MOLECULAR PHARMACOLOGY, 14, 172-184

Molecular Toxicology of (−)-*erythro*-Fluorocitrate: Selective Inhibition of Citrate Transport in Mitochondria and the Binding of Fluorocitrate to Mitochondrial Proteins

EVA KIRSTEN, MANOHAR L. SHARMA, AND ERNEST KUN[1]

Departments of Pharmacology, Biochemistry and Biophysics, and Cardiovascular Research Institute, University of California, San Francisco, California 94143

(Received July 11, 1977)
(Accepted September 6, 1977)

SUMMARY

KIRSTEN, EVA, SHARMA, MANOHAR L. & KUN, ERNEST (1978) Molecular toxicology of (−)-*erythro*-fluorocitrate: selective inhibition of citrate transport in mitochondria and the binding of fluorocitrate to mitochondrial proteins. *Mol. Pharmacol.*, 14, 172-184.

The rate of entry of oxidizable carboxylic acids into the mitoplast compartment of lysosome- and microsome-free mitochondria was determined by monitoring substrate-dependent incorporation of ^{32}P-labeled orthophosphate into ADP to form ATP. The maximal rate of ATP synthesis that was dependent on tricarboxylic acids required the presence of (−)-*erythro*-fluoromalate, replacing the physiological activator L-malate. Previous incubation of mitochondria for 5–15 min (at 25–30°) with 50 pmoles of (−)-*erythro*-fluorocitrate per milligram of mitochondrial protein irreversibly and selectively inhibited citrate-supported ATP synthesis. The efflux of citrate from the mitoplast compartment of mitochondria was measured by incorporation of the acetyl residue of intramitochondrially generated citrate into fatty acids synthesized by the cytoplasmic enzyme system. Previous incubation of mitochondria for 15 min with quantities of (−)-*erythro*-fluorocitrate that inhibited citrate-dependent ATP synthesis also inhibited mitochondrial citrate-dependent fatty acid synthesis. The utilization of added citrate for fatty acid biosynthesis by the cytoplasmic system was not affected by fluorocitrate. Incubation of inner membrane vesicles with 3,4,5,6-^{14}C-labeled (−)-*erythro*-fluorocitrate resulted in the covalent binding of fluorocitrate to protein components of the membrane as determined by molecular filtration in 8 M guanidine hydrochloride and precipitation by 10% trichloracetic acid. The fluorocitrate-containing protein precipitate could be redissolved in 0.1 N NaOH at 0° and reprecipitated by trichloracetic acid. The bond between fluorocitric acid and protein was specifically cleaved by incubation with 0.4 M neutral hydroxylamine, but not by 1 mM *o*-phenanthroline. The hydroxamic acid of fluorocitrate, which was formed by treatment of protein-bound fluorocitrate with

This work was supported by Grants GM-20552 (60%) and Program Project HL-06285 (40%) from the National Institutes of Health. This paper is No. 15 in a series. The preceding paper is reference 12 of this paper. A preliminary report of this work was presented to the annual meeting of the American Society of Pharmacology and Experimental Therapeutics, Chicago [(1977) *Fed. Proc.*, 36, 3837]. A partial account of this work was presented to the International Symposium on Mechanisms of Proton and Calcium Pumps, Padova, September 10–14, 1977.

[1] Recipient of a Research Career Development Award from the United States Public Health Service; to whom requests for reprints should be addressed.

0026-895X/78/0141-0172$02.00/0

hydroxylamine, was detected by high-voltage electrophoresis and thin-layer chromatography. Binding of fluorocitrate to inner membrane vesicles dissolved in 4 M guanidine hydrochloride was prevented by previous incubation of membrane proteins with the organomercurial *o*-{[3-(hydroxymercuri)-2-methoxypropyl]carbamoyl}phenoxyacetic acid sodium salt. The covalent binding of (−)-*erythro*-fluorocitrate to membrane proteins was the result of an enzymatic process that was activated by Mn^{2+}. Oxidizable carboxylic acids did not form covalently bound adducts with inner membrane proteins. It was concluded that (−)-*erythro*-fluorocitrate specifically inhibited citrate transport by its covalent binding to two protein fractions associated with the mitoplast of liver, kidney, heart, and brain tissue.

INTRODUCTION

The identity of the biologically active isomer of fluorocitric acid as (−)-*erythro*-fluorocitrate has been established by enzymatic (1, 2) as well as chemical (3) syntheses, and its structure has been confirmed by X-ray diffraction analysis (4, 5). On the other hand, the exact mode of action of this neurotoxin (6–8) has remained unknown. Enzyme kinetic analyses with purified aconitase [citrate (isocitrate) hydrolase, EC 4.2.1.3] by several groups (9–13) indicated that (−)-*erythro*-fluorocitrate was a linearly competitive, reversible inhibitor of this enzyme with respect to citrate, with a K_i of 60–200 μM. The lethal dose of unresolved fluorocitrate was reported to be approximately 0.2–0.5 μg if injected into the third cerebral ventricle of an adult rat (14), corresponding to an estimated cerebral concentration of the active isomer (3) of less than 10 nM. When isolated mitochondria were incubated with 10–100 nM (−)-*erythro*-fluorocitrate, irreversible inhibition of citrate influx occurred, as monitored by subsequent efflux of isocitrate from the mitoplast compartment[2] (15–17). This effect on citrate transport *in vitro* appeared to be related more directly to the toxic action of fluorocitrate *in vivo* than the inhibition of aconitase. The controversy over the aconitase inhibition- or citrate transport-related mode of action of (−)-*erythro*-fluorocitrate was sustained by experiments performed with crude extracts of mitochondria (18). In crude systems inhibition of aconitase was observed at less than micromolar concentrations of fluorocitrate (18). However, these anomalous results can be attributed to the fortuitous inactivation of aconitase by added Mg^{2+} used in the enzyme assay, not to the enzyme-inhibitory effect of fluorocitrate alone (12, 17).

The present work is concerned with the mode of action of (−)-*erythro*-fluorocitrate on lysosome-free phosphorylating mitochondria (19). The concentration of fluorocitrate chosen was in the same order of magnitude as found to be present in brain mitochondria of rats lethally poisoned with fluoroacetate or fluorocitrate (20). It will be shown that 50 pmoles of (−)-erythro-fluorocitrate per milligram of mitochondrial protein selectively and irreversibly inhibited the mitochondrial metabolism of citrate. The degrees of inhibition of citrate-dependent ATP synthesis and of cytoplasmic fatty acid synthesis that was dependent on intramitochondrially generated citrate were identical. Since aconitase does not participate in the latter process, our results are consistent with the existence of an inhibitory site of (−)-*erythro*-fluorocitrate which is rate-limiting in citrate transport through the inner mitochondrial membrane (15–17). Enzymatically synthesized 3,4,5,6-^{14}C-labeled (−)-*erythro*-fluorocitrate formed a stable macromolecular adduct with inner membrane-associated proteins under the same conditions as those required to inhibit citrate transport.

MATERIALS AND METHODS

Preparation of lysosome-free mitochondria. This method is an improvement of the technique described earlier (19). Freshly removed tissues placed on wetted

[2] The mitoplast compartment is defined as the mitochondrial matrix surrounded by the inner mitochondrial membrane.

filter paper in an ice-cooled Petri dish were finely chopped with a razor blade prior to homogenization in 8 volumes of mannitol-sucrose-buffer medium (19) in a glass-Teflon Potter-Elvehjem grinder (clearance, 0.3 mm). The time of homogenization never exceeded 1–2 min with approximately five to eight slow strokes of the pestle. The coarse particles and nuclei were sedimented by one centrifugation at 3000 × g_{av} (5000 rpm, rotor SS-34, Sorvall refrigerated centrifuge) for 1.7 min. Trapped mitochondria were re-extracted from the sediment by repetition of this step. Mitochondria were sedimented by centrifugation for 5 min at 17,300 × g_{av} (12,000 rpm). The mitochondrial pellet was resuspended in 0.5 ml of medium per gram of original tissue and incubated with frequent stirring in an ice bath with 0.25 mg of digitonin per gram of original tissue weight. Lysosomes were dissolved during this step, while the mitoplast compartment of mitochondria remained intact (see ref. 19). The digitonin used was rendered apparently water-soluble (yielding a clear solution, stable for about 1 hr at room temperature) by three successive recrystallizations of commercial digitonin from hot ethanol (yield, 50%). After 15 min the mitochondrial suspension was diluted at least 10-fold with cold suspending medium. Mitochondria were resedimented by centrifugation at 17,300 × g_{av} for 5 min, then washed once by resuspension and centrifugal resedimentation. The yield of mitochondrial protein as determined by the biuret method ranged from 25 to 30 mg/g in liver and kidney, 15–20 mg/g in heart, and 10–15 mg/g in brain.

Preparation of 3,4,5,6-^{14}C-labeled (−)-erythro-fluorocitrate. [3,4,5,6-^{14}C](−)-*erythro*-Fluorocitrate was synthesized enzymatically with citrate synthase (EC 4.1.3.7) from fluoroacetyl-CoA and [*U*-^{14}C]oxalacetate generated from [*U*-^{14}C]malic acid in the presence of NAD^+ and malate dehydrogenase (EC 1.1.1.37) by a modification of the procedure developed for the enzymatic synthesis of unlabeled (−)-*erythro*-fluorocitric acid (2).

A mixture of sodium fluoroacetate (2.2 g) and phosphorus pentachloride (5.3 g) was distilled under anhydrous conditions, and the fraction that was distilled at 70–72° (755 mm Hg) was collected (yield, 2.0 g). This monofluoroacetyl chloride was converted to monofluoroacetic anhydride as follows: 1.2 g of the product of the preceding step were added dropwise to dry solid sodium fluoroacetate (1.5 g) in a Claisen flask over a period of 10 min. After refluxing for 1.5 hr at 110–120°, the monofluoroacetic anhydride was distilled at 88–90° (12 mm Hg) to yield 520 mg of product.

CoA (86 mg, 0.1 mEq) was dissolved in potassium bicarbonate (0.1 M, 0.5 ml) and chilled at 0°. Excess monofluoroacetic anhydride (14 mg, 0.12 mEq) in 1 ml of ethyl ether was added, and the mixture was shaken vigorously for 5 min. The pH was adjusted to 6.1 by adding solid potassium bicarbonate. After 1 hr the unreacted monofluoroacetic anhydride was extracted with four 1-ml portions of ether, and the remaining traces of ether were removed from the aqueous phase by a stream of nitrogen while the temperature was kept at 10°. Since monofluoroacetyl-CoA is unstable, it was immediately used for the next step. First, 200 μl of citrate synthase (4.8 mg of protein) and 200 μl of malate dehydrogenase (1 mg of protein) were desalted by centrifugal filtration through Sephadex G-50-loaded filter cones. Then 5 μmoles of L-[*U*-^{14}C]malic acid (50 mCi/mmole) were directly dissolved in the mixture of desalted enzymes; 7 μmoles of NAD^+ (in 70 μl, pH 7.0) and 4 μmoles of Tris-HEPES[3] buffer (in 50 μl, pH 8.3) were added, and the total volume was made up to 1 ml with H_2O washes. The reaction mixture, in a 10-ml glass centrifuge tube, was stirred magnetically at room temperature, and the solution of freshly prepared fluoroacetyl-CoA (350 μl) was added stepwise in batches of 50 μl. Fluorocitrate formation was monitored by spectrophotometric analyses for NADH in 5-μl aliquots of the reaction mixture. The reaction came to an end point in 1–2 hr. The reaction

[3] The abbreviations used are: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; FCCP, *p*-trifluoromethoxyphenylhydrazone of carbonyl cyanide.

mixture (final volume, 1.35 ml) was then applied to a small column (1 × 5 cm) of Dowex 50 (H^+) and eluted with distilled water. This procedure removed all enzyme proteins. The clear, acidic eluate (20 ml, containing 98% of the added radioactivity) was freeze-dried, redissolved in 2 ml of water, and treated with 200 mg of charcoal (acid-washed Norit) by stirring for 15 min at room temperature. About 90% of radioactive substances, together with material absorbing at 280 nm, was retained on charcoal. The filtrate, containing the remaining 10% of the radioactivity, was again stirred with 100 mg of charcoal, and the two batches of charcoal were combined. Radioactive carboxylic acids were extracted from charcoal with 2 ml of 2 M formic acid, whereas material absorbing at 280 nm was retained on the charcoal after filtration. This step was repeated four times with 2-ml batches of formic acid, and 95% of the radioactive material was recovered. Formic acid was removed by freeze-drying. The freeze-dried material (containing 2×10^8 cpm as ^{14}C) was redissolved in 1–1.5 ml of H_2O. Since this material also contained unreacted L-[*U*-^{14}C]malic acid (about 25%), fluorocitric acid was isolated by high-voltage electrophoresis on washed strips of Whatman No. 3 filter papers (23 × 57 cm) at 4000 V for 70 min at pH 1.85 and 23° (0.1 M acetate adjusted to pH 1.85 with formic acid). The clearly separated [3,4,5,6-^{14}C]fluorocitrate (as determined by radiochemical analysis of filter paper strips) was eluted with distilled water and concentrated by freeze-drying. The electrophoretically resolved (−)-*erythro*-fluorocitric acid was homogeneous as determined by thin-layer chromatography on silica gel plates with ammonia–isopropyl alcohol (1:10) and butanol–acetic acid–H_2O (4:1:1) as developing solvents. The specific radioactivity of (−)-[3,4,5,6-^{14}C]*erythro*-fluorocitrate was 50 mCi/mmole as calculated from the enzymatically determined quantity of fluorocitrate based on NADH analyses.

Carboxylic acid-dependent phosphorylation of ADP. Mitochondria (1–10 mg of protein per milliliter) suspended in a medium containing 100 mM sucrose, 25 mM KCl, 25 mM Tris-HCl buffer (pH 7.4), 20 mM $MgCl_2$, and 2 mM ADP were incubated in a metabolic shaker (with 70 oscillations/min) at 25° for 5–15 min in the presence and absence of 20–50 pmoles of (−)-*erythro*-fluorocitrate per milligram of mitochondrial protein. Aliquots (20–200 μl, to yield a final mitochondrial protein concentration of 0.1–0.2 mg/ml) were then transferred to vessels containing the same suspending medium without fluorocitrate but containing carboxylic acids and 3.0 mM $^{32}P_i$ (Na^+ salt) (approximately 2×10^5 cpm). Incubation was continued at 30°, and organically bound phosphate was determined (21) in 0.5-ml aliquots by scintillation spectrometry.

Cytoplasmic fatty acid synthesis depending on intramitochondrially generated citrate. Citrate formed intramitochondrially from [*U*-^{14}C]pyruvate, generated from [*U*-^{14}C]alanine and α-ketoglutarate, was coupled to the cytoplasmic fatty acid-synthesizing system of rat liver in a reconstructed system as described (22).

Digitonin fractionation of mitochondria and preparation of inner mitochondrial membrane. Mitoplasts were prepared from lysosome-free mitochondria by published procedures (23, 24). Since the recrystallized digitonin was twice as effective as commercial preparations for the isolation of mitoplasts, 0.6 mg of recrystallized digitonin per 10 mg of mitochondrial protein was used. Inner membrane vesicles were prepared from mitoplasts by disintegration of mitochondria by ultrasonic force (Branson Sonifier W-350) with six to eight bursts for 30 sec each (70 W) in sucrose–mannitol medium, cooled in a −10° ice-salt bath. After sedimentation of unbroken mitoplasts (10 min, 8000 rpm, SS-34 Sorvall refrigerated centrifuge), inner membrane fragments were sedimented at 145,000 × *g* for 1 hr (Spinco 50 Ti rotor at 2°). Soluble extracts from mitoplasts were prepared by stirring (at 0°) a suspension of mitoplasts (70–80 mg of protein per milliliter) in sucrose–mannitol (19) containing 1 mg of Brij-56 per 10 mg of protein for 15 min. Particles were removed by

centrifugation at 145,000 × *g* for 1 hr at 2°.

Molecular filtration. Inner mitochondrial membranes were dissolved in 8 M guanidine hydrochloride (containing 50 mM Tris-HCl, pH 7.2). The solution (1 ml) was placed on a Sephadex G-50 (medium) (1.5 × 50 cm) column and developed at room temperature with the same solvent. Molecular filtration of solubilized proteins of mitoplasts was performed at 4° on a Sephadex G-200 (fine) column (1.5 × 100 cm) with 50 mM Tris-HEPES buffer, pH 7.6. Molecular weight standards were ovalbumin, bovine serum albumin, and immunoglobulin. Acid-precipitable radioactive proteins were collected on glass fiber filters by suction filtration, and radioactivity was measured by scintillation spectrometry with toluene–Triton X-100–Omnifluor (2 liters, 1 liter, 18 g) or Aquasol scintillators.

Radioactive materials. L-[*U*-^{14}C]Malic acid, 50 mCi/mmole, was obtained from Amersham/Searle; citric [1,5-^{14}C]acid, 10 mCi/mmole, and Aquasol, from New England Nuclear; L-[*U*-^{14}C]alanine, 130 mCi/mmole, from Schwarz/Mann; and carrier-free $^{32}P_i$, from ICN Radiochemicals Company.

Other reagents. Citrate synthase (EC 4.1.3.7) from pig heart, coenzyme A, oligomycin, monofluoroacetic acid, Brij-56 (polyoxyethylene 10-cetyl ether), Triton X-100, and mersalyl (*o*-{[3-(hydroxymercuri)-2-methoxypropyl]carbamoyl}phenoxyacetic acid sodium salt) were purchased from Sigma Chemical Company; malate dehydrogenase (EC 1.1.1.37) from pig heart, from Boehringer/Mannheim; ADP and NAD^+, from Pabst Laboratories; and proteinase K, from Beckman Company. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was a gift from Dr. P. Heytler (du Pont Research Laboratories). Other reagents used were of analytical grade. (−)-*erythro*-Fluoromalate and nonradioactive (−)-*erythro*-fluorocitrate were synthesized by published methods (3, 25). Analytical methods were the same as described earlier (see ref. 19).

RESULTS

Effects of prior incubation of mitochondria with (−)-erythro-fluorocitrate and with inhibitors of oxidative phosphorylation on citrate-dependent ATP synthesis. Added citrate alone supported a slow rate of ATP synthesis, which was only slightly above the variable rates maintained by trace amounts of substrates present in isolated liver mitochondria (Table 1, experiments 1 and 2). Addition of 0.5 mM (−)-*erythro*-fluoromalate had no effect on the rate of ATP synthesis (Table 1, experiment 3). The observed rate was of the same order of magnitude as maintained by previously existing substrates in liver mitochondria. The fluoro analogue of L-malate was not oxidized by mitochondria at a detectable rate but was able to activate the transport of tricarboxylic acids

TABLE 1

Effects of (−)-erythro-fluoromalate, inhibitors of ATP synthesis, and prior incubation with (−)-erythro-fluorocitrate on citrate-dependent synthesis of ATP by liver mitochondria

Oligomycin and FCCP were present in both the preliminary (15 min) and final incubation systems (see MATERIALS AND METHODS), but fluorocitrate was added only to the preliminary incubation system. In all reaction media, 20 mM Mg^{2+} was present. Preliminary incubation was caried out at 25°, and final incubation, at 30°. The first set of numbers in parentheses indicates the range of experimental values, and the numbers in the second parentheses indicate the number of experiments performed for each set.

Carboxylic acids and inhibitors	ATP formed
	μmoles/15 min/mg protein
1. No substrate added	0.10 (0.05–0.2) (3)
2. 5 mM citrate	0.32 (0.25–0.58) (5)
3. 0.5 mM (−)-*erythro*-fluoromalate	0.10 (0.06–0.15) (5)
4. 5 mM citrate + 0.5 mM fluoromalate	1.8 (1.30–2.46) (5)
5. 5 mM citrate + 0.5 mM fluoromalate + oligomycin (3 μg/mg of protein)	0.20 (0.15–0.25) (3)
6. 5 mM citrate + 0.5 mM fluoromalate + FCCP (50 pmoles/mg of protein)	0.19 (0.16–0.22) (3)
7. 5 mM citrate + 0.5 mM fluoromalate, prior incubation with (−)-*erythro*-fluorocitrate (50 pmoles/mg of protein)	0.25 (0.18–0.30) (5)

(25) in the same manner as described for other dicarboxylic acids (26). The metabolic inertness of (−)-*erythro*-fluoromalate gave it a significant experimental advantage over L-malate in the assessment of the rate of penetration of tricarboxylic acids, because no correction for L-malate-supported ATP synthesis was necessary with (−)-*erythro*-fluoromalate as an activator (17). When citrate and (−)-*erythro*-fluoromalate were present simultaneously, a large increase in the rate of ATP synthesis occurred, indicating that citrate penetrated the inner mitochondrial membrane and could serve as a substrate of oxidative phosphorylation (Table 1, experiment 4). Known inhibitors of oxidative phosphorylation, oligomycin and FCCP, effectively abolished the (citrate + fluoromalate)-supported ATP synthesis (Table 1, experiments 5 and 6), providing evidence that citrate-dependent ATP synthesis was due predominantly to oxidative phosphorylation. When mitochondria were incubated with 50 pmoles of (−)-*erythro*-fluorocitrate prior to the addition of citrate (see MATERIALS AND METHODS), 77–90% inhibition of citrate-dependent ATP synthesis followed (Table 1, experiment 7). The requirement for prior incubation of isolated mitochondria with (−)-*erythro*-fluorocitrate was stringent. Simultaneous incubation of 5 mM citrate and 100 nM (−)-*erythro*-fluorocitrate (equivalent to 50 pmoles/mg of mitochondrial protein) delayed the onset of inhibition for the experimental period used (15 min). The protective role of citrate against inhibition by (−)-*erythro*-fluorocitrate was specific; no other carboxylic acid, including (−)-*erythro*-fluoromalate, could delay the inhibitory effect of (−)-*erythro*-fluorocitrate when incubated simultaneously with fluorocitrate. The time of incubation with 50 pmoles of (−)-*erythro*-fluorocitrate per milligram of protein that was necessary to achieve complete inhibition of citrate metabolism varied between 5 and 15 min (at 30°), depending on the quantity of mitochondrial protein used, suggesting a reaction between (−)-*erythro*-fluorocitrate and mitochondrial material. The approximately inverse relationship found between the quantity of (−)-*erythro*-fluorocitrate used (between 20 and 50 pmoles/mg of mitochondrial protein) and the period of prior incubation required to achieve substantial (75–95%) inhibition of citrate metabolism supported this assumption.

Identical experimental results (Table 1) were obtained with brain and kidney mitochondria. However, heart mitochondria did not exhibit (citrate + fluoromalate)-supported ATP synthesis (see Fig. 1), suggesting that no appreciable influx of citrate occurred in isolated heart mitochondria. The absence of citrate influx into heart mitochondria was reported (27) using an entirely different technique (28–30), and it was concluded that heart mitochondria possess a system for citrate export but not import (see also Fig. 1F).

Substrate specificity of inhibition of carboxylic acid-dependent ATP synthesis. The time course of ATP synthesis supported by various carboxylic acids is illustrated in Fig. 1. (−)-*erythro*-Fluoromalate was a necessary activator for citrate-, *cis*-aconitate-, and isocitrate-supported ATP synthesis, but only the citrate-supported reaction was inhibited by prior incubation of mitochondria with 50 pmoles of (−)-*erythro*-fluorocitrate. It was also apparent that the inhibition of citrate-dependent ATP synthesis by (−)-*erythro*-fluorocitrate was irreversible. These results were inconsistent with the assumption that the inhibition of citrate metabolism may be due to the effect of (−)-*erythro*-fluorocitrate on aconitase, because inhibition of aconitase was reversible by citrate (12).

Inhibition of extramitochondrial fatty acid synthesis dependent on citrate synthesized in mitochondria. This technique was chosen for the determination of citrate efflux because the experimental design closely resembled existing intracellular metabolic conditions. The incorporation of the acetyl residue of intramitochondrially generated citrate into fatty acids was inhibited after mitochondria had been incubated with 50 pmoles of (−)-*erythro*-fluorocitrate prior to the initiation of citrate-dependent fatty acid biosynthesis in the reconstructed system (Table 2). The magnitude of inhibition of fatty acid synthesis

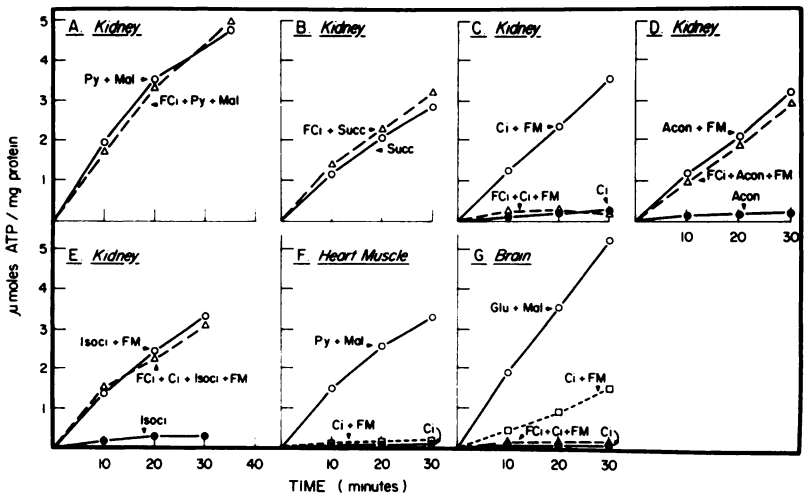

FIG. 1. *Substrate specificity of inhibitory effect of (−)-erythro-fluorocitrate*

Mitochondria of kidney, heart, and brain were incubated with 2 mM ADP and 50 pmoles of (−)-*erythro*-fluorocitrate per milligram of protein for 15 min at 30° prior to measurement of ATP synthesis with various oxidizable substrates, using 0.5 mM (−)-*erythro*-fluoromalate as activator of tricarboxylic acid influx as described in MATERIALS AND METHODS. The concentration of substrates was 5 mM. Mitochondria from kidney and brain were isolated as described in the text. For the isolation of heart mitochondria, the procedure was modified as follows. The finely chopped rat hearts were ground in a glass-Teflon homogenizer with a clearance of 0.15 mm in 8 volumes of medium per gram of tissue at 0°, and coarse particles were sedimented at 2700 rpm (Sorvall rotor SS 34) for 1 min. The nuclear pellet was re-extracted once. The combined supernatants were processed further as described in the text. The rate of substrate-dependent ATP synthesis was measured as described in the text. Py, pyruvate; Mal, L-malate; FCi, (−)-*erythro*-fluorocitrate; Succ, succinate; Ci, citrate; FM, (−)-*erythro*-fluoromalate; Acon, *cis*-aconitate; Isoci, D,-*threo*-isocitrate; Glu, glutamate.

was nearly identical with the inhibition of citrate-supported ATP synthesis (compare 70–90% inhibition by 50 pmoles of fluorocitrate per milligram of mitochondrial protein in Table 2 with 77–93% inhibition by 50 pmoles of fluorocitrate per milligram of mitochondrial protein in Table 1 and Fig. 1). The incorporation of added citrate into fatty acids by the same cytoplasmic system used in the experiments shown in Table 2 was not inhibited even by very high concentrations of (−)-*erythro*-fluorocitrate. The rate of citrate incorporation of fatty acids in 15 min at 37° was 20.9 nmoles, and 21.9 in the presence of 50 μM (−)-*erythro*-fluorocitrate with 5 mM citrate (0.3 mCi of [*U*-^{14}C]citrate per millimole) as added substrate.

Association of (−)-erythro-fluorocitrate with inner membrane proteins. Incubation of purified mitochondria for 10–15 min (pH 7.4, 25–30°) with [3,4,5,6-^{14}C](−)-*erythro*-fluorocitrate at concentrations that produced irreversible inhibition of both influx and efflux of citrate through the inner mitochondrial membrane (see Tables 1 and 2 and Fig. 1) resulted in a stable association of fluorocitrate with inner membrane proteins. When inner membrane vesicles were isolated (see MATERIALS AND METHODS) after incubation of mitochondria with 0.5 μM labeled fluorocitrate, they contained 8–16 pmoles of fluorocitrate per milligram of protein. The nature of the binding of fluorocitrate to proteins was further studied with isolated inner membrane vesicles. From a suspension of inner membrane vesicles incubated (pH 7.2, 30°) for 60 min with 10 μM labeled fluorocitrate in 50 mM Tris-HCl buffer, protein-bound fluorocitrate was readily isolated by molecular filtration on Sephadex G-50 after denaturation of membrane proteins in 8 M guanidine hydrochloride as shown in Fig. 2. The amount of bound fluorocitrate found in the macromolecular

TABLE 2

Inhibition of mitochondrial citrate-dependent cytoplasmic fatty acid synthesis by (−)-erythro-fluorocitrate

Liver and kidney mitochondria from fasted rats (see MATERIALS AND METHODS) were first incubated at 25° in sucrose-mannitol medium for 15 min at a protein concentration of 10 mg/ml, with or without (−)-*erythro*-fluorocitrate (50 pmoles/mg of protein). Aliquots of 300 μl (3 mg of protein) were transferred to incubation vessels containing the cytoplasmic extract of liver homogenates (22) and a substrate-coenzyme mixture in a total volume of 1 ml. Final concentrations of components were: $KHCO_3$, 22 mM; dithiothreitol, 9 mM; $MgCl_2$, 12 mM; L-malate, 4 mM; α-ketoglutarate, 4 mM; L-[*U*-^{14}C]alanine, 6 mM (2×10^5 cpm/μmole); ATP, 2 mM; glucose 6-phosphate, 4 mM; glycylglycine buffer, pH 7.4, 12.5 mM; sucrose, 50 mM; CoA, 0.3 mM; NAD^+, 0.13 mM; $NADP^+$, 0.6 mM; and cytoplasmic extract of rat liver (activated by incubatin with 12 mM dithiothreitol for 30 min at 37°), 4 mg of protein per milliliter. Incubations were carried out at 37° in a metabolic shaker for 15 min. The reaction was terminated by addition of KOH, and fatty acids were extracted after saponification (22) and determined by scintillation spectrometry.

Preparation	Citrate incorporated into fatty acids	
	Control	Fluorocitrate
	nmoles/15 min	
Liver mitochondria + cytoplasmic extract of liver	1.3 (0.9–1.8) (4)	0.32 (0.20–0.52) (4)
Kidney mitochondria + cytoplasmic extract of liver	0.95 (0.80–1.05) (3)	0.25 (0.21–0.30) (3)

fraction was 13.5 pmoles/mg of protein. Increased association of fluorocitrate with isolated inner membrane vesicles (2 mg of protein) occurred when the concentration of fluorocitrate was raised to 50–100 μM. The concentration of fluorocitrate used in the experiments summarized in Table 3 was 89 μM. The same quantities of protein-bound fluorocitrate were isolated either by molecular filtration in 8 M guanidine hydrochloride or by repeated precipitation with trichloracetic acid (experiments 1 and 2 in Table 3). Removal of guanidine hydrochloride by extensive dialysis (against 2 liters of distilled H_2O at 4° for 2 hr) resulted in the precipitation of membrane proteins, but in no loss of protein-bound fluorocitrate. The reactions leading to the stable binding of fluorocitrate to proteins took place in solutions of guanidine hydrochloride up to 4 M (at pH 7.4) (Table 3, experiment 3). Concentrations of guanidine hydrochloride above 4 M completely inhibited binding. Incubation of protein-bound fluorocitrate containing inner membrane vesicles, dissolved in 4 M guanidine hydrochloride, with 0.4 M hydroxylamine at pH 7.4 (1 hr at 37°) released 70% of bound fluorocitrate (experiment 4), whereas no loss of bound fluorocitrate occurred without hydroxylamine (experiment 5) or in the presence of 1 mM *o*-phenanthroline (experiment 6). The thioester bond of succinyl-CoA was almost completely (95%) cleaved by 0.4 M hydroxylamine under the conditions of experiment 4. The ineffectiveness of *o*-phenanthroline indicated that the binding of fluorocitrate was not due to complex formation with Fe^{2+} at an iron-protein site. This possibility was ruled out by the acid stability of the protein-fluorocitrate bond. Prior incubation of membrane vesicles, dissolved in 4 M guanidine hydrochloride, with 1 mM mersalyl inhibited the binding of fluorocitrate to proteins (experiment 7), while this treatment without mersalyl did not interfere with maximal binding (experiment 8). None of the oxidizable carboxylic acids of the citric acid cycle formed stable adducts with membrane proteins under the conditions shown in Table 3.

Inhibition of the binding of fluorocitrate to proteins by prior treatment with mersalyl—a reasonably specific reagent for thiol groups of proteins—and the release of bound fluorocitrate by neutral hydroxylamine suggested the possibility of the formation of a macromolecular thioester of fluorocitrate. The protein-fluorocitrate adduct was precipitated with 10 ml of 10% perchloric acid at 0° and washed free of unreacted fluorocitrate by four successive centrifugations at 4° with four 10-ml portions of 5% perchloric acid. The product, 5.5 mg of protein, contained 155 pmoles of bound fluorocitrate. After freeze-drying, the material was resuspended in 2 ml of a solution of hydroxylamine (625 μmoles) in methanol (31) and sonicated with a Bran-

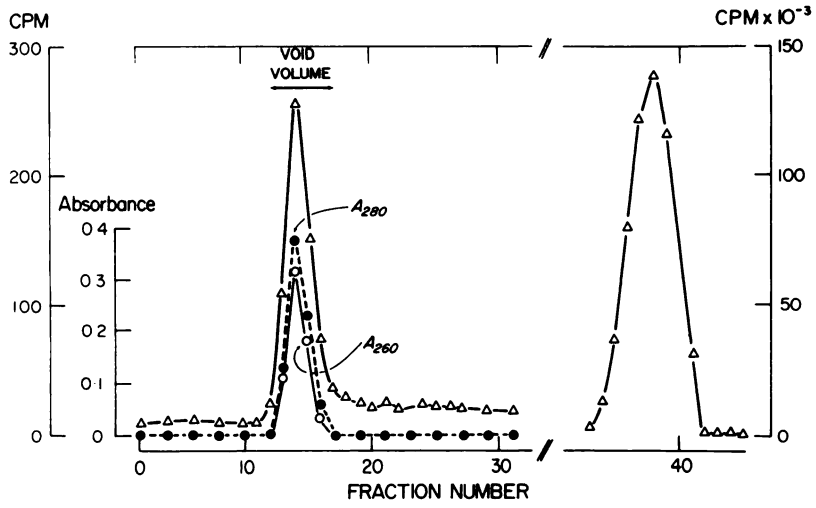

FIG. 2. *Gel filtration of inner membrane proteins labeled with fluorocitrate in the presence of guanidine hydrochloride*

Membrane vesicles (2 mg of protein) were incubated with [^{14}C]fluorocitrate (10 μM; total volume, 200 μl) for 60 min at 30° in 50 mM Tris-HCl buffer, pH 7.2. An aliquot of the incubation mixture (1 mg of protein) was solubilized in 8 M guanidine HCl and 50 mM Tris-HCl, pH 7.2, and applied to a Sephadex G-50 (medium) column (0.95 × 50 cm), equilibrated and developed at room temperature with the same solvent. Fractions (1.6 ml) were collected at a flow rate of 6.5 ml/hr. Absorbances were recorded at 280 nm (●- - -●) and at 260 nm (○——○). Radioactivity (counts per minute) was plotted after subtracting the background (△——△). Gel filtration of macromolecules lasted 14 hr. The first radioactive peak contained fluorocitrate bound to protein (void peak, "CPM" on left ordinate); the second large radioactive fraction was unreacted fluorocitrate ("CPM × 10^{-3}" on right ordinate). It was found in control experiments that there was no measurable nonspecific covalent binding of fluorocitrate to nonmitochondrial proteins (e.g., bovine serum albumin) as tested by molecular filtration or acid precipitation.

son Sonifier (microtip at 50-W output) for 30 successive bursts, each lasting 1 min, in a cooling bath of Dry Ice–acetone. The precipitate, containing 95 pmoles of bound fluorocitrate, was separated by centrifugation, and the clear supernatant after evaporation to dryness was taken up in 50 μl of H_2O and analyzed chromatographically on silica gel (100 μm) thin-layer plates with a developing solvent composed of butanol–acetic acid–H_2O (4:1:1). Authentic labeled fluorocitric acid remained at the origin, whereas the hydroxylamine derivative of fluorocitric acid moved from the origin with an R_F of 0.17. The hydroxylamine derivative of the protein-fluorocitrate covalent compound gave a purple spot test when sprayed with methanolic $FeCl_3$ reagent (31), as is characteristic for hydroxamates. The hydroxamate was also analyzed by high-voltage electrophoresis at pH 1.85 (see MATERIALS AND METHODS). The hydroxamate separated according to its predictably weaker anionic net charge as compared with fluorocitrate itself (Fig. 3).

Incubation of the protein-fluorocitrate adduct at 37° for 1 hr in 0.1 N NaOH resulted in 25% hydrolysis, consistent with known properties of esters. The apparent relatively high stability of the fluorocitrate-protein adduct to 0.1 N NaOH coincided with a concomitant loss of sensitivity of this bond to neutral hydroxylamine (from 70% to 10%), indicating a base-catalyzed rearrangement to a more stable *N*-fluorocitryl-protein compound. We have observed an SN_i type of rearrangement with the succinyl thioester of cysteamine to the stable *N*-succinylcysteamine which occurred above pH 4.5 (33).

Separation of fluorocitrate-labeled proteins by molecular filtration in the absence of denaturing agents. Incubation of an

TABLE 3

Stability of protein-fluorocitrate adduct

In experiments 1 and 2, 89 μM labeled fluorocitrate was incubated for 60 min with 2 mg of membrane vesicles in 50 mM Tris-HEPES buffer, pH 7.4, at 30° in a volume of 200 μl. In experiment 3, 4 M guanidine HCl was present. In experiments 4–6, labeled membrane vesicles were prepared as in experiment 3. Details of experiments are described under RESULTS.

Conditions and procedures	Protein-bound fluorocitrate
	pmoles/mg protein
1. Isolation by molecular filtration in 8 M guanidine HCl	18.3
2. Isolation by precipitation with 10% TCA, a redissolution in 0.1 N NaOH, reprecipitation with 5% TCA	18.6
3. Binding of fluorocitrate (89 μM) in 4 M guanidine HCl for 60 min at 30°; isolation by molecular filtration in 8 M guanidine HCl	18.8
4. Incubation of fluorocitrate labeled vesicles in 4 M guanidine HCl in 0.4 M NH_2OH at pH 7.4, 37°, for 1 hr. Isolation by molecular filtration in 8 M guanidine HCl	6.2
5. Same as experiment 4, except in the absence of NH_2OH	18.8
6. Same as experiment 4, except 0.4 M NH_2OH was replaced by 1 mM *o*-phenanthroline	17.8
7. Incubation of vesicles in 4 M guanidine HCl with 1 mM mersalyl for 15 min, pH 7.4, at 30°, followed by incubation with 89 μM fluorocitrate for 60 min, pH 7.4, at 30° and isolation of protein-fluorocitrate adduct by molecular filtration in 8 M guanidine HCl	4.4
8. Same as experiment 7, except no mersalyl present in first procedure	18.2

extract (4.8 mg of protein) of liver mitoplasts prepared in the presence of Brij-56 (see MATERIALS AND METHODS) with 100 μM [3,4,5,6-^{14}C]fluorocitrate for 15 min at 30° resulted in the binding of 1% of added fluorocitrate to proteins (12 pmoles/mg of protein). The protein solution was directly applied to a column of Sephadex G-200 and developed with 50 mM Tris-HEPES buffer at 4° (pH 7.4) containing no added detergent (see MATERIALS AND METHODS). Acid-precipitable, protein-bound fluorocitrate and total proteins were determined in fractions collected at 4°. Two distinct protein peaks, containing acid-precipitable, protein-bound fluorocitrate, were separated, one with a molecular weight of 175,000, the second with mol wt 71,500 (Fig. 4), indicating that the stable association of fluorocitrate with extractable membrane proteins was not random, but apparently confined to two distinct macromolecular species.

Catalytic nature of fluorocitrate binding to membrane proteins. When a solution of proteins extracted from mitoplasts with Brij-56 (see MATERIALS AND METHODS) was incubated with various concentrations of labeled fluorocitrate for 80 min at 30° and pH 7.39, highly reproducible rates of binding were observed (Fig. 5), which showed a dependence on the concentration of fluorocitrate. At a fixed concentration (100 μM) of fluorocitrate binding was linear with

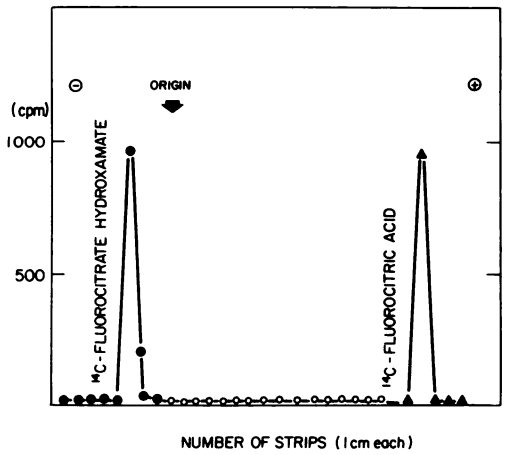

FIG. 3. *Electrophoretic separation at pH 1.85 of an aliquot of (−)-erythro-fluorocitric acid and its hydroxamate derivative isolated from the fluorocitrate-protein adduct*

The time of electrophoresis was 150 min. The technique of electrophoresis is described in MATERIALS AND METHODS, and details of isolation, in RESULTS. The amounts of fluorocitrate and its hydroxamate corresponded to 113 pmoles (1000 cpm). The fluorocitrate hydroxamate migrated slowly toward the cathode, whereas fluorocitric acid migrated toward the anode (for acid-base properties of fluorocitric acid, see ref. 32).

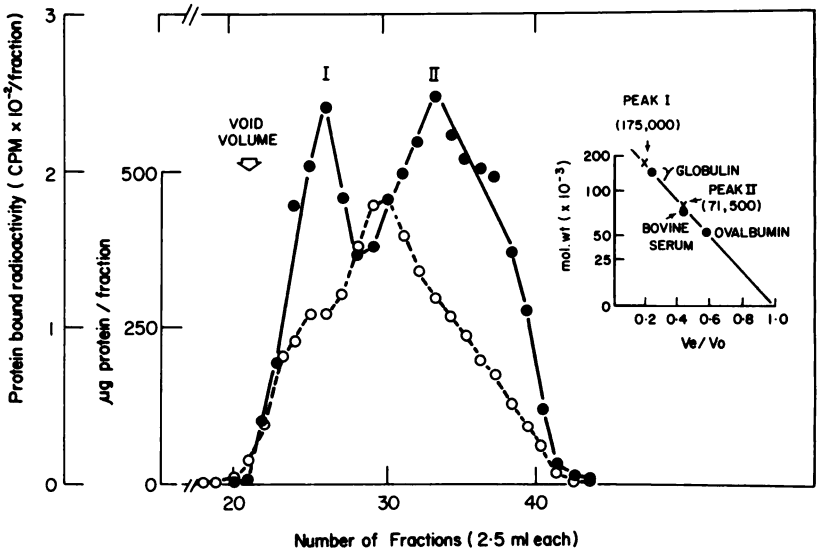

FIG. 4. *Molecular filtration of protein-bound fluorocitrate on Sephadex G-200 in the absence of detergent*

An extract of mitoplasts (in the presence of Brij-56) was concentrated by cone filtration and incubated in a volume of 300 μl (4.8 mg of protein) for 60 min with 100 μM labeled fluorocitrate at 30° and pH 7.39. The solution was directly applied to a Sephadex G-200 column (see MATERIALS AND METHODS) and developed at 4° for 12 hr with 50 mM Tris-HEPES buffer, pH 7.4. Total protein (O- - -O) and radioactive fluorocitrate-containing protein (●——●) were determined in each fraction. Protein-bound fluorocitrate was assayed as described in the legend to Fig. 5. The molecular weights of radioactive fluorocitrate-containing proteins were estimated from a semilogarithmic plot (inset) by comparison with standards.

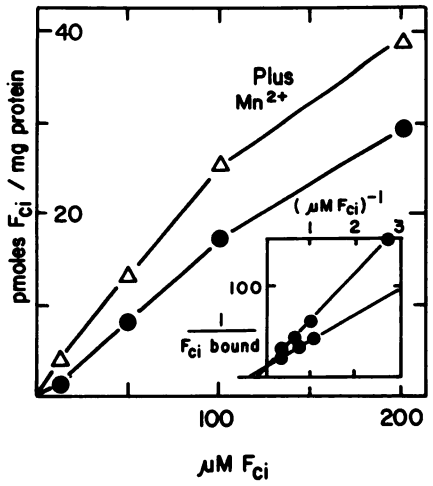

FIG. 5. *Kinetics of binding of fluorocitrate (F_{ci}) to proteins*

The binding of various concentrations of labeled fluorocitrate (between 0 and 200 μM) was determined by incubation of 0.7 mg of membrane protein with fluorocitrate at 30° for 80 min in 50 mM Tris-HEPES buffer, pH 7.39, in the absence and presence of 0.5 mM $MnCl_2$ in a final volume of 50 μl. The reaction was stopped with 2 ml of 10% trichloracetic acid (at 0°), and the precipitated protein, after centrifugation, was redissolved in 0.2 ml of 0.1 N NaOH. After 5 min at 4° the protein-bound fluorocitrate was reprecipitated from 0.1 N NaOH with 20 ml of 5% trichloracetic acid; the finely dispersed protein precipitate was isolated by glass fiber filtration, washed with 30 ml of 5% trichloracetic acid, and, after drying, analyzed for fluorocitrate by scintillation spectrometry (see MATERIALS AND METHODS). In the inset, the ordinate represents the reciprocal of bound fluorocitrate (picomoles per milligram of protein) and the abscissa shows the reciprocal of micromolar concentration of fluorocitrate. The lower curve in the inset represents results in the presence of 0.5 mM Mn^{2+}.

time for at least 2-3 hr. At a fixed time the rates were directly proportional to the concentration of protein in the system. Added Mn^{2+} (at 0.5 mM) augmented binding, an ionic effect that was specific for Mn^{2+}. Half-maximal binding of fluorocitrate occurred at a concentration of 200 μM in the presence of Mn^{2+}, and this value was 400 μM without added Mn^{2+}. The maximal binding of 80 pmoles of fluorocitrate per

milligram of protein was not influenced by Mn^{2+} (Fig. 5, inset). The catalytic binding of fluorocitrate to proteins of the inner mitochondrial membrane was inhibited by mersalyl and other organomercurial compounds, consistent with results shown in Table 3, experiment 7. The catalytic binding of fluorocitrate to mitochondrial proteins was delayed when citrate at millimolar concentrations was present simultaneously. The binding of fluorocitrate was specific for mitochondrial proteins.

DISCUSSION

A new site of action of (−)-*erythro*-fluorocitrate has been identified, which appears to be confined to citrate transport (15–17). Removal of cytoplasmic aconitase (34) by a technique (19) that also eliminates lysosomes and microsomes (35) resulted in the absolute specificity of action of fluorocitrate for citrate transport. Earlier results (16) indicated that the inhibition of isocitrate efflux in mitochondria contaminated by cytoplasmic aconitase occurred not only with citrate as an externally added substrate but also with *cis*-aconitate. In contrast to the inhibitory effect of (−)-*erythro*-fluorocitrate on aconitase, which reflects only the substrate to inhibitor ratio, inhibition of citrate transport depends on the formation of covalent adducts between two mitochondrial proteins and fluorocitrate. The reaction leading to the formation of protein-fluorocitrate adducts is enzymatic, and its rate is modified by previously existing citrate in the system, explaining the failure to demonstrate inhibition of citrate efflux from citrate-loaded mitochondria (18).

The irreversible inhibition of citrate transport may have important metabolic consequences for cellular processes that depend on acetyl-CoA generated from mitochondrial citrate (36). The neurotoxicity of fluorocitrate could be related to predictable inhibition of the synthesis of acetylcholine in nerve cells, a reaction known to depend on mitochondrial citrate as a source of acetyl-CoA (37). It seems more plausible to correlate the irreversible inhibition of citrate transport with the toxic action of fluorocitrate than with the reversible inhibition of aconitase by fluorocitrate. Accumulation of citrate—as known to occur in fluoroacetate poisoning—is not likely to have an effect on the chemically modified citrate transport system, but would displace the inhibition of aconitase according to the known competitive inhibitory mechanism for the enzyme (9, 12).

Detection of a hydroxylamine derivative of fluorocitrate, which was derived from the covalently bound protein-fluorocitrate adduct under conditions characteristic for the formation of carboxylic acid hydroxamates, constitutes a provisional identification of protein-fluorocitrate thioesters. Determination of the precise structure of the macromolecular thioesters requires further studies because it is possible that either one or both carboxyl groups of fluorocitrate, which are activated by fluorine substitution (32), may participate in macromolecular thioester formation. Our results also suggest that a limited amount of base-catalyzed transacylation (38) to *N*-fluorocitryl adducts can occur in proteins. The catalytic activities of the two proteins that form thioesters with fluorocitrate are described elsewhere (39).

The possibility that binding of fluorocitrate to proteins could involve defluorination has been studied with purified cytoplasmic aconitase of heart (33). Partial defluorination of fluorocitrate by aconitase in the presence of added cysteine and Fe^{2+} yielded hydroxycitrate, which was readily separated from protein by molecular filtration, in sharp contrast to the results reported here. We observed that a limited degree of defluorination of fluorocitrate to hydroxycitrate did occur when mitochondria were incubated with fluorocitrate. Electrophoretic reisolation of fluorocitrate from this incubation system revealed the appearance of traces of hydroxycitrate, which at pH 1.85 separated from unreacted fluorocitrate. However, the formation of hydroxycitrate from fluorocitrate is a separate reaction and bears no apparent relationship to the formation of fluorocitryl thioesters of two specific proteins of mitoplasts.

REFERENCES

1. Fanshier, D. W., Gottwald, L. K. & Kun, E. (1962) *J. Biol. Chem.*, 237, 3588–3596.

2. Fanshier, D. W., Gottwald, L. K. & Kun, E. (1964) *J. Biol. Chem.*, 239, 425–433.
3. Dummel, R. J. & Kun, E. (1969) *J. Biol. Chem.*, 244, 2966–2969.
4. Carrell, H. L., Glusker, J. P., Villafranca, J. J., Mildvan, A. S., Dummel, R. J. & Kun, E. (1970) *Science*, 170, 1412–1414.
5. Carrell, H. L. & Glusker, J. P. (1973) *Acta Crystallogr. Sect. B*, 29, 674–682.
6. Chenoweth, M. B. (1949) *Pharmacol. Rev.*, 1, 383–424.
7. Pattison, F. L. M. & Peters, R. A. (1966) in *Handbook of Experimental Pharmacology*, Vol. 20, pp. 387–458, Springer, New York.
8. Peters, R. A. (1969) *Br. Med. Bull.*, 25, 223–226.
9. Villafranca, J. J. & Mildvan, A. S. (1971) *J. Biol. Chem.*, 239, 2360–2364.
10. Gawron, O., Waheed, A., Glaid, A. J., III & Jaklitsch, A. (1973) *Biochem. J.*, 139, 709–714.
11. Gawron, O., Kennedy, M. C. & Rauner, R. A. (1974) *Biochem. J.*, 143, 49–53.
12. Eanes, R. Z. & Kun, E. (1974) *Mol. Pharmacol.*, 10, 130–139.
13. Villafranca, J. J. (1974) *J. Biol. Chem.*, 249, 6149–6155.
14. Morselli, P. L., Garattini, S., Marcucci, F., Mussini, E., Rewersky, W., Valzelli, L. & Peters, R. A. (1968) *Biochem. Pharmacol.*, 17, 195–202.
15. Kun, E. (1972) *Ciba Found. Symp. Carbon Fluorine Compounds*, 70–76.
16. Eanes, R. Z., Skilleter, D. N. & Kun, E. (1972) *Biochem. Biophys. Res. Commun.*, 46, 1618–1622.
17. Kun, E. (1976) in *Biochemistry Involving Carbon-Fluorine Bonds* (Filler, R., ed.), pp. 1–22, American Chemical Society, Washington, D. C.
18. Brandt, M. D., Evans, S. M., Mendes-Morao, J. & Chappell, J. B. (1973) *Biochem. J.*, 134, 217–224.
19. Kun, E. (1976) *Biochemistry*, 15, 2328–2336.
20. Gal, E. M. (1972) *Ciba Found. Symp. Carbon Fluorine Compounds*, 77–99.
21. Sugino, Y. & Myoshi, Y. (1964) *J. Biol. Chem.*, 239, 2360–2364.
22. Watson, J. A. & Lowenstein, J. M. (1970) *J. Biol. Chem.*, 245, 5993–6002.
23. Schnaitman, C. & Greenawalt, H. N. (1968) *J. Cell Biol.*, 38, 158–175.
24. Skilleter, D. N. & Kun, E. (1972) *Arch. Biochem. Biophys.*, 152, 94–104.
25. Skilleter, D. N., Dummel, R. J. & Kun, E. (1972) *Mol. Pharmacol.*, 8, 139–149.
26. Chappell, J. B. (1968) *Br. Med. Bull.*, 24, 150–158.
27. Cheema-Dhadli, S., Robinson, B. H. & Halperin, M. L. (1976) *Can. J. Biochem.*, 54, 561–565.
28. Robinson, B. H., Williams, R. G., Halperin, M. L. & Lezenoff, C. C. (1970) *Eur. J. Biochem.*, 15, 263–272.
29. Robinson, B. H., Williams, R. G., Halperin, M. L. & Lezenoff, C. C. (1971) *Eur. J. Biochem.*, 20, 65–71.
30. Robinson, B. H., Cheema-Dhadli, S. & Halperin, M. L. (1975) *J. Biol. Chem.*, 250, 3639–3643.
31. Knappe, E. & Yekundi, K. G. (1964) *Z. Anal. Chem.*, 203, 87–92.
32. Kun, E. & Dummel, R. J. (1969) *Methods Enzymol.*, 13, 624–672.
33. Villafranca, J. J. & Platus, E. (1973) *Biochem. Biophys. Res. Commun.*, 55, 1197–1207.
34. Eanes, R. Z. & Kun, E. (1971) *Biochim. Biophys. Acta*, 227, 204–210.
35. Kun, E., Kirsten, E. & Piper, W. N. (1978) *Methods Enzymol.*, in press.
36. Srere, P. A. (1965) *Nature*, 205, 766–770.
37. Lipton, M. A. & Barron, E. S. G. (1946) *J. Biol. Chem.*, 166, 367–380.
38. Minaga, T., Sharma, M. L., Kun, E. & Piper, W. N. (1977) *Biochim. Biophys. Acta*, in press.
39. Kun, E., Kirsten, E. & Sharma, M. L. (1977) *Proc. Natl. Acad. Sci. U. S. A.*, in press.