EVIDENCE AGAINST FREE BUTYRYL-CoA AS AN INTERMEDIATE IN LONG CHAIN FATTY ACID BIOSYNTHESIS

Donald B. Martin* and P. Roy Vagelos

Enzyme Section, National Heart Institute
Bethesda, Maryland

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Recent studies on the mechanism of long chain fatty acid biosynthesis by soluble enzyme preparations have indicated that malonyl-CoA is an intermediate in the synthesis of palmitate from acetyl-CoA (Wakil, 1958; Brady, 1958; Lynen, 1959). In addition it has been established that seven molecules of malonyl-CoA sequentially condense with one molecule of acetyl-CoA to form the sixteen carbon atom fatty acid (Wakil, 1959; Lynen, 1960; Brady, 1960; Martin, Horning, and Vagelos, 1961), and that the acetyl-CoA moiety can be replaced by other saturated, even-numbered, fatty acyl-CoA compounds (Wakil, 1959; Lynen, 1960; Brady, 1960; Martin, Horning, and Vagelos, 1961) as well as the odd-numbered and branched-chain fatty acyl-CoA compounds (Horning, Martin, Karmen, and Vagelos, 1961) with the resultant formation of the corresponding long chain fatty acids.

To account for the ready incorporation of the intermediate length fatty acyl-CoA derivatives (butyryl-CoA, hexanoyl-CoA, etc.) into long chain fatty acids, it has been postulated (Wakil, 1959; Lynen, 1960) that these compounds may occur as free intermediates. Lynen (Lynen, 1960) has demonstrated that the yeast fatty acid synthesizing system will catalyze the formation of enzyme-bound acetoacetate from malonyl-CoA and acetyl-CoA. He

*Fellow of the Boston Medical Foundation, Inc.

has proposed that the enzyme-bound product is reduced and dehydrated, and that the resulting butyrate must come off the enzyme before it can re-condense with another malonyl-enzyme complex to further elongate the fatty acid chain.

To determine whether free butyryl-CoA is an obligatory intermediate in the synthesis of long chain fatty acids by a particle-free fatty acid synthesizing system from rat epididymal adipose tissue (Martin, Horning, and Vagelos, 1961), pool experiments were done. The effect of varying amounts of butyryl-CoA pool - when incubated with acetyl-1-C^{14}-CoA, malonyl-CoA, TPNH, and partially purified enzyme - on radioactivity recovered in butyryl-CoA and long chain fatty acids was tested. In addition, the synthesis of butyryl-CoA by this system, and the incorporation of butyryl-CoA into long chain fatty acids, were investigated.

Butyryl-1-C^{14}-CoA and butyryl-CoA were synthesized by a modification of the method of Wieland and Rueff (Goldman, in preparation; Wieland and Rueff, 1953); malonyl-CoA, by the method of Trams and Brady (Trams and Brady, 1960); and acetyl-CoA, by the method of Simon and Shemin (Simon and Shemin, 1953). After standard incubation (Table I) the reaction was stopped by placing the samples in boiling water for three minutes. The CoA esters were hydrolyzed by allowing the samples to stand for several hours following addition of alkali to about pH 12. Carrier butyric and acetic acids were added. The mixtures were acidified and extracted twice with five volumes of n-hexane to remove the long chain fatty acids. The combined hexane extracts were evaporated to a small volume and counted. The aqueous phases were steam distilled, neutralized, and evaporated to dryness. The salt mixtures were acidified and subjected to Celite column chromatography (Swim, 1957), eluting sequentially with n-heptane,

Table I

Butyryl-CoA Synthesis from Acetyl-1-C^{14}-CoA by Rat Adipose Tissue Enzyme

Component omitted	C.p.m. in Butyryl-CoA
None	21,300
Malonyl-CoA	0

The standard incubation mixture contained KPO_4 buffer, pH 6.6, 50 micromoles; TPNH, 0.4 micromole; malonyl-CoA, 0.1 micromole; acetyl-1-C^{14}-CoA, 0.028 micromole (132,080 c.p.m.); 2-mercaptoethanol, 2.5 micromoles; enzyme, 0.3 mg. Final volume adjusted to 1 ml with water. Reaction incubated at 28° C for 10 minutes.

$CHCl_3$, and $CHCl_3$ with 3% butanol, all saturated with 0.2 N H_2SO_4. This choice of solvents separated butyric acid from acetic and caproic and longer chain fatty acids.

Butyric acid was further identified by Duclaux distillation and paper chromatography (Kennedy and Barker, 1951.)

Table I presents data showing the formation of radioactive butyryl-CoA from acetyl-1-C^{14}-CoA in the presence of malonyl-CoA, TPNH, and enzyme. Its formation was dependent on malonyl-CoA. The amount formed varied with different enzyme preparations. The butyrate was present as butyryl-CoA as shown by the formation of the hydroxamic acid derivative which was identified by paper chromatography. Control studies with radioactive butyryl-CoA indicated that insignificant amounts of butyryl-CoA were deacylated to free butyric acid during the incubations with this system.

Addition of unlabeled butyryl-CoA in increasing amounts to the standard incubation mixture (Table II) in every case caused a decrease in the radioactivity found in butyryl-CoA compared to

Table II

Effect of Added Butyryl-CoA on Radioactivity Recovered in Butyryl-CoA (Butyric Acid) and Long Chain Fatty Acids

Expt	Butyryl-CoA Micromoles Added	Butyryl-CoA Difference from Control* C.p.m.	Total C-2 Units in Fatty Acids Micromoles**	Long Chain Fatty Acids C.p.m.
I	0.0	-	0.106	31,560
	0.03	-7,900	0.094	21,160
	0.08	-9,600	0.085	19,060
II	0.0	-	0.070	7,948
	0.03	- 9,200	0.076	11,107
	0.08	- 9,600	0.076	11,079
	0.15	-11,000	0.073	10,392

System and conditions as in Table I, except enzyme, 0.14 mg

*Expressed as difference in counts from control without butyryl-CoA because of variability in butyryl-CoA synthesis with different enzyme preparations.

**Calculated from TPNH oxidized (Martin, Horning, and Vagelos, 1961)

controls without butyryl-CoA pool. Thus butyryl-CoA synthesis was depressed by the presence of butyryl-CoA. Although there was slight depression of the total fatty acids synthesized and the radioactivity incorporated into long chain fatty acids in Experiment I, this was not observed in Experiment II. The decrease in radioactivity even in Experiment I cannot be considered a significant dilution when the size of the butyryl-CoA pool is considered.

An experiment to demonstrate the incorporation of butyryl-1-C^{14}-CoA into long chain fatty acids without prior degradation to acetyl-CoA is shown in Table III. Butyryl-1-C^{14}-CoA was incubated with

Table III

Long Chain Fatty Acid Synthesis from Butyryl-1-C^{14}-CoA

Acetyl-CoA		Long Chain Fatty Acids	
Micromoles Added	After Incubation C.p.m.	Micromoles*	C.p.m.
0.03	0	0.0063	1,561

Complete system contained KPO_4 buffer, pH 6.6, 50 micromoles; TPNH, 0.4 micromole; malonyl-CoA, 0.1 micromole; acetyl-CoA, 0.03 micromole; butyryl-1-C^{14}-CoA, 0.03 micromole (9,900 c.p.m.); 2-mercaptoethanol, 2.5 micromoles; enzyme, 0.14 mg. Conditions same as in Table I.

*Calculated from TPNH oxidized (Martin, Horning, and Vagelos, 1961)

acetyl-CoA, malonyl-CoA, TPNH, and enzyme. After incubation, the acetyl-CoA as well as the long chain fatty acids were examined for radioactivity. Despite significant radioactivity found in long chain fatty acids, none was found in the acetyl-CoA.

These experiments confirm the earlier studies which demonstrated the incorporation of butyryl-CoA into long chain fatty acids. Moreover, they show that butyryl-CoA is incorporated without prior degradation to acetyl-CoA. The observation that free butyryl-CoA is synthesized from acetyl-CoA and malonyl-CoA by this system is being further investigated; the possibility of a separate short chain fatty acid synthesizing enzyme system cannot be excluded. The lack of significant effect of increasing butyryl-CoA pool on the radioactivity from acetyl-1-C^{14}-CoA found in butyryl-CoA and long chain fatty acids leads to the conclusion that free butyryl-CoA is not an obligatory intermediate in the pathway of fatty acid biosynthesis in epididymal adipose tissue. This does not exclude butyryl-CoA as an intermediate, but suggests that it might be enzyme-bound.

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