Inhibition of Enzymes which Interact with Citrate by (—)Hydroxycitrate and 1,2,3,-Tricarboxybenzene

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The effects of two citrate analogues, (—)hydroxycitrate and 1,2,3-tricarboxybenzene, were studied using rat liver enzymes which interact with citrate.

The most pronounced effect of 1,2,3-tricarboxybenzene was inhibition of acetyl-CoA carboxylase (K_i 20 μM). It also inhibited the mitochondrial citrate transporter (50% inhibition at 3 mM), but was not a substrate for this transporter.

ATP-citrate lyase was markedly inhibited by both free (—)hydroxycitrate (K_i 8 μM) and (—)hydroxycitrate lactone (K_i 50 to 100 μM). Acetyl-CoA carboxylase was activated by both the forms of (—)hydroxycitrate (K_a 0.7 mM and 1.6 mM, respectively). (—)Hydroxycitrate is a substrate for the mitochondrial citrate transporter, but its rate of transport is less than 10% of that of citrate.

Other citrate metabolizing enzymes also were inhibited by 1,2,3-tricarboxybenzene and (—)hydroxycitrate but much higher concentrations were required.

The importance of citrate as a regulator and substrate in intermediary metabolism has received considerable attention. It is an intermediate in the tricarboxylic acid cycle and in the pathway for biosynthesis of lipids. It is an inhibitor of phosphofructokinase [1—3] and, therefore, a potential regulator of glycolysis. Activation of acetyl-CoA carboxylase [4—6] by citrate may stimulate the fatty acid biosynthetic pathway. Citrate has also been shown to inhibit pyruvate dehydrogenase [7,8], an action which could limit pyruvate oxidation in fasting.

Two analogues of citrate which inhibit specific steps in citrate metabolism have recently been reported [9,10]. (—)Hydroxycitrate is an inhibitor of ATP-citrate lyase [9] and 1,2,3-tricarboxybenzene is an inhibitor of the mitochondrial citrate transporter [10]. We have studied the effects of these inhibitors on the enzymes and transport system known to be involved in the intermediary metabolism of citrate. These studies were undertaken to allow more rational interpretation of their effects in biological systems.

Enzymes. Acetyl-CoA carboxylase (EC 6.4.1.2); citrate-(isocitrate)hydro-lyase or aconitase (EC 4.2.1.3); ATP-citrate lyase (EC 4.1.3.8); citrate synthase (EC 4.1.3.7); NAD-isocitrate dehydrogenase (EC 1.1.1.41); NADP-isocitrate dehydrogenase (EC 1.1.1.42); phosphofructokinase (EC 2.7.1.11) pyruvate dehydrogenase (EC 1.2.4.1).

MATERIALS AND METHODS

Male Wistar rats weighing 110—150 g were fed *ad libitum* until sacrificed. (—)Hydroxycitrate lactone was a kind gift from Dr Y. S. Lewis (Mysore, India). 1,2,3-Tricarboxybenzene was obtained from K. and K. Laboratories (Plainview, N. Y.). [1-^{14}C]Pyruvate and sodium [^{14}C]bicarbonate were purchased from Amersham-Searle (Don Mills, Ontario). Aldolase, glycerol-l-phosphate dehydrogenase, triosephosphate isomerase, NADP$^+$-linked isocitrate dehydrogenase, metabolic intermediates and the pyridine nucleotides were obtained from Boehringer Mannheim GmbH (New York, N.Y.). Acetyl-CoA and bovine serum albumin were obtained from Sigma Chemical Co. (St. Louis, Missouri) and coenzyme A was obtained from Calbiochem (Los Angeles, California).

Preparation and Assay of Enzymes from Rat Liver

Protein was determined by the method of Warburg and Christian [11] or Lowry *et al.* [12]. Enzymes were used for kinetic studies within 1—2 days after their preparation.

Citrate synthase was prepared up to the first $(NH_4)_2SO_4$ fractionation with a specific activity of 0.63 units/mg protein, and assayed by the method of Shepherd and Garland [13].

Aconitase was prepared by the method of Fansler and Lowenstein [14], with slight modifications. The intramitochondrial enzyme was obtained by suspending a mitochondrial pellet [15] in four times its volume of 15 mM tricarballylate-Tris buffer, pH 7.8. The suspension was sonicated at 20 kHz/s for 8 min (5–10 °C). Insoluble material was removed by centrifugation at 13000×g for 15 min. Solid $(NH_4)_2SO_4$ was added to the supernatant to 50% of saturation. After centrifugation at 13000×g for 15 min the precipitate was discarded and the supernatant assayed for aconitase activity. The enzyme had a specific activity of 24 units/mg protein. The kinetic parameters were determined using a spectrophotometric assay [14] or a fluorometric assay coupling this reaction to NADP+-linked isocitrate dehydrogenase.

ATP-citrate lyase was purified to the 25–45% $(NH_4)_2SO_4$ fractionation with a specific activity of 0.39 unit/mg protein by the method of Takeda *et al.* and assayed fluorometrically [16].

NADP+-linked isocitrate dehydrogenase (intramitochondrial and extramitochondrial) was purified as follows. Rat livers were homogenized in 0.1 M potassium phosphate buffer pH 7.6 containing 0.5 M sucrose and centrifuged at 1000×g for 10 min. The supernatant was centrifuged again for 10 min at 13000×g to obtain a mitochondrial pellet. This pellet was suspended in 3 volumes of 0.1 M potassium phosphate buffer pH 7.6, sonicated at 20 kHz/s for 6 min (5–10°C) and centrifuged at 13000×g for 30 min. Dithiothreitol (0.3 mM) and EDTA (1 mM) were added to this supernatant, then $(NH_4)_2SO_4$ was added to 50% saturation, After centrifugation at 10000×g for 15 min, the pellet was discarded and additional ammonium sulphate added to 80% of saturation. After centrifugation, the precipitate was dissolved in a minimum volume of 0.1 M Tris-HCl pH 7.4 containing 0.3 mM dithiothreitol and 1 mM EDTA. The specific activity of the enzyme was 203 units/mg protein. The supernatant obtained from the initial 13000×g centrifugation was centrifuged again at 105000×g for 60 min. A 50–80% ammonium sulphate fractionation was carried out, as just described for the soluble mitochondrial enzyme, to a specific activity of 330 units/mg protein. Both enzymes were assayed fluorimetrically [17].

NAD+-linked isocitrate dehydrogenase was prepared from rat liver mitochondria (specific activity 17 units/mg) and assayed as described by Martin and Denton [17].

Acetyl-CoA carboxylase was assayed in an 80000 ×g supernatant by the sodium [^{14}C]bicarbonate fixation method [18].

Phosphofructokinase was prepared as a 30–50% $(NH_4)_2SO_4$ fraction (specific activity 7.9 units/mg protein) by the method of Kemp [19], with the exception that the heat step was omitted. It was assayed fluorometrically [3].

Pyruvate dehydrogenase was assayed in rat liver mitochondria after homogenization of the mitochondrial pellet in hypotonic solution (10 mM potassium phosphate, 1 mM EDTA, 1 mM dithiothreitol, 1% fatty-acid-poor bovine serum albumin, pH 7.4) [20].

Mitochondrial-tricarboxylate-exchange studies were carried out using the methods of Robinson *et al.* [21] for both [^{14}C]citrate loading and [^{14}C]citrate/citrate exchanges.

The Purification of (–)Hydroxycitric Acid

(–)Hydroxycitric acid was dissolved in ether and filtered to remove insoluble impurities. Purified (–)hydroxycitric acid lactone, m.p. 178 °C, was recrystallized from ether as described by Lewis and Neelakanton [22]. Aqueous solutions of potassium salts of the free acid were obtained by raising the pH to 12 with KOH for 10 min at 25 °C prior to neutralization.

1 unit of enzyme activity is defined as the amount causing transformation of 1 μmol substrate per min at 25 °C under optimal conditions of measurement.

RESULTS AND DISCUSSION

Phosphofructokinase

As shown previously [1–3], citrate inhibited phosphofructokinase (Table 1). Phosphofructokinase was also inhibited by 1,2,3-tricarboxybenzene and (–)hydroxycitrate, but the concentrations required for 50% decrease in activity were approximately 1.2-fold greater than for citrate (3.9 mM) (Table 1).

Acetyl-CoA Carboxylase

Activation of acetyl-CoA carboxylase by citrate was inhibited by 1,2,3-tricarboxybenzene is a manner competitive with citrate (K_i approx. 20 μM, Fig. 1). In the absence of citrate, 1,2,3-tricarboxybenzene had no effect. (–)Hydroxycitrate activated acetyl-CoA carboxylase (Fig. 2), as reported previously [23,24].

The Mitochondrial Tricarboxylate Carrier and the Intramitochondrial Enzymes

Previous studies have shown that 1,2,3-tricarboxybenzene is a competitive inhibitor of citrate transport in rat-liver mitochondria with a K_i of 3 mM [10] (Table 2B). (–)Hydroxycitrate did not inhibit the carrier but was a weak substrate. The K_m appears to be of the same order of magnitude as that for citrate, but the V was much lower (Table 2A). As a weak substrate for this carrier, (–)hydroxycitrate may enter the mitochondria where it could affect the intramitochondrial citrate-metabolizing enzymes. Free (–)hydroxycitrate inhibited intra-

Table 1. *Effects of citrate, 1,2,3-tricarboxybenzene and (−)hydroxycitrate on phosphofructokinase from rat liver*
The assay mixture (1 ml) contained 0.1 M Tris-HCl pH 7.0, 0.03 mM EDTA, 10.0 mM $MgCl_2$, 0.06 mM NADH, 0.06 mM dithiothreitol, 0.01 M NH_4Cl, 0.2 mM ATP, 0.3 mM fructose-6-phosphate, 30 µg aldolase, 5 µg glycerol-phosphate dehydrogenase and 5 µg triosephosphate isomerase in addition to the extract from rat liver. The reaction was started by the addition of fructose 6-phosphate. Each assay was studied in duplicate using six concentrations of each inhibitor

Addition to assay mixture	Concentration for 50 % inhibition
	mM
Citrate	3.9
1,2,3-Tricarboxybenzene	4.7
(−)Hydroxycitrate (free)	5.0
(−)Hydroxycitrate (lactone)	3.6

Table 2. *Effect of citrate, (−)hydroxycitrate and 1,2,3-tricarboxybenzene on the mitochondrial citrate transporter of rat-liver mitochondria*
Incubations were performed at 8 °C as described by Robinson *et al.* [21]. The exchanges were started by the addition of [^{14}C]citrate-loaded mitochondria. After 45 s, citrate transport was terminated by the rapid addition of 1,2,3-tricarboxybenzene (50 mM). The constants reported were calculated from Lineweaver-Burk plots, using 7 concentrations of each substrate

A

Addition	*Km*	Rate of citrate exchange, V_{max}
	mM	nmol × min^{-1} × mg protein^{-1}
Citrate	0.22	3.5 to 8.0 (12)
(−)Hydroxycitrate (free)	0.35	0.32
(−)Hydroxycitrate (lactone)	0.30	0.17

B

Addition	Type of inhibition	Concentration required for 50 % inhibition
		mM
Citrate + 1,2,3-tricarboxybenzene	competitive	3.0
Citrate + (−)hydroxycitrate (free)	no inhibition	—
Citrate + (−)hydroxycitrate (lactone)	no inhibition	—

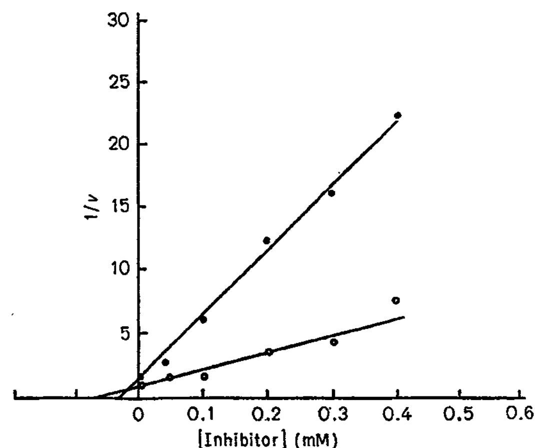

Fig. 1. *Dixon plot to show the effect of 1,2,3-tricarboxybenzene on citrate-activated acetyl-CoA carboxylase activity.* Acetyl-CoA carboxylase was assayed by the [^{14}C]bicarbonate fixation assay [18]. Supernatant (0.02 ml) was preincubated at 37 °C for 30 min in the presence of potassium citrate. The preincubation mixture also contained 50 mM Tris-HCl pH 7.5, 20 mM $MgCl_2$, 1.0 mM 2-mercaptoethanol, and 0.9 mg/ml bovine serum albumin in a total volume of 0.7 ml. The reaction was started by adding 0.40 mM $NaH^{14}CO_3$ (10-µCi), 2 mM ATP, and 0.14 mM acetyl-CoA to the preincubation mixture and then incubated for 2 min. Velocity (*v*) was measured as $NaH^{14}CO_3$ final (nmol min^{-1} µg^{-1}). 1,2,3-Tricarboxybenzene (inhibitor) was added to the preincubation mixture in the presence of potassium citrate, 3 mM (●) and 7 mM (○)

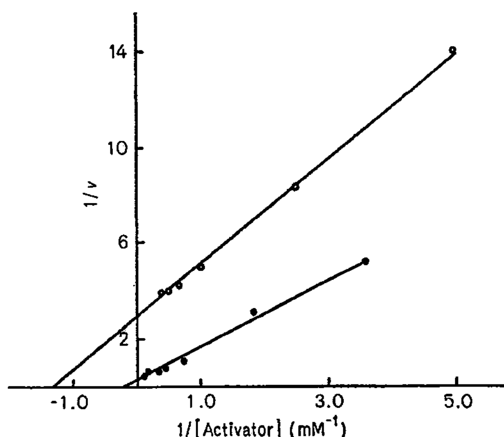

Fig. 2. *Effect of citrate and (−)hydroxycitrate on acetyl-CoA carboxylase activity from rat liver.* Conditions of assay were those described for Fig. 1. The K_m values were calculated to be 6.7 mM for citrate (●——●) and 0.7 mM for free (−)hydroxycitrate (○——○). Not shown in the figure, the K_m for the lactone (−)hydroxycitrate was 1.6 mM

mitochondrial NADP$^+$-linked isocitrate dehydrogenase with a K_i of 0.25 mM and aconitase with a K_i of 0.28 mM (Table 3). Intramitochondrial aconitase was also inhibited by (−)hydroxycitrate lactone (K_i 0.3 mM). Neither (−)hydroxycitrate nor 1,2,3-tricarboxybenzene inhibited the NAD$^+$-linked isocitrate dehydrogenase. In fact, slight activation of this enzyme by (−)hydroxycitrate was observed at low isocitrate concentrations. This enzyme can also be activated by citrate [25—27]. The K_i values for the other intramitochondrial enzymes that are inhibited by these compounds are very high (Table 3),

Table 3. K_i *values for inhibition by (—)hydroxycitrate and 1,2,3-tricarboxybenzene of enzyme with citrate and isocitrate as substrate or product*
The various enzymes were assayed as described in Methods. The kinetic constants were calculated from Dixon plots using a minimum of six inhibitor concentrations

Enzyme	Substrate	(—)Hydroxycitrate (free)	(—)Hydroxycitrate (lactone)	1,2,3-Tri-carboxybenzene
		mM	mM	mM
Citrate synthase	oxalacetate	3.0	5.0	1.5
	acetyl-CoA	3.5	3.0	0.7
Aconitase	*cis*-aconitate	0.28	0.3	3.7[a]
Citrate lyase	citrate	0.008	0.05—0.1	no effect
Isocitrate dehydrogenase NADP+-linked (mitochondrial)	isocitrate	0.25	2.4	1.6
Isocitrate dehydrogenase NADP+-linked (extramitochondrial)	isocitrate	0.4	2.0	2.2
Isocitrate dehydrogenase NAD+-linked	isocitrate	no inhibition (slight activation at low isocitrate)	no inhibition (slight activation at low isocitrate)	no effect

[a] All inhibitors were competitive except this one, which was non-competitive.

e.g. citrate synthase, aconitase and NADP+-linked isocitrate dehydrogenase.

ATP-Citrate Lyase

Competitive inhibition of ATP-citrate lyase by (—)hydroxycitrate [9] at low concentrations (8 μM) has been confirmed in this study. 1,2,3-Tricarboxybenzene did not affect this enzyme (Table 3).

Pyruvate Dehydrogenase

As reported previously [7,8], pyruvate dehydrogenase prepared from rat-liver mitochondria was inhibited completely by citrate (4.5 mM, Table 4). The concentrations of (—)hydroxycitrate and 1,2,3-tricarboxybenzene required to inhibit pyruvate dehydrogenase were greater than that required for citrate (Table 4). (—)Hydroxycitrate was somewhat more inhibitory than 1,2,3-tricarboxybenzene. Since neither inhibitor should be expected to reach these concentrations inside the mitochondria, these inhibitors are unlikely to affect pyruvate dehydrogenase in intact cell experiments.

Table 4. *Inhibition of rat-liver pyruvate dehydrogenase by citrate. (—)hydroxycitrate and 1,2,3-tricarboxybenzene*
The enzyme was first converted to the phosphorylated (inactive) form by preincubation in the presence of 1 mM ATP for 30 min at room temperature. The enzyme was then activated in the presence of 10 mM $MgCl_2$ and 9 mM $CaCl_2$ for 5 min at room temperature. The control enzyme value was 17.8 ± 2.7 nmol [1-^{14}C]pyruvate → $^{14}CO_2$ per 2 min. The tricarboxylates were added to the activation mixture and the enzyme was then assayed 5 min later. Results represent the mean ± S.E.M. of 4 experiments, each experiment with quadruplicate observations

Addititon of tricarboxylate to activation mixture	Concentration	Activity
	mM	%
No addition (control)		100
Citrate	0.5	77 ± 10.8
	1.5	36 ± 11.8[a]
	4.5	−6 ± 8.2[a]
(—)Hydroxycitrate (hydrolyzed or lactone)	0.5	97 ± 18.2
	0.5	97 ± 18.2
	1.5	75 ± 17.0
	4.5	59 ± 10.0[a]
1,2,3-Tricarboxybenzene	0.5	86 ± 7.2
	1.5	84 ± 6.6
	4.5	71 ± 8.2[a]

[a] $P < 0.01$.

General Comments

There are major problems with the use of metabolic inhibitors in the study of regulatory phenomenon. A compound is required whose site and mode of action are specific. Also, the concentration of this agent required to achieve this action should be known as higher concentrations of these inhibitors might have additional sites of action.

1,2,3-Tricarboxybenzene is a very potent inhibitor of acetyl-CoA carboxylase (K_i 20 μM, Fig. 1). If its cytosolic concentration were to rise to the mM range, inhibition of the mitochondrial citrate transporter (Table 2), phosphofructokinase (Table 1), extramitochondrial NADP+-linked isocitrate dehydrogenase and aconitase (Table 3) would occur. Since this agent is unlikely to enter rat-liver mito-

chondria (Table 2), one would not anticipate direct effects on intramitochondrial enzymes.

(−)Hydroxycitrate is a potent inhibitor of ATP-citrate lyase (K_i 8 μM, Table 3) and has been used in studies on the control of lipogenesis [11,28−31]. Higher cytosolic concentrations might activate acetyl-CoA carboxylase (K_a 700 μM, Fig. 2) and also enter the mitochondrion on the citrate transporter (K_m 350 μM, Table 2). Once inside the mitochondrion, inhibition of $NADP^+$-linked isocitrate dehydrogenase and aconitase (Table 3) could occur. At concentrations in the mM range, citrate synthase (Table 3), pyruvate dehydrogenase (Table 4) and phosphofructokinase (Table 1) might also be inhibited.

In addition, 1,2,3-tricarboxybenzene and (−)-hydroxycitrate, like citrate, can chelate divalent metal cations like Mg^{2+}, Mn^{2+} and Ca^{2+} which could produce other non-specific metabolic actions.

Conclusions

1,2,3-Tricarboxybenzene may be a suitable agent to study the cholesterol biosynthetic pathway in rat liver with fatty acid synthesis inhibited. This would require a cytosolic concentration 1,2,3-tricarboxybenzene which was high enough to inhibit acetyl-CoA carboxylase (K_i approx. 20 μM) but low enough so as not to inhibit the mitochondrial citrate transporter (K_i approx. 2 mM) or phosphofructokinase (K_i approx. 5 mM). This agent is unlikely to enter rat-liver mitochondria because it is not a substrate for the citrate transporter.

In studies using (−)hydroxycitrate as a specific inhibitor of ATP-citrate lyase (K_i approx. 8 μM), activation of acetyl-CoA carboxylase (K_a approx. 700 μM) and entry into the mitochondrion on the citrate transporter (K_m 350 μM) should be considered in interpreting results. Once inside the mitochondrion, this agent could inhibit aconitase and $NADP^+$-linked isocitrate dehydrogenase (K_i values 250−280 μM). High cytosolic concentrations could also inhibit phosphofructokinase.

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