Fluorinated Substrate Analogues as Stereochemical Probes of Enzymatic Reaction Mechanisms†

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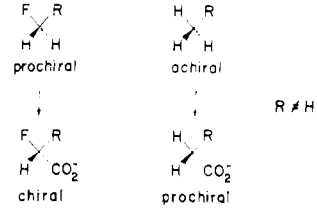
ABSTRACT: This paper explores the utility of the fluoromethyl group ($-CH_2F$) as a probe of enzyme stereospecificity in transformations converting substrate methyl groups to product methylene groups. Two reactions have been examined in detail: the enolization of fluoropyruvate by pyruvate kinase (EC 2.7.1.40) assayed by NMR measurements in D_2O and the carboxylation of fluoropyruvate to 3-fluorooxalacetate catalyzed by the biotin-dependent enzyme transcarboxylase (EC 2.1.3.1). In the presence of inorganic phosphate, pyruvate kinase mediates enolization and hydrogen exchange at carbon 3 of fluoropyruvate some 20-fold slower than the corresponding V_{max} rate with pyruvate. Both the two prochiral hydrogens of fluoropyruvate and the three equivalent hydrogens of pyruvate are enolized at equivalent rates, indicating that the fluoromethyl group is processed torsiosymmetrically in this reaction; that is, the enzyme does not act chirally on the two chemically distinguishable hydrogens. In contrast, transcarboxylase converts fluoropyruvate to only one (>99%) isomer of 3-fluorooxalacetate, identified as the 3*R* isomer by in situ reduction with NADH and malate dehydrogenase to *erythro*-L-fluoromalate (2*R*,3*R*) and by comparison with authentic threo and erythro standards. In a control experiment it was determined that both (3*R*)- and (3*S*)-3-fluorooxalacetate are reduced quantitatively by high concentrations of malate dehydrogenase to the (2*R*,3*R*)-*erythro*- and (2*R*,3*S*)-*threo*-fluoromalate diastereomers, respectively. Thus the biotin-dependent transcarboxylase must remove one of the two prochiral hydrogens of the fluoromethyl group specifically in generation of the attacking nucleophilic enolate anion at the active site.

The use of fluorinated substrate analogues and inhibitors in the investigation of biological reactions has received considerable attention in the last few years (CIBA Foundation Symposium, 1972; Filler, 1976; Schlosser, 1977). The small size fluorine and its short internuclear distance to carbon in a carbon–fluorine covalent bond have made it a favorite element for pharmacologists and enzymologists alike; fluorine has been introduced into many biologically active molecules, from monosaccharides to polypeptides. Most of these studies have centered on the ability of the analogues to serve as metabolic inhibitors, to substitute for substrates in enzymatic reactions or as physical probes of structure and conformation of proteins.

Much effort has been also devoted to certain stereochemical aspects of fluorinated compounds in biological reactions. Kun has been a pioneer in this field; his classical investigations with fluorine as a hydrogen analogue in fluorinated mono-, di-, and tricarboxylic acids included the elegant proof that (−)-*erythro*-fluorocitrate, and not its (+) enantiomer or threo diastereomers, is the active metabolic poison in fluoroacetate toxicity (Kun, 1976). Other workers, such as Briley & Barnett have used fluorine as an oxygen analogue in carbohydrate derivatives and have obtained interesting information concerning the stereochemical and geometric requirements of binding sites and reaction mechanisms involving carbohydrates (Barnett, 1972; Briley et al., 1977).

There is an area of fluorine stereochemistry, however, that has received relatively little attention: the use of fluoromethyl and fluoromethylene groups as prochiral and chiral probes in enzymatic reactions which normally involve methyl or methylene groups. The stereochemical pathways for such reactions as carboxylations, decarboxylations, condensations, or enolizations have been of long standing interest to biochemists. The now classical methodology to examine the stereochemistry of these reactions has been pioneered by Cornforth et al. (1969) and Arigoni (1969) and then by Rose (1970); their methods depend on the use of isotopically chiral methyl or methylene groups containing ^1H, ^2H, and/or ^3H (Cornforth, 1969; Arigoni, 1969). The potential advantage of using a fluoromethyl group instead of a methyl group arises from the fact that a transformation (such as, for example, a carboxylation) of a fluoromethyl group involves a change from prochiral to chiral configuration; the equivalent change with a methyl group is from achiral to prochiral:



Determination of stereochemistry in a chiral product is generally much easier than in a prochiral product. For example, with an isotopically chiral [^1H,^2H,^3H]pyruvate which undergoes stereospecific carboxylation, the selective enrichment of tritium in the 3*R* or 3*S* positions of the product oxalacetate is determined only by enzymic reduction to malate and subsequent treatment with fumarase exhaustively to remove the 3*R* hydrogen of malate. (The degree of enrichment depends on the intramolecular isotope effect in the carboxylase catalysis.) But, with fluoropyruvate, stereospecific carboxylation would yield a 3-fluorooxalacetate which on enzymic reduction would yield a 3-fluoromalate diastereomer (now two

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asymmetric centers) which can be separated physically from its diastereomer with opposite chirality at carbon 3.

This paper presents our initial studies on the feasibility of using fluoromethyl groups as probes of enzyme stereospecificity. We have examined enzyme-catalyzed enolization of fluoropyruvate by pyruvate kinase as well as enolization and carboxylation of fluoropyruvate by transcarboxylase. We have also briefly examined the specificity of malate dehydrogenase for reduction of *R,S*-3-fluorooxalacetate and its susceptibility to transamination by L-aspartate transaminase.

Experimental Section

UV-visible spectrophotometry was carried out on a Gilford 220 with a Beckman recorder; NMR[1] measurements were taken on a Varian T-60 spectrometer; pH was measured on a Beckman 3500 Digital pH meter. Liquid scintillation spectrophotometry was carried out on a Beckman LS-100 spectrometer; samples of up to 1 mL were dissolved in 10 mL of aqueous counting scintillant (ACS, from Amersham/Searle); a relative quench correction was applied to the measurements when necessary; this correction was obtained from a calibration curve constructed by the method of external standard-channels ratio. Fluoride ion analyses were carried out with a fluoride electrode from Orion (no. 94-06); the solutions for these measurements were prepared according to instructions provided by the manufacturer. β-Fluoropyruvic acid, sodium salt, was from Sigma (grade II); stoichiometric calculations using this material were carried out using a molecular weight of 164 (dihydrate). Sodium pyruvate, malic and lactic dehydrogenases, and rabbit muscle pyruvate kinase (type III) were also Sigma products. Silicic acid (100 mesh, powder) was from Mallinckrodt. All other commercial materials, such as buffers, enzymes, and coenzymes, were of the best available purity. Propionyl-CoA carboxylase from pig heart was prepared as in Cheung et al. (1975); transcarboxylase from *Propionibacterium shermanii* was a gift of Professor H. G. Wood.

Fluorooxalacetate Acid (FOAA). Diethyl fluorooxalacetic acid was prepared according to Blank et al. (1955). A variety of methods was attempted to prepare the free acid; it is worth noting that we were unable to reproduce the deprotection of diethyl fluorooxalacetate by trifluoroacetic acid (Dummel, 1971); no free acid could be detected by NMR under conditions indicated by these authors. The method that proved successful consisted of dissolving the oily diester in a 2:1 mixture of acetic and hydrochloric acids and allowing the solution to stand for 3 days (Kun et al., 1958). Work-up and crystallization were according to Dummel et al. (1971): all operations were performed in the absence of heat to prevent the decarboxylation of the acid. In spite of this precaution, our material appeared to contain about 10% fluoropyruvic acid (see Results). After crystallization (Dummel et al., 1971), the product melted at 82–85 °C (decomposition) (lit., Dummel et al., 1971: 86–87 °C); UV 265 nm (400 M^{-1} cm^{-1}) in 1 M NaOH (lit., Dummel et al., 1971: 270 nm (200 M^{-1} cm^{-1})); $FeCl_3$ test, positive; ^1H NMR in D_2O, δ 5.25 ppm (d, J = 46 Hz); upon raising the pH to ~13 with NaOD, the doublet disappeared rapidly. We determined the maximal velocity of MDH-catalyzed reduction of FOAA by NADH (50 mM potassium phosphate, pH 7.5); the rate is about 14 μmol min^{-1} mg^{-1}. V_{max} for reduction of oxalacetate under identical conditions is 250 times higher (lit. (Kun, 1976); V_{max} OAA/V_{max} FOAA ~100).

(R,S)-[β-^3H]-β-Fluoropyruvic Acid, Sodium Salt. Fluoropyruvic acid, sodium salt (384 mg, 2.35 mmol), was dissolved in 2.8 mL of water in a constricted tube and 0.2 mL of ^3H_2O (1 Ci/mL) was added to the solution; the tube was sealed and heated to 110 °C for 60 min. It was then opened and the solution was frozen and lyophilized overnight; the solid residue was dissolved in 10 mL of water at 0 °C for 2 min, refrozen, and lyophilized. This aqueous reequilibration step is necessary since the water of crystallization (2 mol of water per mol of salt, see below) is of higher specific activity than the acid itself and in fact contributes to 75% of the available counts before reequilibration. After reequilibration the solid was dried for 2 days at 100 °C over Drierite under vacuum; the specific radioactivity at this point was 50 μCi/mmol. The [β-^{3}H]fluoropyruvate thus obtained was quantitatively assayed by reducing it to L-fluorolactate with LDH; 73–78% of the weighed sample was NADH reducible. The same assay for commercial fluoropyruvate yielded a value of 78%. These values suggest that fluoropyruvate is a stoichiometric dihydrate and that the exchange with radioactive water occurred with minimal decomposition. The same conclusion can be reached by assaying the radioactivity of our synthetic β-fluoropyruvate; passing a freshly prepared aqueous solution of this material through a Dowex 1 column (chloride) and washing thoroughly with water, yielded about 20–30% of the counts in the water wash. This "instantaneous" detritiation was observed in all kinetic experiments carried out with [^{3}H]fluoropyruvate. Assuming that the specific radioactivities of the water of crystallization and fluoropyruvate are the same, then a value of 20–30% of the total counts in the H_2O wash is consistent with the dihydrate formulation.

erythro- and threo-Fluoromalates. Samples of authentic D,L-*erythro*- and D,L-*threo*-3-fluoromalates were prepared by borohydride reduction essentially according to the procedure of Krasna (1961) starting from (*R,S*)-3-fluorooxalacetate (10 g). The synthetic mixture of both diastereomers (1.0 g) was separated by silicic acid column chromatography with an elution profile essentially identical with that noted by Krasna (1961), the threo form eluting before the erythro form. On evaporation of the titrated fluoromalate peaks to dryness, semicrystalline material was obtained (150 mg of erythro; 50 mg of threo). Each diastereomer gave a molecular ion of 152 (weak) and intense peak of 149 (− 3-H) and at 129 (− HF) on mass spectroscopic analysis. The 60-MHz ^1H NMR in D_2O showed for the threo diastereomer a doublet (0.5 H, J = 2 Hz) at δ 4.6, an apparent triplet (1 H, J = 2 Hz) at δ 5.15, and a doublet (0.5 H, J = 2 Hz) at δ 5.9. The erythro diastereomer showed a doublet (0.5 H, J = 3 Hz) at δ 4.7, a doublet (1 H, J = 3 Hz) at 5.15, and a doublet (0.5 H, J = 3 Hz) at δ 5.9. Analytical separation of the *erythro*- and *threo*-3-fluoromalate diastereomers was achieved by using two stainless steel HPLC columns (Waters Associates Inc.) attached head to tail (7 mm × 122 cm). The columns were packed dry with silicic acid which was pretreated with 0.5 N H_2SO_4 according to Krasna (1961) and eluted according to his procedure. The flow rate of 1.0 mL/min was maintained by a Waters HPLC pump Model 6000A.

Deuterated Buffers. Buffers for NMR measurements were prepared in D_2O (Merck, >99%) and titrated to the required pH with concentrated HCl or KOH; the pD of the solution was calculated using the equation pD = pH + 0.4. The solution was then evaporated under reduced pressure at 40 °C and redissolved in D_2O, and the whole cycle was repeated three times. Addition of the final required volume of D_2O was done in a volumetric flask and gave solutions of the appropriate concentration and pD and which contained acceptable spectro-

[1] Abbreviations used: NMR, nuclear magnetic resonance; MDH, malate dehydrogenase; NADH, nicotinamide adenine dinucleotide (reduced form); LDH, lactate dehydrogenase; Me_4Si, tetramethylsilane.

photometric signals of HDO in the NMR spectrum (δ 4.8 ppm).

NMR Measurements. As described above, NMR measurements were carried out on a Varian T-60, equilibrated in a steadily air conditioned room at 25 °C. (The temperature in the NMR probe was observed to be 34 °C.) The results did not vary whether the tube was kept in the probe throughout the experiment or whether the tube was removed and repositioned periodically. Rate measurements were typically carried out as follows: a thin-walled NMR tube was charged with the required amount of solid pyruvate derivative (usually 10–70 mg) and solid $MgCl_2$; the solids were rapidly dissolved by adding 0.5 mL of deuterated buffer and shaking. At time zero, 100 μL of an enzyme solution of the required concentration in deuterated buffer was added with a syringe and the tube was rapidly, but gently, mixed by inversion. The rate of disappearance of a peak was followed by carrying out its integration periodically and plotting the values of $(A_t - A_\infty)/(A_0 - A_\infty)$ on semilogarithmic paper vs. time; A_t = integral at time t; A_∞ = integral at infinite time (usually more than 20 half-lives); A_0 = integral at time zero. Linear plots were obtained at all times (except when indicated, see Results) and reproducibility was within 10%. All crucial measurements were carried out in duplicate and most points were collected to more than 2–3 half-lives. The rate of exchange was expressed as micromoles of keto acid processed per minute per milligram of enzyme. Observed enzymatic rates were corrected by the appropriate concentration-dependent, nonenzymic rate.

Detritiation Experiments. The rate of loss of tritium from (*R,S*)-[β-^{3}H]-β-fluoropyruvate was typically measured as follows: to a test tube were added solid fluoropyruvate (nonradioactive, 61 mg; radioactive, 5 mg), $MgCl_2$ (4 mg), and 0.5 mL of deuterated buffer. At time zero, 100 μL of an enzyme solution of the required concentration was added and the tube was shaken gently. Fifty-μL aliquots were removed periodically, diluted into 1 mL of water, and loaded on a Dowex 1 column (chloride, 0.5 × 2 cm). The resin bed was washed with 4 mL of water; each milliliter was dissolved in 10 mL of ACS cocktail, counted, and quenched corrected. The sum of the five fractions was taken as the water wash.

Optical Quantitation. (*R,S*)-3-Fluorooxalacetate and fluoropyruvate were typically determined by incubating 2–3 optical density units of 340 nm of NADH (0.32 or 0.48 mM) and the unknown amount of keto acid in 3 mL of 200 mM sodium pyrophosphate buffer, pH 7.5; the decrease at 340 nm was monitored upon addition of MDH or LDH to the solution. The amount of keto acid was then calculated using the extinction coefficient of NADH, 6.22×10^3 at 340 nm. Oxalacetate was determined by means of its borate complex, according to Kun et al. (1960).

Enzymatic Preparation of L-Fluoromalate (2*R*). (A) [^{14}C]Fluoromalate from Fluoropyruvate. The incubation contained (in micromoles) Tris buffer (40, pH 8.0, KCl (4), ATP (2, pH 7), Na[^{14}C]HCO_3 (10, 2 μCi/μmol), $MgCl_2$ (1), propionyl-CoA (0.2), 50 units of MDH, 0.4 mg of transcarboxylase (specific activity = 26 U/mg), 0.16 mg of propionyl-CoA carboxylase (specific activity = 0.74 U/mg), fluoropyruvate (2), $(NH_4)_2SO_4$ (1), and NADH (0.2) in a total volume of 0.55 mL. The reaction was run at 30 °C and followed by the decrease of A_{340} on a spectrophotometer; NADH was added accordingly. After 1 h, about 0.5 μmol of L-fluoromalate had formed. The reaction was stopped by the addition of 0.1 mL of 2 M HCl and air was bubbled through the solution to remove excess $^{14}CO_2$. The solution was diluted fivefold and titrated to pH 7, then passed through a Dowex 1 column (chloride, 0.5 × 5 cm). After washing with 10 mL of water, the column was developed with 50 mM HCl; the radioactive fractions were collected and lyophilized to dryness and the residue was taken up in 30% ethanol. About 1.2×10^6 cpm of ^{14}C-containing material was obtained. The chirality of this [^{14}C]fluoromalate sample was determined by silicic acid column chromatography as noted in the Results section.

In order to show that the counts were in fluoromalate derivative, a sequence of reactions was carried out destined to convert the radioactive material to an aspartate derivative. The incubation contained (in μmol): Tris (20, pH 8), NAD (0.5), L-glutamate (1), aspartate aminotransferase (10 units), MDH (50 units), and 3300 cpm (about 1 nmol) of presumed fluoromalate. The solution was incubated for 1.5 h. A control experiment was performed without MDH. The solutions were then loaded onto a Dowex 50 column (H^+ form, 0.5 × 2 cm), washed with 2 mL of water, and developed with 3 mL of 2 M NH_4OH. In the control experiment all radioactivity came out in the aqueous wash, while in the full experiment (with MDH) 85% of the radioactivity was retained in the column and subsequently only eluted with 2 M NH_4OH. This suggests that fluoromalate was oxidized to fluorooxaloacetate and subsequently transaminated to yield a radioactive aspartate derivative (this material is in fact aspartate and not fluoroaspartate, see Results).

(B) L-2-[^{3}H]Fluoromalate from Fluorooxalacetate. To a vial was added (4*R*)-[^{3}H]NADH (3.4 μCi/μmol), 2 μmol (determined by A_{340}), in 0.5 mL of a 0.1 M ammonium bicarbonate buffer, pH 7.5. Fluorooxalacetate (10 mg) was dissolved in the same buffer and 0.1 mL (1 μmol) was added to the vial. MDH (50 μL, ~600 units) was then added to initiate the reaction. After 3 min the reaction solution was passed through a column of charcoal/Celite (50:50) (2 × 0.5 cm) and lyophilized. The diastereomeric content was then determined by silicic acid column chromatography as noted above.

Results

The Enolization of β-Fluoropyruvic Acid. Hydrate Composition. The NMR spectrum of fluoropyruvate, freshly dissolved in 0.5 M potassium phosphate buffer, pD 8.5, shows two doublets (J = 48 Hz) centered at δ 4.53 ppm and 5.60 ppm downfield from external Me_4Si, in a ratio of 5.5 to 1, respectively. We have assigned, in agreement with Kokesh, the upfield doublet to the hemiketal form (hydrate) and the downfield doublet to the keto form (Kokesh, 1976); our conclusion is that the keto form exists in solution only to the extent of about 15%. We cannot detect any signals due to a possible enol tautomer; this species must then exist as less than 5% under these conditions. The equilibrium constant for the reaction hydrate $\rightleftharpoons$ keto can be calculated to be K_{eq} = 0.18 at 25 °C.

Nonenzymic Rate. When a solution of fluoropyruvate and $MgCl_2$ in the same buffer was monitored periodically in the NMR spectrometer at 25 °C, it was possible to measure the rate of disappearance of both doublets. Since the ratio of the signals remains constant through the experiment,[2] it was found convenient to simply follow the disappearance of the upfield branch of the upfield doublet (sharp signal at 4.13 ppm) instead of scanning the whole spectrum each time. This technique proved useful when measuring faster, enzyme-catalyzed rates. Figure 1A shows the decrease of the signal at δ 4.13 ppm with no pyruvate kinase present in the tube. Table I gives the kinetic data obtained under various conditions of fluoropyruvate and $MgCl_2$ concentration. The measurements show that, in the absence of enzyme, there is a slight catalytic effect by Mg^{2+}

[2] This observation indicates that keto–hydrate interconversion is fast compared with hydrogen exchange.

TABLE I: Rate Measurements on the Enolization of Various Ketoacids.[d]

buffer	[substrate] (M)	[$MgCl_2$] (mM)	pyr kinase (mg/mL)	$k^{obsd} \times 10^3$ (min^{-1})	v_H (μmol/(min mg))	comments
			I. Fluoropyruvate			
A	0.244	0	0	2.40		
A	0.183	40	0	2.85		
A	0.671	40	0	3.53		
A	0.299	0	17[a]	2.09		
A	0.254	123	14.2[b]	18.8[c]	0.285	curved
A	0.122	40	12.0	21.6[c]	0.193	curved
A	0.122	40	25.0	36.1[c]	0.163	curved
A	0.183	40	24.0	31.5[c]	0.219	curved
A	0.254	40	8.3	12.0[c]	0.279	curved
A	0.254	40	14.2	16.4[c]	0.243	curved
A	0.254	40	14.2	18.8[c]	0.286	curved
A	0.289	40	25.0	25.6	0.262	linear
A	0.671	40	8.33	8.77	0.423	linear
A	0.671	40	25.0	21.6	0.486	linear
A	1.000	40	25.0	17.3	0.530	linear
A	0.671 β-[^{3}H]	40	0.0	0.36		by detritiation
A	0.671 β-[^{3}H]	40	25.0	2.16	0.048	by detritiation
B	0.671	40	0.0	9.36		
B	0.671	40	28.3	16.5	0.169	linear
			II. Pyruvate			
A	0.272	40	0.0	5.68		
A	0.860	40	0.0	5.13		
A	0.106	40	2.9	158.0	5.57	linear
A	0.136	40	5.0	173.0	4.56	linear
A	0.202	40	0.12	8.88	5.94	linear
A	0.259	40	0.08	7.45	6.95	linear
A	0.515	40	2.5–3.0	41.5	6.2–7.4	linear
A	0.860	40	5.0	24.8	3.33	linear
A	1.000	40	5.83	15.2	1.66	linear

[a] Contains EDTA, 2.7 mM. [b] Contains EDTA, 2.25 mM. [c] Rate for the initial phase of the reaction. [d] All rates were determined by NMR unless indicated. Buffer A: 0.5 M potassium phosphate, 40 mM $MgCl_2$, pD 8.5. Buffer B: 0.5 M Tris-DCl, 0.03 M KCl, 30 mM ATP, 40 mM $MgCl_2$, pD 8.50.

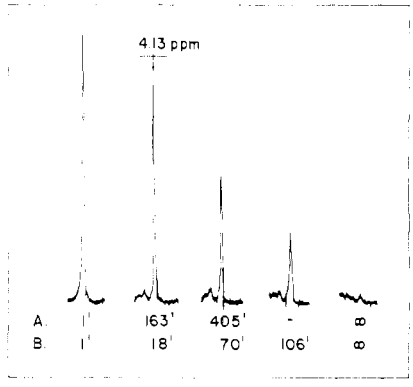


FIGURE 1: Time dependence (in minutes) of the signal at 4.13 ppm in the NMR spectrum of fluoropyruvate in the presence and absence of pyruvate kinase. Conditions: 671 mM fluoropyruvate, 40 mM $MgCl_2$, 0.5 M potassium phosphate, pD 8.50. (A) No enzyme; (B) 25 mg/mL pyruvate kinase.

ions and a slight dependence on the concentration of fluoropyruvate: increasing the concentration of fluoropyruvate by a factor of 3.66 increases the rate of enolization by a factor of 1.23. This effect could be due to general base catalysis of proton removal. Assuming that hydrogen exchange occurs solely through the keto form, one can, using the equilibrium constant of 0.18 calculated above, calculate a "true" rate of enolization, k^F ($k^F = 6.7k^{obsd}$). At 671 mM fluoropyruvate, k^F is 2.36×10^{-2} min^{-1} and this rate of exchange represents both hydrogens at C_3.

Enzymic Rate. Figure 1B shows how the disappearance of the signal at 4.13 ppm was accelerated when the NMR tube contained, in addition to the components described above, 25 mg/mL of pyruvate kinase. It is clear from this figure that the enzyme catalyzed the exchange reaction; further experiments confirmed this observation and defined the process as an active site process; the data are presented in Figure 2A and Table I. A few important features can be pointed out:

1. Rose has shown that a variety of phosphate analogues can replace ATP in promoting enolization of pyruvate by pyruvate kinase; inorganic phosphate was one of the most successful of these analogues (Rose, 1960); he also showed that potassium ions greatly stimulated the enolization activity. Our results with fluoropyruvate are in agreement with his observations. By using a potassium phosphate buffer, complemented with $MgCl_2$ we have provided the essential cofactors necessary for the reaction. The results cannot be explained by assuming that exchange of hydrogens occurs via the formation and dephosphorylation of fluoro-PEP; while inorganic phosphate will promote enolization, it will not function as a cofactor in the phosphotransferase activity. (ATP could also serve as a cofactor in this enolization reaction. We cannot, in this case, however, exclude the possibility that β-hydrogen exchange occurred via sequential formation and dephosphorylation of fluorophosphoenolpyruvate.)

2. It has also been established by Rose that magnesium cations are essential for pyruvate kinase in both the transferase and enolization activities (Rose, 1960). Figure 2A shows that a concentration of 17 mg/mL of enzyme without complement of $MgCl_2$ yielded a rate of enolization which was essentially equal to the nonenzymic rate. Upon addition of 123 mM

TABLE II: Kinetic Parameters for the Enolization Reaction.

compound	K_{rel}[a]	V_{max}[b] (μmol/(min mg))	V_{max} (%)	K_m (M)	V_{max}/K_m	chirality
pyruvate	1.00	8.33	100	0.056	149	nonchiral
fluoropyruvate	2.78	0.62	4.96	0.047[c]	13.1[c]	nonchiral

[a] Relative, hydration corrected, nonenzymic rate, per hydrogen. [pyruvate] = 860 mM; [fluoropyruvate] = 671 mM. [b] The V_{max} values are uncorrected; that is, for pyruvate the value reflects three equivalent hydrogens and for fluoropyruvate two equivalent hydrogens as processed by pyruvate kinase. [c] K_m corrected for hydration, as noted in the text.

$MgCl_2$ to this solution, the rate increased by ninefold; this rate increase cannot be due solely to the nonenzymatic effect of Mg^{2+} ions. The dependence of the rate of enzymatic enolization of fluoropyruvate on magnesium ions is a good indication that the process is, in fact, a specific, active site process.

3. At a concentration of 671 mM fluoropyruvate, and with a complement of 40 mM $MgCl_2$, the rate is directly proportional to the enzyme concentration.

4. At concentrations of fluoropyruvate *below* 254 mM—regardless of enzyme concentration—the semilog plots were nonlinear; at concentrations of substrate above 289 mM, the plots were completely linear to 90% conversion. The rate of enolization follows saturation kinetics and the value of the observed K_m determined from these data is 0.313 M (total of hydrate and keto forms), which agrees with the approximate ranges where we observe nonlinear and linear kinetics. (The K_m, corrected for hydration, for the keto form of fluoropyruvate is 47 mM, close to the value of 57 mM for pyruvate, Table II.)

5. Under saturating conditions of substrate, the rates are perfectly monophasic, indicating that the *fluoromethyl group behaves torsiosymmetrically* in the pyruvate kinase mediated enolization reaction. This is observed regardless of whether the promoting cofactor is inorganic phosphate or ATP.

Isotope Effects. The rate of detritiation of (*R,S*)-[^{3}H]fluoropyruvate was measured in 0.5 M potassium phosphate buffer in D_2O, both in the presence and absence of enzyme. The isotope effect can be computed for the enzymic and nonenzymic reactions by comparing the detritiation rates with the rates obtained by NMR under similar conditions of enzyme and substrate concentrations. From Table I the nonenzymic isotope effect can be computed to be 9.8 and the enzymic effect is 9.5 ± 1. These values indicate C–H bond breaking is rate limiting in both the spontaneous and inorganic phosphate promoted enzyme catalyzed reactions. The measurement is consistent with Rose's observation that C–H bond breaking is the rate-determining step in the inorganic phosphate assisted, pyruvate kinase catalyzed enolization of pyruvic acid (Robinson & Rose, 1972).

Enolization of Pyruvic Acid. Hydrate Composition. The NMR spectrum of freshly prepared solutions of pyruvic acid in 0.5 M potassium phosphate buffer, pD 8.5, shows a signal for the keto form at δ 2.33 ppm and a signal for the hydrate form at δ 1.50 ppm, in a ratio of 9 to 1, respectively. We concluded, in accord with Kokesh, that the equilibrium constant K_{eq} is about 9, favoring the keto form (Kokesh, 1976).

Nonenzymic and Enzymic Enolization. The disappearance of the singlet at δ 2.33 ppm was followed vs. time in the absence and presence of pyruvate kinase; the data were calculated and plotted as for fluoropyruvate. Figure 2B and Table I show the results for various experiments of this type. There is a very small nonenzymic rate dependence on the concentration of pyruvate; if we assume again that the hydrogen exchange occurs solely through the keto form then the "true" rate of nonenzymic enolization, k^P, can be obtained. At 860 mM

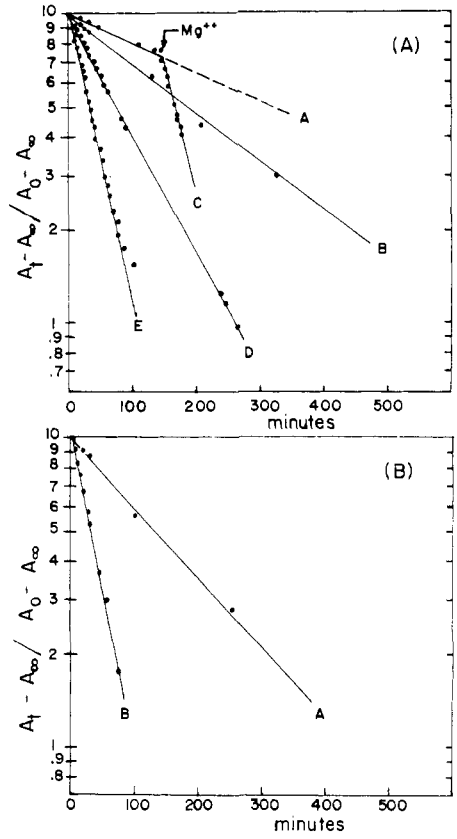


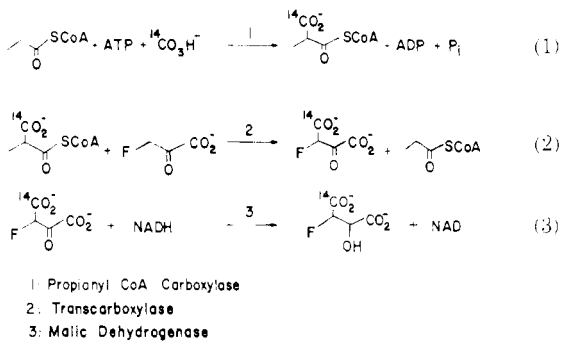
FIGURE 2: (A) Semilog plots for the enolization of fluoropyruvate in the presence and absence of pyruvate kinase and/or $MgCl_2$. All experiments were carried out in 0.5 M potassium phosphate buffer, pD 8.50. The following concentrations were used (fluoropyruvate, mM; pyruvate kinase, mg/mL; $MgCl_2$, mM): (A) 299, 17, 0; (B) 671, 0, 40; (C) 254, 14.2, 123; (D) 671, 8.33, 40; (E) 671, 25, 40. Run C is derived from run A by the addition of $MgCl_2$ to the NMR tube at 185 min. (B) Semilog plots for the enolization of pyruvate by pyruvate kinase. Conditions: 0.5 M potassium phosphate, pD 8.50; 40 mM $MgCl_2$, 25 °C. The disappearance of the singlet at 2.33 ppm in the NMR spectrum was followed vs. time and the data were plotted as explained in the Experimental Section. The following concentrations were used (pyruvate, mM; pyruvate kinase, mg/mL): (A) 860, 0; (B) 860, 5.

pyruvate, k^P is 5.69×10^{-3} min^{-1} and the rate of enolization represents three hydrogens undergoing exchange at the same rate.

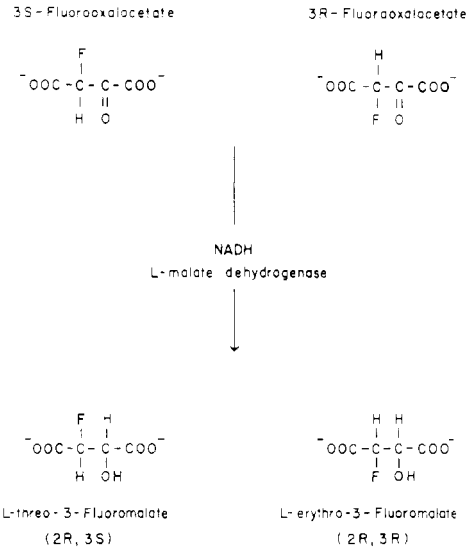
The enolization rate was accelerated by the presence of enzyme and, as expected, the semilog plots yielded linear graphs consistent with the torsiosymmetry of the methyl group. A Lineweaver–Burk plot was obtained for the enzymic reaction. At high substrate concentrations we observed marked substrate inhibition; the kinetic parameters were thus obtained by extrapolating the line obtained for the lower concentrations of substrate (Table II).

Carboxylation of β-Fluoropyruvic Acid by Transcarboxylase. To test for stereochemical selectivity in processing of the fluoromethyl group in other enzymatic reactions we in-

SCHEME I



SCHEME II



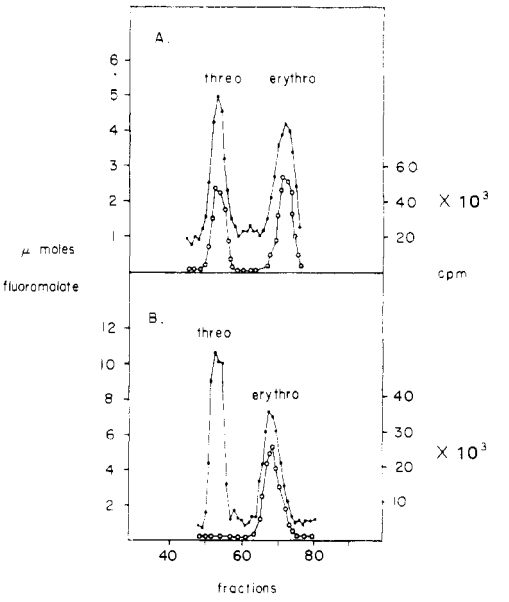


FIGURE 3: (A) Silicic acid chromatogram of authentic *erythro*- and *threo*-fluoromalate and the products from the reduction of (*R,S*)-3-fluorooxalacetate with (4*R*)-[^{3}H]NADH by malate dehydrogenase: (●—●) authentic fluoromalates; (○—○) counts per min. The elution profile was 300 mL of 15% 1-butanol in $CHCl_3$ followed by 600 mL of 35% 1-butanol in $CHCl_3$. Ten-milliliter fractions were collected; 10 mL of water was added to each and titrated to pH 7 with 1 mM NaOH. (B) Product from Scheme I chromatographed with *erythro*- and *threo*-fluoromalate standards. All other conditions the same as in a: (●—●) authentic fluoromalates and (○—○) counts per min.

vestigated the transcarboxylation between methylmalonyl-CoA and fluoropyruvate, catalyzed by transcarboxylase from *P. shermanii* (Wood, 1972; Cheung et al., 1975).

The coupled reaction scheme utilized in our experiments is shown in eq 1, 2, and 3 of Scheme I.

In this scheme, [^{14}C]methylmalonyl-CoA was generated in situ according to reaction 1 and the reaction was coupled with transcarboxylase and fluoropyruvate to give 3-fluorooxalacetate as carboxylation product (reaction 2); 3-fluorooxalacetate was then reduced immediately to fluoromalate with malic dehydrogenase (reaction 3). We reasoned that *if malate dehydrogenase showed little or no selectivity in the reduction of either* (3*R*)- *or* (3*S*)-3-*fluorooxalacetate*—if it reduced both of these enantiomers essentially equally well—then we could determine the selectivity and the chirality (including chiral purity) of the initial carboxylation (reaction 2). If only one isomer of 3-fluorooxalacetate were formed (indicative of recognition of the fluoromethyl group as a prochiral center by transcarboxylase), then reduction by MDH would yield only one of the expected fluoromalate diastereomers; if, however, the carboxylation yielded both isomers of 3-fluorooxalacetate, then it follows that we would observe both erythro and threo diastereomers of 3-fluoro-L-malate. Furthermore, it would be possible, from the identity of the isomer and a knowledge of the stereoselectivity of L-malate dehydrogenase to determine the absolute configuration of the initial carboxylation product 3-fluorooxalacetate (Scheme II).

We carried out the reactions of Scheme I with radioactively labeled sodium bicarbonate as starting material, then isolated radioactive fluoromalate as described in the Experimental Section, and identified the material by silicic acid column chromatography (Krasna, 1961) in the presence of synthetic *threo*- and *erythro*-fluoromalates. As shown in Figure 3B, radioactivity coelutes with one and only one (the erythro) fluoromalate peak. As an upper limit no more than 1% of the radioactive enzyme-generated fluoromalate can be the threo diastereomer.

Two obvious controls to be performed were (a) to find out if the rate of racemization (via enolization) of (*R,S*)-3-fluorooxalacetate was slow enough to make it noncompetitive with enzymatic reduction by L-malate dehydrogenase, and (b) if the enzymic reduction was in fact nonselective toward both enantiomers of 3-fluorooxalacetate (i.e., that both could be detected by this reductive assay).

Control A: The Rate of Enolization of 3-Fluorooxalacetate. The rate of hydrogen exchange of 3-fluorooxalacetate was determined independently by following the disappearance, in 0.5 M potassium phosphate buffer, pD 8.5, of the doublet due to the hydrate form centered at δ 5.25 ppm. The results indicated that the half-life for enolization has a lower limit of 9–12 min under these conditions (NMR probe temperature = 34 °C); the rate is probably somewhat lower at pH 7.5. The V_{max} for the reduction of 3-fluorooxalacetate by L-malate dehydrogenase is 0.011 μmol min^{-1} per unit at pH 7.5, 25 °C; the half-life for reduction of 0.1 μmol in the presence of 600 units of enzyme is thus about 1 s. Clearly, the nonenzymic enolization poses no problem in scrambling stereochemical information at carbon 3 in such incubations coupled to L-malate dehydrogenase.

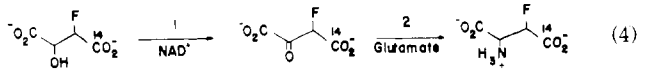
Control B: Lack of Selectivity of L-Malate Dehydrogenase for (*R*)- *or* (*S*)-3-*Fluorooxalacetate.* Upon incubation of freshly prepared solutions of synthetic (*R,S*)-3-fluorooxalacetate in limiting amount with L-malate dehydrogenase, rapid oxidation of NADH was observed; this was indicated by a

monophasic decrease at 340 nm, equal to 90% of the expected 3-fluorooxalacetate dihydrate present by weight, suggesting both *R* and *S* isomers were reduced.

The selectivity (or lack thereof) of L-malate dehydrogenase for (*R,S*)-3-fluorooxalacetate was then examined by direct diastereomeric product analysis: the reduction of 3-fluorooxalacetate by the enzyme in the presence of (4*R*)-[4-^{3}H]NADH and subsequent analysis of the radioactivity products (Experimental Section). Upon rapid reduction of 3-fluorooxalacetate (600 units of enzyme) and chromatographic analysis, two clearly separated, distinct peaks of radioactivity were obtained which were coincident with the peaks corresponding to the authentic *erythro*- and *threo*-fluoromalates. As shown in Figure 3A, of the 5×10^5 cpm of total [2-^{3}H]-3-fluoromalate recovered, 40% was in the threo diastereomer and 60% in the erythro diastereomer, indicative of a modest kinetic preference of malate dehydrogenase for the 3*R* isomer of 3-fluorooxalacetate.

The reduction of oxalacetic acid by L-malate dehydrogenase occurs to give (2*S*)-malate only;[3] assuming that the stereoselectivity is the same for 3-fluorooxalacetate, then, on the basis of the identification of *erythro*-fluoromalate (2*R*,3*R*) as the sole (>99%) isomer produced in the reaction sequence, it follows that 3-fluorooxalacetate synthesized in the enzymic carboxylation of fluoropyruvate had the 3*R* configuration only, as noted in Scheme II.

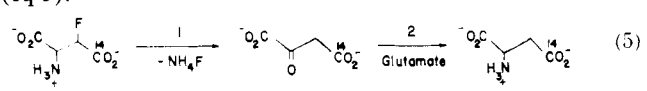
The Transamination of 3-Fluorooxalacetate. In order to further characterize the L-fluoromalate (erythro) obtained enzymatically from the carboxylation of fluoropyruvate (vide supra), we carried out its reoxidation to 3-fluorooxalacetate by L-malate dehydrogenase/NAD followed by a coupled transamination with aspartate aminotransferase in the presence of glutamate (eq 4):



(4)

1. Malic Dehydrogenase
2. Aspartate Aminotransferase

In the presence of L-malate dehydrogenase and a 1000-fold excess of glutamate, radioactive material was retained on a column of Dowex 50 (H^+) resin; in the absence of L-malate dehydrogenase all of the starting radioactivity washed through the same column without being retained. These results are consistent with the reactions proposed in eq 4. However, it appeared unlikely that the material adhering to the Dowex 1 column was β-fluoroaspartate; Kun has shown that β-fluoroaspartate is unstable and decomposes readily to ammonium fluoride and oxalacetate under such conditions (Kun et al., 1960). We believe that the radioactive material is in fact aspartic acid itself, obtained from decomposition of β-fluoroaspartate and further transamination of the oxalacetate thus formed in the presence of excess glutamate and transaminase (eq 5):



(5)

1. α–β Elimination
2. Aspartate Aminotransferase

A control experiment was carried out to test this hypothesis.

Control: Release of Fluoride Ion during Transamination of 3-Fluorooxalacetete. Independently synthesized (*R,S*)-3-fluorooxalacetate was transaminated by aspartate aminotransferase using either aspartate or glutamate as amine donors; oxalacetate and fluoride ion were determined separately, the latter with a specific fluoride ion electrode. We confirmed Kun's results that fluoride ion is released in this reaction in equivalent amounts. With a 1:1 stoichiometry of glutamate to 3-fluorooxalacetate, 1 equiv of oxalacetate and 1 equiv of fluoride were obtained.[4] However, using a 100:1 stoichiometry of glutamate to 3-fluorooxalacetate, 1 equiv of fluoride but less than 0.1 equiv of oxalacetate were obtained; furthermore in this last experiment, the concentration of oxalacetate—as followed spectroscopically—went through a maximum point and then decreased. This result is consistent with the postulate that, after oxalacetate is produced from the decomposition of β-fluoroaspartate, it undergoes further transamination to aspartic acid itself (eq 5).

Discussion

The aim of this work has been investigation of the fluoromethyl group of fluoropyruvate as a probe of enzyme specificity: for reactions where the torsiosymmetric methyl group of pyruvate is converted to a prochiral methylene group, use of the prochiral fluoromethyl group and its conversion to a chiral fluoromethylene linkage could provide increased (and more easily obtained) stereochemical information and indicate directly the degree of rotational immobilization of the bound $-CH_2F$ group at the active site of a given enzyme. We have evaluated some aspects of the nonenzymatic and enzymatic properties of fluoropyruvate and some derived fluorinated compounds in this work.

Nonenzymatic Reactions. Hydration. It has been known for some time that a fluorine atom or atoms α to a carbonyl group substantially decrease the rate of dehydration of the hemiketal derived from the carbonyl; the hemiketal form is then stabilized relative to the keto or aldehyde form. The extreme example of this effect is the observation that hexafluoroacetone hydrate is a stable crystalline solid (Middleton & Lindsey, 1964). As observed in this work, even one fluorine atom is enough to increase the hydrate composition from 10–14% in pyruvate, 85–95% in fluoropyruvate and 3-fluorooxalacetate.

Enolization. Since an equilibrium is established between the hydrate and keto forms in solution, we will assume in our discussion that the keto form is the reactive species involved in the hydrogen exchange reactions. The knowledge that fluoropyruvate is hydrated to an extent of 85% at pD 8.5 is of great importance in comparing the kinetic parameters for the enolization of various keto acids. Table II shows that the enolization is essentially the same for both keto acids, within a factor of 2.8. We might have expected a fluorine atom to increase the acidity of the enolizable hydrogens by a larger factor than the 2.8-fold observed but the relatively small factor seen for fluorine here may be compared with the data of Hine & Adolph in similar systems.

Hine has shown that the kinetic acidity of methyl difluoroacetate is 300 times smaller than that of methyl fluoroacetate which in turn is comparable to that of methyl acetate (Hine et al., 1967). Adolph & Kamlet demonstrated independently that a similar, inverted scale of acidities occurs by increasing the fluoride substitution in a series of nitromethanes (Adolph & Kamlet, 1966). Their conclusion—which can also be applied to our case—is that, when a highly electronegative element is bonded to a carbon center undergoing a change in hybridization from sp^3 to sp^2 during a reaction, there is a net destabili-

[3] L-Malate is (2*S*)-malate, but L-fluoromalate diastereomers are 2*R* chiral species. The L center is now *R* because of the fluorine substituent at carbon 3 which reverses the usual atomic number priority of α-hydroxy acids.

[4] These ratios were determined after correcting the initial 3-fluorooxalacetate concentrations for hydration and contamination by fluoropyruvate.

zation of the transition state due to the increase in electronegativity of the carbon center (Hibbert, 1977). It may be this effect that rationalizes the slower than expected enolization rate of fluoropyruvate and that probably also explains the low equilibrium (<1%) enol contents of fluoropyruvate and fluorooxalacetate (Hine et al., 1967).

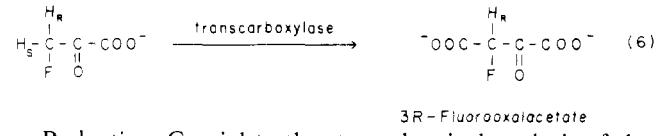
Enzymatic Reactions. Enolization. We initially attempted to prepare chiral β-D-fluoropyruvate by the pyruvate kinase catalyzed enolization of fluoropyruvate in D_2O. Our hope was that the enzyme would selectively exchange only one of the two prochiral hydrogens of that methylene group; our results, however, show that this is not the case.

Pyruvate kinase greatly accelerates the rate of exchange of the β-hydrogen of pyruvate and fluoropyruvate. This enzyme-catalyzed enolization can be considered as the first step in the overall reaction sequence leading from free α-keto acid to the phosphoenol derivative; it suggests that fluoropyruvate should be a competent substrate in the complete back reaction (not directly tested). Our results are compatible and complementary to the elegant investigations of Stubbe and Kenyon who showed that (*Z*)-methyl-PEP and (*Z*)-fluoro-PEP are indeed substrates for pyruvate kinase and transfer their phosphate group to ADP (Stubbe & Kenyon, 1971, 1972).

We can compare the effectiveness of pyruvate kinase catalysis for hydrogen exchange in the two three-carbon keto acids, by comparing the ratios of V_{max}/K_m[5] for each; this ratio can be used as a measure of the "second-order rate constant" for the enzymatic reaction and as a relative index for the efficiency of one substrate vs. another (Knowles & Albery, 1977). The effectiveness of pyruvate kinase for its natural substrate, pyruvate, is 11.4 times higher. We considered that maybe a step other than C–H bond breaking might be rate limiting for fluoropyruvate so we investigated the tritium isotope effect of the inorganic phosphate assisted pyruvate kinase catalyzed enolization of fluoropyruvate. Our results show that k_H/k_T for both enzymatic and nonenzymatic reactions has a value of about 10 which, although possibly smaller than maximally observed values (Cleland et al., 1977), indicates that C–H bond breaking is (at least partially) rate limiting in both cases. Our finding is consistent with the observation of Robinson and Rose that, in the similar enolization of pyruvate in the presence of inorganic phosphate, C–H bond breaking is rate limiting (while in ATP assisted reactions, C–H bond breaking is fast compared with a different step, presumably product release) (Robinson & Rose, 1972).

Apparently both the methyl group of pyruvate and the fluoromethyl group of fluoropyruvate are small enough to rotate freely, while the molecules are bound to this enzyme. Thus, the prochiral methylene hydrogens of the fluoromethyl group thereby become indistinguishable to the active-site base-catalyzed enolization. In the NMR studies shown with 671 mM fluoropyruvate and 25 mg/mL pyruvate kinase, there was no detectable fluoropyruvate-induced loss of activity. The complete exchange observed requires at least 2000 turnovers so the fluoro substituent of this fluoromethyl ketone is quite inert to S_N2-type displacement and the enzyme alkylation.

Carboxylation. The carboxylation of fluoropyruvate from methylmalonyl-CoA, catalyzed by transcarboxylase, to produce fluorooxalacetate and propionyl-CoA proceeds at a V_{max} one-sixth of that of pyruvate carboxylation and shows a V_{max} isotope effect of ~1.5 with [3-$^2H_2$]-3-fluoropyruvate (Cheung & Walsh, 1976). This enolization/carboxylation sequence is stereospecific and yields only the 3*R* isomer of fluorooxalacetate. Our previous studies with chiral [3-^{1}H, ^{2}H, ^{3}H]pyruvate indicate carboxylation of that keto acid proceeds with retention of configuration. If the carboxylation of fluoropyruvate also proceeds with retention, then we anticipate that the *pro*-S hydrogen at C_3 of fluoropyruvate is removed (eq 6). This expectation will be testable directly when chiral fluoropyruvate samples become available and should then allow transcarboxylase to be a test enzyme for analysis of chirality of tritiated fluoropyruvate samples.



(6)

Reduction. Crucial to the stereochemical analysis of the carboxylation of fluoropyruvate is the requirement that malate dehydrogenase be able to reduce both enantiomers of 3-fluorooxalacetate. Our experiments show that this is indeed the case. Essentially full reduction of limiting amounts of 3-fluorooxalacetate was obtained rapidly with high concentrations of malate dehydrogenase; this is substantiated by our chromatographic data and monophasic optical assays. These results appear to be in some contradiction to the observations of Skilleter et al. that only L-*erythro*-fluoromalate is obtained (on crystallization in preparative scale experiments) from the L-malate dehydrogenase catalyzed reduction of 3-fluorooxalacetate (Skilleter et al., 1971). The basis for this discrepancy is unclear[6] but Figure 3A clearly shows both fluoromalate diastereomers are produced.

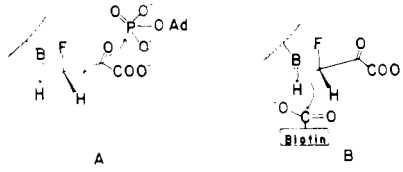
Transamination. We have confirmed Kun's original observations and shown that (*R,S*)-3-fluorooxalacetate is a substrate for aspartate aminotransferase; it appears to yield fluoroaspartate which then decomposes to ammonium fluoride and oxalacetate (Kun et al., 1960). The amount of fluoride released is stoichiometric with the amount of oxalacetate formed and 3-fluorooxalacetate reacted. These results indicate that the enzyme processes both enantiomers of 3-fluorooxalacetate and yields initially *erythro*- and *threo*-fluoroaspartates which subsequently eliminate fluoride. In contrast to these results, Briley has recently shown that difluorooxalacetate is also a substrate for aspartate aminotransferase yielding difluoroaspartate which is stable and does not decompose (Briley et al., 1977).

Comparisons between Processing of Fluoropyruvate by Pyruvate Kinase and Transcarboxylase. Pyruvate kinase catalyzes the enolization of fluoropyruvate nonchirally, while transcarboxylase catalyzes it carboxylation—and presumably the prior enolization step—in a totally chiral fashion. In one enzyme the fluoromethyl group behaves torsiosymmetrically while in the other enzyme it does not. The difference might be found in steric reasons; while both enzymes require a base at the active site to abstract a proton from the position of fluoropyruvate, the group transfer occurs at two different positions of the fluoropyruvate molecule: phosphoryl transfer occurs at the keto oxygen while carboxyl transfer occurs at the carbon atom. It is possible that given a recognition site for the fluorine atom, such as a hydrogen bond donor, the rotation of the flu-

[5] Note that K_m for fluoropyruvate corrected for hydration is 0.313 M × 0.15 = 47 mM.

[6] Skilleter and co-workers actually precipitated the lead salt of the enzymatic product. One can imagine that isolation of only the erythro diastereomer was due to the differential solubility of these two diastereomeric salts.

oromethyl group is hindered (to the point of total immobilization?) at the transcarboxylase active site by both the base and the biotin-bound carboxylate group; in pyruvate kinase only the base might be positioned next to the fluoromethyl group and there might be less steric (or negligible) hindrance to rotation:



A B

We have demonstrated that it cannot be assumed a priori that a fluoromethyl group will be processed in a totally chiral fashion by an enzyme; our results with pyruvate kinase show that care must be taken before making this assumption. However, when the prochiral group is processed stereospecifically, we have shown that it can be used to provide direct stereochemical information as in the carboxylation reaction studies. With due regard for the particular electronic characteristics of fluorinated substrates, such as increased hydration and/or lower turnover rates, it should become possible to extend our stereochemical studies to other enzymatic systems and get information on the degree of immobilization of the reacting fluoromethyl group at a given enzyme active site.

Acknowledgments

We wish to thank Professor H. Wood for his generous gift of pure *P. shermanii* transcarboxylase.

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