

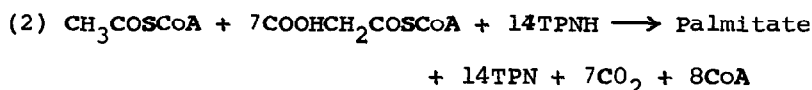
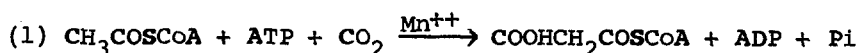
MECHANISM OF TRICARBOXYLIC ACID CYCLE REGULATION OF FATTY ACID SYNTHESIS

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The biosynthesis of long chain fatty acids from acetyl-CoA can be represented by the following reactions:



Reaction 1, the carboxylation of acetyl-CoA to form malonyl-CoA, is catalyzed by acetyl-CoA carboxylase, a biotin-enzyme (Wakil 1958, Brady 1958, Lynen 1959). Reaction 2, the reaction of acetyl-CoA with seven moles of malonyl-CoA and fourteen moles of TPNH to form palmitate, is an incompletely understood complex reaction catalyzed by palmitate synthetase (Wakil 1961, Martin, Horning, and Vagelos 1961, Lynen 1961).

The observation by Brady and Gurin (Brady and Gurin 1952) that certain tricarboxylic acid cycle intermediates stimulate fatty acid synthesis has been confirmed in many laboratories. However the mechanism of this stimulation has remained obscure. The present communication presents evidence that the tricarboxylic acid cycle intermediates that stimulate fatty acid synthesis in a partially purified enzyme preparation from rat adipose tissue do so by increasing the rate

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of reaction 1, a rate-limiting step in fatty acid synthesis from acetyl-CoA (Ganguly 1960, Numa, Matsuhashi and Lynen 1961).

The synthesis of long chain fatty acids from acetyl- 1-C^{14} -CoA (combined reactions 1 and 2) was assayed as follows: 100 μ moles of imidazole-HCl buffer, pH 6.5, 0.2 μ mole of acetyl- 1-C^{14} -CoA (110,000 cpm), 3 μ moles of ATP, 1 μ mole of MnCl_2 , 30 μ moles of NaHCO_3 , 2 μ moles of TPNH, 2 μ moles of 2-mercaptoethanol and 3.0 mg of enzyme were added in a total volume of 1.1 ml. The rat adipose tissue enzyme was prepared through the ammonium sulfate step as previously reported (Martin, Horning and Vagelos 1961). The reaction mixture was incubated for one hour at 30°C and stopped by the addition of H_2SO_4 . The radioactive long chain fatty acids were extracted and counted as previously reported (Horning, Martin, Karmen and Vagelos 1960). The addition of citrate, fumarate, cis-aconitate, α -ketoglutarate, malate, succinate, and oxaloacetate at 10^{-2} M final concentration increased fatty acid synthesis from 3.7 to 60 times the control with no addition. Citrate and fumarate were the most stimulatory.

Palmitate synthetase was present in more than 100-fold excess over the carboxylase in the adipose tissue extract; therefore the latter was the rate-limiting step of fatty acid synthesis in this preparation. As can be seen in Table I, citrate at a concentration of 12 mM stimulated reaction 1 approximately 23-fold (experiment 1); on the other hand there was no effect on reaction 2 (experiment 2).

Table I

Citrate Effect on Acetyl-CoA Carboxylase
and Palmitate Synthetase

Expt.No.	Citrate (mM)	Enzyme assay		Percent stimulation
		React. 1*	React. 2**	
		(cpm)		
1	0	839		-
	12	20100		2300
2	0		0.068	-
	6		0.064	0
	12		0.064	0

* Reaction 1 was assayed as follows: 20 μ moles of imidazole-HCl buffer, pH 6.5, 0.05 μ mole of acetyl-CoA, 3 μ moles of ATP, 1 μ mole of $MnCl_2$, 20 μ moles of $NaH^{14}O_3$ (5,500,000 cpm), 1.4 mg of enzyme and citrate as indicated in a volume of 0.33 ml. Reactions started by addition of enzyme and incubated 1 hour at 30°C. Reactions stopped by adding Dowex-50-H until pH fell to 2. Suspensions centrifuged and 0.1 ml of supernatant solutions plated and counted.

** Reaction 2 was assayed spectrophotometrically by measuring decrease in absorbancy at 340 m μ associated with the oxidation of TPNH as described previously (Martin, Horning and Vagelos 1961).

The requirements of the acetyl-CoA carboxylation reaction in the presence of citrate are shown in Table 2. It is seen that fixation of $C^{14}O_2$ is dependent upon acetyl-CoA, ATP, and Mn^{++} . Up to 95% of the fixed radioactivity from such an experiment was isolated as malonyl-CoA. Furthermore it was found that the added citrate neither diluted the radioactivity of $C^{14}O_2$ that was fixed in malonyl-CoA nor did citrate become labeled during the carboxylation reaction. It is, therefore, clear that citrate was not acting as a carboxyl donor in a transcarboxylation reaction. Avidin, a specific biotin-enzyme inhibitor, markedly inhibited the carboxylase even in the presence of citrate. Prior mixing of the avidin with biotin partially prevented the inhibition.

Table 2
Requirements of Carboxylation Reaction

<u>Components</u>	<u>Malonyl-CoA</u> (cpm)
Complete	26,247
- Acetyl-CoA	357
- ATP	165
- Mn ⁺⁺	246
Complete + 0.5 mg Avidin	1,956
Complete + 0.5 mg Avidin + 5 mg Biotin*	10,017

Carboxylase assayed as in Table 1 with the addition of 12 mM citrate. Experiments contained 1.1 mg enzyme.

* Avidin and biotin were mixed prior to addition to assay mixture.

Citrate was not rapidly metabolized when it was incubated alone with the enzyme preparation. When citrate-1,5-C¹⁴ was incubated under the conditions of Table 2 and the tricarboxylic acid cycle intermediates isolated by celite column chromatography all the intermediates together contained only 1% of the added radioactivity. Over 90% of the radioactivity was recovered as citrate.

Prior incubation of enzyme with 10 mM citrate alone for 30 minutes was found to produce an 8-40 fold increase in the initial rate of malonyl-CoA synthesis when the remaining components of the carboxylase assay were added. Therefore the activation of the carboxylase by citrate was determined in the standard carboxylase assay after a prior incubation of the enzyme in the presence or absence of citrate.

Using this assay procedure experiments were done to determine if a stable, activating intermediate accumulated during the prior incubation with citrate. It was found that acidifying or boiling the enzyme that had been pretreated with citrate completely abolished

its stimulatory effect when this was added to fresh control enzyme. Furthermore, the addition of enzyme pretreated with citrate to control enzyme caused only an additive increase in the rate of carboxylation; a doubling of the rate of the activated preparation would be expected if an activating intermediate were formed in the prior incubation. Finally control enzyme and enzyme pretreated with citrate were subjected to ultracentrifugation at $114,000 \times g$ for two hours. The enzyme was sedimented as a pellet in both cases. Essentially all the citrate pretreated enzyme activity was found in the pellet. When supernatant solution from this experiment was added to control enzyme pellet, no stimulation of the carboxylation reaction was observed.

All these experiments favor the view that the carboxylase enzyme itself is activated during the preincubation and that no free, activating intermediate is formed from citrate. The nature of the enzyme activation is still obscure, but several possibilities exist. Citrate may be altering the enzyme configuration or structure in a fashion analogous to the recently reported hormone effects on glutamic dehydrogenase (Tomkins, Yielding and Curran 1961); citrate, a known chelating agent, may by this or other means remove an inhibitor from the enzyme; or citrate may give rise to an intermediate in catalytic amounts that remains firmly attached to the carboxylase and acts in a transcarboxylating role. These possibilities are under investigation.

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