The present study has demonstrated that the analysis of progress-curve data can be a useful procedure for studying enzymes with Michaelis constants that are micromolar or lower. Although the results did not permit an unequivocal choice of mechanism, nevertheless, they did allow elimination of several mechanisms from further consideration. The results also provided good estimates of parameter values which were of use in connection with the definitive elucidation of the mechanism of the dihydrofolate reductase reaction by steady-state techniques.

References

Birdsall, B., Griffith, D. V., Roberts, G. C. K., Feeney, J., & Burgen, A. (1977) *Proc. R. Soc. London, Ser. B 196*, 251–265.

Blakley, R. L. (1960) *Nature (London) 188*, 231.

Blakley, R. L. (1969) *The Biochemistry of Folic Acid and Related Pteridines*, North-Holland Publishing Co., Amsterdam.

Blakley, R. L., Schrock, M., Sommer, K., & Nixon, P. F. (1971) *Ann. N.Y. Acad. Sci. 186*, 119–130.

Burchall, J. J., & Chan, M. (1969) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 28*, 352.

Cha, S. (1968) *J. Biol. Chem. 243*, 820–825.

Cha, S., Kim, S. Y. R., Kornstein, S. G., Kantoff, P. W., Kim, K. H., & Naguib, F. N. M. (1981) *Biochem. Pharmacol. 30*, 1507–1515.

Cleland, W. W. (1970) *Enzymes, 3rd Ed. 2*, 1–65.

Dawson, R. M. C., Elliott, D. C., Elliott, W. H., & Jones, K. M. (1969) *Data for Biochemical Research*, Oxford University Press, Oxford.

Duggleby, R. G., & Morrison, J. F. (1977) *Biochim. Biophys. Acta 481*, 297–312.

Duggleby, R. G., & Morrison, J. F. (1978) *Biochim. Biophys. Acta 526*, 398–409.

Duggleby, R. G., & Morrison, J. F. (1979) *Biochim. Biophys. Acta 568*, 357–362.

Gready, J. E. (1980) *Adv. Pharmacol. Chemother. 17*, 37–102.

Hillcoat, B. L., Nixon, P. F., & Blakley, R. L. (1967) *Anal. Biochem. 21*, 178–189.

Matthews, D. A., Alden, R. A., Bolin, J. T., Filman, D. J., Freer, S. T., Hamlin, R., Hol, W. G. J., Kisliuk, R. L., Pastore, E. J., Plante, L. T., Xuong, N.-H., & Kraut, J. (1978) *J. Biol. Chem. 253*, 6946–6954.

McCullough, J. L., Nixon, P. F., & Bertino, J. R. (1971) *Ann. N.Y. Acad. Sci. 196*, 131–142.

Pollard, J. H. (1977) *Numerical & Statistical Techniques*, pp 309–311, Cambridge University Press, Cambridge.

Rood, J. I., & Williams, J. W. (1981) *Biochim. Biophys. Acta 660*, 214–218.

Rood, J. I., Laird, A. J., & Williams, J. W. (1980) *Gene 8*, 255–265.

Selwyn, M. J. (1965) *Biochim. Biophys. Acta 105*, 193–195.

Smith, E., & Morrison, J. F. (1969) *J. Biol. Chem. 244*, 4224–4234.

Smith, E., & Morrison, J. F. (1971) *J. Biol. Chem. 246*, 7764–7772.

Viola, R. E., Cook, P. F., & Cleland, W. W. (1979) *Anal. Biochem. 96*, 334–340.

Williams, J. W., Morrison, J. F., & Duggleby, R. G. (1979) *Biochemistry 18*, 2567–2573.

Williams, J. W., Duggleby, R. G., Cutler, R., & Morrison, J. F. (1980) *Biochem. Pharmacol. 29*, 589–595.

Zakrzewski, S. F., & Sansone, A. M. (1971) *Methods Enzymol. 18B*, 728–731.

3-Fluoro-3-deoxycitrate: A Probe for Mechanistic Study of Citrate-Utilizing Enzymes†

Steven E. Rokita, Paul A. Srere, and Christopher T. Walsh*

ABSTRACT: The interaction of a novel fluorinated analogue of citrate, 3-fluoro-3-deoxycitrate (3-fluorocitrate), with the four known citrate-processing enzymes is described in this report. Three of the citrate-processing enzymes, citrate synthase, ATP citrate lyase, and citrate lyase, catalyze reversible aldol-type condensations. The fate of 3-fluorocitrate with each enzyme is uniquely related to their mechanisms of action. For citrate synthase, 3-fluorocitrate is a competitive inhibitor. 3-Fluorocitrate is a substrate for the carboxylate activation half-reaction catalyzed by ATP citrate lyase and induces a net ATPase action during conversion to 3-fluorocitryl-S-coenzyme A. Because of the unusual mechanism of citrate cleavage catalyzed by bacterial citrate lyase, 3-fluorocitrate is a mechanism-based inhibitor, acting at two points during turnover of the acetyl enzyme. The fourth citrate-processing enzyme, aconitase, does turn over 3-fluorocitrate catalytically. This enzyme, catalyzing a dehydration and rehydration of citrate, also catalyzes the elimination of HF from 3-fluorocitrate, yielding *cis*-aconitate and fluoride.

Citrate, **1**, is a central component in the primary metabolism of prokaryotes and eukaryotes. It is processed by only four enzymes and undergoes two types of transformations (Goodwin, 1968). Three enzymes carry out reversible aldol-type cleavage of the 3-hydroxytricarboxylate skeleton to yield the C_2 (acetate or acetyl-CoA)[1] and the C_4 (oxalacetate) fragments. These are bacterial citrate lyase (EC 4.1.3.6), mam-

† From the Departments of Chemistry and Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139 (S.E.R. and C.T.W.), and the Department of Biochemistry and Veterans Administration Medical Center, The University of Texas Health Science Center at Dallas, Dallas, Texas 75235 (P.A.S.). *Received December 28, 1981.* This investigation was supported in part by National Institutes of Health Grant GM 20011.

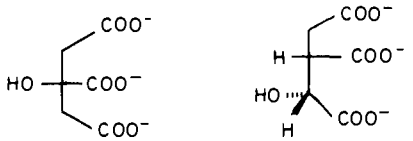
[1] Abbreviations: HPLC, high-performance liquid chromatography; CoA, CoASH, or $CoAS^-$, coenzyme A; NADH, reduced nicotinamide adenine dinucleotide; $NADP^+$, nicotinamide adenine dinucleotide phosphate; cpm, counts per minute; 3-fluorocitrate, 3-fluoro-3-deoxycitrate; unit, enzyme unit defined to be the quantity of enzyme necessary to transform 1 μmol of substrate in 1 min; Tris, tris(hydroxymethyl)-aminomethane.

0006-2960/82/0421-3765$01.25/0

malian ATP citrate lyase (EC 4.1.3.8), and the ubiquitous citrate synthase (EC 4.1.3.7). The fourth enzyme, aconitase (EC 4.2.1.3), catalyzes the reversible dehydration and rehydration of citrate designated as the second step of the citric acid cycle. In this reaction, rehydration occurs with the opposite regiospecificity to yield isocitrate, **2**, from citrate.

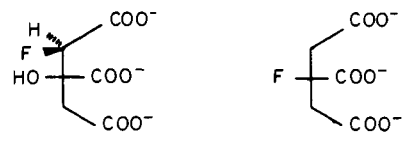
This laboratory has previously investigated the mechanistic and stereochemical consequences of the replacement of a substrate's C–H bond by a C–F bond (Goldstein et al., 1978; Marletta et al., 1981, 1982). Most recently, we have studied the citrate derivative where one of the two prochiral methylene hydrogens has been replaced by fluorine, resulting in a well-known metabolic poison, 2-fluorocitrate (Peters, 1972). The condensation of fluoroacetyl-CoA with oxalacetate catalyzed by citrate synthase yields only one of the four possible stereoisomers, (−)-*erythro*-2-fluorocitrate (Fanshier et al., 1964). We have recently used an ATP citrate lyase mediated cleavage to establish the absolute chirality of the (−)-erythro isomer as (2*R*,3*R*)-2-fluorocitrate, **3** (Marletta et al., 1981).

Although an sp^3 C–F bond is relatively short (C–H, 1.09 Å in methane; C–F, 1.39 Å in fluoromethane) and the van der Waals radius of F is similar to that of H (1.35 and 1.2 Å, respectively) the polarity of the C–F bond is much more analogous to that of a C–OH bond (Peters, 1972). One further similarity between a hydroxyl substituent and a fluorine substituent is their ability to be hydrogen-bond acceptors. However, only a hydroxyl can be a hydrogen-bond donor. Thus, replacement of the sp^3 C–OH substituent by an sp^3 C–F substituent should produce a citrate analogue, 3-fluoro-3-deoxycitrate (3-fluorocitrate), **4**, that has structural and



1

2



3

4

electronic similarity to citrate. However, the replacement of the C–OH by a C–F should have distinct and predictable mechanistic consequences for the two types of enzymes that process citrate. First, 3-fluorocitrate cannot undergo retroaldol reactions. Second, depending on the nature of the elimination mechanism, aconitase could catalyze HF elimination from **4** in analogy to HOH elimination from **1**.

By employment of a fluorination procedure developed by Dr. Kollonitsch (Kollonitsch et al., 1975, 1979), the novel 3-fluorocitrate as well as its ^{14}C-labeled form was prepared from triethyl citrate by Drs. Kollonitsch and S. Marburg and G. Doldouras at the Merck Sharp & Dohme Research Laboratories (unpublished experiments). In this paper we report on the interactions of 3-fluorocitrate with the four citrate-processing enzymes.

Materials and Methods

3-Fluorocitrate and 3-fluoro[1,5-^{14}C]citrate (6.76 μCi/μmol) were gifts of Drs. J. Kollonitsch and S. Marburg and G. Doldouras of Merck Sharp & Dohme Research Laboratories (Rahway, NJ). The procedure for the chemical analysis of 3-fluorocitrate is identical with that used for citrate by Hartford (1962). The (+)- and (−)-*erythro*-2-fluorocitrate enantiomers have been previously synthesized (Dummel & Kun, 1969). [Caution: handle (−)-*erythro*-2-fluorocitrate with extreme care. It is highly toxic.] The resolved (−)- and (+)-*threo*-hydroxycitrates and (−)- and (+)-*erythro*-hydroxycitrates were the generous gift of Dr. Ann Sullivan from the Department of Biochemical Nutrition of Hoffmann-La Roche Inc. (Nutley, NJ). All other chemicals and reagents were of the highest quality commercially available.

Citrate synthase, isolated from pig heart, was purchased from Sigma (80 units/mg) and assayed by monitoring the disappearance of the thiol ester (Srere & Kosicki, 1961). ATP citrate lyase was purified to homogeneity from rat liver as described by Srere and colleagues (Singh et al., 1976). This enzyme was assayed in two ways: the cleavage of the citrate carbon skeleton was assayed by coupling the formation of oxalacetate to its subsequent reduction catalyzed by malate dehydrogenase (Cottam & Srere, 1969), and the cleavage of ATP was followed by coupling the ADP produced to pyruvate kinase and lactate dehydrogenase (Srere, 1961). Citrate lyase from *Klebsiella aerogenes* was purchased from Sigma (8 units/mg) and assayed by a malate dehydrogenase couple (Bergmeyer, 1974). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis revealed that by weight 64% of the protein was bovine serum albumin and 36% was citrate lyase. The addition of 3 μL of reagent-grade acetic anhydride to a routine activity assay was sufficient for the acetic anhydride reactivation of citrate lyase. Inactivated citrate lyase was reactivated with acetate by incubating the enzyme in 100 mM sodium acetate for 5 min at 25 °C. Aconitase (pig heart, mainly cytosolic form) was the generous gift of Claire Monti and Dr. Jenny Glusker of the Institute for Cancer Research (Philadelphia, PA). Unless otherwise indicated, all solutions of aconitase were reactivated immediately before use by incubating a 1:1 mixture of a solution composed of a 1:1:1 dilution of 90 mM L-ascorbate (pH 7.8–8.1), 30 mM L-cysteine (pH 7.5–8.0), and 15 mM $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ with the stock enzyme solution (3.8 mg/mL) for 30 min at room temperature (J. Glusker, personal communication). If large amounts of the enzyme were used in an assay, the reactivation reagents were removed from the enzyme by passing the incubation through a Sephadex G-10 column to give enzyme with specific activity of 2.8 units/mg (250 min^{-1}) for the citrate → isocitrate reaction. When aconitase was separated from the reactivation reagents, it had an active-form half-life of more than several hours at 0 °C. The production of isocitrate by this enzyme was followed by monitoring the reduction of $NADP^+$ by the isocitrate dehydrogenase ($NADP^+$) catalyzed oxidation of isocitrate under conditions similar to those of Gawron (Gawron & Jones, 1977). The coupled assay contained 1 unit of isocitrate dehydrogenase in 1 mL of 13.5 mM triethanolamine, pH 8.0, 0.2 mM $NADP^+$, and 2 mM $MgCl_2$ with the appropriate substrate concentration. Unless noted, all enzyme assays were performed at 25 °C. All coupling enzymes were purchased from Sigma.

For separation and isolation of acyl-CoA compounds in an ATP citrate lyase activity assay, HPLC was used with a C-18 μBondapak column eluted with 100 mM potassium phosphate, pH 5.8, and 12% methanol at 2 mL/min. The HPLC separation of the tricarboxylates is described in Figure 4. A Waters Associates HPLC (including differential refractometer and fixed-wavelength detectors) was used together with a Mi-

Table I: ATPase Activity of ATP Citrate Lyase in the Presence of Citrate Analogues[a]

substrate	K_m (mM)	rel ATPase V_{max}
citrate	0.53	1.0
3-fluorocitrate	0.57	0.089
tricarballylate	1.6	0.48
DL-isocitrate	8.7	0.64
cis-aconitate	47	0.24

[a] Activity assays contained 1-20 μL of 10 mg/mL ATP citrate lyase, 5.0 mM ATP, 0.2 mM CoA, 10 mM dithiothreitol, 10 mM $MgCl_2$, 10 mM KCl, 0.2 mM NADH, 1.4 mM phosphoenolpyruvate, the tricarboxylic acid, and 80-100 mM Tris (pH 8.0) in 1 mL. The coupling enzymes added were pyruvate kinase (5 units) and lactate dehydrogenase (5 units). The reactions, run at 25 °C, were initiated with the tricarboxylic acid and monitored by the consumption of NADH at 340 nm due to the reduction of pyruvate.

cromeritics variable wavelength detector set at 210 nm. The reverse-phase column (C-18) and column packing material were purchased from Waters Associates. Fluoride concentration was analyzed with a fluoride electrode (Orion 94-06); the buffer and solutions for the standardization of these measurements were prepared according to instructions provided by the manufacturer.

Results and Discussion

The processing of 3-fluorocitrate by each of the four citrate-processing enzymes, citrate synthase, ATP citrate lyase, bacterial citrate lyase, and aconitase, will be taken up in the order listed.

(I) Citrate Synthase. Citrate synthase catalyzes the familiar condensation of acetyl-CoA and oxalacetate to form citrate. The equilibrium is displaced far in the direction of citrate formation due to the hydrolysis of the thiol ester (Srere, 1975), and thus, it is quite difficult to run the synthase reaction in the direction of cleavage (Srere, 1975). 3-Fluorocitrate was shown to be a competitive inhibitor of the synthase with a K_i of 550 μM in the citrate formation reaction compared to the citrate K_m of 250 μM (Bergmeyer, 1974). We have not prepared 3-fluorocitryl-CoA to see if it is hydrolyzed by the synthase. The retroaldol cleavage pathway is not available to 3-fluorocitryl-CoA.

(II) ATP Citrate Lyase. ATP citrate lyase effects the aldol-like cleavage of citrate with a K_{eq} = 0.0985 M under physiological conditions by hydrolyzing a cosubstrate molecule of ATP as a thermodynamic driving force (Srere, 1975). ATP is utilized first to generate a covalent phosphoenzyme, which in turn activates the C-1 carboxylate of citrate by forming a mixed citric–phosphoric anhydride (Srere, 1975). The phosphoryl group is then displaced by an enzyme nucleophile, which in turn is displaced by $CoAS^-$ to yield the enzyme-bound citryl–S–CoA that then may undergo an aldol-type cleavage as shown in eq 1–5 (Walsh & Spector, 1969).

If 3-fluorocitrate were recognized as citrate for carboxylate activation, then any of the steps from eq 2 leading to eq 4 might be observed. Again, 3-fluorocitryl-CoA could not be cleaved as in eq 5 since it cannot undergo a retroaldol cleavage.

ATPase Activity of Citrate Cleavage Enzyme in the Presence of 3-Fluorocitrate. When 3-fluorocitrate replaced citrate in an ATPase assay of a homogeneous preparation of ATP citrate lyase, catalytic activity was detected. This enzymic cleavage of ATP required the presence of both 3-fluorocitrate and CoA. Table I lists the V_{max} relative and K_m for 3-fluorocitrate and citrate in this ATPase assay. The V_{max} for 3-fluorocitrate-dependent ATP cleavage is 8.9% that of citrate. Also included for comparison in Table I are three additional tricarboxylates. Tricarballylate, propane-1,2,3-tricarboxylate, is the C-3 hydrogen analogue of citrate and exhibits 48% the V_{max} for ADP production compared to citrate. Two naturally occurring analogues, isocitrate and *cis*-aconitate, have much higher K_m values than either the C-3 fluoro or hydrogen analogue of citrate but also induce significant ATPase activity.

If the 3-fluorocitrate-dependent ATP cleavage follows the normal enzymic pathway, turnover must be aborted after eq 2 or 4 since free enzyme, required for subsequent catalytic cycles, can be regenerated by diffusion of 3-fluorocitryl phosphate or of 3-fluorocitryl-CoA away from the active site. The requirement for CoASH to effect ATP fragmentation suggests that 3-fluorocitryl-CoA is the likely enzymic product as does the precedented formation of tricarballylyl-CoA by this enzyme (Inoue et al., 1969).

Formation of 3-Fluorocitryl-CoA. Evidence for 3-fluorocitryl–S–CoA formation was derived from HPLC separations

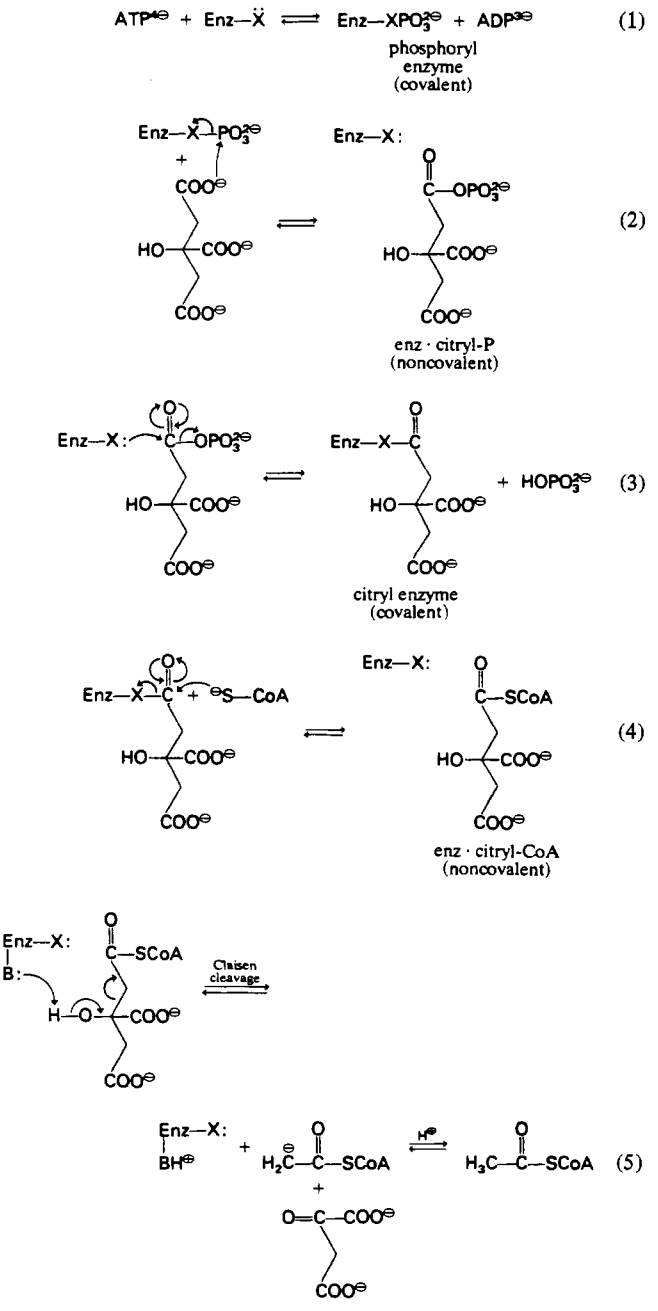
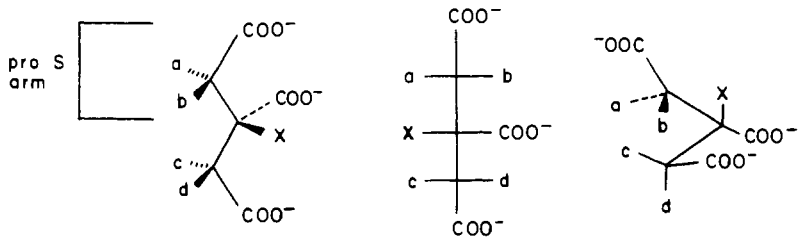


Table II



compound	a	b	c	d	X	Cahn-Ingold-Prelog[a]	cit_{pn}[b]	referred to in the text by
(−)-*erythro*-2-fluorocitrate	H	F	H	H	OH	2*R*,3*R*	(4*R*)-4	3
(+)-*erythro*-2-fluorocitrate	H	H	H	F	OH	2*S*,3*S*	(2*S*)-2	
(−)-*erythro*-2-hydroxycitrate	H	OH	H	H	OH	2*R*,3*S*	(4*R*)-4	
(+)-*erythro*-2-hydroxycitrate	H	H	H	OH	OH	2*S*,3*R*	(2*S*)-2	
(−)-*threo*-2-hydroxycitrate	OH	H	H	H	OH	2*S*,3*S*	(4*S*)-4	
(+)-*threo*-2-hydroxycitrate	H	H	OH	H	OH	2*R*,3*R*	(2*R*)-2	
citrate	H	H	H	H	OH			1
D(+)-isocitrate	H	H	OH	H	H	2*R*,3*S*		2
3-fluoro-3-deoxycitrate	H	H	H	H	F			4
tricarballylate	H	H	H	H	H			

[a] Because of the priority system of the *R*/*S* designations, a fluoro group and a hydroxyl group may share an analogous position in space but not have the same *R*/*S* assignment. [b] For a discussion of the stereochemical designation based on the parent citrate molecule, see Glusker & Srere (1973). In this numbering system, the *pro-S* branch of citrate, biosynthetically derived from acetate, has higher numbering than the *pro-R* branch.

of enzymic incubations. From an HPLC chromatograph of an activity assay a compound clearly distinct from 3-fluorocitrate (not retained) or CoASH migrates at the position typical of a CoA-derivatized tricarboxylic acid (2.5 min). For comparison, synthetic and enzymic tricarballylyl-CoA has a retention time on this column of 3 min. On reinjection of this putative 3-fluorocitryl-CoA after mild basic hydrolysis (pH 8.0, 20 h) to hydrolyze a labile acyl thiol ester, free CoASH was identified under the same column conditions. From this hydrolysate, 3-fluorocitrate was also identified with an acetic anhydride/pyridine colorimetric assay analogous to the Furth–Hermann reaction for citrate (Hartford, 1962). ATP citrate lyase therefore processes the C-3 hydrogen and fluoro analogues of citrate all the way up to the last step (eq 4) in catalysis. Since skeletal cleavage is not chemically possible, the acyl-CoA intermediates are released into solution. It is as yet unclear whether release of tricarballylyl-CoA or 3-fluorocitryl-CoA is a rate-determining step in turnover of those analogues. In vivo, if 3-fluorocitrate acted this way and if 3-fluorocitryl-CoA is as unstable as other CoA derivatives of a tricarboxylic acid (Buckel & Eggerer, 1969; Walsh, 1970), it would turn ATP citrate lyase into a wasteful ATPase.

Ratios of ATPase Activity to Carbon Skeletal Cleavage. Given the ability of noncleavable citrate analogues to induce catalytic ATP fragmentation by ATP citrate lyase, we reexamined the behavior of (+)- and (−)-*erythro*-2-fluorocitrate and the various 2-hydroxycitrates with this enzyme. This laboratory has recently reported the regiospecific cleavage of (−)-*erythro*-(2*R*,3*R*)- and (+)-*erythro*-(2*S*,3*S*)-2-fluorocitrates at 0.1% and 3% the velocity of citrate, respectively, using a coupled malate dehydrogenase assay (Marletta et al., 1981). Sullivan and colleagues have also noted the slow catalytic cleavage of some of the 2-hydroxycitrate stereoisomers (Sullivan et al., 1977) (Table II). Table III summarizes enzymic flux data by the ATPase assay and the coupled malate dehydrogenase assay. Under these experimental conditions, we now find the cleavage rate of (2*S*,3*S*)-2-fluorocitrate [(+)-erythro isomer] is only 0.5%, not 3%, the rate of citrate, but it is clear that the malate dehydrogenase assay for carbon–carbon bond cleavage gives 1 order of magnitude lower value than the ATPase assay for each 2-fluorocitrate isomer. With the toxic (−)-*erythro*-2-fluorocitrate, for example, 10 out of 11 times an ATP molecule is cleaved without the subsequent fragmentation of the C_6 tricarboxylate into C_2 and C_4 fragments. Similar uncoupling is seen with the (+)-*erythro*-2-fluorocitrate.

Table III: Ratio of ATPase Activity to Carbon Skeletal Fragmentation for 2-Fluoro- and 2-Hydroxycitrates[a]

substrate	K_i (μM)	rel V_{max} skeletal cleavage	rel ATPase act.	ATPase/ C–C bond cleavage
citrate		1.0	1.0	1.0
(−)-*erythro*-2-fluorocitrate[b]	190	0.0013	0.013	10
(+)-*erythro*-2-fluorocitrate	73	0.005	0.063	13
(−)-*threo*-2-hydroxycitrate[c]	0.52	0.0	0.0037	
(+)-*threo*-2-hydroxycitrate	8.7	0.0009	0.08	88
(−)-*erythro*-2-hydroxycitrate	3.0	0.0013	0.0048	3.7
(+)-*erythro*-2-hydroxycitrate	13	0.000095	0.045	470

[a] ATPase activity measured in the same manner as in Table II; the skeletal cleavage assay differs from the ATPase assays only by substituting malate dehydrogenase for pyruvate kinase and lactate dehydrogenase. Malate dehydrogenase will catalyze the reduction of oxalacetate, fluorooxalacetate, and hydroxyoxalacetate (Marletta et al., 1981; Sullivan et al., 1977). [b] These rates are not the result of contamination by other citrate analogues or by small amounts of citrate as shown by Marletta et al. (1981). [c] Data on the skeletal cleavage and inhibition constants of hydroxycitrates from Sullivan et al. (1977).

The uncoupling ratios are even more striking with two of the 2-hydroxycitrate isomers. Although cleavage rates for (+)-threo and (+)-erythro are exceedingly low, the ATPase V_{max} rates are 8% and 4.5% that of citrate, leading to uncoupling ratios of 88/1 and 470/1, respectively, for ATP cleavage vs. retroaldol fragmentation.

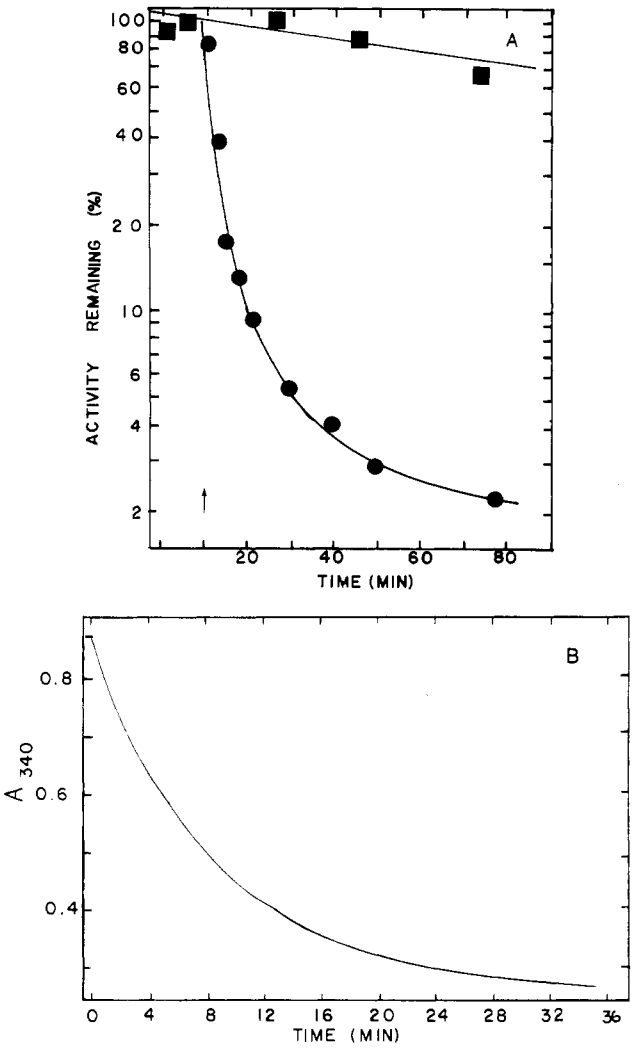
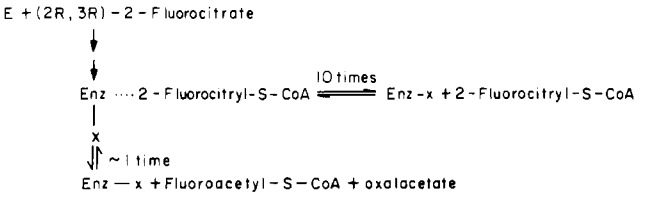
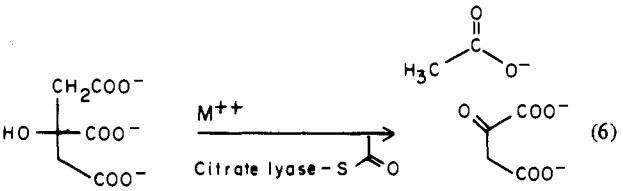


FIGURE 1: (A) Time-dependent inactivation of citrate lyase. At indicated times, aliquots (10 μL) of enzymic incubations were assayed for activity remaining in the fashion described under Materials and Methods. The control incubation (■) was maintained at 25 °C and contained 0.48 mg of citrate lyase, 1.0 mM $ZnCl_2$, and 95 mM triethanolamine, pH 7.6 in 1.0 mL. To an incubation with the same composition was added 30 μM 3-fluorocitrate where indicated (●). (B) Typical time course for citrate cleavage catalyzed by citrate lyase in the presence of Zn^{2+}. Citrate lyase (0.58 μg of the Sigma preparation) is added to initiate the reaction at t_0. The assay contains 2 mM $ZnCl_2$, ca. 100 mM triethanolamine, pH 7.6, 9 mM sodium citrate, 0.2 mM NADH, and 1 unit of malate dehydrogenase. The decrease in NADH resulting from the reduction of the cleavage product, oxalacetate, is monitored at 340 nm. For ca. 1.9×10^{-12} mol of enzyme, there is ca. 100 nmol of oxalacetate (calculated from the change in the absorbance at 340 nm) produced before the enzyme is inactive. With a turnover number of 2700 min^{-1}, citrate lyase exhibits a partition ratio of 27 000 turnovers per inactivation.

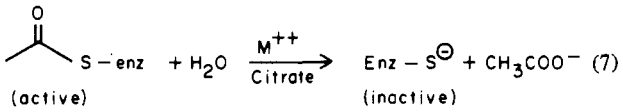
The data for the 2-fluoro- and 2-hydroxycitrates can be explained by a kinetic competition between normal skeletal cleavage of an enzyme-bound activated substrate molecule and adventitious diffusion from the active site, after either eq 2 or eq 4 of the ATP citrate lyase mechanism. Given the 3-fluorocitryl-CoA and tricarballylyl-CoA precedents above, we feel that the product of eq 4, in this case 2-fluorocitryl-CoA or 2-hydroxycitryl-CoA, is the likely species that dissociates from the enzyme as illustrated below for (2*R*,3*R*)-2-fluorocitrate. When the carbon skeletal cleavage steps become very slow, the dissociation steps predominate and lead to uncoupling.[2] With citrate, dissociation does not compete kinetically with skeletal cleavage.



(*III*) *Bacterial Citrate Lyase.* The inducible citrate lyase from such bacteria as *Streptococcus diacetilactis* or *K. aerogenes* has three unusual features: (1) the resting enzyme is an acetyl-S-enzyme; (2) the active site sulfhydryl (of the thiol ester) is part of a covalently attached CoA derivative; (3) during normal turnover (eq 6), the enzyme autoinactivates

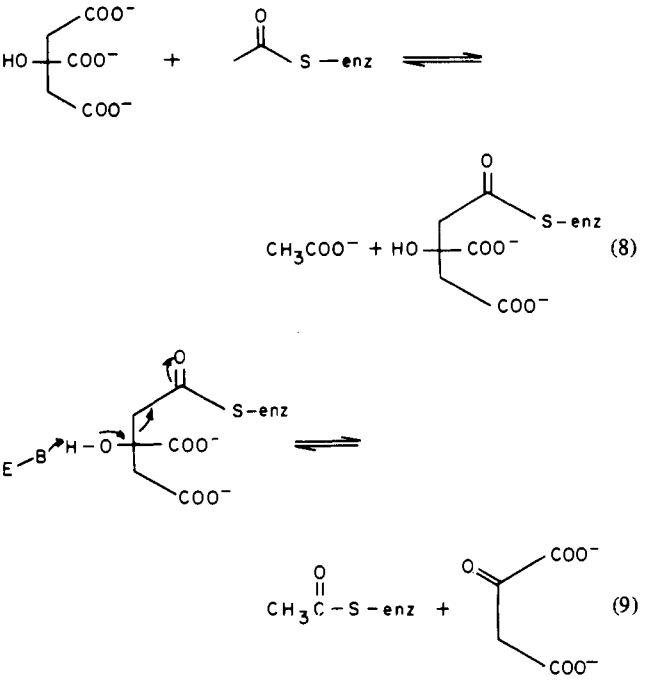


(6)

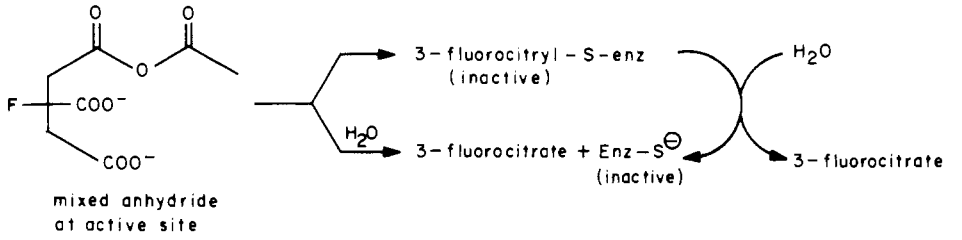


(7)

with a frequency dependent on the divalent metal ion present by acyl transfer to water, leaving inactive deacetyl enzyme (eq 7) [for a review, see Srere (1975)]. The process of catalytic turnover is best described as an acyl exchange process to form a citryl-S-enzyme (eq 8), followed by an aldol-type cleavage (eq 9) to release oxalacetate and regenerate the starting acetyl-S-enzyme [e.g., see Srere (1975)].



[2] The partitioning of the hydroxycitrates might also be affected by the instability of the acyl-CoA to hydrolysis via lactonization. If the ATP citrate lyase only processes one carboxylate of the citrate analogue (as it does with citrate) corresponding to the *pro-S* arm of citrate, then two of the hydroxycitrate stereoisomers may readily lactonize while the other two would not. In fact, the (+)-*threo*- and (+)-*erythro*-hydroxycitrates, both with the hydroxyl group on the corresponding *pro-R* arm, do exhibit a larger ATPase activity than the other two stereoisomers. Thus, in this case lactonization may add to the uncoupling between the carboxylate activation and carbon–carbon bond cleavage.



(10)

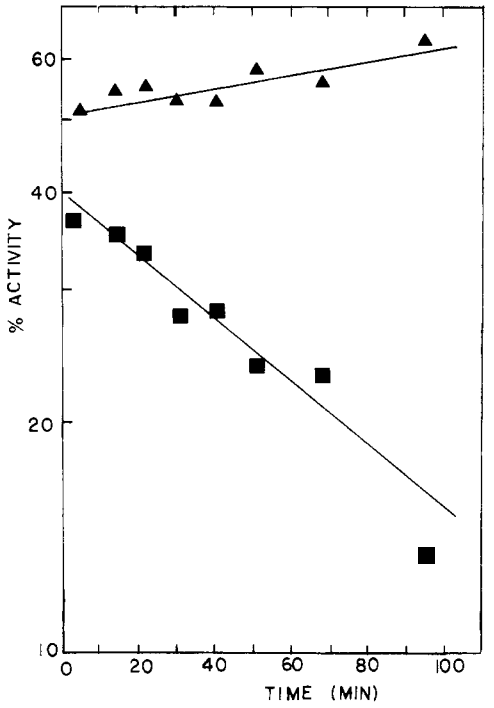
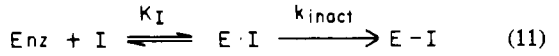


FIGURE 2: Relative change in capacity for acetic anhydride and acetate to reactivate citrate lyase treated with 3-fluorocitrate. So that reactivation characteristics could be monitored clearly, subsequent to inactivation, citrate lyase was inactivated quickly by adding 30 μM 3-fluorocitrate as in Figure 1. If the percent of the reactivation of lyase relative to the total initial activity is called a, percent of reactivation by acetate b, and the percent of residual activity c, then the line denoted by (▲) is a plot of the function $a/(a + b + c)$, demonstrating a larger proportional ability for acetic anhydride to reactivate lyase over the time of an inactivation incubation. The line denoted by (■) is a plot of $b/(a + b + c)$, demonstrating the decreasing ability of acetate to reactivate lyase. Acetic anhydride reactivation was accomplished by adding 3 μL of acetic anhydride to a normal activity assay of citrate lyase–3-fluorocitrate incubation. Inactivated citrate lyase was reactivated by acetate by incubating 20 μL of the enzyme inhibition incubation with 20 μL of a 0.2 M solution of sodium acetate for 5 min at 25 °C. The activity of 20 μL of this reactivation incubation was analyzed in a normal activity assay.

On the basis of this information, one might expect that 3-fluorocitrate will act as a suicide substrate by two possible routes. First, in analogy to the above studies with mammalian ATP citrate lyase, a mixed acetic–3-fluorocitric acid anhydride (eq 10) intermediate might diffuse away from the active site and leave the enzyme stranded in a deacetylated, inactive form. Second, if the 3-fluorocitryl–S–enzyme intermediate is subsequently formed, it cannot be cleaved between the C-2 and C-3 positions, and the enzyme would accumulate in this inactive form. Hydrolysis of this acyl enzyme could ensue; however, the enzyme would still be inactive (eq 10).

Suicidal Inactivation by 3-Fluorocitrate. If 3-fluorocitrate is indeed an effective time-dependent inactivator of this bacterial enzyme, one would expect the kinetics described by eq 11. Figure 1 shows the time-dependent loss of activity with

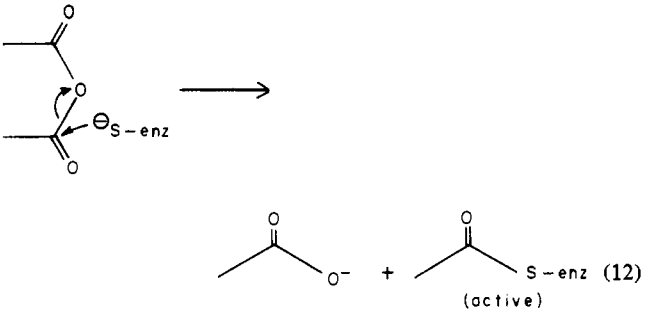


(11)

30 μM 3-fluorocitrate to be very rapid until ca. 2–3% of the activity remains. Also shown is the time course of enzyme activity in the presence of citrate to show that the autoinactivation process with the physiological substrate has a frequency of ca. 1 in 27 000 turnovers. The kinetic constants for the interaction between 3-fluorocitrate and citrate lyase were extrapolated from a double-reciprocal plot of the observed k_{inact} vs. 3-fluorocitrate concentration. The observed k_{inact} constants were measured in incubations similar to those described in Figure 1. The K_i equals 43 μM for 3-fluorocitrate and a limiting k_{inact} of 0.67 min^{-1} in eq 11.

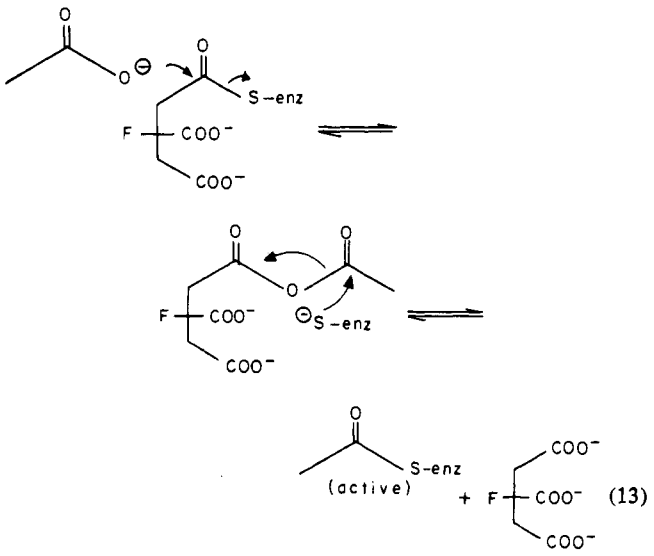
Figure 1 emphasizes two as yet unexplained phenomena during this enzymic inactivation: deviation from first-order inactivation and attainment of a plateau level of residual activity, which has been found to be dependent on the concentration of 3-fluorocitrate only. Lyase is multimeric as noted below; thus, these unusual inactivation characteristics may be explained by negative cooperativity between inactive subunits. Still, the inactivation behavior of 3-fluorocitrate is difficult to analyze. A careful HPLC analysis for products with 3-fluoro[1,5-^{14}C]citrate failed to reveal any other compounds after exposure to and inactivation of bacterial citrate lyase. There is no enzymic processing of 3-fluorocitrate to a stable derivative.

Analysis of Deacetyl vs. 3-Fluorocitryl Enzyme as Inactive Enzyme Species. In order to distinguish between deacetyl enzyme and 3-fluorocitryl enzyme as the inactive enzyme species, we exploited the different abilities of acetic anhydride and acetate to reactivate inactive enzyme. It is known that acetic anhydride will specifically activate deacetyl enzyme (eq 12) (Buckel et al., 1971; Srere et al., 1972). The fractional

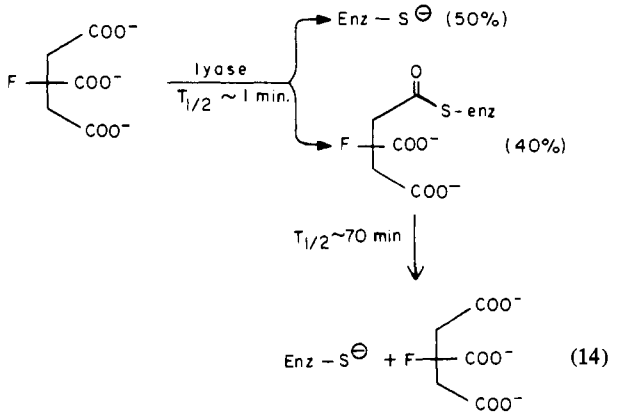


(12)

regain of initial activity is an indicator of the proportion of enzyme left in the enzyme–S$^-$ state. 3-Fluorocitryl–S–enzyme will not be reactivated by this electrophilic reagent. However, we postulated that 3-fluorocitrate could be displaced by nucleophilic acetate ion in an analogous fashion to the normal acyl exchange reaction (eq 13). In fact, treatment of 3-fluorocitrate-inactivated citrate lyase with either sodium acetate or acetic anhydride led to the restoration of up to about 50% of the initial activity. This suggests a 1:1 distribution of inactive enzyme initially between deacetyl enzyme and 3-fluorocitrylated enzyme. As shown in Figure 2, an analysis



of the reactivation characteristics over time indicated that the acetate ion reactivatable component disappeared over time with a half-life of ca. 70 min, suggesting subsequent hydrolysis of the 3-fluorocitryl–S–enzyme to deacetyl citrate lyase (eq 14).



Stoichiometry of Enzyme Labeling with 3-Fluoro[1,5-^{14}C]citrate. Under the conditions described in Figure 3, 3-fluoro-[1,5-^{14}C]citrate inactivated 50% of the citrate lyase in 20 min. The radioactivity associated with the precipitated protein increased from a background value of 400 to 2500 cpm in 20 min but then did not increase further but rather began to decrease slowly. We presume that ^{14}C radioactivity represents 3-fluoro[^{14}C]citryl groups bound by a thiol ester linkage at the enzyme active site. The acid stability is consistent with this type of linkage as are several control experiments. The ^{14}C binding to protein requires active lyase. Lyase pretreated with either Mg^{2+} and citrate or NH_2OH, each yielding inactive deacetyl enzyme (Buckel et al., 1971; Srere et al., 1972), did not bind any 3-fluoro[^{14}C]citrate in this trichloroacetic acid precipitation/filtration assay.

The ^{14}C label can be displaced from the 3-fluoro[^{14}C]citryl–S–enzyme in several ways. Incubation with 1 M NH_2OH for 15 min displaced most (70%) of the ^{14}C label from the protein. Similarly, 0.3 M β-mercaptoethanol removed >95% of the ^{14}C label, possibly by an acyl exchange process. Finally, in conjunction with the above acetic anhydride vs. acetate ion diagnostic tests, acetate ion (0.1 M) displaced the ^{14}C label in 5 min while acetic anhydride at the concentration used in reactivation assays had no effect. Plotted in Figure 3B is the quantitative ability of acetate ions to reactivate the catalytic activity in the 3-fluoro[^{14}C]citrylated lyase, reflecting acyl exchange to reform active acetyl enzyme (eq 13). Again there

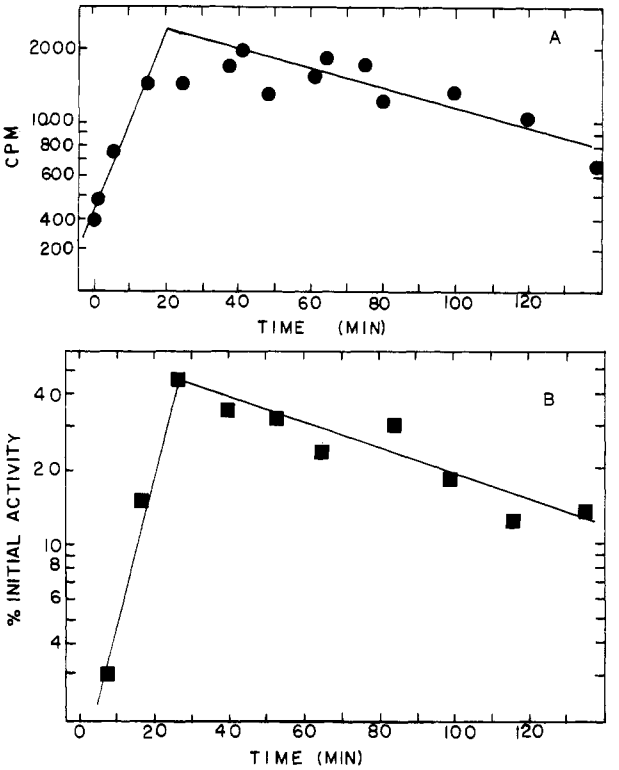
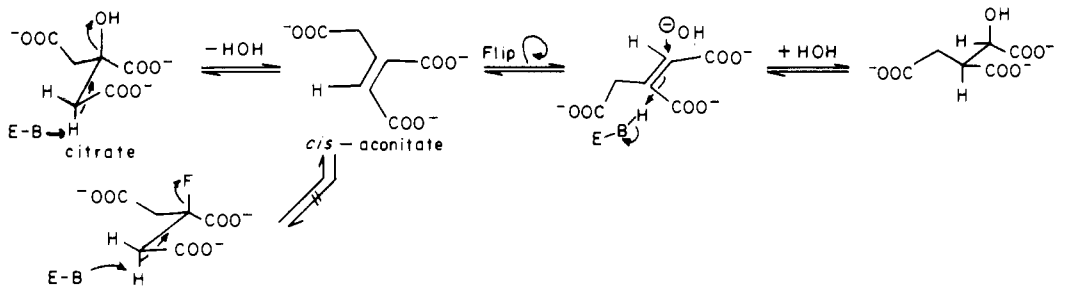


FIGURE 3: Incubations of 3-fluoro[1,5-^{14}C]citrate with citrate lyase. (A) The incubations contained 9 μM 3-fluoro[1,5-^{14}C]citrate (ca. 6.76 μCi/μmol), 0.36 mg of citrate lyase (1 mg of protein from the Sigma preparation), 1 mM $ZnCl_2$, and 100 mM triethanolamine at pH 7.6 at 22 °C. At the intervals indicated, 200-μL aliquots were removed and mixed with 400 μL of 5% trichloroacetic acid, and the precipitated protein was collected on a Millipore 0.45-μm filter. Filters were then washed with 1 mL of 5% trichloroacetic acid and counted for ^{14}C radioactivity. (B) A parallel incubation to (A) was assayed for the ability of acetate to reactivate the enzyme as described earlier in Figure 2. The reactivation of citrate lyase is plotted as a function of the percent of activity induced by acetate incubation over the initial activity of citrate lyase.

is an initial ability to resuscitate dead enzyme, but then this capacity decreases, an observation consistent with subsequent defluorocitrylation to yield free, inactive deacetyl lyase. As expected, the release of radioactivity paralleled the loss of acetate-induced reactivation of the enzyme.

Given the deviation from first-order inactivation kinetics and the lability of 3-fluorocitryl–S–enzyme, stoichiometry calculations are perilous, but a rough estimate can be made. At 50% inactivation (20 min in Figure 3A), 2500 dpm (80% counting efficiency) are protein associated. Extrapolating to 100% inactivation, 5000 dpm would be bound. The incubation contained 0.64 nmol of hexamer (3.8 nmol of active sites) postulated to have a molecular weight of 560 000 (Singh et al., 1974). By considering the specific radioactivity and correcting for aliquot sizes, our calculations imply that there are 2.1 nequiv of radioactive label/3.8 nmol of active site. As expected from the partitioning data, approximately half of the active sites are modified.

Comparison of 3-Fluorocitrate with Another Citrate Analogue in Citrate Lyase Inactivation. Table IV compares the inactivation behavior of 3-fluorocitrate vs. the C-3 hydrogen analogue, tricarballylate. This analogue also ought to acylate the enzyme but be recalcitrant to cleavage and regeneration of resting, active acetyl enzyme. In fact, tricarballylate does inactivate but at a K_i value 25-fold higher (1.09 mM vs. 43 μM) and with a first-order inactivation rate constant 40-fold slower (0.017 min^{-1} vs. 0.67 min^{-1}) than those of 3-fluoro-



(15)

(16)

Table IV: Citrate Analogues as Suicide Substrates of *K. aerogenes* Citrate Lyase[a]

compound	K_i or K_m (μM)	k_{cat} (min^{-1})	k_{inact} (min^{-1})
citrate	160	10^3	0.1
3-fluorocitrate	43	none	0.67
tricarballylate (3-deoxycitrate)	1090	none	0.017

[a] Activity assays for citrate lyase were initiated by adding citrate lyase to 1 mL containing 20 mM $ZnCl_2$, 0.2 mM NADH, the appropriate tricarboxylate (for V_{max}, 9 mM citrate was used), and ca. 80 mM triethanolamine, pH 7.6. The rate was followed by the consumption of NADH at 340 nm due to a malate dehydrogenase catalyzed reduction of oxalacetate. Time inactivation experiments normally consisted of assaying the citrate lyase activity remaining in aliquots of a 1-mL incubation containing 10 mg/mL bovine serum albumin, 0.6 mM $ZnCl_2$, 0.6 mg of lyase, and 80 mM triethanolamine, pH 7.6.

Table V: Stoichiometric Production of F^- and Isocitrate from 3-Fluorocitrate Catalyzed by Aconitase[a]

time (min)	reduced $NADP^+$ concn (μM)	F^- concn (μM)
0	0.0	<1.0
60	8.2	10.5
150	18.8	14.8
240	25-33	21.0

[a] The reduction of $NADP^+$ by isocitrate dehydrogenase coupled to the aconitase reaction (49 μg of aconitase) was monitored in the manner described under Materials and Methods. At the times indicated, 250 μL of this assay was removed and diluted with 250 μL of a constant ion buffer (IV, pH 5–6) for assay for fluoride content with an Orion fluoride electrode (see Materials and Methods).

citrate. By these criteria, 3-fluorocitrate is dramatically (1000-fold) superior at mechanism-based inactivation of *K. aerogenes* citrate lyase to tricarballylate. Citrate itself inactivates as shown in Figure 1. Under these experimental conditions citrate will show a k_{inact} of about 0.1 min^{-1}, some 6.7-fold slower than the 3-fluorocitrate-induced process. Lyase inactivated with tricarballylate, as opposed to 3-fluorocitrate, does not regain partial activity after incubation with acetate, although it does with acetic anhydride treatment. At least in the time frame of our assays either the covalent modification of lyase by tricarballylate is too slow to detect or its subsequent hydrolysis is too rapid.

(IV) Aconitase. The reactivity of 3-fluorocitrate with the fourth enzyme that processes citrate, aconitase, is of specific interest for at least two reasons. This enzyme carries out a reversible dehydration (anti) and then rehydration with opposite regiospecificity (eq 15) rather than effecting changes in the substrates' carbon skeleton (Glusker, 1971). While the C-3 fluoro substituent in 3-fluorocitrate prevents retroaldol chemistry, base-catalyzed HF eliminations by an initial proton abstraction are well precedented in chemical and enzymic cases (Kollonitsch et al., 1978; Walsh, 1978, 1979; Silverman & Abeles, 1976). On this basis, 3-fluorocitrate should be a substrate for dehydrofluorination to *cis*-aconitate if aconitase can effect such proton abstraction reactions. Once HF is eliminated, the resulting aconitate would then mesh into the normal reaction pathway (eq 16). HF elimination, in contrast to HOH loss, should be effectively irreversible. The second reason for our interest in aconitase stems from its sensitivity to another fluoro analogue of citrate. Peters had assigned aconitase to be the site where (−)-*erythro*-2-fluorocitrate, **3**, acts. This metabolic poison is itself produced from fluoroacetate via citrate synthase (Peters, 1972). Differing modes of irreversible inhibition of aconitase by this stereoisomer have been discussed in the literature, and the release of fluoride has been associated with the inactivation of aconitase in at least one laboratory (Villafranca & Platus, 1973).

3-Fluorocitrate Turnover via Coupled Isocitrate Dehydrogenase Assay. The most convenient assay for aconitase activity involves coupling the isocitrate produced to isocitrate dehydrogenase, resulting in the reduction of $NADP^+$. Although 3-fluorocitrate was shown to be neither a substrate for nor an irreversible inhibitor of this coupling enzyme, it does behave as a noncompetitive reversible inhibitor (K_i = 2.5 mM) of isocitrate dehydrogenation. Enough isocitrate dehydrogenase was added to this coupled assay to avoid an underestimation of the ability of aconitase to turn over 3-fluorocitrate.

Since the turnover of 3-fluorocitrate was determined to be much slower than that of citrate (see below), reversible inhibition of aconitase could be studied. 3-Fluorocitrate behaved as a competitive inhibitor of citrate processing in the coupled assay with a K_i of 120–160 μM, indicating binding recognition by aconitase. By addition of 0.14 unit of aconitase to a standard assay containing 3-fluorocitrate (2.2 mM) instead of citrate, an initial velocity of 0.27 nmol of $NADP^+$ reduced/min was detected over a background value of 0.06 nmol/min in the absence of aconitase. This rate was approximately linear for 60 min with no evidence of aconitase or isocitrate dehydrogenase inactivation. This velocity is 0.1% the V_{max} for citrate processing, reflecting turnover numbers of 0.25 min^{-1} vs. 250 min^{-1} for 3-fluorocitrate vs. citrate, with a 90000 subunit molecular weight for aconitase (Glusker, 1971). These data indicate that 3-fluorocitrate does indeed undergo catalytic HF elimination by aconitase, albeit at 1000-fold reduced V_{max}.

Catalytic Production of Fluoride from 3-Fluorocitrate and Aconitase. The reaction scheme shown in eq 16 suggests that aconitase-mediated production of inorganic fluoride ion should be stoichiometric with $NADP^+$ reduction in the isocitrate dehydrogenase coupled assay. The results of fluoride ion electrode measurements (Table V) validate this prediction. Since 3-fluorocitrate does not inactivate aconitase, the reported fluoride ion generated by (−)-*erythro*-2-fluorocitrate turnover could not be responsible for its toxic effect.

Table VI: Summary of Interactions between Citrate and Its Analogues with Citrate-Processing Enzymes

compound	enzyme			
	citrate synthase	ATP citrate lyase	*K. aerogenes* citrate lyase	aconitase
citrate	substrate	substrate, C–C bond cleavage 1:1 with ATP splitting	substrate, low turnover inactivator	substrate for dehydration
3-fluorocitrate	competitive inhibitor	substrate for ATP splitting only	no cleavage, efficient inactivator	substrate for HF elimination
tricarballylate	competitive inhibitor	substrate for ATP splitting only	no cleavage, slow inactivator	competitive inhibitor
(−)-*erythro*-2-fluorocitrate	product from fluoroacetyl-CoA and oxalacetate	10% coupling ATP hydrolysis to C–C bond cleavage	slow cleavage, turnover inactivator[a]	proposed metabolic target for this toxin
(+)-*erythro*-2-fluorocitrate	reversible inhibitor[a]	13% coupling ATP hydrolysis to C–C bond cleavage	extremely slow turnover, slow irreversible inhibition[a]	no reported irreversible inhibition

[a] S. E. Rokita and C. T. Walsh, unpublished observations.

Conversion of 3-Fluoro[1,5-^{14}C]citrate to [1,5-^{14}C]Citrate by Aconitase. The equilibrium ratios for citrate/*cis*-aconitate/isocitrate are 88/4/8 (Glusker, 1971), so incubation of 3-fluoro[^{14}C]citrate with aconitase in the absence of isocitrate dehydrogenase should lead to production of radioactive citrate as the predominant component. This would occur via enzymic HF elimination, followed by enzyme-catalyzed HOH addition. Figure 4A documents this behavior by using small amounts of 3-fluoro[1,5-^{14}C]citrate (2.6 μM, recall K_i = 120–160 μM) to increase detection sensitivity and increase the mole fraction converted to citrate. Over a 6-h incubation of 45% of the radioactive label is converted to citrate. Note that this experiment requires an effective separation of citrate and 3-fluorocitrate. Figure 4B describes HPLC conditions used to separate these compounds as well as *cis*- and *trans*-aconitate and isocitrate. In addition, the starting 3-fluorocitrate must be scrupulously free not only of [^{14}C]citrate but also of [^{14}C]isocitrate and *cis*-[^{14}C]aconitate since these contaminants would be enzymically equilibrated to citrate. The 3-fluoro-[^{14}C]citrate used in these enzymic experiments, as well as for the incubations with citrate lyase, had been first purified by the analytical HPLC system of Figure 4B. Finally, it is imperative to ensure that nonenzymic HF loss resulting in the conversion of 3-fluorocitrate to *cis*-aconitate is not a significant competing reaction. As a control, a parallel incubation was run by adding inactive enzyme to the assay mixture already containing the proportional amounts of reactivation reagents. After 24 h, no radioactivity was associated with citrate, isocitrate, or either isomer of aconitate above the t_0 time points. We also analyzed for nonenzymic production of aconitate (λ_{max} = 240 nm) from pH 1 to pH 13 with 3-fluorocitrate and saw no detectable elimination. Similarly, HPLC analysis of 3-fluoro[^{14}C]citrate at pH 13 (phosphate buffer, 24 °C) for 2.5 h indicated that 3-fluorocitrate decomposed less than 10%. In the acetic anhydride/pyridine colorimetric assay used for analytical estimation of citrate and many of its analogues, 3-fluorocitrate did lose HF as expected. Thus, there is no doubt that 3-fluorocitrate is a low V_{max} substrate of aconitase, losing fluoride during turnover but not inactivating the enzyme to any detectable extent.

This paper describes the behavior of each of the four known citrate-processing enzymes with a novel fluorinated analogue of citrate, 3-fluorocitrate, **3**. As collated in Table VI, each enzyme shows a distinct type of interaction conditioned by the mechanism of catalysis and the distinctive consequences that arise on substituting a C–F bond for a C–OH bond. For completion of the comparison, the behavior of the deoxy analogue of citrate, tricarballylate, is also summarized in Table VI. A comparison is also made between 3-fluorocitrate, not

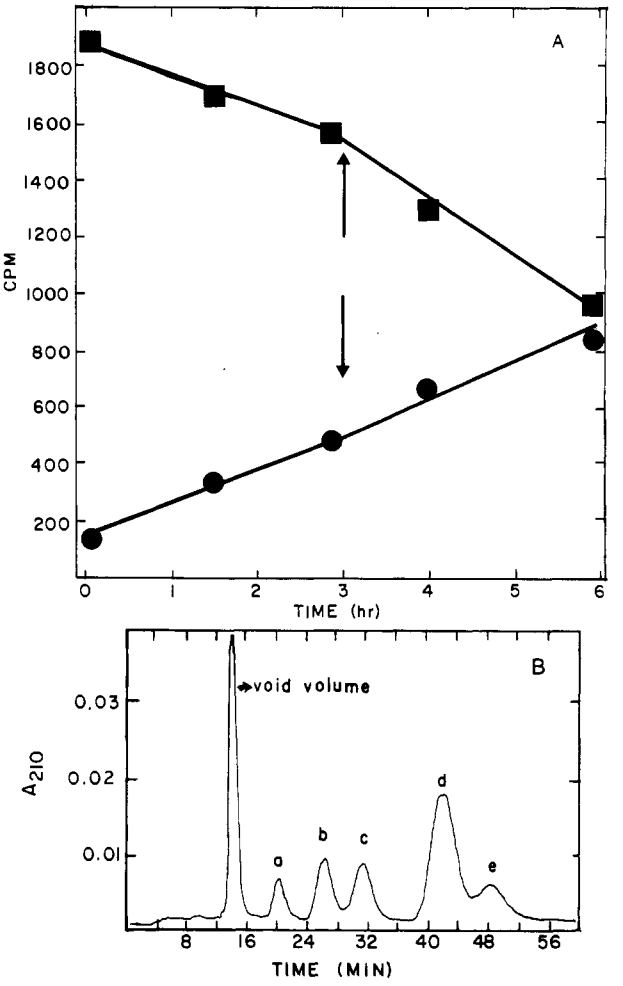


FIGURE 4: Dehydrofluorination and rehydration of 3-fluoro[1,5-^{14}C]citrate to [1,5-^{14}C]citrate catalyzed by aconitase as seen by HPLC analysis. (A) The enzyme incubation, maintained at 22 °C, contained 1.2 mM $MgCl_2$, 20 mM Tris, pH 7.4, and 2.5 μM 3-fluoro[1,5-^{14}C]citrate in a total volume of 420 μL. The reaction was initiated with ca. 0.1 mg of aconitase, and after 175 min another 0.1 mg of aconitase was added as indicated by the arrow. Aliquots (60 μL before second enzyme addition, 70 μL after) were removed at the times indicated, mixed with 40 μL of a solution containing 2.5 mM citrate, isocitrate, 3-fluorocitrate, and aconitate (cis and trans), and separated on an HPLC under the conditions noted below. Citrate (●) and 3-fluorocitrate (■) were collected and measured for ^{14}C radioactivity. (B) A 40-μL mixture of 2.5 mM citrate, isocitrate, 3-fluorocitrate, and aconitate (0.02 mM, cis and trans) was separated on a 10 mm × 25 cm column packed with μBondapak C-18 Porasil B (37–75 μm) by eluting with 1.5% potassium phosphate buffer at pH 2.9 at 1 mL/min. The tricarboxylates (a) isocitrate, (b) 3-fluorocitrate, (c) citrate, (d) *cis*-aconitate, and (e) *trans*-aconitate were detected at 210 nm.

demonstrably toxic, and two citrate analogues with fluorine substitutions at another site, (2*R*,3*R*)-2-fluorocitrate, the poisonous condensation metabolite of fluoroacetate, and (2*S*,3*S*)-2-fluorocitrate, supposedly not toxic (Fanshier et al., 1964).

While the C–F bond is a very short bond, akin to the C–H bond, and H and F atoms are also nearly the same size, the extreme electronegativity of fluorine polarizes the C–F bond much more akin to a C–OH linkage. Thus, a C–F group is a steric and electronic hybrid between C–H and C–OH linkages. The reactivity of the strong C–F bond and the persistent monovalent character of fluorine dictate the response of the four citrate-utilizing enzymes to 3-fluorocitrate.

The fact that HF is eliminated catalytically from 3-fluorocitrate by aconitase lends more credence to the possibility that aconitase effects the transformation of citrate to *cis*-aconitate by proton abstraction, followed by hydroxyl elimination. Evidence for a carbanion mechanism for aconitase has recently been presented by Schloss, Cleland, and co-workers (Schloss et al., 1980). They found that the carbanions of nitrocitrate and nitroisocitrate, models of a citrate or isocitrate carbanion transition state, show extremely tight binding to aconitase on the order of 10^{-8} M.

Unexplained is the very low V_{max} (0.2%) for HF loss compared to HOH loss from citrate. Once a carbanion is formed β to a fluoro substituent, facile fluoride elimination is expected. Furthermore, the protons β to a fluoro substituent should be even more activated for deprotonation than those β to a hydroxy substituent (Hine, 1975). Whether the low turnover reflects suboptimal orientation for the anti elimination or some electronic effect is currently unclear.

Acknowledgments

We owe a special acknowledgment to Dr. Janos Kollonitsch, Dr. Steve Marburg, and George Doldouras at Merck Sharp & Dohme Research Laboratories for the provision of 3-fluorocitrate samples, nonradioactive and radioactive, without which these experiments could not have been performed. We also acknowledge the generous provision of 2-hydroxycitrate isomers from Dr. A. Sullivan, Hoffmann-La Roche, Inc., of purified aconitase from Drs. C. Monti and J. Glusker, Institute for Cancer Research (Fox Chase, PA), and of 2-fluorocitrate isomers from Dr. M. Marletta, Department of Nutrition and Food Science, Massachusetts Institute of Technology.

References

Bergmeyer, H., Ed. (1974) *Methods of Enzymatic Analysis*, pp 442–443, Academic Press, New York.

Buckel, W., & Eggerer, H. (1969) *Hoppe-Seyler's Z. Physiol. Chem. 350*, 1367–1376.

Buckel, W., Buschmeier, V., & Eggerer, H. (1971) *Hoppe-Seyler's Z. Physiol. Chem. 352*, 1195–1205.

Cottam, G. L., & Srere, P. A. (1969) *Arch. Biochem. Biophys. 130*, 304–311.

Dummel, R. J., & Kun, E. (1969) *J. Biol. Chem. 244*, 2966–2969.

Fanshier, D. W., Gottwald, L. K., & Kun, E. (1964) *J. Biol. Chem. 239*, 425–434.

Gawron, O., & Jones, L. (1977) *Biochim. Biophys. Acta 484*, 453–464.

Glusker, J. (1971) *Enzymes, 3rd Ed. 5*, 413–439.

Glusker, J. P., & Srere, P. A. (1973) *Bioorg. Chem. 2*, 301–310.

Goldstein, J. A., Cheng, Y., Marletta, M. A., & Walsh, C. (1978) *Biochemistry 17*, 5567–5575.

Goodwin, T. W. (1968) *The Metabolic Role of Citrate*, Biochemical Symposium No. 27, Academic Press, New York.

Hartford, C. G. (1962) *Anal. Chem. 34*, 426–428.

Hine, J. (1975) *Structural Effects of Equilibria in Organic Chemistry*, pp 192–194, Wiley, New York.

Inoue, H., Tsunemi, T., Szuki, F., & Takeda, Y. (1969) *J. Biochem. (Tokyo) 65*, 889–900.

Kollonitsch, J., Marburg, S., & Perkins, L. M. (1975) *J. Org. Chem. 40*, 3808–3809.

Kollonitsch, J., Patchett, A. A., Marburg, S., Maycock, A. L., Perkins, L. M., Doldouras, G. A., Duggan, D. E., & Aster, S. D. (1978) *Nature (London) 274*, 906–908.

Kollonitsch, J., Marburg, S., & Perkins, L. M. (1979) *J. Org. Chem. 44*, 771–777.

Marletta, M. A., Srere, P. A., & Walsh, C. (1981) *Biochemistry 20*, 3719–3723.

Marletta, M. A., Cheung, Y.-F., & Walsh, C. (1982) *Biochemistry 21*, 2637–2644.

Peters, R. (1972) *Carbon-Fluorine Compds: Chem., Biochem. Biol. Act., Ciba Found. Symp., 1971*, pp 55–70, 95.

Schloss, J. V., Porter, D. J. T., Bright, H. J., & Cleland, W. W. (1980) *Biochemistry 19*, 2358–2362.

Silverman, R. B., & Abeles, R. H. (1976) *Biochemistry 15*, 4718–4723.

Singh, M., Carpenter, D. E., & Srere, P. A. (1974) *Biochem. Biophys. Res. Commun. 59*, 1211–1218.

Singh, M., Richards, E. G., Mukherjee, A., & Srere, P. A. (1976) *J. Biol. Chem. 251*, 2911–2915.

Srere, P. A. (1961) *J. Biol. Chem. 236*, 50–53.

Srere, P. A. (1975) *Adv. Enzymol. Relat. Areas Mol. Biol. 43*, 57–101.

Srere, P. A., & Kosicki, G. W. (1961) *J. Biol. Chem. 236*, 2557–2559.

Srere, P. A., Bottger, B., & Brooks, G. C. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1201–1202.

Sullivan, A., Singh, M., Srere, P. A., & Glusker, J. (1977) *J. Biol. Chem. 252*, 7583–7590.

Villafranca, J., & Platus, E. (1973) *Biochem. Biophys. Res. Commun. 55*, 1197–1207.

Walsh, C. T. (1970) Ph.D. Thesis, The Rockefeller University.

Walsh, C. T. (1978) *Annu. Rev. Biochem. 47*, 881–931.

Walsh, C. T. (1979) *Enzymatic Reaction Mechanisms*, Chapter 24, pp 777–827, W. H. Freeman, San Francisco.

Walsh, C. T., & Spector, L. B. (1969) *J. Biol. Chem. 244*, 4366–4374.