The Effects of 2-Ethylcitrate and Tricarballylate on Citrate Transport in Rat Liver Mitochondria and Fatty Acid Synthesis in Rat White Adipose Tissue

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2-Ethylcitrate and propane 1,2,3 tricarboxylic acid (tricarballylate) were found to be inhibitors of the citrate transporting system as monitored by *cis*-aconitate and isocitrate oxidation. Kinetic data showed the inhibitions to be competitive with tricarboxylate anion. When the exchange of intramitochondrial [^{14}C]citrate with added extramitochondrial citrate or L-malate was measured at low temperatures, 2-ethylcitrate and 2-propylcitrate were found to be good inhibitors, while tricarballylate did not inhibit and in fact was found to be a good substrate for exchange on the transporting system. Neither 2-ethylcitrate nor tricarballylate at low concentrations (< 10 mM) were found to inhibit fatty acid synthesis with pyruvate as precursor in rat white adipose tissue but both were potent inhibitors of fatty acid synthesis with glucose as precursor. The implications of these findings are discussed.

The rate of egress of citrate from the mitochondrial to the cytoplasmic compartment is of fundamental importance to two major pathways. Firstly, it has been postulated that cytoplasmic citrate levels control the flow through the glycolytic pathway by influencing the activity of phosphofructokinase in some but not all mammalian tissues [1—4]. Secondly the pathway for fatty acid synthesis in rat white adipose tissue and rat liver is thought to involve exit of citrate from the mitochondria to acetyl-CoA when cleaved by citrate-ATP-lyase [5—7]. It has been shown by a variety of techniques that the transporting system for citrate, *cis*-aconitate and *threo*-D$_s$-isocitrate in mitochondria operates by an exchange diffusion carrier which catalyses a stoichiometric exchange of tricarboxylate anion for L-malate [8—15]. No inhibitors for this transporting system have so far been reported.

Unusual Abbreviations. FCCP, carbonyl-cyanide-*p*-trifluoromethoxy-phenyl-hydrazone.

Enzymes. Aconitate hydratase (EC 4.2.1.3); NAD-isocitrate dehydrogenase (EC 1.1.1.41); NADP-isocitrate dehydrogenase (EC 1.1.1.42); acetyl-CoA carboxylase (EC 6.4.1.2); acetyl CoA synthetase (EC 6.2.1.1); citrate lyase (EC 4.1.3.6).

EXPERIMENTAL PROCEDURE

Rats

Epididymal fat pads and livers obtained from male Wistar rats (150 g) fed on a purina chow diet. In all experiments the animals were allowed free access to food before the time of killing (approximately 10:00 a. m.). Rat liver mitochondria were isolated by methods described previously [7] in a medium containing 0.25 M sucrose, 3.4 mM Tris chloride and 1 mM methylenedioxy-(bis)-(ethylamino)-tetra-acetic acid, pH 7.4. The final pellet was resuspended in preparation medium to give a final concentration of 40—80 mg protein per ml for use in experiments.

Chemicals

Enzymes, coenzymes, glycolytic intermediates and adenine nucleotides were obtained from Boehringer Mannheim GmbH (Mannheim) Corp. (N. Y.). Bovine serum albumin (fraction V) was obtained from Sigma Chemical Corp. (St. Louis, Mo.). Tritium labelled H_2O and D-[U-^{14}C]glucose were obtained from New England Nuclear Corp. (Boston, Mass.).

Other radioactive compounds were obtained from Amersham Searle. Carbonyl-cyanide-*p*-trifluoromethoxy-phenyl-hydrazone (FCCP) was provided by Dr. P. G. Heytler. *Threo*-D_s-isocitrate was obtained from D. G. Wilson (University of Western Ontario, London, Canada). 2-Ethylcitrate (S 40017-3) and 2-propylcitrate (S 40008-4) were obtained in crude form from the Rare Chemicals Division of the Aldrich Chemical Co. (Milwaukee, Wisc.). Pentane 1,4,5-tricarboxylate was obtained from K & K Laboratories Inc. (Plainview, N. J.).

Media

Fat pads were incubated in a Krebs bicarbonate buffered medium [16], gassed with oxygen and CO_2 (95:5) in a volume of 2.5 ml/fat pad (300 mg). Isolated cells were prepared by the method of Rodbell [17] and suspended in the above buffer plus albumin (20 mg/ml). The concentrations of various additions are given in the tables or text.

Methods

Analysis of the incubation medium for pyruvate, lactate and glucose and the incorporation of D-[$U^{14}C$]-glucose or ^3H-labelled H_2O into fatty acid were measured as summarized in Halperin and Robinson [18]. The assay of various metabolites was carried out by the following methods: L-malate, Hohorst [19]; citrate, Moellering and Gruber [20]; *threo*-D_s-isocitrate, Siebert [21]; protein was measured by the method of Gornall, Bardawill and David [22].

[^{14}C]Citrate Loading

Rat liver mitochondria (200 mg protein) were incubated in 10 ml of a medium containing 125 mM KCl, 20 mM Tris chloride pH 7.4 at 10° for 10 min in the presence of 2 μM rotenone, 0.4 μg/ml antimycin A, 10 mM ATP, 1 mM $NaHCO_3$, 5 mM 2-oxoglutarate and 20 μC $NaH^{14}CO_3$. After this time the volume was made up to 100 ml with ice-cold buffer containing 0.25 M sucrose and 5 mM Tris pH 7.4 and the mitochondria separated by centrifugation at 18000 × *g* for 10 min. The adhering supernatant was decanted off as much as possible and the mitochondria resuspended in 3—4 ml of the sucrose/Tris buffer for use in isotopic exchange experiments.

When the mitochondria were treated in such a way it was found that ^{14}C label was incorporated into citrate and isocitrate in the 6 position. When the [^{14}C]bicarbonate had been removed by acidification and neutralisation with potassium carbonate followed by evacuation over KOH, the radioactivity contained in [6-^{14}C]citrate and [6-^{14}C]isocitrate was determined by the method of McElroy and Williams [25]. 99% of the radioactivity incorporated by the mitochondrial incubation was accounted for as being present in citrate or isocitrate and less than 0.5% in L-malate as judged by enzymic decarboxylation [25]. The specific activity of the resulting citrate (+ isocitrate) varied between 30 and 200 counts/nmole and the citrate content of the final mitochondria suspension between 30 and 70 nmoles/mg mitochondrial protein. The variability was thought to be due in part to variable ATPase activity in the mitochondrial suspension.

When ATP or 2-oxoglutarate was omitted from the incubation medium no counts were incorporated into citrate.

When ATP and antimycin were omitted, counts were incorporated into citrate, but a considerable amount (40 nmoles/mg protein) of unlabelled L-malate was produced compared with the endogenous complements of less than 10 nmoles/mg protein. When oligomycin was added to the incubation medium no counts were incorporated. The probable mechanism is as follows:

$$\text{2-oxoglutarate} + {}^*CO_2 + \text{NADPH} \rightleftharpoons {}^*\text{isocitrate} + \text{NADP} \quad (1)$$

$$\text{isocitrate}^* \rightleftharpoons \text{citrate}^* \quad (2)$$

$$\text{NADH} + \text{NADP} + \text{ATP} \rightleftharpoons \text{NADPH} + \text{NAD} + \text{ADP} + P_i \quad (3)$$

$$\text{2-Oxoglutarate} + \text{NAD} + \text{CoA} \rightleftharpoons \text{Succinyl CoA} + \text{NADH} + CO_2 \quad (4)$$

$$\text{ATP} + 2\ \text{2-oxoglutatate} + {}^*CO_2 \rightleftharpoons \text{succinyl CoA} + \text{citrate}^* + CO_2 + \text{ADP} + P_i \quad (5)$$

So that the final exchange reaction is as shown in Equation (5).

The Purification of 2-Ethylcitric Acid

The nuclear magnetic resonance spectrum was determined using a Varian A60 spectrometer and ^2H_2O as solvent. Sodium 2, 2-dimethyl-2-silapentane-5 sulfonate ($\delta = 0$ ppm) was used as an internal reference. The mass spectrum was obtained with a Perkin-Elmer RMU-6 mass spectrometer at 70 e.v.

The trisodium salt of 2-ethylcitric acid as obtained commercially was not guaranteed as to its chemical structure or purity. The material required further purification to remove a fluorescent contaminant and the assigned structure was confirmed in the following manner.

An aqueous solution of 1.0 g of trisodium 2-ethylcitrate was passed over a column of Dowex-50W-X8 (acid form) ion exchange resin to convert the trisodium salt to the free acid. Evaporation of the aqueous eluant gave 2-ethylcitric acid as an oil [23]. The oil was allowed to stand for 2 days at room temperature during which time crystallization had occurred.

The crystalline mass was briefly stirred with a small portion of diethylether to remove some adhering oil. Filtration gave 160 mg of pure, non-fluorescent, 2-ethylcitric acid, m. p. 161—162, obtained for the first time as a crystalline solid. The mother liquors were evaporated to give an oil, which on further standing for 24 hours crystallized to a solid mass. The residue was taken up in acetonitrile and a further crop of 240 mg of the insoluble 2-ethylcitric acid, m. p. 158—162° was obtained on filtration. The total recovery was 52%.

The mass spectrum of 2-ethylcitric acid was very similar to citric acid itself. It did not give a parent ion at 220 $[M]^+$ but did give peaks at 175 $[M\text{-}CO_2H]^+$, at 157 $[M\text{-}CO_2H, H_2O]^+$ and confirms the identity of 2-ethylcitric acid.

The spectrum of 2-ethylcitric acid showed a triplet at 0.73 (3 H, $J = 7.2$), a quintet at 1.51 (2 H, $J = 6.6$), a doublet at 2.47 ($J = 3$), a doublet at 2.63 ($J = 3$) and a singlet at 2.84. The spectrum is entirely consistent with the assigned structure [24].

RESULTS

Mitochondrial Oxidation Kinetics

When rat liver mitochondrial NAD(P) redox changes were followed by fluorimetry [26] a rapid oxidation of pyridine nucleotides occurred on addition of uncoupling agent (FCCP). After the addition of respiratory inhibitors such as rotenone or antimycin, addition of L-malate caused little reduction, but when *cis*-aconitate was added, rapid reduction of intramitochondrial NAD(P) occurred (Fig. 1). If the *cis*-aconitate was added before the L-malate no reduction occurred until the L-malate was added since a dicarboxylate anion is required as an activator of the tricarboxylate transporting system [8, 9, 27, 28]. When increasing concentrations of 2-ethylcitrate (2, 4, and 6 mM), were added after the antimycin, the rates of reduction resulting from the addition of *cis*-aconitate were progressively slower. The order of addition did not effect the inhibition observed. Similar observations were made when citrate or isocitrate oxidation was monitored in the presence of L-malate.

A plot of the reciprocal of the rate of NAD(P) reduction against increasing inhibitor concentration at two different L-malate concentrations and two different *cis*-aconitate concentrations showed that the inhibition was competitive with respect to *cis*-aconitate and not L-malate [29], the K_i value being 0.7 mM (Fig. 2).

A similar plot for two concentrations of *threo*-D_s-isocitrate at two different concentrations of L-malate gave the same indication with a K_i of 0.35 mM (Fig. 3).

It was found that propane-1,2,3,-tricarboxylate (tricarballylate) was also inhibitory to the oxidation of tricarboxylate anions as judged by fluorimetric measurement of intramitochondrial NAD(P) reduction. However, although the inhibition proved to be competitive with isocitrate and not L-malate, the K_i

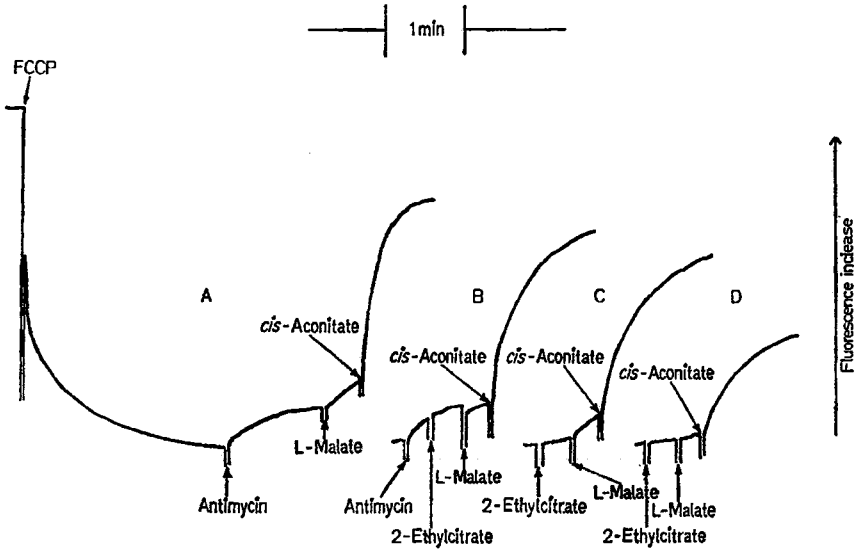

Fig. 1. *The inhibition of* cis-*aconitate oxidation by 2-ethylcitrate.* Rat liver mitochondria (2—5 mg protein) were suspended in 3.0 ml of a medium containing 125 mM KCl, 20 mM Tris-Cl and 2 mM inorganic phosphate, pH 7.4 at 30°. Pyridine nucleotide oxidation/reduction changes were followed fluorimetrically. Only experiment A is shown in full since the initial oxidation of intramitochondrial NAD(P) by uncoupling agent was the same for A, B, C, and D. Additions were as follows: Expt. A. FCCP, 1 μM; antimycin 0.2 μg/ml; L-malate, 200 μM; *cis*-aconitate 80 μM. Expts. B. C. and D; additions as for Expt. A but with 2, 4 and 6 mM 2-ethylcitrate added respectively at the points shown

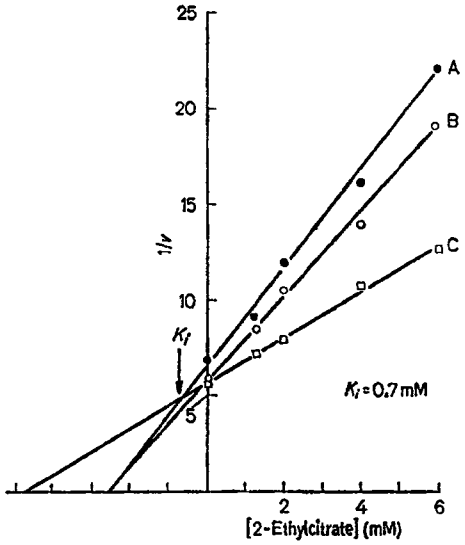

Fig. 2. *The kinetics of 2-ethylcitrate inhibition of* cis-*aconitate oxidation.* The reciprocal of the velocity of intramitochondrial NAD(P) reduction by *cis*-aconitate is plotted against inhibitor concentration under three sets of conditions. Rat liver mitochondria (2.6 mg protein) were suspended in 3.0 ml of a medium containing 125 mM KCl, 20 mM Tris-Cl and 2 mM inorganic phosphate pH 7.4 at 30°. 1 μM FCCP was added and after 3 min 0.2 μg/ml antimycin followed by 2-ethylcitrate (final concentrations shown), *cis*-aconitate and L-malate. The rate of intramitochondrial NAD(P) reduction on the addition of L-malate was monitored fluorometrically. The three sets of conditions were (A) 200 μM L-malate, 80 μM *cis*-aconitate (●); (B) 600 μM L-malate, 80 μM *cis*-aconitate (○); (C) 200 μM L-malate, 200 μM *cis*-aconitate (□)

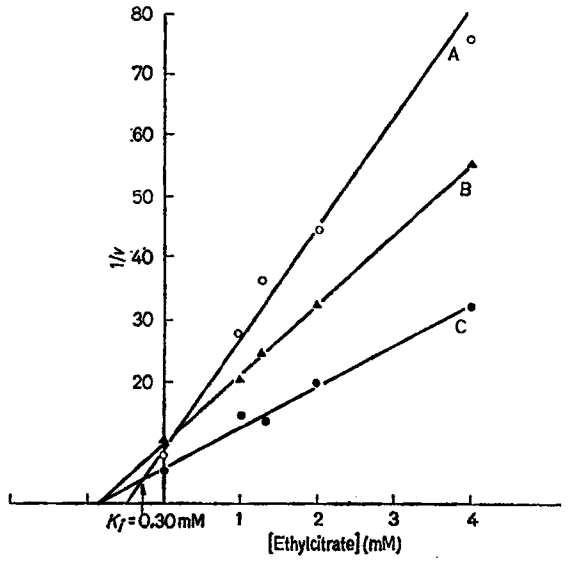

Fig. 3. *The kinetics of 2-ethylcitrate inhibition of* threo-D_s-*isocitrate oxidation in the presence of L-malate.* The experiment was carried out using *threo*-D_s-isocitrate as described for *cis*-aconitate in Fig. 2 under the following sets of conditions: (A) 1 mM L-malate, 100 μM *threo*-D_s-isocitrate (○); (B) 0.2 mM L-malate, 250 μM *threo*-D_s-isocitrate (▲); (C) 1 mM L-malate, 250 μM *threo*-D_s-isocitrate (●). Mitochondrial protein was 2.5 mg per incubation. The initial velocity, v, was measured as the rate of fluorescence increase in arbitrary units

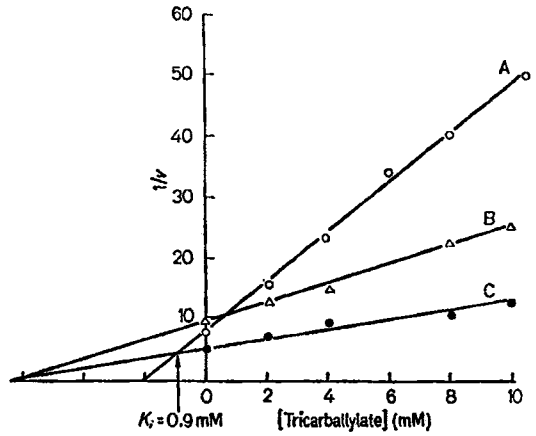

Fig. 4. *The kinetics of tricarballylate inhibition of* threo-D_s-*isocitrate oxidation in the presence of L-malate.* The experiment was carried out as described for Fig. 3 except that tricarballylate was used instead of 2-ethylcitrate under the following sets of conditions: (A) 1 mM L-malate, 100 μM *threo*-D_s-isocitrate (○); (B) 0.2 mM L-malate, 250 μM *threo*-D_s-isocitrate (△); (C) 1 mM L-malate, 250 μM *threo*-D_s-isocitrate (●). Mitochondrial protein was 2.5 mg per incubation

for this compound was somewhat higher than for 2-ethylcitrate being 0.9 mM (Fig. 4). Fig. 5 shows a Lineweaver-Burk plot for L-malate activated isocitrate oxidation as a function of isocitrate concentration and in the presence of 4 mM 2-ethylcitrate, 4 mM tricarballylate and 4 mM 1,4,5-pentane tricarboxylate. It can be seen that the pentane 1,4,5-tricarboxylate had no effect on the rate of isocitrate oxidation while tricarballylate had some effect giving a K_i value of 1.5 mM and 2-ethylcitrate was quite inhibitory giving a K_i of 0.48 mM. Again the competition with tricarboxylate anion rather than L-malate was confirmed since the V_{max} remained unchanged by the inhibitors [30].

When the oxidation of isocitrate (250 μM) by rat liver mitochondrial NADP-isocitrate dehydrogenase was monitored in a broken preparation, very little inhibition was evident when high concentrations of tricarballylate or 2-ethylcitrate were used (Table 1) whereas substantial inhibition was observed in whole mitochondria at these same concentrations of inhibitors. However, when *cis*-aconitate oxidation (80 μM) was monitored in a broken mitochondrial preparation, inhibition by high concentrations of tricarballylate and 2-ethylcitrate was evident though inhibition was greater at these concentrations when used on whole mitochondria. Tricarballylate was found to activate the NAD-linked isocitrate dehydrogenase, while 2-ethylcitrate was found to inhibit this enzyme. The inhibition was again less than that observed when the same concentrations of inhibitor and substrate were used in whole mitochondria.

2-Ethylcitrate and tricarballylate at 5 mM concentration had no affect on the reduction of intra-

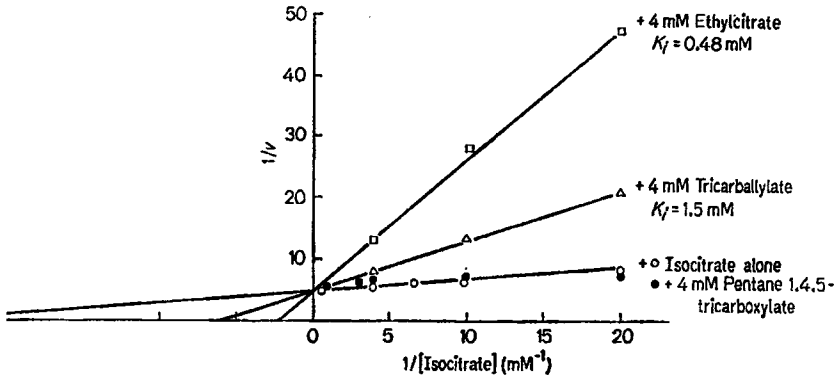

Fig. 5. *Lineweaver-Burk plots of* threo-D_s-*isocitrate oxidation in the presence of* L-*malate and showing the effects of 2-ethylcitrate and tricarballylate on the observed kinetics.* Rat liver mitochondria (3 mg protein) were suspended in 3.0 ml of a medium containing 125 mM KCl, 20 mM Tris-Cl and 2 mM inorganic phosphate pH 7.4 at 30°. 1 µM FCCP was added, followed 3 min later by 0.2 µg/ml antimycin. *Threo*-D_s-isocitrate was added (final concentrations as shown on the graph) and the rate of intramitochondrial NAD(P) reduction monitored fluorimetrically on the addition of 0.8 mM L-malate (○). The concentration dependence was then investigated in the presence of 4 mM pentane 1,4,5-tricarboxylate (●), 4 mM tricarballylate (△) and 4 mM 2-ethylcitrate (□). The reciprocal of the rate of fluorescence increase v was plotted against the reciprocal of the *threo*-D_s-isocitrate concentration

Table 1. *Inhibition of* cis-*aconitate and isocitrate oxidation in whole and broken mitochondria by tricarballylate and 2-ethylcitrate*

Experiments to estimate the inhibition by analogues in whole mitochondria were performed as described in Fig. 1 at the concentrations of tricarboxylate anion shown in the presence of 0.8 mM L-malate. For the experiments to estimate the inhibition of aconitase and NADP-isocitrate dehydrogenase by analogues in a broken preparation, sonicated mitochondria (2 mg protein) were suspended in 2.5 ml of a medium containing 125 mM KCl, 20 mM Tris chloride, pH 7.4 at 30°. 0.2 mM NADP and 1 mM $MnCl_2$ were added and the reaction started by the addition of either *cis*-aconitate or isocitrate (concentrations shown) and NADP reduction was monitored at 340 nm spectrophotometrically. The experiments were then repeated in the presence of tricarballylate and 2-ethylcitrate. When NAD-isocitrate dehydrogenase activity was monitored, mitochondria were sonicated in the presence of 2 mM ADP, 2 mM $MgCl_2$ and 10 mM reduced glutathione. Sonicated mitochondria (2 mg protein) were suspended in 2.5 ml of a medium containing 95 mM KCl, 20 mM Tris chloride and 20 mM potassium phosphate, pH 7.4 in the presence of 2 mM NAD, 2 mM $MgCl_2$ and 2 mM ADP. The reaction was started by the addition of isocitrate, and NAD reduction followed spectrophotometrically

Inhibitor (concn.)	Enzyme	Substrate (concn.)	Inhibition	
			Broken Mitochondria	Whole Mitochondria
mM		µM	%	%
Tricarballylate (10)	Aconitate hydratase	*cis*-Aconitate (80)	48	84
Tricarballylate (10)	NADP-isocitrate-dehydrogenase	*threo*-D_s-Isocitrate (250)	0	48
Tricarballylate (10)	NAD-isocitrate-dehydrogenase	*threo*-D_s-Isocitrate (250)	(two-fold activation)	
2-Ethylcitrate (7)	Aconitate hydratase	*cis*-Aconitate (80)	33	73
2-Ethylcitrate (7)	NADP-isocitrate-dehydrogenase	*threo*-D_s-Isocitrate (250)	3.0	83
2-Ethylcitrate (7)	NAD-isocitrate-dehydrogenase	*threo*-D_s-isocitrate (250)	27	

mitochondrial NAD(P) by 0.5 mM glutamate or 0.5 mM 3-hydroxybutyrate. Tricarballylate inhibited the reduction of intramitochondrial NAD(P) by 0.5 mM L-malate in the presence of cysteine sulphinate. The explanation for this is in the fact that tricarballylate is able to displace L-malate from the mitochondria (see Table 2).

The kinetics of inhibition by 2-ethylcitrate and tricarballylate were established using the type of experiment shown in Fig. 1 where the addition of inhibitor was made after substrate depletion. Fig. 6 shows the striking difference observed, especially with tricarballylate between the experiments in which the transport inhibitor was added before the uncoupling agent and those in which it was added after substrate depletion. Thus, when tricarballylate was added after antimycin (expt. B) the rate of reduction on the addition of L-malate was somewhat slower than with the control experiment (A). When tricarballylate was added before incubation with FCCP, then the addition of isocitrate caused a rapid reduction when no L-malate was present. 2-Ethyl-

Table 2. *Correlation between isotopic exchange data and exchange as determined by enzymic assay*
See text for description of the experiment

Additions	Radioactivity		Exchange	L-Malate		Citrate		Exchange
	Supernatant	Total-supernatant = pellet		Supernatant	Total-supernatant = Pellet	Supernatant	Total-supernatant = Pellet	
	counts/min		%	nmoles		nmoles		%
None	419	4511	0	31.8	45.4	74.5	166.5	0
10 mM Tricarballylate	1380	3540	21.8	44.5	22.7	115.5	125	25.5
10 mM Citrate	1360	3560	21.4	64.0	13.2	—	—	—
10 mM 2-Ethylcitrate	501	4419	2.3	30.2	47.0	79.0	161	2.3
10 mM Pentane 1, 4, 5 Tricarboxylate	430	4490	0.6	31.4	45.8	76.0	165	1.2
1 mM Citrate	796	4124	8.4	60.4	16.8	—	—	—
1 mM Citrate + 10 mM Tricarballylate	1165	3755	16.5	61.3	15.9	—	—	—
1 mM Citrate + 10 mM 2-Ethylcitrate	610	4310	4.2	51.0	26.2	—	—	—
1 mM L-Malate	720	4200	6.2	—	—	85.6	156.4	6.7
1 mM L-Malate + 10 mM Tricarballylate	1090	3830	14.9	—	—	101.2	139.8	15.9
1 mM L-Malate + 10 mM 2-Ethylcitrate	611	4319	4.2	—	—	82.5	158.5	4.7
1 mM Citrate + 10 mM Pentante 1, 4, 5 Tricarboxylate	800	4120	8.4	65.0	12.2	—	—	—

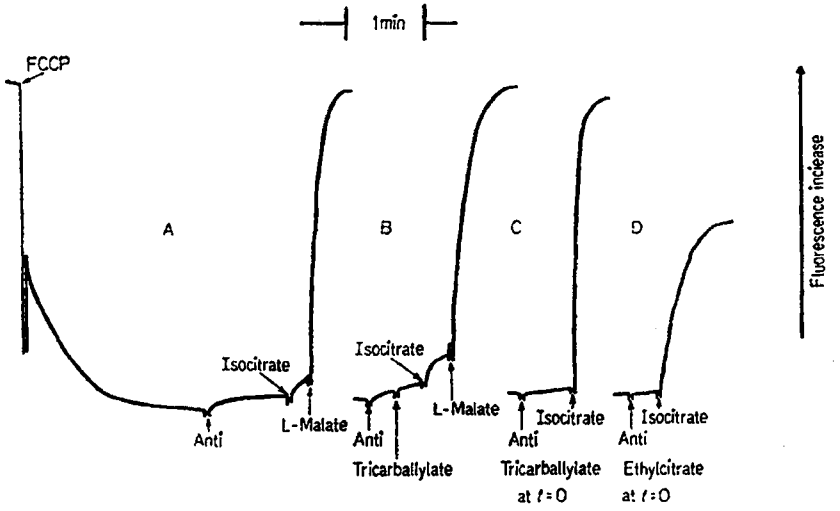

Fig. 6. *The activation of isocitrate oxidation by tricarballylate and 2-ethylcitrate.* Rat liver mitochondria (2–6 mg protein) were added to 2.5 ml of a medium containing 125 mM KCl, 20 mM Tris-chloride and 2 mM inorganic phosphate pH 7.4 at 30°. 1 μM FCCP was added and after 3 min 0.2 μg/ml antimycin followed by isocitrate, and L-malate. Only experiment A is shown in full since the initial oxidation of intramitochondrial NAD(P) by uncoupling agent was the same for A, B, C, and D. Pyridine nucleotide oxidation/reduction changes were followed fluorimetrically. Additions were as follows. Expt. A. FCCP, 1 μM, antimycin 0.2 μg/ml; *threo*-D_s-isocitrate, 200 μM; L-malate, 600 μM. Expt. B. FCCP, 1 μM; antimycin, 0.2 μg/ml; tricarballylate, 5 mM; *threo*-D_s-isocitrate, 200 μM; L-malate, 600 μM. Expt. C. tricarballylate, 5 mM; FCCP, 1 μM; antimycin 0.2 mg/ml; *threo*-D_s-isocitrate, 200 μM. Expt. D. 2-Ethylcitrate, 5 mM; FCCP, 1 μM; antimycin, 0.2 μg/ml; *threo*-D_s-isocitrate, 200 μM

citrate added at time zero also allowed isocitrate to cause a reduction in the absence of L-malate but the rate and extent of reduction observed were lower then with tricarballylate.

[^{14}C]Citrate Exchange

Aliquots of rat liver mitochondria (containing 7 mg protein) loaded with [^{14}C]citrate as described in the Methods section were pipetted into 10 ml centrifuge tubes containing 1 ml incubations of a buffer containing 125 mM KCl, 20 mM Tris chloride pH 7.4 at 0°. After 2 min the tubes were centrifuged for 4 min at 18000 × *g* to separate the mitochondria and 0.9 ml samples of the supernatant taken into 0.5 ml 1.5 M perchloric acid. After centrifuging the acidified supernatant to remove small amounts of precipitated protein, the samples were neutralised with base containing 6 N K_2CO_3 and 0.5 N triethanolamine. The tubes were then evacuated over KOH in a vacuum dessicator for 15 min to remove any remaining labelled CO_2 before counting the samples in a scintillator composed of 1:4 dioxane, naphthalene, 2-5 diphenyl oxazol and 1,4 bis (5-phenyloxazol-2-yl) benzene [30], using a Nuclear Chicago MKI scintillation counter. The samples were also assayed for citrate and L-malate as described in the Methods section and samples of unseparated mitochondrial incubations was acidified and neutralised as described for counting and assay of the total [^{14}C]citrate, citrate and L-malate. The results of making various additions to the 1 ml incubations before the mitochondrial addition is set out in Table 2.

When no additions were made about 10 % of the total counts were extramitochondrial, *i. e.* appeared in the supernatant, while 41 % of the L-malate and 33 % of the citrate was extramitochondrial. This was taken as the reference point for the other incubations. The presence of 10 mM tricarballylate in the medium caused an efflux of citrate as measured by radioactivity and by enzyme assay and an efflux of L-malate from the mitochondria. When the ratio of the difference in supernatant citrate between this and the control to the total intramitochondrial citrate available for exchange was expressed as percentage exchange, the radioactive and enzyme assay data gave good agreement. 10 mM citrate, as might be expected, gave a good exchange with [^{14}C]citrate and L-malate as did 1 mM citrate and 1 mM L-malate. 10 mM 2-ethylcitrate caused a small exchange and 10 mM pentane 1,4,5,-tricarboxylate an even smaller one. 10 mM 2-ethylcitrate inhibited the exchange of 1 mM L-malate and 1 mM citrate with [^{14}C]citrate while 10 mM tricarballylate caused their exchange to be increased presumably due to the addition of tricarballylate/[^{14}C]citrate exchange. Throughout the table good agreement is shown between radioactive and enzyme assay data. The advantage of the radioactive method is that citrate/[^{14}C]citrate exchange can be monitored. Table 3 shows the effect of 25 mM concentrations of inhibitors on citrate/[^{14}C]citrate exchange. As can be seen 25 mM 2-ethylcitrate reduces the exchange to one-fifth of its inhibited value but the value without citrate present is the same as the exchange produced by 2-ethylcitrate alone. This indicated that 2-ethylcitrate is a weak substrate for the transporting system. On the other hand 2-propylcitrate on its own caused no exchange. Fig. 7 shows the effect of increasing 2-ethylcitrate

Table 3. *The effect of analogues on citrate/citrate exchange* The experiment was carried out as described for Table 2. The proton was 6 mg per incubation and the specific activity of intramitochondrial [^{14}C]citrate was 163 counts × min^{-1} × nmole^{-1}

Additions	Radioactivity		Exchange
	Supernatant	Total-supernatant = Pellet	
	counts/min		%
None	1890	19520	0.0
Citrate 1 mM	4750	16660	13.4
Citrate 1 mM + 25 mM 2-Ethylcitrate	2470	18940	2.7
Citrate 1 mM + 25 mM 2-Propylcitrate	2750	18660	3.1
25 mM 2-Ethylcitrate	2460	18950	2.7
25 mM 2-Propylcitrate	1880	19530	0.0

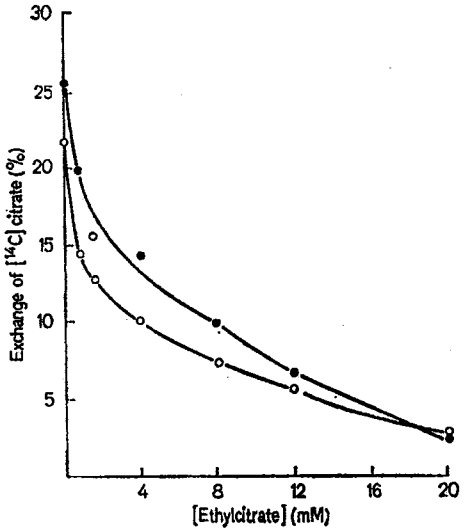

Fig. 7. *2-Ethylcitrate inhibition of citrate/[^{14}C]citrate and malate/[^{14}C]citrate exchange.* Incubations were performed as described for Table 1 using increasing concentrations of 2-ethylcitrate in the presence of 0.5 mM L-malate (○) and 0.5 mM citrate (●) as exchanger for the [^{14}C]citrate in the mitochondria. 6 mg mitochondrial protein was used in each incubation and per cent exchange calculated as in Table 1. The specific activity of intramitochondrial [^{14}C]citrate was 146 counts × min^{-1} × nmole^{-1}

Table 4. *Effects of 2-ethylcitrate and tricarballalyte on glucose uptake, outputs of lactate and pyruvate and glucose conversion to fatty acid in rat epididymal adipose tissue*

Fat pads were preincubated for 30 min in medium containing 10 mM glucose and then transferred for 60 min to fresh medium containing 10 mM glucose, D-[U-^{14}C]glucose (0.1 μC/ml) and insulin 10 mU/ml and the additions as noted below in each experiment. Mean values ± SEM for 4 paired groups of fat pads

* $P < 0.01$ for paired observations. ** $P < 0.05$ for paired observations

Experiment	Additions	Glucose uptake	Lactate output	Pyruvate output	Rate of fatty acid synthesis
		μatoms C × h^{-1} × g wet wt^{-1}			μatoms Glc-C × h^{-1} × g wet wt^{-1}
1	None	58.1 ± 4.8	5.85 ± 0.17	0.63 ± 0.06	27.8 ± 5.0
	2-Ethylcitrate (10 mM)	45.7 ± 4.8**	2.84 ± 0.15*	0.39 ± 0.03*	19.8 ± 2.8*
2	None	60.3 ± 5.8	4.86 ± 0.24	0.69 ± 0.04	45.3 ± 6.6
	Tricarballylate (5 mM)	47.1 ± 6.4	3.45 ± 0.17*	0.45 ± 0.02*	32.5 ± 5.3*

Table 5. *Effects of 2-ethylcitrate and tricarballylate on fatty acid synthesis from pyruvate or acetate in isolated rat white adipocytes*

Isolated adipocytes (50 mg/ml) were incubated in KRB plus albumin (20 mg/ml), insulin (100 μU/ml), glucose (2 mM) and ^{3}H-labelled H_2O (0.1 mC/ml) for 60 min and the additions as noted below. Results are expressed as mean ± SEM for 4 observations. *$P < 0.01$ **$P < 0.05$

Additions	Rate of fatty acid synthesis	
	Pyruvate (10 mM)	Acetate (10 mM)
	μatoms H × h^{-1} × g lipid^{-1}	
None	50.6 ± 1.4	42.0 ± 4.6
2-Ethylcitrate (5 mM)	57.0 ± 2.1**	44.6 ± 3.5
2-Ethylcitrate (10 mM)	16.8 ± 1.7*	18.6 ± 1.6*
Tricarballylate (2.5 mM)	60.6 ± 3.2**	48.4 ± 4.0
Tricarballylate (10 mM)	49.0 ± 3.3	38.8 ± 2.2

concentration on the L-malate/[^{14}C]citrate and citrate/[^{14}C]citrate exchanges. The sensitivity of the two exchanges to the inhibitor are markedly similar, and this may have implications from the mechanistic point of view of the exchange diffusion carrier.

Effect of Tricarboxylate Inhibition on Fatty Acid Synthesis

Since both tricarballylate and 2-ethylcitrate inhibited the transport of citrate from the mitochondrion into the cytoplasm the effects of these agents on glucose conversion to fatty acid was studied and the results reported in Table 4. Both agents inhibited the conversion of glucose to fatty acid by 30% but they also decreased the pyruvate and lactate outputs to a similar degree. In additional studies [33] both these agents were found to inhibit phosphofructokinase as indicated by the increased (1.5×) glucose 6-phosphate and fructose 6-phosphate levels and normal tissue ATP contents (76 nmoles/g wet wt.) in the adipocyte.

In order to determine whether the inhibition of this transport of citrate could inhibit fatty acid synthesis, the rates of conversion of pyruvate (requiring the citrate permease but by passing phosphofructokinase) and acetate (not requiring either the citrate permease or phosphofructokinase) to fatty acid were compared and the results reported in Table 5. Under the conditions studied, tricarballylate at low concentrations did not decrease and in fact caused a small increase in the rate of fatty acid synthesis with pyruvate or acetate as substrate, whereas only 2-ethylcitrate at very high concentrations (20 mM) caused an inhibition of fatty acid synthesis from both pyruvate and acetate.

DISCUSSION

The fluorimetric measurements of intramitochondrial NAD(P) reduction by *cis*-aconitate and *threo*-D_S-isocitrate in the presence of L-malate provided two important pieces of information. Firstly the kinetic data established that the competition by 2-ethylcitrate and tricarballylate was with the tricarboxylate anion and not with the L-malate. Secondly it was established that the concentrations of inhibitor had to be reactively high compared with the tricarboxylate concentration in order that significant inhibition be observed. The values of the K_i for 2-ethylcitrate and tricarballylate inhibition of isocitrate oxidation (0.45 and 0.9 mM, respectively) were appreciably higher than the K_m of 67 μM reported for isocitrate oxidation in whole mitochondria as measured by intramitochondrial NAD(P) reduction [11]. Any suspicion of non-specific tricarboxylate anion effects were ruled out by the lack of inhibition observed with pentane 1,4,5-tricarboxylate.

The inhibition of aconitase observed when high concentrations of tricarballylate and 2-ethylcitrate were used suggested that perhaps the effects of these inhibitors on *cis*-aconitate oxidation may have been a combination of inhibition at the level of the membrane and at the level of the mitochondrial aconitase. The lack of inhibition of NADP-isocitrate dehydrogenase in a broken mitochondrial preparation indi-

cated that the observed inhibition of isocitrate oxidation in whole mitochondria was at the level of the membrane. The design of the experiments was such however that effects other than those of permeation were minimised since the activator (L-malate) was always added after the substrate for the oxidation so that inhibitor permeation on the tricarboxylate anion carrier prior to permeation of substrate was not possible.

When tricarballylate was added at zero time (*i. e.* before depletion of endogenous substrate) it was able to exchange for L-malate so that when isocitrate was added after substrate depletion the tricarballylate could permit rapid entry of isocitrate by tricarballylate/isocitrate exchange even though L-malate had been removed by the substrate depletion (Fig. 6). 2-Ethylcitrate at the same concentrations as tricarballylate permitted slow entry of isocitrate into the mitochondria.

The site of action of the inhibitors was confirmed by the [^{14}C]citrate exchange data. Citrate, L-malate and tricarballylate all appeared to exchange with [^{14}C]citrate. 2-Ethylcitrate showed a slight exchange and 2-propylcitrate no exchange at all when used alone, but both caused inhibition of citrate/[^{14}C]-citrate and malate/[^{14}C]citrate exchange as judged both from radioactive and the enzyme assay data. This evidence would suggest that tricarballylate is a substrate for the transporting system and inhibits by competing for actual transport on the carrier protein. 2-Ethylcitrate on the other hand is only a weak substrate, but effectively blocks the transport of citrate or L-malate on the tricarboxylate anion transporting system. 2-Propylcitrate is not a substrate at all for this transporting system but is an inhibitor of citrate transport. The fact that 2-ethylcitrate appears to be equally potent as an inhibitor of L-malate/citrate and citrate/citrate exchanges is very interesting. If there were separate sites for L-malate and citrate binding on the transporting system then it would be expected that citrate present at the extramitochondrial surface would be able to compete for its site and thus relieve the inhibition whereas extramitochondrial L-malate would be ineffective ar a competitor for that site. However since 2-ethylcitrate appears to inhibit L-malate/[^{14}C]citrate and citrate/[^{14}C]citrate exchanges equally well, it is more likely that the binding sites for citrate and L malate on the transporting system are the same or are in close proximity.

The citrate permease, citrate lyase pathway has been suggested as the principle pathway for the transport of acetyl CoA from the mitochondria to cytoplasm in the fatty acid synthesis pathway [5—7]. The evidence supporting this is derived from either measurements of enzyme activity of citrate lyase and comparing this to the rates of fatty acid synthesis, or from radio isotope data. Lowenstein has suggested in a recent publication [31] that hydroxycitrate and tricarballylate inhibit citrate lyase and fatty acid synthesis. Both 2-ethylcitrate and tricarballylate (which are citrate analogues) like citrate, seemed to inhibit phosphofructokinase and, as a result of this, glucose conversion to fatty acid. Pyruvate and acetate conversion to fatty acid by-pass the phosphofructokinase step. Further, since 80 % of acetyl-CoA synthetase is of cytoplasmic origin in this tissue [34] acetate by-passes the citrate permease and citrate lyase steps. The failure of tricarballylate (10 mM) to inhibit fatty acid synthesis from pyruvate and acetate suggests that either it did not inhibit the citrate pathway to a sufficient degree to limit fatty acid synthesis or that there are additional pathways available for acetyl-CoA transport from the mitochondria to the cytoplasm. 2-Ethylcitrate (20 mM) inhibited both pyruvate and acetate conversion to fatty acid and therefore it is unlikely that inhibition of the citrate pathway could be implicated.

Tricarballylate (2.5 mM) stimulated the conversion of pyruvate and acetate to fatty acid (Table 5). Since tricarballylate is a known activator for acetyl-CoA carboxylase [32] it is likely that activation of this enzyme is responsible for the observed increase in the rate of fatty acid synthesis.

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