Synthesis of Butyric and Other Short-Chain Acids by a Partially Purified Enzyme Preparation*

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ABSTRACT: The fatty acid synthetase from lactating goat mammary supernatant was partially purified. Fatty acid synthesis by this enzyme preparation, in contrast to cruder preparations, was totally dependent upon the presence of malonyl coenzyme A and reduced nicotinamide–adenine dinucleotide phosphate. In the spectrophotometric assay the requirement for acetyl-CoA could not be observed, although tracer experiments showed the incorporation of acetyl-CoA and malonyl-CoA into butyric, hexanoic, octanoic, and longer-chain acids by a pathway similar to the one described for the synthesis of palmitic acid in other systems. The enzyme preparation is inactivated on storage in the absence of citrate. Preincubation of this enzyme with citrate results in partial restoration of the enzyme activity.

Fatty acid synthesis in lactating mammary glands of certain species, notably ruminants, differs from that occurring in other tissues in the formation of significant amounts of short- and intermediate-chain acids of four to fourteen carbon atoms. Extensive investigation in recent years using enzyme systems from animal, plant, and microbial sources have clarified our understanding of the nature of the main process involved, e.g., the malonyl-CoA[1] pathway in the synthesis of palmitic acid (Majerus *et al.*, 1965; Lynen *et al.*, 1964; Wakil *et al.*, 1964; Vagelos, 1964). From the incorporation of the label of malonyl-CoA into all the fatty acids of milk fat by the particle-free supernatant of bovine mammary gland, Ganguly (1960) concluded that the synthesis of these acids also occurs via the malonyl-CoA pathway. In this investigation the possibility remained that the malonyl-CoA provided as substrate may have been decarboxylated and the acetyl-CoA formed might have been incorporated via a nonmalonyl-CoA pathway into some of the fatty acids, perhaps the short-chain ones. Abraham *et al.* (1961) and Dils and Popják (1962) reported that the malonyl-CoA pathway was the most important route for the synthesis of milk fatty acids in rat mammary gland. Goat and rabbit mammary supernatants were found by Singh and Kumar (1963) and Kumar *et al.* (1965) to synthesize hexanoic and longer-chain acids, but not butyric, by the malonyl-CoA pathway. The present investigation describes a partial purification of the fatty acid synthetase of the goat mammary tissue and shows that this enzyme preparation synthesizes butyric as well as the other fatty acids via the malonyl-CoA pathway, although there appears to be an independent route for the synthesis of butyrate in the homogenate and the supernatant.

Experimental Procedure

Materials. Acetyl-CoA and acetyl-[1-^{14}C]CoA were prepared by the method of Simon and Shemin (1953) and malonyl-CoA and malonyl-[2-^{14}C]CoA by that of Trams and Brady (1960). All of the CoA derivatives were purified by paper chromatography. Labeled acetic anhydride and malonic acid were obtained from New England Nuclear Corp. and all other reagents were obtained from various commercial sources.

Methods. The particle-free supernatant was prepared from lactating goat mammary tissue as previously described (Kumar *et al.*, 1965). Solid ammonium sulfate was added slowly to the supernatant fraction to obtain a 30% saturation. The precipitate formed during the following 30 minutes was centrifuged and dissolved in a minimum volume of 0.05 M Tris-HCl buffer containing 0.01 M 2-mercaptoethanol, and was dialyzed for 4 hours against 4 liters of 0.01 M Tris-HCl buffer, *p*H 7.5, containing 0.01 M 2-mercaptoethanol. The dialyzed enzyme was recentrifuged at 40,000 rpm for 40 minutes and the supernatant (fraction 1), after dilution to a protein content of 3–4 mg/ml, was added to a suspension of alumina-c-γ to obtain a 1:4 gel-protein ratio. After equilibrium the gel was discarded. The supernatant was added to a fresh suspension of alumina-c-γ to obtain a final gel-protein ratio of 2:1. The fatty acid–synthesizing enzyme system was eluted with 0.1 M potassium phosphate buffer, *p*H 7.8, containing 0.01 M 2-mercaptoethanol. The eluate was con-

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[1] Abbreviations used in this work: CoA, coenzyme A; NAD$^+$ and NADH, the oxidized and reduced forms of nicotinamide-adenine dinucleotide, respectively; NADP$^+$ and NADPH, the oxidized and reduced forms of nicotinamide-adenine dinucleotide phosphate, respectively.

centrated using polyethylene glycol, 20,000 (Fisher Scientific Co.), to obtain fraction II.

The fatty acids were extracted from the incubation mixture and fractionated into butyric, hexanoic, octanoic, and a mixture of longer-chain acids as described previously (Kumar *et al.*, 1965). Butyric acid was decarboxylated according to the method of Goldfine and Bloch (1961) and the stepwise degradation of butyric acid was carried out by the procedure of Robinson *et al.* (1962).

Protein was estimated by the method of Gornall *et al.* (1949). Radioactivity was determined by liquid scintillation counting using the scintillation mixture described by Bray (1960), corrections being made for quenching by hyamine in the decarboxylation experiments.

Results

The particle-free supernatant of lactating mammary glands was previously shown to synthesize hexanoic and longer-chain acids via the malonyl-CoA pathway. The synthesis of butyrate appeared to occur by an independent pathway, presumably by the reversal of the oxidation. In order to study the synthetic processes involved, the supernatant was fractionated further, and the nature of the acids synthesized and the cofactor requirements were determined. Table I shows the extent of incorporation of acetyl-CoA into butyrate and the longer chain acids by the 0–30% ammonium sulfate fraction. The incubation mixture contained all the cofactors required for the β-oxidative as well as the malonyl-CoA pathways. Addition of malonyl-CoA to the mixture increased the incorporation of the label from acetyl-CoA into C_6 and longer-chain acids but the synthesis of butyrate was unaffected. The data also show that the synthesis of the longer-chain acid fraction was dependent largely on the presence of the NADPH-generating system. The requirement for the reduced pyridine nucleotide for the synthesis of butyrate was inconclusive, normal synthesis being obtained with either of the nucleotides.

Further purification of the enzyme by adsorption on calcium phosphate gel was unsuccessful. Several different gel preparations under varying conditions failed to adsorb significant amounts of protein. Purification of the enzyme using alumina-c-γ as protein adsorbant yielded fraction II, which had a specific activity only one and one-half times that of fraction I. This purification step, however, did remove an $NADP^+$-reducing activity found to be present in fraction I, thereby making it possible to follow the synthesis of fatty acid spectrophotometrically from the oxidation of NADPH. Table II summarizes the results of the purification process.

TABLE I: Incorporation of Acetyl-[1-^{14}C]CoA into Butyric Acid and the Longer-Chain Fatty Acids by the 0–30% Ammonium Sulfate Fraction.[a]

Incubation Mixture	Per Cent Incorporation	
	Butyric Acid	C_6 and Longer-Chain Acids
Complete	2.6	13.5
Complete + malonyl-CoA	2.4	18.5
Complete − NADPH generating system	3.2	4.8
Complete − NADH generating system	3.5	13.3

[a] The complete system contained glycylglycine buffer, *p*H 7.2 (240 μmoles); $MnCl_2$ (0.2 μmole); $KHCO_3$ (10 μmoles); ATP (10 μmoles); coenzyme A (0.1 μmole); $NADP^+$ (0.5 μmole); glucose 6-phosphate (8 μmoles); glucose 6-phosphate dehydrogenase (0.5 Kornberg unit); NAD^+ (0.5 μmole); lactic acid (2 μmoles); lactic acid dehydrogenase (3.8 μg); Na citrate (50 μmoles); acetyl-[1-^{14}C]CoA (2 μmoles, 25,000 cps); and enzyme protein (12.5 mg). Total volume was 3.0 ml; 2 μmoles of malonyl-CoA was added where indicated. Incubation period was 2 hours at 37°.

TABLE II: Partial Purification of the Fatty Acid Synthetase from Lactating Goat Mammary Gland.

Fraction	Volume (ml)	Protein (mg)	Total Units[a]	Specific Activity (units/mg)
Supernatant	90	990	16,560	17
Ammonium sulfate 0–0.30 (fraction I)	22	100	18,700	187
Alumina-c-γ 0.10 M eluate (fraction II)	7	42	11,643	277

[a] The unit of enzyme activity is the number of millimicromoles of labeled acetyl-CoA incorporated into total fatty acids in the complete system (Table I) during a 2-hour incubation period.

Figure 1 shows that no oxidation of NADH occurs in this system while NADPH is rapidly oxidized. Figure 2 shows that the oxidation of NADPH was dependent on the presence of malonyl-CoA. A requirement for acetyl-CoA, which contributes the two methyl-terminal carbon atoms, could not be demonstrated. The acetyl-CoA required could have been formed either by the action of malonyl-CoA decarboxylase reported to be present in all but very highly purified enzyme preparations (Brady *et al.*, 1960; Bressler and Wakil, 1961) or

TABLE III: Incorporation of Acetyl-[1-^{14}C]CoA and Malonyl-[2-^{14}C]CoA into Butyric and the Longer-Chain Fatty Acids.[a]

Incubation No.	Substrate	Generating System	Labeled Substrate Incorporated (mμmoles) C_4	C_6 + Above
1	Acetyl-[1-^{14}C]CoA + malonyl-CoA	NADPH	45.8	156.4
2	Malonyl-[2-^{14}C]CoA + acetyl-CoA	NADPH	57.3	442.6
3	Acetyl-[1-^{14}C]CoA	NADPH	1.3	4.2
4	Acetyl-[1-^{14}C]CoA	NADH	0.5	4.6
5	Acetyl-[1-^{14}C]CoA	NADPH + NADH	1.5	3.4

[a] The incubation mixture (1.30 ml) contained glycylglycine buffer, *p*H 7.6 (200 μmoles); 2-mercaptoethanol (10 μmoles); the enzyme, fraction II (2 mg), preincubated with citrate (50 μmoles/ml enzyme solution); acetyl-[1-^{14}C]CoA (2 μmoles, 129,376 cps) or malonyl-[2-^{14}C]CoA (2 μmoles, 121,640 cps). Unlabeled acetyl-CoA or malonyl-CoA (2 μmoles) was added where indicated. NAD^+- and $NADP^+$-reducing systems were as given in Table I.I ncubation period was 2 hours.

by the action of the citrate-cleavage enzyme (Spencer and Lowenstein, 1962; Formica, 1962; Bhaduri and Srere, 1963). The role of citrate was investigated in another experiment (Figure 3). It can be seen that no fatty acid synthesis occurred with an enzyme preparation that had been stored without citrate for 3 days at −18° even when acetyl-CoA was provided. A 15-minute preincubation of this enzyme preparation with citrate resulted in the restoration of the activity to a significant extent.

The nature of the fatty acids synthesized by fraction II was determined from the incorporation of labeled substrates into different fatty acid fractions. The data presented in Table III show that ^{14}C from both acetyl-CoA and malonyl-CoA was incorporated into butyric and the longer-chain acid fraction. The data confirm the absolute dependence of the fatty acid synthesis on malonyl-CoA and NADPH. The fraction consisting of hexanoic and the longer-chain acids obtained with each of the substrate pair, labeled acetyl-CoA–unlabeled malonyl-CoA and unlabeled acetyl-CoA–labeled malonyl-CoA, with NADPH as electron donor (incubations 1 and 2), was further fractionated into hexanoic, octanoic, and a fraction consisting of decanoic and longer-chain acids. From the radioactivity in each of these fractions the extents of formation of these acids were determined and are shown in Table IV. The data show that similar amounts of acetyl-CoA and malonyl-CoA were involved in the synthesis of butyrate, suggesting the condensation of 1 mole of acetyl-CoA and malonyl-CoA. That such a condensation did occur and

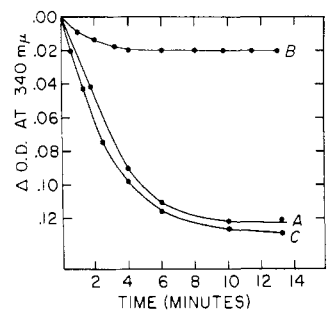

FIGURE 1: Spectrophotometric demonstration of the reduced pyridine nucleotide requirement. The complete system contained 240 μmoles of glycylglycine buffer, *p*H 7.6; 1 μmole of malonyl-CoA; 0.075 μmole of NADPH; 0.075 μmole of NADH; 1 μmole of acetyl-CoA, and 0.6 mg of enzyme, fraction II, which had been preincubated with citrate at 37° for 15 minutes. Final volume was 0.9 ml. Addition of the enzyme initiated the reaction, and the decrease in optical density at 37° was followed. Curve A represents the complete system; curve B, the complete system minus NADPH; curve C, the complete system minus NADH.

TABLE IV: Incorporation of Acetyl-[1-^{14}C]- and Malonyl-[2-^{14}C]CoA into Butyric, Hexanoic, Octanoic, and Longer-Chain Acids.

Incubation No.	Substrate	Labeled Substrate Incorporated (mμmoles) C_{10}+ Above	C_8	C_6	C_4
1	Acetyl-[1-^{14}C]CoA + malonyl-CoA	46.4	1.2	9.2	45.8
2	Malonyl-[2-^{14}C]CoA + acetyl-CoA	423.2	6.4	12.4	57.3

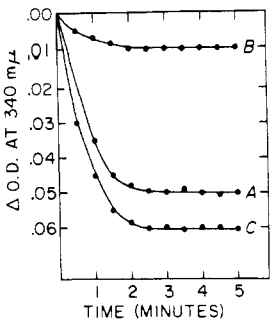

FIGURE 2: Spectrophotometric evidence for the requirement for malonyl-CoA. Curve A represents the complete system of Figure 1, curve B the complete system minus malonyl-CoA, and curve C the complete system minus acetyl-CoA.

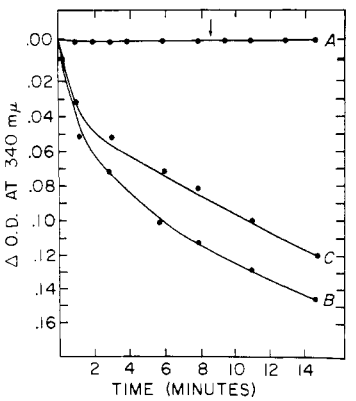

FIGURE 3: Spectrophotometric evidence for the activation of the enzyme with citrate. The reaction mixture contained 240 μmoles of glycylglycine buffer, *p*H 7.6; 1 μmole of malonyl-CoA; 0.075 μmole of NADPH; and 0.6 mg of enzyme fraction II. Curve A represents the enzyme preincubated at 37° for 15 minutes but containing no citrate, curve B the enzyme which had been stored with citrate (50 μmoles/ml) and preincubated, and curve C the enzyme in curve A which was preincubated with citrate. The arrow indicates the point where 1 μmole of acetyl-CoA was added.

that acetyl-CoA contributed carbons 3 and 4 and malonyl-CoA contributed carbons 1 and 2 are seen from the distribution of ^{14}C in the butyrate synthesized (Table V).

TABLE V: Distribution of ^{14}C in Butyric Acid.

Incubation No.	Labeled Substrate	Carbon Atom	^{14}C in Carboxyl Carbon[a] (%)
1	Acetyl-[1-^{14}C]CoA + malonyl-CoA	1	0.5
		2	1.3
		3	86.0
2	Malonyl-[2-^{14}C]CoA + acetyl-CoA	1	0.2
		2	97.0
		3	0.0

[a] ^{14}C in carboxyl carbon/^{14}C in fatty acid × 100 = % ^{14}C in carboxyl carbon.

Discussion

Butyric acid synthesis by a pathway other than the malonyl-CoA pathway has been reported in a few systems (Goldman *et al.*, 1961; Lennarz, 1963; Kumar *et al.*, 1965). Of these only the mammary tissue extracts have been shown to synthesize significant amounts of butyric, hexanoic, and octanoic acids, besides the longer chain acids. Reduction of acetoacetyl-CoA, D(−)-β-hydroxybutyryl-CoA, or crotonyl-CoA to butyrate by rat brain and adipose enzyme was observed by Robinson *et al.* (1963a,b). However, in the presence of malonyl-CoA, only long-chain acids were formed. The present investigation shows the formation of butyrate as well as the longer chain by the partially purified mammary enzyme preparation involving malonyl-CoA and NADPH (Table III and Figures 1 and 2). With the particle-free supernatant and even the ammonium sulfate–precipitated fraction, the synthesis of butyrate from acetyl-CoA was unaffected by the addition of malonyl-CoA and appears to be independent of NADPH (Table I). The spectrophotometric data, not presented here, revealed oxidation of NADPH as well as NADH with acetyl-CoA alone. These observations suggest that the cruder preparations contained an enzyme system which synthesized butyrate from acetyl-CoA, and which required NADH and also, perhaps, NADPH. This could be the result of a contamination of the preparations with mitochondrial subfragments which synthesize butyrate by the reversal of β-oxidation (Seubert *et al.*, 1957). The occurrence of the two independent pathways could account for the lack of agreement regarding the cofactor requirements of the process (Hele *et al.*, 1957; Dils and Popják, 1962; Spencer and Lowenstein, 1962; Matthes *et al.*, 1963).

The results of the incorporation data (Tables III, IV, and V) suggest that a condensation of acetyl and malonyl residues occurred to give rise to butyrate, which then underwent chain elongation by successive condensation with malonyl residues. The peculiarity of the mammary enzyme system lies in significant quantities of the covalently linked short-chain acids dissociating from the enzyme system, assuming the reaction to be analogous to those which culminate in the synthesis of palmitate.

From its behavior on DEAE-cellulose and Sephadex gels, the enzyme system appears to be a multifunctional complex similar to that found in yeast (Lynen *et al.*, 1964) and other mammalian tissues (Vagelos, 1964).

The presence of citrate during storage at −18° has been observed invariably to prevent the development of a turbidity in the enzyme solution, and perhaps thereby preserving the enzyme activity. Reactivation of the enzyme stored without citrate varied with different preparations and with the period of storage. The effect of citrate is not explicable on the basis of the known actions of citrate on fatty acid synthesis (Martin and Vagelos, 1962; Waite and Wakil, 1962; Vagelos *et al.*, 1963; Matthes *et al.*, 1963). Spencer and Lowenstein (1962) have shown that citrate is a regulator of fatty acid synthesis and that it exerts its effect by making available the acetyl-CoA needed for the synthesis. The observation (Figure 3) that acetyl-CoA did not stimulate fatty acid synthesis in the absence of citrate, while not ruling out its role as a precursor of acetyl-CoA, indicates that it may have a role other than that of providing acetyl-CoA. A 30% stimulation of the rat adipose fatty acid synthetase by prior incubation with citrate was observed by Martin and Vagelos (1962), but it was considered of little consequence when compared with the effect of a similar treatment on acetyl-CoA carboxylase. Perhaps citrate exerts a more pronounced effect on the synthetase from the mammary gland.

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