlabeled H_2O. For proof that malate synthase does indeed yield both fluoromalate products, the experiment was repeated with [^{14}C]glyoxalate but no ^3H_2O so that enzymatic fluorocitrate cleavage yielded fluoro[^1H]acetyl-CoA. Again both fluoromalates formed in about equal amounts, and this is shown in Figure 2. The finding of tritium in both fluoromalate diastereomers appears to require racemization of the isotope in either the ATP citrate lyase or malate synthase reactions. This step may be distinct from the achiral processing of fluoroacetyl-CoA by malate synthase.

The fluoromalate product distribution represents achiral recognition by malate synthase of one of its two substrates, either C_2 of fluoroacetyl-CoA or the aldehyde carbon of glyoxalate. Addition of chiral 2-fluoroacetyl-CoA carbanion to either *re* or *si* face of bound glyoxalate could give, for example, (2S,3S)- and (2R,3S)-3-fluoromalates. The recent results of Retey and colleagues (Keck et al., 1980) confirm that the fluoromalate diastereomers result from achiral recognition of fluoroacetyl-CoA. Only *si* attack at the carbonyl carbon is observed. In their experiments, which were analyzed by NMR, they found a threo to erythro ratio of 4:3. When the same experiment was carried out with (2R)-fluoro[2-^2H]acetyl-CoA, the ratio shifted to 3:7, allowing the calculation of k_H/k_D of ~3. Our results with the coupled enzyme generation of tritiated fluoroacetyl-CoA do not show this increase in *erythro*-fluoromalate production; however, as already discussed, the fluoroacetyl-CoA in our reaction apparently was racemic. Even though malate synthase does not catalyze random addition to the glyoxalate carbonyl face, it is worth pointing out the recent bizarre precedent that citrate synthase acting on propionyl-CoA and oxalacetate is chiral in 2S H abstraction from propionyl-CoA but does indeed catalyze addition to both *re* and *si* faces of oxalacetate (Brandange et al., 1977). We are currently investigating where the apparent racemization of the fluoroacetyl-CoA occurred. Until further details of the processing of fluoroacetyl-CoA are known, the only safe enzyme for the analysis of the absolute chirality of a chiral fluoroacetyl-CoA is, in fact, citrate synthase with its 2S specificity.

Acknowledgments

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