

TITLE

EXPERIMENTS WITH (-)-HYDROXYCITRATE

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CONCLUSION OF STUDY

The experiments provide new evidence that the citrate cleavage pathway is the major source of carbon for the synthesis of fatty acids in rat. Transfer of citrate carbon from the mitochondria into the cytoplasm probably occurs in the form of tricarboxylic acids, and not via α -ketoglutarate or glutamate.

Experiments were also conducted to determine the level of fatty acid synthesis in non-ruminant animals.

HOW THIS STUDY IS RELEVANT TO OUR PRODUCT

These experiments demonstrated some of the mechanisms of action of (-)-HCA and further underpinned the safety of the various salts of (-)-HCA. They form much of the referenced basis in many subsequent articles and experiments with (-)-HCA.

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INTRODUCTION

Hydroxycitrate was first reported to occur as a minor constituent of sugar beets by E. von Lippman (1883). The first biochemical investigation of the stereoisomers of hydroxycitrate was conducted by Martius and Maué (1941). Using the methylene blue test for dehydrogenase activity, these authors demonstrated that racemic hydroxycitrate is attacked by what was presumed to be isocitrate dehydrogenase from various sources. On the other hand, racemic *allo*-hydroxycitrate is not attacked. Racemic hydroxycitrate was resolved, and it was shown that only the (—)-isomer is attacked. These results have recently been confirmed in my laboratory by Jean White. She found that highly purified preparations of isocitrate dehydrogenase from beef heart and beef liver catalyze the reduction of TPN to TPNH when the (+) but not the (—)-isomer of hydroxycitrate is tested as substrate.

The (—)-isomer of hydroxycitrate was first isolated from *Garcinia cambogia* by Lewis and Neelakantan (1964, 1965). The (+)-*allo*-isomer was first isolated from *Hibiscus sabdariffa* by Griebel (1939, 1942). Isolation procedures for both isomers have recently been described in detail by Lewis (1969). The absolute configurations of the lactones of these compounds have been determined by X-ray crystallography (Glusker, 1969). This work confirms the predictions of the absolute configurations made earlier by Lewis.

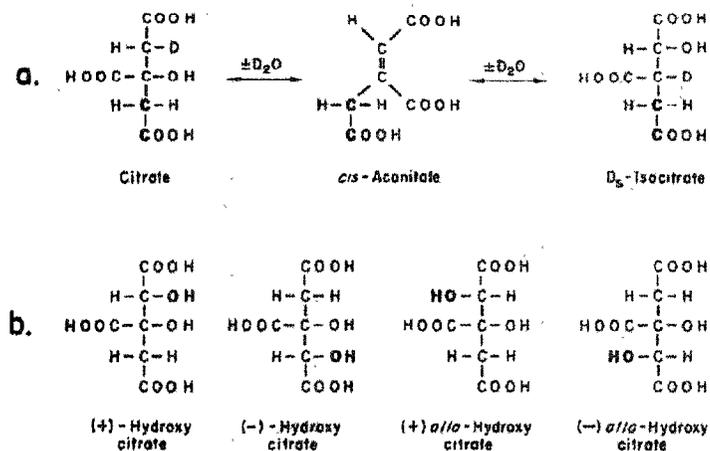
The steric relation of citrate and 'natural' isocitrate to the four stereoisomers of hydroxycitrate is shown in Scheme 1. The absolute configuration of citrate derived from oxaloacetate and acetyl CoA via the citrate synthase reaction are shown in Scheme 1a, as are the absolute configurations of *cis*-aconitate and D₃-isocitrate derived from such citrate via the aconitase reaction (Lowenstein, 1967). Scheme 1b shows that the stereochemistry of (+)-hydroxycitrate corresponds to that of D₃-isocitrate, the

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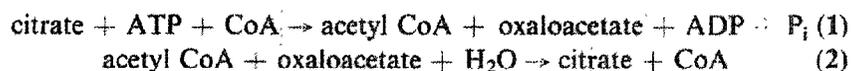
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Scheme 1. Fischer projection formulas of the absolute configuration of the stereoisomers of hydroxycitrate in relation to the absolute configuration of citrate, *cis*-aconitate and D₅-isocitrate. In a, the carbon atoms from the acetyl group of acetyl-CoA which become citrate in the citrate synthase reaction are shown in heavy outline. The stereochemistry of the aconitase reaction is shown by depicting the reaction as it occurs in D₂O. In b, the hydroxyl group shown in heavy outline is unique to the particular stereoisomer of hydroxycitrate. The configuration of (+)-hydroxycitrate corresponds to that of D₅-isocitrate. (Note that in the projection formulas the horizontal bonds point from the vertical bonds towards the reader)

'natural' substrate of isocitrate dehydrogenases from animal sources. This is in harmony with the finding that (+)-hydroxycitrate is a substrate for isocitrate dehydrogenase.

In the reaction catalysed by citrate cleavage enzyme (reaction 1), the carbon atoms of citrate which become the acetyl group of acetyl CoA are the same stereochemically as the carbon atoms of the acetyl group of acetyl CoA which become citrate in the reaction catalysed by citrate synthase (reaction 2)



(Spencer and Lowenstein, 1962). This finding has been confirmed (Bhaduri and Srere, 1963). The carbon atoms of citrate in question are shown in heavy outline in Scheme 1a. Comparison with the four compounds in Scheme 1b shows that (-)-hydroxycitrate and (-)-*allo*-hydroxycitrate carry a hydroxyl group on the carbon atom of citrate which normally becomes the methyl group of acetyl CoA in the citrate cleavage reaction.

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It might be mentioned that from the mechanistic view, the presence of an additional hydroxyl group in the 2-position of citrate would be expected to facilitate cleavage of the carbon-carbon bond between the two hydroxyl-carrying carbons. However, (–)-hydroxycitrate does not appear to be cleaved by citrate cleavage enzyme, at least not as far as can be ascertained through hydroxamate assays. Instead, we have found that (–)-hydroxycitrate is a potent inhibitor of citrate cleavage enzyme (Watson, Fang and Lowenstein, 1969). Apparently the steric positioning of the additional hydroxyl group enhances binding to the enzyme but prevents a subsequent step in the catalytic sequence. The other stereoisomer, (–)-*allo*-hydroxycitrate, has not so far been available for testing in the citrate cleavage reaction.

INHIBITION OF CITRATE CLEAVAGE ENZYME*
BY HYDROXYCITRATE

(–)-Hydroxycitrate is a powerful inhibitor of the reaction catalysed by citrate cleavage enzyme (reaction 1). The inhibition is competitive with respect to citrate. At a KCl concentration of 300 mM, the K_m for citrate is about 200 μ M, while the K_i for (–)-hydroxycitrate is about 0.57 μ M (Figure 1). At a KCl concentration of 85 mM the K_m and K_i values are 70 and 0.15 μ M respectively (Watson, Fang and Lowenstein, 1969). Under similar conditions, (+)-*allo*-hydroxycitrate has little inhibitory effect on the enzyme.

FATTY ACID SYNTHESIS IN NON-RUMINANT MAMMALS

In the breakdown of foodstuffs, pyruvate derived from carbohydrate and fatty acids derived from fat are converted to acetyl-CoA by intramitochondrial enzyme systems. Under normal conditions of carbohydrate utilization, the rate of oxidation of the acetyl group of acetyl-CoA via the citric acid cycle is determined by the energy demands of the tissue, or its equivalent, the availability of ADP. When the carbohydrate intake of an animal is in excess of its energy requirements the glycogen stores become filled. Thereafter excess carbohydrate is broken down to pyruvate. The reactions of glycolysis occur in the extramitochondrial space of the cell. However, the oxidation of pyruvate to acetyl-CoA occurs in the mitochondria. Acetyl groups not required for energy production are converted into fatty acids. In the rat, fatty acid synthesis occurs predominantly in the extramitochondrial space of the cell. The transfer of the acetyl group of acetyl-CoA from the intramitochondrial space into the cytoplasm is thus

* ATP: citrate-oxaloacetate lyase (CoA-acetylating and ATP-dephosphorylating), EC 4.1.3.8.

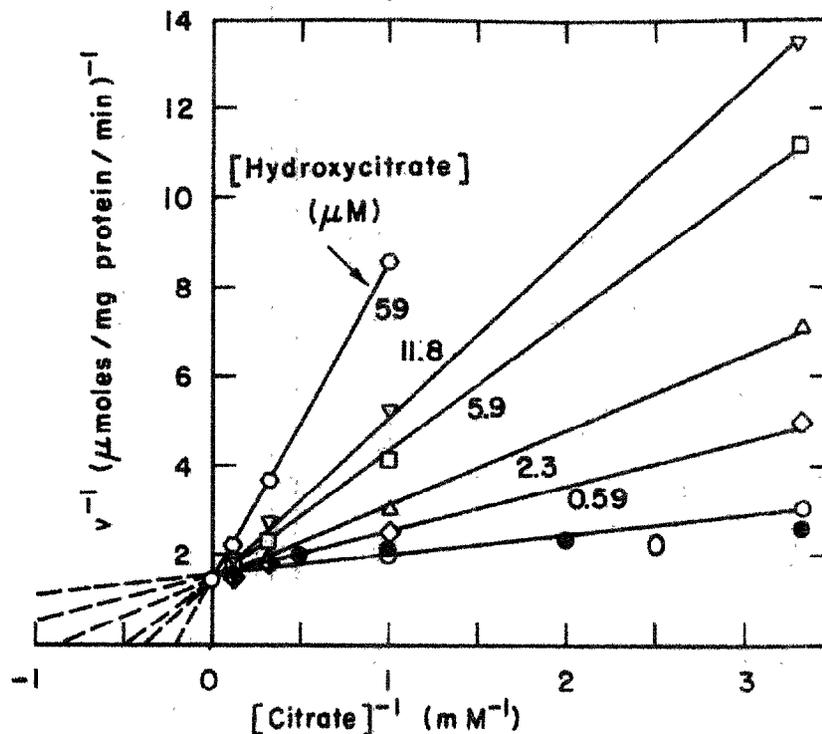


Figure 1. Inhibition of citrate cleavage by (-)-hydroxycitrate. The reaction mixture contained 20 mM MgCl₂, 300 mM KCl, 0.34 mM CoA, 93 mM Tris-HCl buffer, pH 8.2, 10 mM dithiothreitol, 3.33 mM ATP, 0.16 mM DPNH, malate dehydrogenase (0.33 unit/ml) citrate cleavage enzyme (1.7 milliunits/ml, specific activity about 2), and citrate and (-)-hydroxycitrate as indicated. The final volume was 3.0 ml and the temperature was 23° C. The assay was started by addition of ATP, and the reaction was followed by measuring the decrease in absorbance at 340 mμ (Watson, Fang and Lowenstein, 1969)

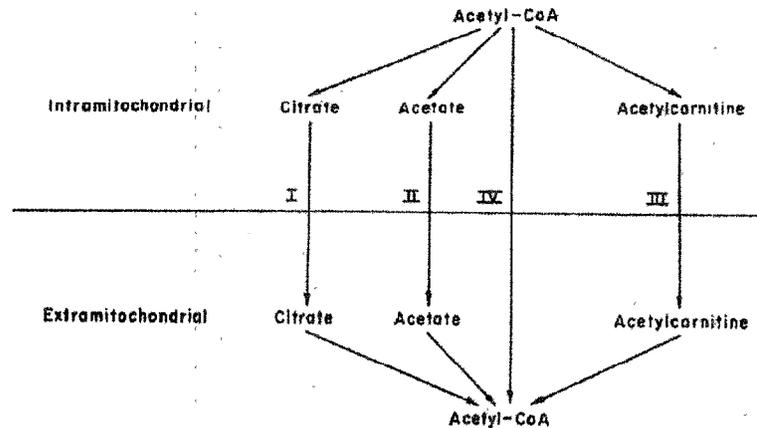
an important step in the conversion of carbohydrate into fat by non-ruminant mammals (Lowenstein, 1968).

Various ways in which the acetyl group of intramitochondrial acetyl-CoA might be transferred into the extramitochondrial space of the cell are summarized in Scheme 2 (Spencer and Lowenstein, 1962; Lowenstein, 1963). Each of the possible pathways shown in the Scheme was examined in terms of rates of diffusion of metabolites and of intra- and extramitochondrial enzyme levels. On this basis it was concluded that *citrate is the major source of the acetyl group of acetyl CoA which is used for the extramitochondrial synthesis of fatty acids in non-ruminant mammals*

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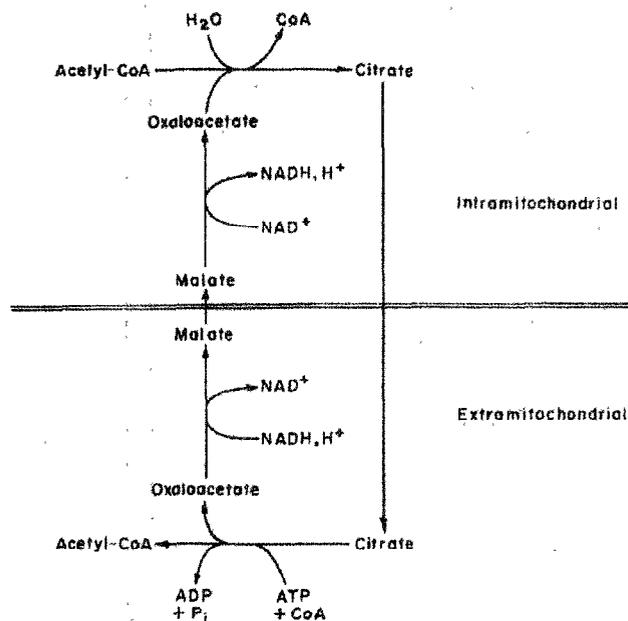
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Scheme 2. Four possible pathways for the transfer of the acetyl group of acetyl-CoA from the intra- to the extramitochondrial space of the cell. In I, citrate is formed from acetyl-CoA by the citrate synthase reaction. Citrate is then transferred into the extramitochondrial space before being converted back to acetyl-CoA by the citrate cleavage enzyme reaction. In II, acetyl-CoA is hydrolysed to acetate which is transferred into the extramitochondrial space and is converted back to acetyl-CoA by the acetate thiokinase reaction. In III, acetyl-CoA is converted to acetyl carnitine, which after diffusing from the mitochondria is converted back to acetyl-CoA. The enzyme which catalyses both reactions is acetyl-CoA-carnitine acetyl transferase. In IV, acetyl-CoA is depicted as leaving the mitochondria by direct diffusion of the intact molecule. (From Kornacker and Lowenstein, 1965)

(Lowenstein, 1968). Scheme 3 shows an elaboration of the citrate pathway indicated in outline in Scheme 2. Starting with intramitochondrial acetyl-CoA this pathway involves the formation of citrate via the citrate synthase reaction, the diffusion of intramitochondrial citrate into the extramitochondrial space (possibly as a magnesium citrate chelate), and the formation of acetyl-CoA via the citrate cleavage reaction. Oxaloacetate formed in the citrate cleavage reaction must be returned to the intramitochondrial space. Mitochondria are exceedingly impermeable to oxaloacetate at the low concentrations of this substance which prevail in the cell (Lardy, 1966). The extramitochondrial conversion of oxaloacetate to malate, and the intramitochondrial conversion of malate to oxaloacetate are shown in Scheme 3 as a possible way to overcome the permeability barrier presented to oxaloacetate. This proposal is based on the ubiquitous occurrence of both intra- and extramitochondrial malate dehydrogenases. However, other means of returning the carbon skeleton of oxaloacetate into the mitochondria are not ruled out. For example,

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Scheme 3. Transfer of the acetyl group of acetyl-CoA from the intra- to the extramitochondrial space of the cell via citrate. The 'carrier' in this scheme is oxaloacetate, which is used up in the mitochondria and is regenerated in the extramitochondrial space. Unless the carrier is returned to the mitochondria, it will accumulate in the extramitochondrial space. The return of oxaloacetate is shown to proceed through the intermediate formation of malate, but other intermediate reactions are not ruled out

oxaloacetate may be converted into aspartate, which may diffuse into the mitochondria and then be converted back to oxaloacetate. This pathway is feasible because of the ubiquitous occurrence of extra- and intramitochondrial glutamate-aspartate transaminase (Borst, 1961; Lardy, 1966; Chappell and Robinson, 1968). Another possibility is the conversion of malate to pyruvate. The pyruvate may diffuse into the mitochondria and then be converted back to oxaloacetate. This pathway is feasible because of the occurrence of malic enzyme in the extramitochondrial space of the cell, particularly under conditions when fatty acid synthesis is high (Lowenstein 1961; Wood and Utter, 1965; Ballard and Hanson, 1967). Much material has been published in the last five years that is pertinent to the entry and egress from mitochondria of citric acid cycle compounds and of reducing equivalents. A comprehensive treatment of various facets

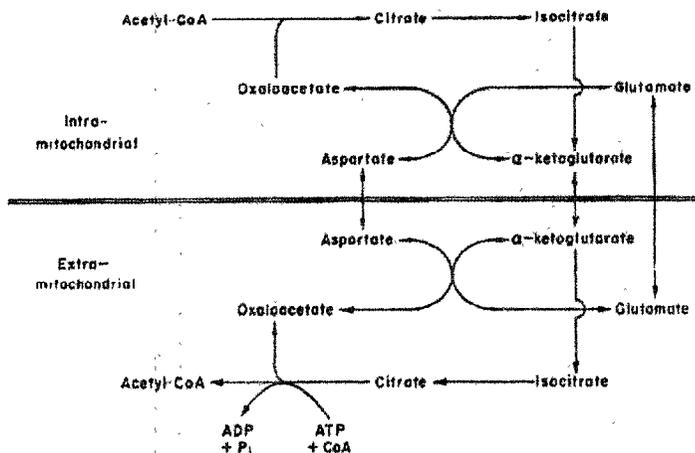
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of these topics is given in the recent reviews by Greville (1969), Utter (1969), and Tager, de Haan and Slater (1969).

The role of the citrate cleavage reaction in the formation of extramitochondrial acetyl-CoA was also investigated by Daikuhara, Tsunemi, and Takeda (1968). A reconstructed system was used which consisted of mitochondria and particle-free supernatant prepared from rat liver. The acetylation of either sulfanilamide or *p*-toluidine was studied using either ¹⁴C-labelled or unlabelled pyruvate as the source of carbon. Treatment of the supernatant with antiserum to pure citrate cleavage enzyme (Inoue, Suzuki, Fukunishi, Adachi and Takeda, 1966) reduced the acetylation of sulfanilamide and *p*-toluidine to about 15% of the amounts observed in controls. Neither L- nor D-carnitine affected the rates of acetylation in the presence or absence of antiserum. The authors concluded that more than 80% of the extramitochondrial acetyl-CoA derived from pyruvate is supplied through the citrate cleavage pathway.



Scheme 4. Transfer of the acetyl group of acetyl-CoA from the intra- to the extramitochondrial space of the cell via α -ketoglutarate and glutamate. Acetyl-CoA is converted to α -ketoglutarate through the intermediate formation of citrate and isocitrate. The scheme is intended to show that α -ketoglutarate can diffuse into the extramitochondrial space as such or after conversion to glutamate via transamination. Either glutamate or α -ketoglutarate or both are then converted back to citrate, which is converted to acetyl-CoA and oxaloacetate by the citrate cleavage reaction (adapted from D'Adamo and Haft, 1962). The return of oxaloacetate into the mitochondria is shown to proceed through the intermediate formation of aspartate, but other intermediate reactions are not ruled out

In 1962 D'Adamo and Haft reported experiments which showed that the radioactive carbon of [5-¹⁴C]glutamate, but not that of [2-¹⁴C]glutamate, is incorporated into fatty acids (see also Abraham, Madsen and Chaikoff, 1964; Madsen, Abraham, and Chaikoff, 1964; D'Adamo and Haft, 1965; Leveille and Hanson, 1966a, 1966b). On the basis of these observations, and of the presumed impermeability of mitochondria to polyanions such as citrate, it was proposed that extramitochondrial citrate is derived from intramitochondrial citrate via the intermediate formation of α -ketoglutarate or glutamate, as shown in Scheme 4. According to this view intramitochondrial citrate is converted to α -ketoglutarate or glutamate or both. α -Ketoglutarate or glutamate or both then diffuse into the extramitochondrial space where they are converted back to citrate by a reversal of the intramitochondrial reactions. Oxaloacetate formed in the citrate cleavage reaction must then be returned into the mitochondria by one of the pathways discussed above. Scheme 4 shows this to take place via transamination reactions, but other pathways are not ruled out. The enzymes necessary for the pathway proposed by D'Adamo and Haft are found both in the intra- and extramitochondrial space of the cell. However, a *de novo* synthesis of fatty acids from glutamate was not demonstrated in the experiments referred to above, and caution must be exercised in interpreting isotope incorporation experiments in terms of net synthesis along a pathway.

A NEW ASSESSMENT OF THE CITRATE PATHWAY OF FATTY ACID SYNTHESIS IN RAT LIVER

The finding that (—)-hydroxycitrate is a powerful inhibitor of citrate cleavage enzyme has made it possible to test in a new way the hypothesis that citrate is the major precursor of the acetyl groups used for the extramitochondrial synthesis of fatty acids when the major source of carbon is pyruvate. The experiments presented below involved the use of a reconstituted system consisting of mitochondria and high speed supernatant (cytoplasmic protein) prepared from rat liver. In initial experiments we used [2-¹⁴C]pyruvate to generate intramitochondrial acetyl-CoA. Subsequently we used [¹⁴C]alanine for this purpose, since it is much stabler than pyruvate and can be stored readily in a state of purity.

Figure 2 shows that fatty acid synthesis from [¹⁴C]alanine requires the presence of both mitochondria and high speed supernatant. In the reconstituted system employed by us, *fatty acid synthesis can be severely depressed by the addition of a low concentration of (—)-hydroxycitrate* (Figure 3). As little as 3.5 μ M hydroxycitrate suppresses fatty acid synthesis by 44%; 14 μ M suppresses it by 88%; and 48 μ M suppresses it

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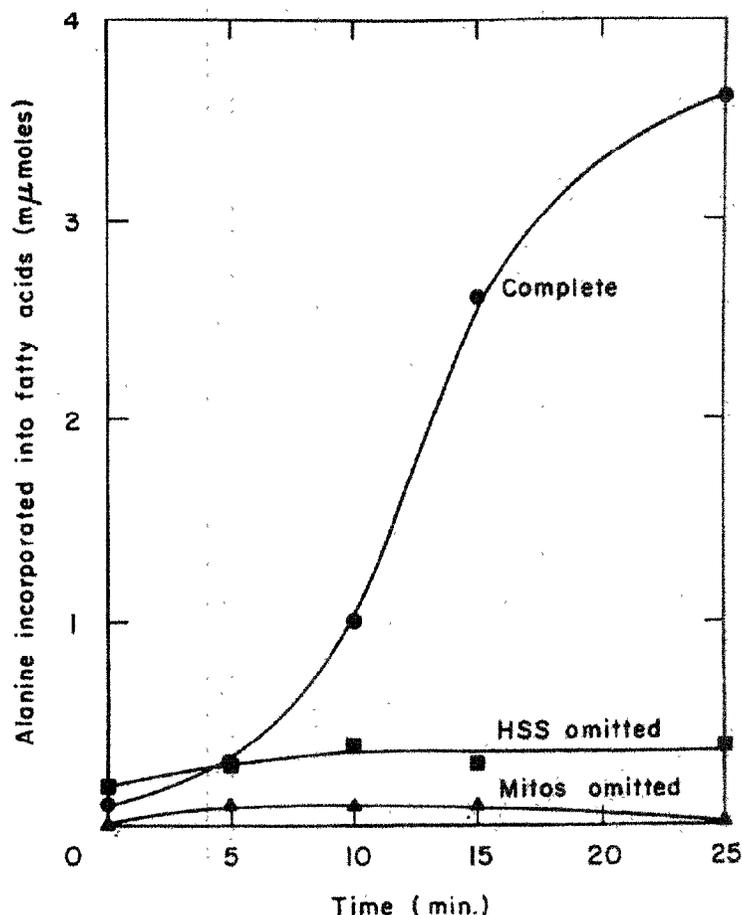


Figure 2. Requirement for mitochondria and high speed supernatant for fatty acid synthesis from [¹⁴C]alanine. The complete system contained mitochondria, 2.3 mg protein per ml and activated high speed supernatant, 3.0 mg protein per ml, prepared from rat liver. The high speed supernatant was activated by incubation with 20 mM MgCl₂, 10 mM dithiothreitol, and 50 mM glycylglycine buffer, pH 7.4, at 38° C for 20 minutes, just before use (Fang and Lowenstein, 1967). In addition the complete system contained 22 mM KHCO₃, 4 mM L-malate, 4 mM α-ketoglutarate, about 0.13 mM CoA, 2 mM ATP, 4 mM MgCl₂, about 0.13 mM NAD, about 0.5 mM NADP, 4 mM glucose-6-phosphate, 4 mM potassium phosphate, pH 7.3, and 1 mM [U-¹⁴C]alanine (200 cpm per mμ mole). The mixture had a final pH of 7.3. The initial volume was 6 ml, and the incubations were carried out in conical flasks which were shaken at 125 strokes per minute in a water bath at 38° C. Aliquots of 1.0 ml were removed at the times indicated, pipetted into 2.0 ml 5N NaOH and analysed as described previously (Fang and Lowenstein, 1967; Watson and Lowenstein, unpublished)

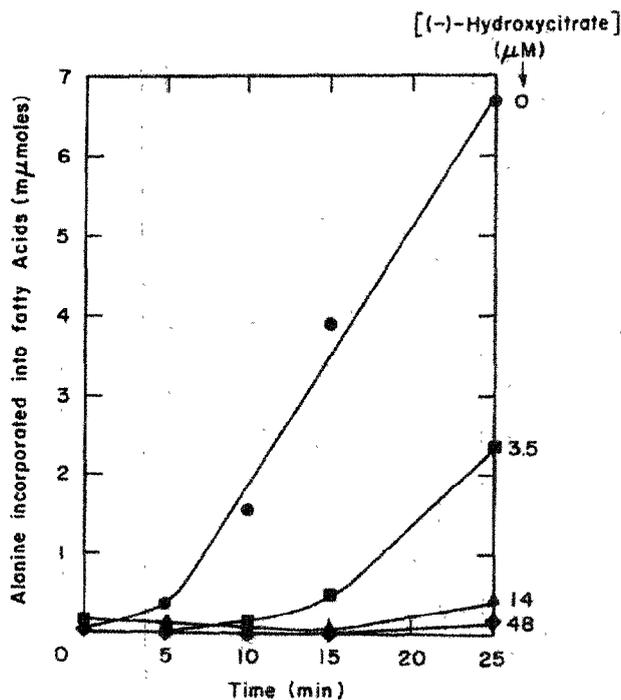


Figure 3. Effect of (-)-hydroxycitrate on fatty acid synthesis from [¹⁴C]alanine. The complete system described in the legend to Figure 2 was used, except that (-)-hydroxycitrate was added to the reaction mixture as indicated (Watson and Lowenstein, unpublished)

by over 95%. By comparison, 100 μ M (-)-hydroxycitrate has been found to be without effect on two key enzymes which catalyse reactions in pathways 2 and 3 of Scheme 2, namely acetate thiokinase and acetyl-CoA-carnitine acetyl transferase. A possible alternative explanation for the inhibition of fatty acid synthesis in the presence of (-)-hydroxycitrate is that this substance inhibits some vital part of intramitochondrial metabolism. This possibility may be ruled out on the basis of the observation that 75 μ M (-)-hydroxycitrate has no effect on mitochondrial respiration (Table 1). Moreover, this amount of hydroxycitrate does not affect the rate of ¹⁴CO₂ production from [2-¹⁴C]pyruvate. The experiment shown in Figure 3 therefore provides new evidence that the acetyl group of acetyl-CoA is transferred from the intra- into the extramitochondrial space in the form of citrate, or a near relative of citrate, and not in the form of either acetyl-CoA, acetyl carnitine, or free acetate.

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Table 1. Lack of effect of (–)-hydroxycitrate on mitochondrial respiration. The suspending medium consisted of a solution containing 0.245 M sucrose, 20 mM Tris-HCl buffer, pH 7.3, 2.5 mM MgCl₂, and 2 mM potassium phosphate. It was saturated with air and stored at 25° while experiments were in progress. Suspending medium (1.8 ml) was added to the reaction chamber and was allowed to equilibrate. Mitochondria (0.1 ml, about 3.0 mg protein) were added to the medium. After the endogenous rate of oxygen consumption was established this was followed by either 20 μmoles pyruvate plus 10 mM malate, or 5 μmoles succinate, or 8 mM citrate (with and without 10 μmoles malate). All substrates were added in the form of their potassium salts which had been neutralized to pH 7.3. Where indicated, 0.2 to 1.0 μmoles ADP was added with the substrates. Oxygen consumption was measured at 28° with a Clark electrode in a magnetically stirred chamber with a total capacity of 2.2 ml

Substrate	ADP	[(–)-Hydroxycitrate]	
		None	75 μM
<i>Oxygen uptake (nμ atoms/mg protein/minute)</i>			
Pyruvate plus malate	absent	13.9	13.9
	present	31.2	31.2
Succinate	absent	30	30
	present	95	95
Citrate	absent	13.3	13.2
	present	53.7	53.7
Citrate plus malate	absent	16.6	13.3
	present	53.7	53.7

The question of whether the transfer of the acetyl group occurs via a tricarboxylic acid such as citrate itself, or via glutamate or α-ketoglutarate, was put to experimental test. If the pathway of acetyl group transfer involves α-ketoglutarate then the addition of a pool of unlabelled α-ketoglutarate should lead to a trapping of [4,5-¹⁴C]α-ketoglutarate formed from [U-¹⁴C]alanine via the reactions: alanine → pyruvate → acetyl-CoA → citrate → isocitrate → α-ketoglutarate.* Such a trapping should be reflected in a reduction of ¹⁴C incorporated into fatty acids. The results in Figure 4 show that addition of α-ketoglutarate does not

* [U-¹⁴C]Alanine gives rise via pyruvate to [1,2-¹⁴C]acetyl labelled acetyl-CoA. The latter gives rise via the citrate synthase reaction to stereospecifically-labelled [1,2-¹⁴C]citrate, which in turn gives rise to [4,5-¹⁴C]α-ketoglutarate. These steric interrelations have been discussed fully elsewhere (Lowenstein, 1967).

diminish the formation of labelled fatty acids. At the highest concentration of α -ketoglutarate added (12 mM), the total amount of α -ketoglutarate present exceeded the amount of acetyl group incorporated into fatty acids by a factor of about 1200. Some of the α -ketoglutarate added was converted to glutamate by transamination with alanine; this probably accounts for the increase in fatty acid synthesis from [14 C]alanine which occurred as the concentration of α -ketoglutarate was increased. Even so, α -ketoglutarate was present in great excess. Since it failed to dilute the radioactive carbon pool which serves to supply precursors for the synthesis

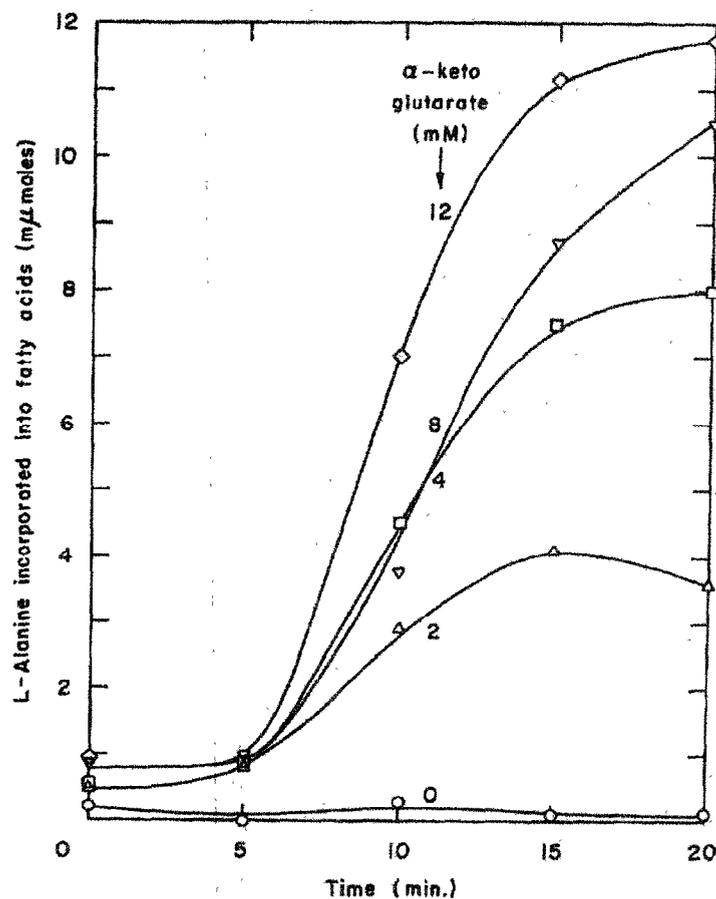


Figure 4. Effect of α -ketoglutarate on fatty acid synthesis from [14 C]alanine. The complete system described in the legend to Figure 2 was used, except that α -ketoglutarate was added to the reaction mixture as indicated, and the alanine concentration was 6 mM (Watson and Lowenstein, unpublished)

of fatty acids, it is concluded that the acetyl group of intramitochondrial acetyl-CoA is most probably not exported from the mitochondria as α -ketoglutarate or glutamate but as citrate.*

It is possible that in the *in vitro* system employed by us the reductive carboxylation of α -ketoglutarate by isocitrate dehydrogenase does not occur, whereas in the intact cell conditions for this reaction are more favourable. The *in vitro* system described here contained a NADPH regenerating system, which makes it unlikely that the reductive decarboxylation did not occur for lack of NADPH.

In conclusion, the experiments presented here provide new evidence that the citrate cleavage pathway is the major source of carbon for the synthesis of fatty acids in rat. Transfer of citrate carbon from the mitochondria into the cytoplasm probably occurs in the form of tricarboxylic acids, and not via α -ketoglutarate or glutamate.

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* The egress from the mitochondria of *cis*-aconitate and isocitrate is not excluded by these experiments. The equilibrium mixture of these tricarboxylic acids contains about 91% of citrate (Krebs, 1953).