

Fluorocitrate Inhibition of Aconitate Hydratase and the Tricarboxylate Carrier of Rat Liver Mitochondria

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1. The effect of biologically synthesized and purified fluorocitrate on the metabolism of tricarboxylate anions by isolated rat liver mitochondria was investigated, in relation to the claim by Eanes *et al.* (1972) that this fluoro compound inhibits the tricarboxylate carrier at concentrations at which it has little effect on the aconitate hydratase activity. 2. That the inhibitory action of fluorocitrate is at the level of the aconitate hydratase and not at the level of the tricarboxylate carrier is indicated by the following findings. Although the oxidation of citrate and *cis*-aconitate, but not that of isocitrate, was inhibited by fluorocitrate, the exchange of internal citrate for external citrate or L-malate was not. Had the tricarboxylate carrier been affected, these latter exchange reactions would have been inhibited. 3. By using aconitate hydratase solubilized from mitochondria it was found that with citrate as substrate the inhibition by fluorocitrate was partially competitive ($K_i = 3.4 \times 10^{-8} M$), whereas with *cis*-aconitate as substrate the inhibition was partially non-competitive ($K_i = 3.0 \times 10^{-8} M$).

It is well established, mainly as a result of the early observations of Peters and his co-workers (Lotspeich *et al.*, 1952; Morrison & Peters, 1954) that fluorocitrate is a potent inhibitor of aconitate hydratase (EC 4.2.1.3) derived from rat liver mitochondria. With this inhibitor, K_i values of $300 \mu M$ (Guarriera-Bobyleva & Buffa, 1969), $64 \mu M$ (Eanes *et al.*, 1972) and $2.5 \mu M$ (Fanshier *et al.*, 1964) have been reported for inhibition of the enzyme reaction for the conversion of citrate into *cis*-aconitate. The variation in K_i values may reasonably be attributed, at least in part, to a difference in the proportion of the inhibiting isomer of fluorocitrate, (-)-*erythro*-fluorocitrate, contained in the fluorocitrate samples used by these workers (Dummel & Kun, 1969).

However, Eanes *et al.* (1972) have proposed that the inhibition of citrate oxidation by fluorocitrate is not at the level of aconitate hydratase, but at the level of the tricarboxylate carrier (see Chappell, 1968). Their interpretation is based on the observation that concentrations of fluorocitrate that abolish isocitrate efflux from whole mitochondria with citrate as substrate have no effect on isocitrate formation when mitochondrial structure is disrupted with Triton X-100.

Fluorocitrate is often added to experiments on the tricarboxylate carrier to inhibit metabolism of citrate through aconitate hydratase (Chappell, 1968). Since an effect of fluorocitrate on the carrier has repercussions on the interpretation of such experiments, we

have investigated the effect of (-)-*erythro*-fluorocitrate on this system in greater detail.

Experimental

Materials

Enzymes, coenzymes and adenine nucleotides were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. [U - ^{14}C]Sucrose, 3H_2O , [$1,5$ - ^{14}C]citric acid and $NaH^{14}CO_3$ were from The Radiochemical Centre, Amersham, Bucks., U.K. Substrates and bovine serum albumin were from Sigma Chemical Co., St. Louis, Mo., U.S.A. Triton X-100 was from BDH Chemicals, Poole, Dorset, U.K. (-)-*erythro*-Fluorocitrate (cycloheximide salt) was a generous gift from Professor E. Kun.

Methods

Rat liver mitochondria. These were prepared from albino Wistar rats by the method of Chappell & Hansford (1972).

Protein. This was assayed by a modified biuret method, including deoxycholate to solubilize the mitochondria. A standard curve was constructed by using bovine serum albumin (fraction V).

Oxygen uptake. This was followed in a Clark-type oxygen electrode as described by Chappell (1961, 1964).

Citrate spaces. The method is basically the same as that described by Chappell (1968). [U - ^{14}C]Sucrose was used to determine the extra-matrix space. In each experiment mitochondria (5 mg of protein)

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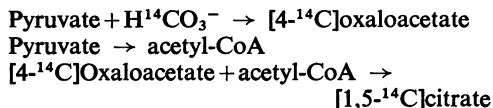
were incubated in 1 ml of 120 mM-KCl-20 mM-Tris-HCl, pH 7.4, medium with rotenone (10 μ g), antimycin A (5 μ g), potassium phosphate (1 mM), malate (1 mM), 10-15 μ Ci of $^3\text{H}_2\text{O}$ and 0.1 μ Ci of ^{14}C ([1,5- ^{14}C]citrate or [U- ^{14}C]sucrose) at 20°C.

The samples were then spun in an Eppendorf 3200 bench centrifuge for 4 min. Portions (0.5 ml) of the supernatant were added to equal amounts of 3% (w/v) HClO_4 and the pellets were resuspended in water and 3% (w/v) HClO_4 and re-centrifuged. Samples of the acidified pellet and supernatant fractions were counted for radioactivity in a Packard Tri-Carb liquid-scintillation counter (with an internal standard), by using a toluene scintillator containing in 2 litres, 800 ml of methoxyethanol, 160 g of naphthalene and 12 g of 5-(4-biphenyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole.

[^{14}C]Citrate loading. (a) From pyruvate. Rat liver mitochondria were incubated in a medium containing 120 mM-KCl, 20 mM-Tris-HCl, pH 7.4, 3 mM-sodium pyruvate, 3 mM-ATP, 20 mM-KHCO₃ and 10 μ Ci of $\text{H}^{14}\text{CO}_3^-$ at 20°C for 10 min. *N*-Ethylmaleimide was then added to this to a final concentration of 4 mM and the mixture was spun at 38000g for 10 min in an

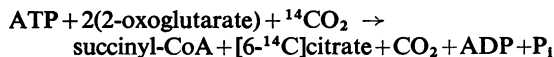
MSE 18 centrifuge. The supernatant was discarded. The pellet was resuspended in a medium of 120 mM-KCl, 20 mM-Tris-HCl, pH 7.4, and 10 mM-*N*-ethylmaleimide.

The reaction was presumed to be:



(b) From 2-oxoglutarate. The method was similar to that of Robinson *et al.* (1970), but *N*-ethylmaleimide was used and the experiment was carried out at 20°C. The medium contained 75 mM-KCl, 20 mM-Tris-HCl, pH 7.4, rotenone (1 μ g/ml), antimycin A (0.5 μ g/ml), 10 mM-ATP, 20 mM-KHCO₃, 5 mM-2-oxoglutarate, 10 μ Ci of $\text{H}^{14}\text{CO}_3^-$, 1 mM-potassium phosphate and 1 mM-malate.

The probable mechanism is:



(see Robinson *et al.*, 1970).

The advantage of using 2-oxoglutarate as the

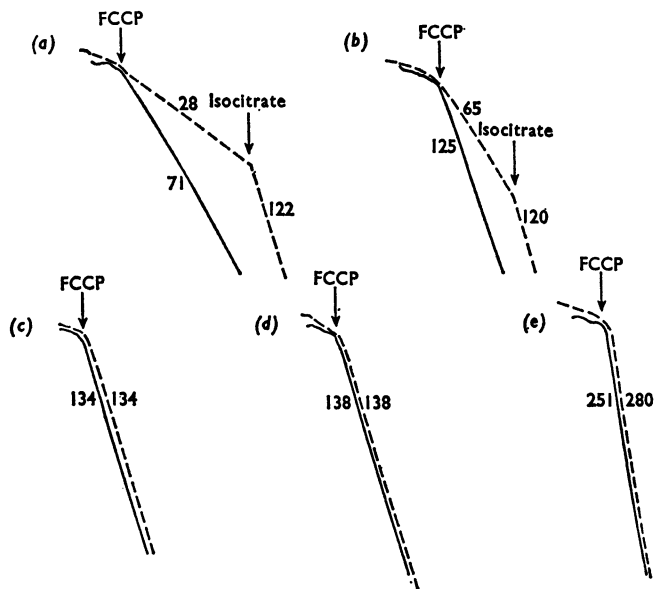


Fig. 1. Effect of fluorocitrate on the oxidation of (a) citrate, (b) cis-aconitate, (c) isocitrate, (d) 2-oxoglutarate and (e) glutamate

Mitochondria (4 mg of protein) were suspended in 120 mM-KCl, 20 mM-Tris-HCl, pH 7.4, 25 mM-potassium phosphate; the concentration of substrates was 3.13 mM, except for 2-oxoglutarate and glutamate, which were 5 mM. In all cases 1 mM-malate was present. In the experiments with fluorocitrate, the mitochondria were preincubated with fluorocitrate (1 μ M) for 2 min before addition of substrate(s), then 1 min further was allowed before simultaneous addition of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (2 μ M) and ADP (0.25 mM). Rates are in ng-atoms of O/min per mg of protein. —, No fluorocitrate; ----, 1 μ M-fluorocitrate.

source of carbon skeleton is that metabolism of labelled citrate during later experiments produces labelled CO_2 which is removed during the experiment, whereas from pyruvate the label can become dispersed among other metabolites.

[^{14}C]Citrate exchange. Rat liver mitochondria (3–4 mg of protein) loaded with [^{14}C]citrate were pipetted into tubes containing 1 ml of 120 mM-KCl, 20 mM-Tris-HCl, pH 7.4, rotenone (10 μg), antimycin A (5 μg) and 2 mM-*N*-ethylmaleimide and preincubated in this medium with fluorocitrate (2.5 μM) for 2 min where appropriate. To exchange the citrate, 2 min incubation with malate (2.3 mM or 6.6 mM) or citrate (6.6 mM) was allowed in the presence and absence of fluorocitrate. Separation and determination of radioactivity was carried out as described for 'citrate spaces'.

Results and Discussion

Oxidation of tricarboxylic acids

The effect of 1 μM -fluorocitrate on oxygen uptake by mitochondria in the presence of citrate, *cis*-aconitate and isocitrate (substrates for the tricarboxylate carrier) and by 2-oxoglutarate and glutamate was investigated as shown in Fig. 1. The rate of oxidation uncoupled by carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (2 μM) was measured

in the presence of ADP (0.25 mM) to activate the mitochondrial isocitrate dehydrogenase (EC 1.1.1.41).

The uncoupled oxidation of citrate (3.13 mM) was inhibited by 60% in the presence of fluorocitrate, whereas that of *cis*-aconitate (3.13 mM) was inhibited by 50%. In both cases subsequent addition of isocitrate caused a high rate of oxygen utilization, although this was not as high as with isocitrate alone. However, uncoupled oxidation of isocitrate (6.25 mM-DL mixture) was not inhibited by fluorocitrate. The difference between rates of isocitrate oxidation in the presence and absence of citrate or *cis*-aconitate is probably due to competition for the tricarboxylate carrier between isocitrate and citrate or *cis*-aconitate.

During mitochondrial oxidation citrate and *cis*-aconitate are substrates for aconitate hydratase, but isocitrate, 2-oxoglutarate and glutamate are not. This would therefore suggest that fluorocitrate has no effect on the tricarboxylate carrier and that the inhibition of the rates of citrate and *cis*-aconitate oxidation is due to inhibition of the aconitate hydratase reactions. 2-Oxoglutarate and glutamate enter the mitochondrion by different carriers (Chappell, 1968) and as predicted fluorocitrate had no effect on their rates of uncoupled oxidation.

No inhibition by fluorocitrate was demonstrated when it was added after substrate (not shown); this is probably due to competition between substrate and inhibitor for the carrier.

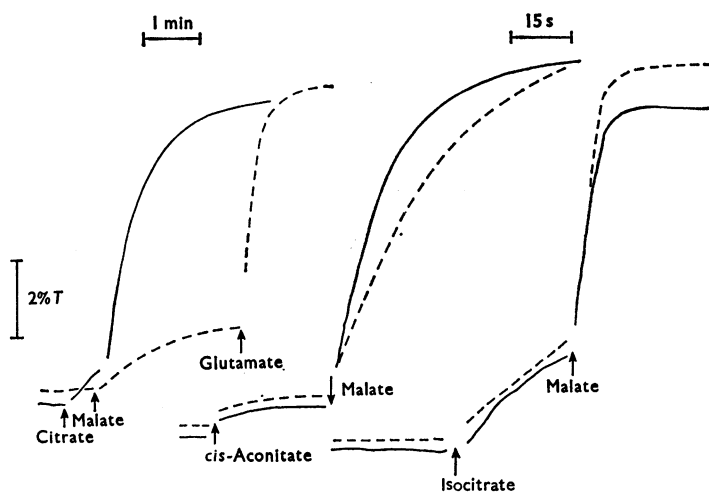


Fig. 2. Effect of fluorocitrate on reduction of mitochondrial nicotinamide nucleotides

Mitochondria (6 mg of protein) were suspended in 2.5 ml of a medium containing 120 mM-KCl, 20 mM-Tris-HCl pH 7.4, and 2 μM -carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, at 30°C. NAD(P)H formation was followed at 340–373 nm. After 10–15 min 0.25 mg of antimycin A/ml and 2 mM-Tris-phosphate, pH 7.4, were added, together with 2 μM -fluorocitrate, where present. After 2 min substrate was added as shown. Additions: 2 mM-citrate, 2 mM-*cis*-aconitate, 2 mM-DL-isocitrate, 10 mM-glutamate, 0.4 mM-malate (all as potassium salts). —, No fluorocitrate present; ----, 2 μM -fluorocitrate present.

Reduction of mitochondrial nicotinamide nucleotides

Mitochondria were depleted of endogenous substrates by incubation for 10–15 min with uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, and then the respiratory chain was blocked with antimycin A. Under these conditions the addition of substrate anions has little effect on the redox state of the intra-mitochondrial nicotinamide nucleotides. Subsequent addition of activators of transport (0.4 mM-malate for tricarboxylic acids) causes reduction of internal NAD(P)⁺, which was monitored at 340–373 nm on a double-beam spectrophotometer (Chappell, 1968). The results are shown in Fig. 2.

Fluorocitrate (3 μM) added 2 min before substrate almost completely inhibited reduction by citrate with catalytic malate, although subsequent addition of glutamate still caused reduction. Under the same conditions reduction by *cis*-aconitate with catalytic malate was inhibited by 50% in the presence of fluorocitrate. No inhibition was seen with isocitrate (and catalytic malate) as reductant, again indicating that the tricarboxylate carrier was unaffected by fluorocitrate. If malate was added before fluorocitrate, inhibition of reduction by citrate was still seen (not shown), indicating that this inhibition was not acting

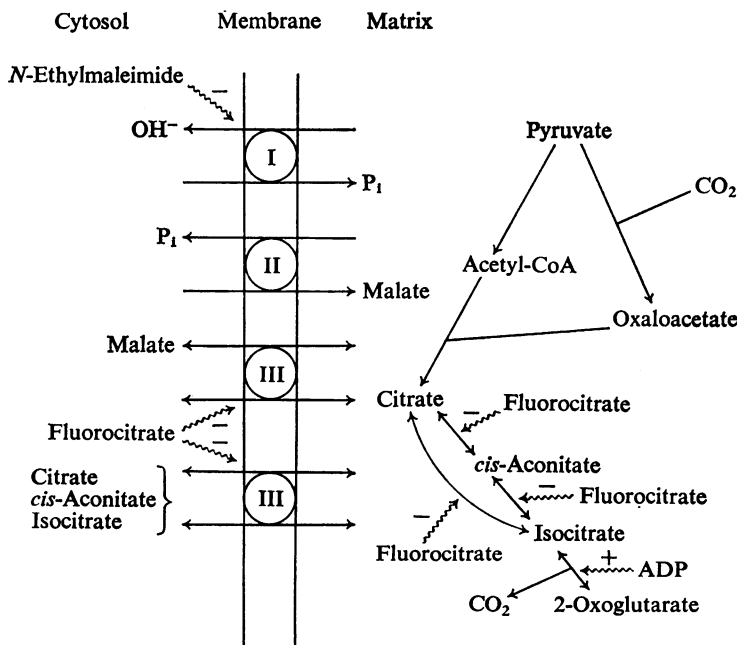
through inactivation of the coupled malate carrier (II, Scheme 1).

Swelling of mitochondria

A decrease in $E_{\epsilon_{10}}$ of mitochondria suspended in iso-osmotic solutions of NH₄⁺ salts indicates permeation of the mitochondria by the anion (Chappell, 1968). Rotenone-inhibited mitochondria with 3 μM-fluorocitrate exhibit the same swelling characteristics in iso-osmotic ammonium citrate and *cis*-aconitate as controls (not shown). This is inconsistent with inhibition of the carrier.

Citrate spaces

By measuring the penetration of a mitochondrial pellet by ³H₂O and [¹⁴C]sucrose the volume of the matrix can be estimated. The volume of the matrix available to citrate (Table 1) in the absence of fluorocitrate was apparently greater than the estimated volume. This indicates an accumulation of citrate inside the matrix. In the presence of fluorocitrate (3 μM) the accumulation was greater, showing that fluorocitrate does not inhibit the entry of citrate. The difference in accumulation was probably due to



Scheme 1. Possible sites of inhibition by fluorocitrate

I, Phosphate-hydroxyl antiporter; II, malate-phosphate antiporter; III, tricarboxylate-tricarboxylate or malate antiporter. Inhibition is indicated by \dashrightarrow , activation by \dashrightarrow^+ .

Table 1. Penetration of mitochondria by citrate in the presence and absence of fluorocitrate

For details see the Experimental section.

Citrate added (mM)	Fluorocitrate added (μ M)	Apparent volume of matrix available (% of sucrose space)	Concn. of citrate in matrix (mM)
1.53	0	210	3.06
1.53	3	230	3.50
2.64	0	140	3.54
2.64	3	160	4.06

inhibition of aconitate hydratase, and therefore of citrate metabolism.

[¹⁴C]Citrate exchange

Mitochondria were loaded with [¹⁴C]citrate and this label was exchanged by incubating with external malate or citrate in the presence and absence of fluorocitrate (see under 'Methods'). The results are shown in Table 2. *N*-Ethylmaleimide was present to prevent phosphate transport (I, Scheme 1) and thus activation by catalytic amounts of malate (II, Scheme 1). Fluorocitrate increases the radioactivity in the pellet relative to the control, probably owing to inhibition of aconitate hydratase and therefore suppression of metabolism and ¹⁴CO₂ formation. The radioactivity in the pellet with fluorocitrate present is therefore expressed as a percentage of the value for control plus fluorocitrate. The results show that internal citrate exchanges readily with external malate or citrate, and that this exchange is not inhibited by fluorocitrate.

Isocitrate efflux and formation

Aconitate hydratase will catalyse the conversion of citrate or *cis*-aconitate into isocitrate; added citrate and *cis*-aconitate will therefore exchange on the tricarboxylate carrier with the isocitrate formed inside the mitochondria (III, Scheme 1). Table 3 shows the results of experiments in which isocitrate produced was coupled to external NADPH formation by added isocitrate dehydrogenase (EC 1.1.1.42) and the rates of reduction of NADP⁺ were measured at 340–373 nm on a double-beam spectrophotometer.

The results for 1 mM-citrate or *cis*-aconitate as substrate confirm those of Eanes *et al.* (1972), although we could not show complete inhibition, in agreement with Peters & Shorthouse (1970). In contrast to Eanes *et al.* (1972) we found that addition of Triton X-100 diminished but did not abolish the inhibition by fluorocitrate. Similar results were obtained with 0.5 mM substrate.

When mitochondria were sonicated (six 15 s bursts at 4°C) and the fragments removed by centri-

Table 2. Effect of fluorocitrate on exchange of intramitochondrial [¹⁴C]citrate with extramitochondrial malate and citrate

For details see the Experimental section: 2.3 mM-malate was added to the mitochondria loaded from pyruvate, and 6.6 mM-malate to those loaded from 2-oxoglutarate.

Additions	Radioactivity in pellet (% of control)	
	From pyruvate	From 2-oxoglutarate
None	100	100
Malate	98	74
6.6 mM-Citrate	81	84
	Radioactivity in pellet (% of control + fluorocitrate)	
Fluorocitrate	100	100
Fluorocitrate and malate	60	51
Fluorocitrate and 6.6 mM-citrate	45	53

fugation (40 min at 38000g in an MSE 18 centrifuge), the supernatant fraction showed the same sensitivity to fluorocitrate with citrate as substrate as the mitochondria treated with Triton X-100, indicating that fluorocitrate inhibited soluble aconitate hydratase at these concentrations. With *cis*-aconitate as substrate, addition of Triton to the supernatant from sonicated mitochondria had a variable effect; in this case, therefore, the results in Table 3 for *cis*-aconitate and Triton may be less reliable.

A more detailed investigation of the kinetics of this system was made. Fig. 3 shows substrate inhibition of aconitate hydratase by fluorocitrate at citrate concentrations greater than 1 mM; this is lower than the value of 2–3 mM taken from the results of Fanshier *et al.* (1964). These workers, using concentrations of

Table 3. Effect of fluorocitrate on the rate of isocitrate efflux and formation with citrate and *cis*-aconitate as substrate

Rat Liver mitochondria (1–2 mg of protein) were suspended in 2.5 ml of a medium containing 120 mM-KCl, 20 mM-Tris-HCl, pH 7.4, 10 μ g of rotenone, 50 μ g of NADP⁺, 25 μ g of isocitrate dehydrogenase and 1 mM-MgCl₂. This suspension was incubated for 1 min with fluorocitrate before the addition of substrate and the rate of NADPH formation was measured at 340–373 nm. After 4 min 60 μ l of 5% (w/v) Triton X-100 was added and the new rate was determined. Citrate and *cis*-aconitate were added to a final concentration of 1 mM.

Fluorocitrate added (nM)	Inhibition of isocitrate efflux (%)		Inhibition of isocitrate formation after addition of Triton X-100 (%)	
	Citrate	<i>cis</i> -Aconitate	Citrate	<i>cis</i> -Aconitate
10	9	12	6	6
25	24	19	20	11
50	37	24	22	15
100	66	50	42	18
500	81	72	52	28

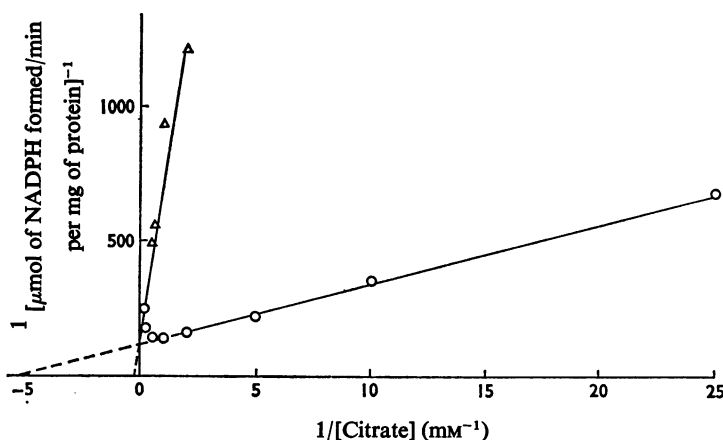


Fig. 3. Lineweaver-Burk plot of fluorocitrate and citrate inhibition of aconitate hydratase

Mitochondria were diluted 1:1 (v/v) with isolation medium and sonicated for six 15 s bursts at 4°C. The fragmented mitochondria were spun down at 38000g for 40 min and samples of supernatant (1 mg of protein) were treated as described in Table 3, with different substrate concentrations. ○, No fluorocitrate; △, 10 μ M-fluorocitrate present.

citrate greater than 2 mM, and 10 μ M- and 100 μ M-fluorocitrate, estimated a K_i for (–)-erythro-fluorocitrate of 2.5×10^{-6} M. Fig. 3 shows our results for these conditions, giving an apparent K_i of 6×10^{-7} M. However, since substrate inhibition at these concentrations of citrate is marked, these values are unreliable. Consequently we have repeated this work at lower substrate concentrations. Addition of 1 mM-isocitrate caused a rate of reduction five times that of the highest rate recorded in these experiments, showing that the assay system was not limiting.

Fig. 4 is a Lineweaver-Burk plot showing the rate of formation of isocitrate by lysed mitochondria, with

citrate as substrate. The K_m for citrate in intact mitochondria is 2.0×10^{-4} M and in Triton-treated mitochondria it is 2.2×10^{-4} M, agreeing closely with the value of 2×10^{-4} M found by Henson & Cleland (1967) using ox liver. Fig. 4 indicates competitive inhibition by fluorocitrate; however, plots of $1/v$ against inhibitor concentration were curved, indicating that the inhibition was partially competitive. The values of apparent K_m in the presence of fluorocitrate obtained from Fig. 4 were therefore used in the appropriate equation (Dixon & Webb, 1964). This gave consistent values of K_i at different fluorocitrate concentrations. In intact mitochondria the K_i is 1.2×10^{-8} M;

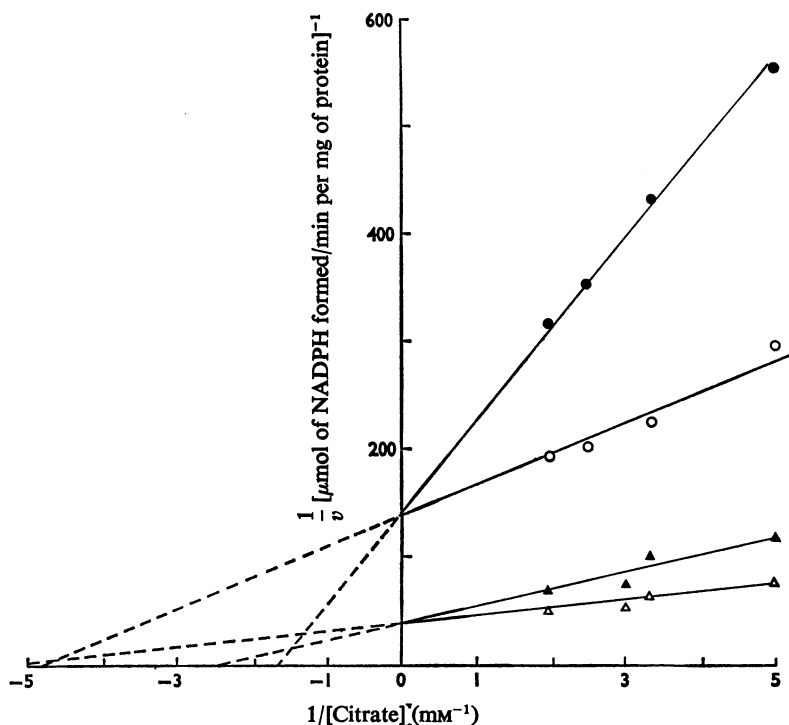


Fig. 4. Fluorocitrate inhibition of isocitrate efflux from whole mitochondria and formation in lysed mitochondria, with citrate as substrate

For experimental details see Table 3. ○, Whole mitochondria; ●, whole mitochondria + 50 nM-fluorocitrate; △, lysed mitochondria; ▲, lysed mitochondria + 50 nM-fluorocitrate.

in lysed mitochondria it is $3.4 \times 10^{-8} M$. The values of V_{max} were 8.7 and 24.3 nmol of NADPH formed/min per mg of protein respectively.

Similar experiments with *cis*-aconitate as substrate (see Fig. 5) gave values of K_m of $1.2 \times 10^{-4} M$ and $1.1 \times 10^{-4} M$ for intact and lysed mitochondria respectively, agreeing with the value of $9.0 \times 10^{-5} M$ found by Tomizawa (1953) with rabbit liver. In this case fluorocitrate inhibition appeared to be non-competitive; again plots of $1/v$ against inhibitor concentration were curved, indicating partial non-competitive inhibition. Substitution in the appropriate equation (Dixon & Webb, 1964) gave consistent values of K_i . The K_i and V_{max} for intact mitochondria were $5.8 \times 10^{-8} M$ and 26.9 nmol of NADPH formed/min per mg of protein respectively, and for Triton-treated mitochondria they were $3.0 \times 10^{-8} M$ and 79.6 nmol of NADPH formed/min per mg of protein respectively.

The values of K_i reported here are very much lower than the values in the literature. The types of inhibition we have observed agree with those reported by

Fanshier *et al.* (1964) and J. J. Villafranca (unpublished work, quoted by Glusker, 1971).

Eanes *et al.* (1972) propose that fluorocitrate inhibits the tricarboxylate carrier, on the basis of three observations. The K_i for inhibition of isocitrate formation from citrate is very much lower in intact mitochondria than that for aconitate hydratase; the concentrations of fluorocitrate which inhibit 100% in intact mitochondria do not inhibit isocitrate formation in broken mitochondria; and the activation of the tricarboxylate carrier by fluoromalate is abolished by fluorocitrate.

We propose that their interpretation is incorrect because we find that the values of K_i for intact and broken mitochondria are the same, and the rate of isocitrate formation in broken mitochondria is inhibited by the low fluorocitrate concentrations used by Eanes *et al.* (1972), in spite of the claim to the contrary in the text. Activation of isocitrate efflux by fluoromalate would be abolished if aconitate hydratase was inhibited, since the tricarboxylate carrier is not rate-limiting.

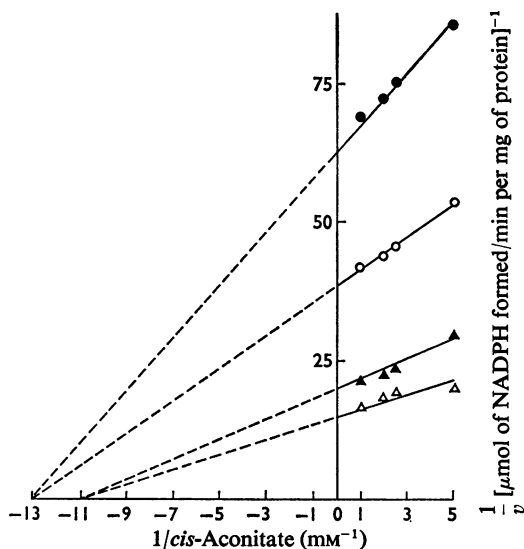


Fig. 5. Fluorocitrate inhibition of isocitrate efflux from whole mitochondria and formation in lysed mitochondria with *cis*-aconitate as substrate

For experimental details see Table 3. ○, Whole mitochondria; ●, whole mitochondria + 50 nm-fluorocitrate; △, lysed mitochondria; ▲, lysed mitochondria + 500 nm-fluorocitrate.

We therefore propose that fluorocitrate does not inhibit the tricarboxylate carrier, and that the results of Eanes *et al.* (1972) can be adequately explained by inhibition of aconitate hydratase.

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