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Review

Mechanism of fatty acid synthesis*

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For many years one of the predominating concepts in biochemistry has been the general assumption that synthetic and degradative processes are alternate aspects of a reversible mechanism. The thesis was widely accepted and was thought to include processes involved in the synthesis of proteins, carbohydrates, fats, etc. However, this hypothesis has lost most of its attractiveness; in every instance in which it was thought to apply, experiments proved the contrary.

Fatty acid synthesis is an excellent example. Until a few years ago (1 to 12), the predominant concept of the mechanism of fatty acid synthesis was that it occurred via the reversal of the enzymatic reactions involved in β-oxidation. This concept was advocated by Lynen early in 1953 (13), when he stated, "The β-oxidation of fatty acids proposed by Knoop is nothing else but the reversal of this (synthetic) cyclic process."

This concept of fatty acid synthesis was generally accepted by the biochemical community despite earlier observations by Gurin and his colleagues (14, 15, 16) on the possible presence of two separate and distinct systems for synthetic and degradative processes. Experimental evidence supporting fatty acid synthesis via "a modified scheme for reversal of β-oxidation sequence" came from two independent observations. The first was the discovery by Langdon (17) that crotonyl CoA can be reduced by TPNH in the presence of an enzyme found in the soluble extracts of rat liver; the second was the demonstration by Stumpf and Barber (18) and by Wakil *et al.* (19) that stearic acid synthesis from acetyl CoA and palmityl CoA was catalyzed by mitochondrial enzymes in the presence of DPNH and TPNH. Pyridoxal phosphate (19) appears to be a cofactor in this conversion, a fact which is supported by nutritional studies relating to the *in vivo* synthesis of long-chain fatty acids.

The afore-mentioned system is located in the mitochondria and is concerned primarily with the elongation of fatty acids of moderate chain length. Thus the name "mitochondrial," or "elongation," system for fatty acid synthesis has been proposed for this series of reactions.

Evidence for a second pathway of fatty acid synthesis was obtained at the Enzyme Institute at the University of Wisconsin (5, 6, 7, 9, 10) by the isolation of a new enzyme system from avian liver that was free of the key enzymes of the β-oxidation sequence. This system converted acetyl CoA to long-chain fatty acids in the presence of ATP, Mn^{++}, CO_2, and TPNH. It appeared to be associated with particles of a size smaller than microsomes (the whole system may be separated as a pellet on centrifugation at 140,000 $\times$ g for 2 hours) since neither mitochondria nor microsomes contain this system. Furthermore, malonyl CoA is the key intermediate (9, 10) in this system and is the compound which contributes all but one of the C_2 units going into the final acyl CoA product. For these reasons, the name "non-mitochondrial system" or " malonyl CoA pathway" for fatty acid synthesis has been proposed.

This review deals with some new aspects of these two systems and summarizes our present views as to the mechanism of synthesis of fatty acids.

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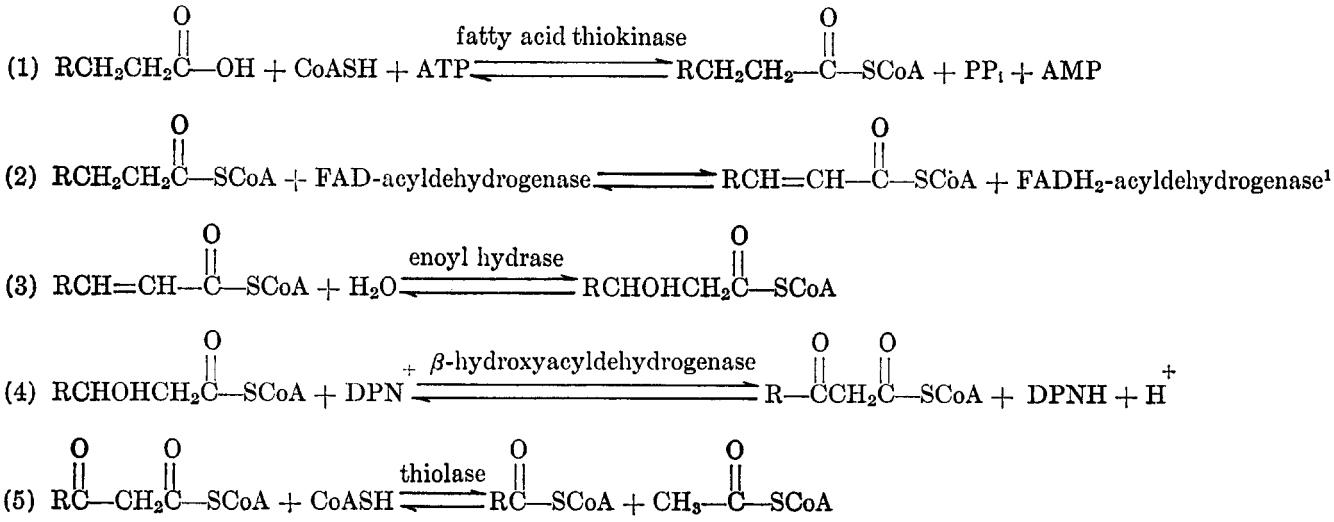
THE "MITOCHONDRIAL SYSTEM" FOR FATTY ACID SYNTHESIS

In 1953, several laboratories (20 to 23) announced the elucidation of the mechanism of fatty acid oxidation, demonstrating that fatty acids are oxidized via a pathway very similar to the β-oxidation scheme proposed by Knoop (24) fifty years earlier. The sequence of the reactions and the enzymes involved are as follows:



Each of the enzymes involved in this sequence of reactions has been prepared in a highly purified state and each step has been shown to be reversible. The equilibrium constants for the first four reactions were shown to be near unity, whereas the thiolase reaction has an equilibrium constant of 1.6×10^{-5} at pH 7.0 (25); this means that in the presence of CoASH there is very little β-ketoacyl CoA present. Nevertheless, the equilibrium can be shifted toward condensation in the presence of DPNH and β-hydroxyacyl dehydrogenase.

Stadtman and Barker (26 to 32) were the first to demonstrate the conversion of labeled acetate into short-chain fatty acids by water-soluble enzyme preparations which they obtained from *Clostridium Kluyveri*. Short-chain fatty acids (butyrate and hexanoate) were synthesized by the *Kluyveri* extract and the reactions from acetyl CoA to butyryl CoA and hexanoyl CoA were catalyzed presumably by the enzymes of the β-oxidation sequence.

[1] The reduced acyldehydrogenase is then oxidized by the electron transferring flavoprotein to an FAD-acyldehydrogenase and reduced electron transferring flavoprotein (77). The latter enzyme then transfers its electron to a suitable acceptor (cyctochrome C or a dye).

Later, Stansly and Beinert (33) demonstrated the conversion of acetyl CoA to butyryl CoA in the presence of DPNH, a reduced dye, and the purified enzymes of the fatty acid oxidation cycle. They were unable to demonstrate the formation of significant amounts of longer acyl CoA derivatives. This appeared to be the result of a preferential condensation of acetyl CoA with another molecule of acetyl CoA rather than with a higher homologue (butyryl CoA, hexanoyl CoA, etc.). An additional weakness in these experiments was the requirement of reduced dye as an electron donor rather than a natural electron donor such as reduced pyridine nucleotides, reduced flavins, reduced cytochromes, etc.

In 1955, Langdon (17, 34) discovered, in soluble extracts of rat liver, an enzyme (TPNH crotonyl CoA reductase) that catalyzed the reduction of crotonyl CoA by TPNH. Later, Seubert *et al.* (35) were able to isolate this enzyme from pig liver mitochondria and to purify it extensively by differential centrifugation in the presence of cholate. The purified particulate enzyme was free from the various enzymes of the β-oxidation cycle and had a wide range of specificity, ranging from crotonyl CoA to α,β-unsaturated stearyl CoA. The localization of this enzyme in the mitochondria is of extreme interest to our knowledge of the cellular distribution of the fatty acid synthesizing systems. With the aid of this enzyme, Seubert *et al.* (35) were able to reconstitute a fatty acid synthesizing system from the purified enzymes of the β-oxida-

tion cycle (thiolase, enoyl hydrase, and β-hydroxyacyl dehydrogenase), a source of DPNH (alcohol and alcohol dehydrogenase), and a source of TPNH (glucose-6-phosphate and glucose-6-phosphate dehydrogenase). With this system they were able to demonstrate the synthesis of octanoyl and capryl CoA from hexanoyl CoA and acetyl-1-C^{14} CoA. However, the poor yield of naturally occurring longer chain fatty acids, e.g., stearic and palmitic, suggested that this pathway for fatty acid synthesis may not be the operational one *in vivo*.

Stumpf and Barber (18) were the first to show the fatty acid synthesis in intact mitochondria derived from avocado mesocarp. Adenosine triphosphate, CoA, and Mn^{++} were essential components for the conversion of C^{14}-acetate into the long-chain fatty acids (palmitic and oleic) in this system.

Recently, Wakil *et al.* (19) were able to demonstrate that intact mitochondria isolated from pigeon, rat, or beef livers can synthesize long-chain fatty acids from acetyl CoA (Table 1). Mitochondria were prepared in either 0.25 M or 0.88 M sucrose according to the general procedures of Hogeboom *et al.* (36). When the mitochondria were incubated anaerobically with acetyl-1-C^{14} CoA, ATP, DPNH, and TPNH, C^{14}-labeled long-chain fatty acids were isolated. Under these conditions, the conversion of acetyl CoA to fatty acids was completely dependent upon and directly related to the presence of ATP (Fig. 1). In the presence of 10 μmoles of ATP, 10% to 20% of the acetyl CoA was converted to fatty acids. Neither butyryl CoA nor GTP could substitute for ATP, whereas ADP, CTP, and UTP could replace ATP at relatively high levels.

TABLE 1. FATTY ACID SYNTHESIS BY MITOCHONDRIA

	Acetyl CoA Incorporated into Long-Chain Fatty Acids
	μmoles
Complete system*	4.5
No ATP	0.3
No TPNH	2.4
No DPNH	2.0
No TPNH; no DPNH	0.0
Complete system + HCO_3^-	3.0
No acetyl CoA + malonyl CoA	2.4
No acetyl CoA + malonyl CoA, without ATP	0.8

* The reaction mixture contained 34.5 mμmoles acetyl CoA (55,200 cpm), 8 μmoles ATP, 0.25 μmole TPNH, 0.25 μmole DPNH, 2.8 mg of mitochondrial protein, 50 μmoles phosphate (pH 6.5), and H_2O to a total volume of 0.5 ml. Four μmoles $KHCO_3$ and 30 mμmoles malonyl CoA (HOOC $CH_2C^{14}O$ CoA 70,000 cpm) were used where indicated. The reaction mixture was incubated anaerobically at 38° for 1 hour.

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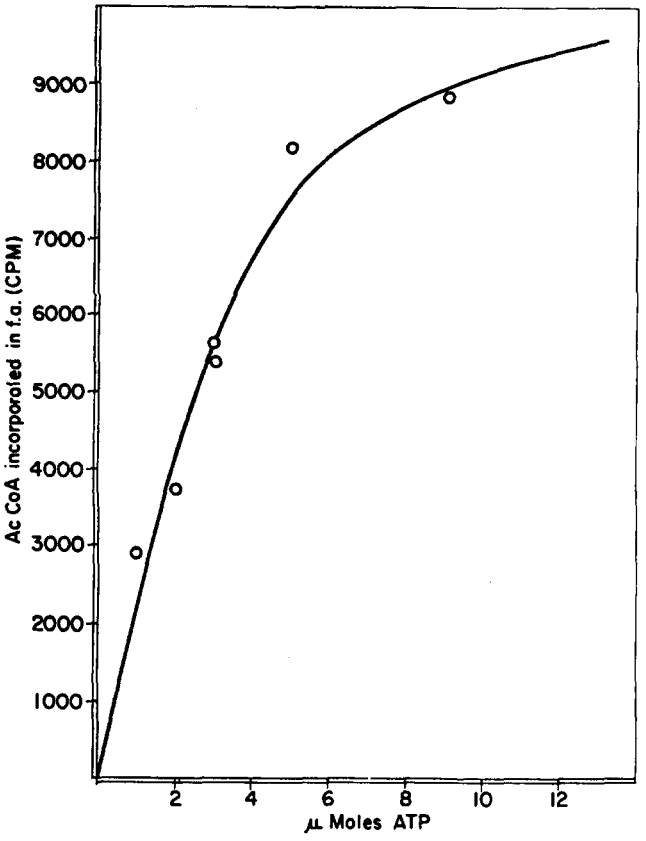


FIG. 1. ATP requirement in fatty acid synthesis by mitochrondria. The reaction mixtures contained 34.5 mμmoles of acetyl CoA (55,200 cpm), 0.25 μmole TPNH, 0.25 μmole DPNH, 2.8 mg of mitochrondrial protein, 50 μmoles phosphate (pH 6.5), ATP as indicated, and H_2O to a final volume of 0.5 ml. The reaction mixture was incubated anaerobically at 38° for 1 hour.

The incorporation of acetyl CoA into the fatty acids was proportional to the amounts of mitochondria added, as is shown in Figure 2. Anaerobic conditions were necessary to minimize the oxidation of DPNH and TPNH by molecular oxygen. Both reduced pyridine nucleotides (TPNH and DPNH) were required for optimum synthesis; in the absence of either reduced nucleotide there was 60% to 80% inhibition of synthesis, while in the absence of both nucleotides there was no synthesis at all.

The omission of HCO_3^- from this system did not affect the synthesis, in contrast to the non-mitochondrial system which is absolutely HCO_3^- dependent

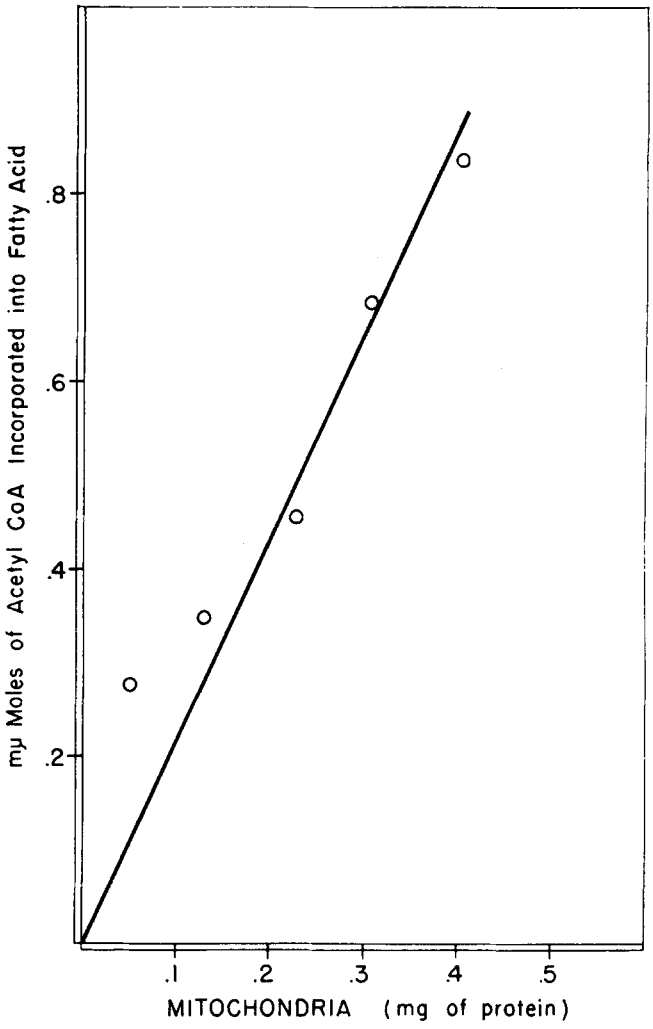


FIG. 2. The relationship between C^{14}-acetyl CoA incorporation in fatty acids and the amount of mitochondria. Each reaction contained 58.6 mμmoles 1-C^{14}-acetyl CoA (152,000 cpm), 0.1 μmole TPNH, 0.1 μmole DPNH, 2 μmoles ATP, 30 μmoles phosphate buffer (pH 6.5), and water to a final volume of 0.5 ml. The reaction was started with the addition of freshly prepared rat liver mitochondria as indicated. The reaction mixture was incubated anaerobically at 38° for 1 hour.

(Table 5). Furthermore, C^{14}-malonyl CoA was incorporated into the fatty acids only in the presence of ATP (Table 1). Malonyl CoA is known to be decarboxylated to acetyl CoA by a mitochondrial enzyme (37), and it seems possible that this accounts for the utilization of malonyl CoA in the presence of ATP.

The products from the incorporation of acetyl-1-C^{14} into fatty acids by mitochondria were separated from the reaction mixture by the procedure of Wakil *et al.* (1) and were identified by a variety of techniques (38, 39). Figure 3 shows the distribution of the radioactivity among the various acids (C_{12} to C_{20}) when separated by reverse-phase chromatography according to the procedure of Kaufmann and Nitsch (39). The major components of the synthesized fatty acids were stearic acid (40%) with C_{20} acid, palmitate, myristate, and laurate constituting the remainder. This is in sharp contrast to the non-mitochondrial system where palmitic acid is the only end product of the reaction.

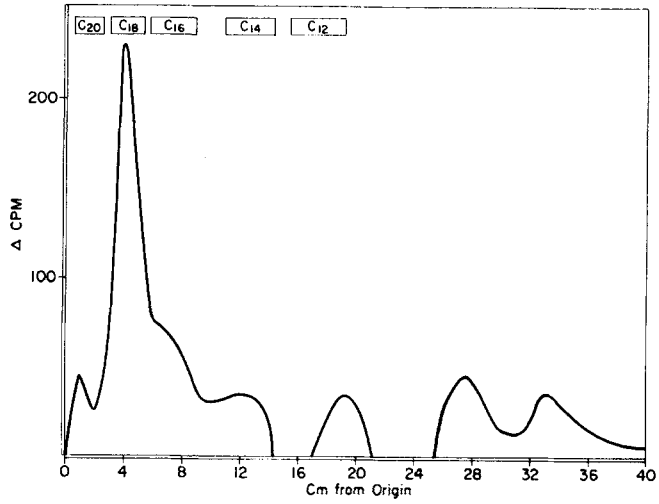


FIG. 3. Distribution of the mitochondria-synthesized fatty acids in the paper chromatographic system of Kaufmann and Nitsch (39).

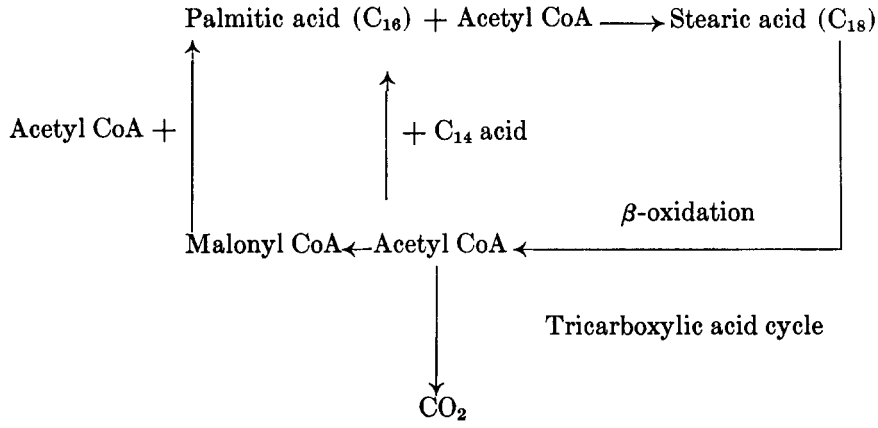
Stearic acid synthesized from 1-C^{14}-acetyl CoA was isolated by dilution with unlabeled stearic acid, recrystallized from various solvents to a constant specific activity, and the resulting acid decarboxylated by the Schmidt reaction as described by Phares (40). The specific activity (cpm per microatom carbon) of the liberated CO_2 was slightly over twice the specific activity of stearic (expressed as cpm per microatom carbon), indicating either *de novo* synthesis of stearic acid from acetyl CoA or the elongation of a pre-existing short-chain fatty acid by the successive additions of acetyl CoA.

It is too early to ascertain which of these mechanisms is operative. Most evidence, especially that obtained from the soluble enzyme system (see below), points to an elongation of short-chain fatty acids rather than total synthesis from acetyl CoA. This again contrasts with the synthesis of palmitate from acetyl CoA by the non-mitochondrial system which is true *de novo* synthesis (3). The presence of intermediate length fatty acids (C_{16}, C_{14}, and C_{12}) in the mitochondrial system is of special significance in reference to the synthetic mechanism in the mitochondria.

These results are in complete agreement with reported experimental data on the synthesis of stearate and palmitate in whole animals (41 to 50). The significant finding by many investigators was that pal-

mitic acid (C_{16}) can be converted to stearic acid (C_{18}) by the addition of one C_2-unit, while the reverse does not appear to be possible. In other words, the conversion of stearic acid to palmitic acid is not achieved by the mere cleavage of one C_2-unit, but instead, is brought about by the complete oxidation of stearic acid to acetyl CoA and the subsequent conversion of acetyl CoA to palmitic acid via either the malonyl CoA pathway or by condensation on a pre-existing myristic acid (C_{14}). This is illustrated by the following scheme:

SCHEME 1. Relationship between palmitic and stearic acids.

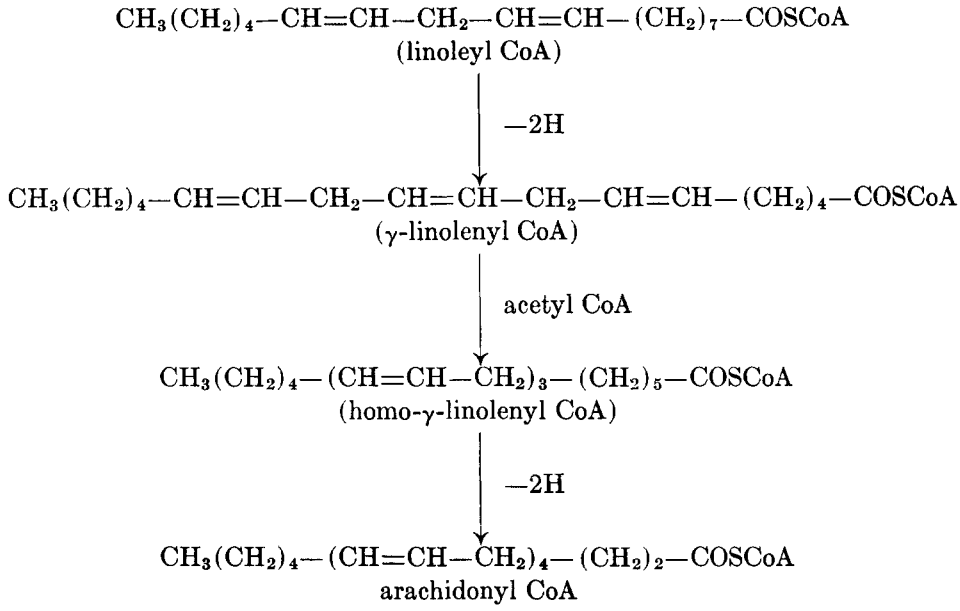


The inability of stearic acid to be directly converted to palmitic acid by the loss of one "C_2-unit" may be explained by the well-known observation that a fatty acid molecule being oxidized by the β-oxidation enzymes (mitochondria or whole animal systems) is completely converted to acetyl CoA with very little, if any, chance of leaving the enzyme prior to complete oxidation (20, 50, 51, 52). Thus, once stearic acid is on the path of oxidation, it continues until it is completely converted to acetyl CoA. The resulting acetyl CoA can be either oxidized to CO_2 by the tricarboxylic acid cycle or can be channeled into the various synthetic reactions, e.g., palmitic acid, amino acids, carbohydrates, purines, etc. Palmityl CoA is synthesized primarily by the non-mitochondrial system (the malonyl pathway) and can be elongated by the mitochondria by the addition of acetyl CoA to form stearyl CoA. On this basis, therefore, the addition of C^{14}-1-acetyl CoA would result in the formation of stearic acid labeled predominantly in the carboxyl group of the molecule. This, indeed, appeared to be the case in the stearic acid synthesized by rat liver slices as reported by Zabin (49), who found that the C^{14} content of the carboxyl carbon of stearic acid derived from acetate-1-C^{14} was higher than the rest of the carbon atoms of the molecule, while the remaining C^{14} in carbon atoms 3 to 18 of stearic acid appeared to be uniformly distributed along the chain. On the other hand, palmitic acid which was isolated simultaneously from the liver slices (49) showed isotope concentration uniformly distributed along the entire molecule. These results suggest that the addition of the last two carbon atoms to palmitate (C_{16}) to form stearate (C_{18}) is accomplished by a different mechanism from the multiple condensation of the "C_2-units" to form the C_{16} molecule. The mitochondrial system appears to be concerned with such elongation of C_{16} acid and to form C_{18} acid and may account for the variation of the isotope ratio in carbons 1 and 2 of C_{18} acid as compared to the remaining carbon atoms.

The incorporation of acetyl CoA into C_{20} fatty acids (Fig. 3) is extremely significant and may indicate that the mitochondrial system is the site for synthesis of these acids by the addition of one acetyl CoA unit onto a C_{18} fatty acid. When stearyl CoA was incubated anaerobically with 1-C^{14}-acetyl CoA, mitochondria, TPNH, and DPNH, the long-chain fatty acids were isolated and tentatively identified as a C_{20} fatty acid (possibly arachidic acid) by paper chromatography. Similar condensation of 1-C^{14}-acetyl CoA can be demonstrated with oleyl CoA as substrate instead of stearyl CoA and the formation of C_{20} acid. We have not as yet identified the C_{20} acid produced from either stearyl CoA or oleyl CoA. Neither have we any information as to whether the C_{20} produced from oleyl CoA still retains its double bond. If the C_{20} acid derived from oleyl CoA is unsaturated, and if other un-

saturated fatty acyl CoA's can substitute for oleyl CoA in this system, then the *in vivo* experiments of Mead and his colleagues (53 to 57) on the synthesis of arachidonic acid from C^{14}-labeled stearic, linoleic, and linolenic acids would be in accordance with this hypothesis. According to Mead, linoleic acid is dehydrogenated to γ-linolenic acid, which then adds acetyl CoA to homolinolenic acid; the latter acid is subsequently dehydrogenated to arachidonic acid. The over-all scheme proposed by Mead and Howton is as follows: (CoA derivatives of these acids are probably the forms participating in these reactions):

SCHEME 2. Possible pathway for the conversion of linoleyl CoA to arachidonyl CoA.



Elongation of Fatty Acids by Soluble Extracts of Mitochondria. When an acetone powder of liver mitochondria was extracted with buffer a particle-free extract was obtained which catalyzed the incorporation of acetyl CoA into higher chain fatty acids in the presence of ATP, DPNH, and TPNH, as shown in Table 2. In the absence of any one of these components there was little or no incorporation of C^{14}-acetyl CoA into the fatty acids, in agreement with the results obtained from intact mitochondria. The product of synthesis in the soluble system was essentially similar to that obtained by whole mitochondria, except that there was more incorporation in the relatively shorter chain acids, compared to the higher chain acids.

Dialysis of the soluble extracts and their subsequent treatment with charcoal resulted in a sharp decline in enzymatic activity, as shown in Table 3. The enzymatic activity can be completely restored by addition of a boiled extract of the enzyme preparation. We were able to show that the boiled extract can be completely replaced by two distinct compounds: an intermediate-chain fatty acid (C_8, C_{10}, C_{12}, C_{14}, etc.) and pyridoxal phosphate (Table 3).

The requirement for the addition of fatty acid acceptor indicated that this system causes the elongation of fatty acids by the addition of an acetyl CoA unit to an appropriate fatty acid. For example, when C_8 acid was used as an acceptor, we were able to isolate C_{10} acid; when C_{10} acid was used, a C_{12} acid was isolated, and so on. The higher fatty acids isolated in these experiments (i.e., C_{10} in the first example and C_{12} in the second) contained over 80% of the incorporated C^{14}-acetyl CoA.

When the corresponding acyl CoA derivatives (C_8 CoA, C_{10} CoA, C_{12} CoA, etc.) were used, ATP was no longer necessary (Table 3), demonstrating that the ATP was required for the formation of the acyl CoA's of these endogenous fatty acids. Butyryl CoA and hexanoyl CoA are very poor replacements for the higher chain acyl CoA's in this system, indicating a specificity for the elongation of intermediate- and long-chain fatty acids, but not for the shorter chain acids. This may be the result of either the lack of an enzyme that would catalyze the condensation of acetyl

CoA with butyryl CoA and hexanoyl CoA, or to the absence of the specific TPNH-α,β-unsaturated acyl CoA reductase for these fatty acids (19). Indeed, we could not detect the TPNH-crotonyl CoA reductase when the extract was assayed with crotonyl CoA as substrate (19). (We have not attempted, as yet, an assay for the reduction of longer chain α,β-unsaturated acyl CoA derivatives by TPNH in the soluble extract.)

The requirement for DPNH and TPNH may be explained by the need for DPNH to reduce the condensation product (β-ketoacyl CoA), while TPNH is needed for the reduction of the α,β-unsaturated acyl CoA to the saturated derivative. The over-all scheme may be written as follows, illustrating the conversion of palmityl CoA to stearyl CoA.

(6) palmityl CoA (C_{16}CoA) + acetyl CoA $\xrightarrow{\text{I}}$ β-keto-stearyl CoA + CoA

(7) β-keto-stearyl CoA + DPNH + H^+ $\xrightarrow{\text{II}}$ β-hydroxystearyl CoA + DPN^+

(8) β-hydroxystearyl CoA $\xrightarrow{\text{III}}$ α,β-unsaturated stearyl CoA + H_2O

(9) α,β-unsaturated stearyl CoA + TPNH + H^+ $\xrightarrow{\text{IV}}$ TPN^+ + stearyl CoA

I = condensing enzyme possibly containing pyridoxal phosphate

II = β-hydroxyacyl CoA dehydrogenase

III = enoyl hydrase

IV = α,β-unsaturated acyl CoA reductase

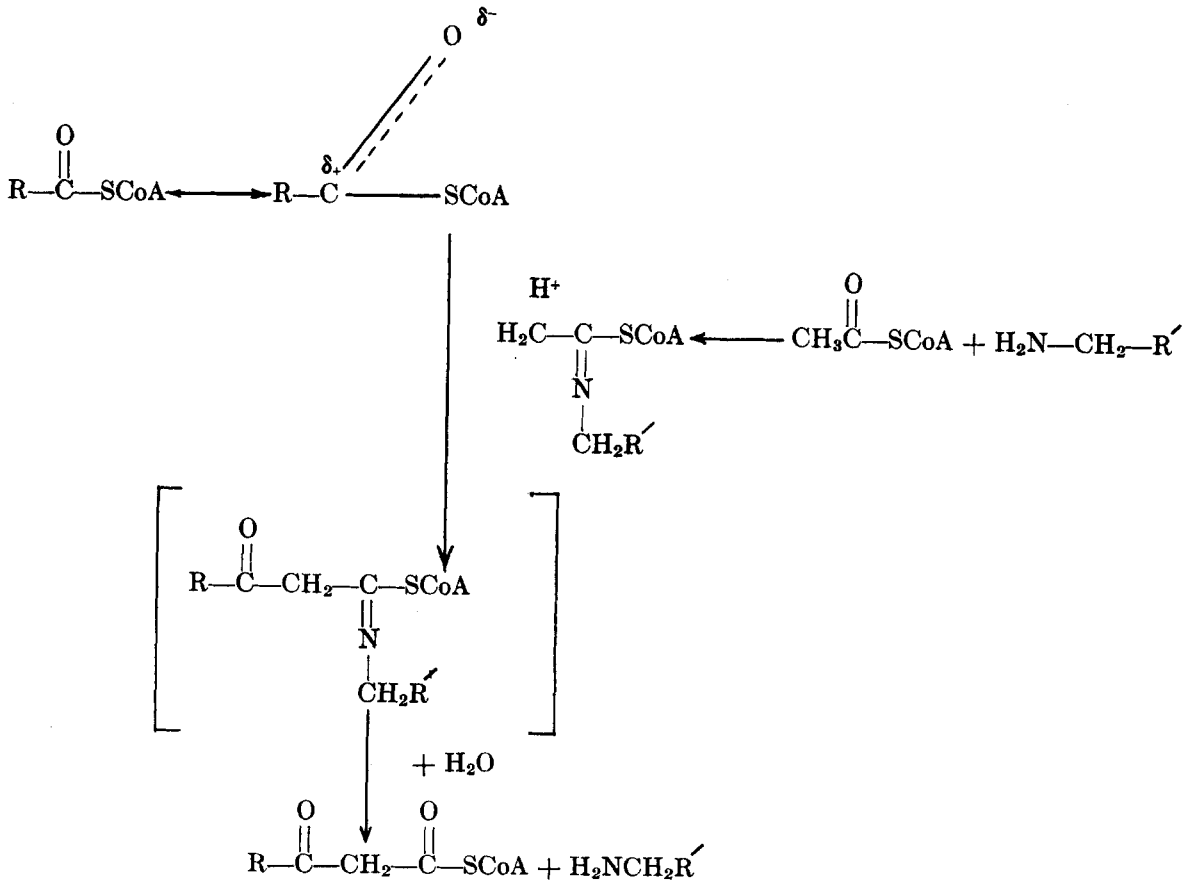
The Role of Pyridoxal Phosphate in Fatty Acid Synthesis. Birch and György in 1936 (58) found that rats suffering from vitamin B_6 deficiency developed symptoms of acrodynia similar to those obtained earlier by Burr and Burr (59, 60, 61) with fatty acid deficient rats; they also observed that unsaturated fatty acids exercised a sparing effect on vitamin B_6. These initial observations were confirmed by many workers and were extended to show that the acrodynia caused by pyridoxine deficiency can be overcome by feeding arachidonic acid or linoleic acid (62, 63, 64). Sherman *et al.* (64) were able to demonstrate an increase in the synthesis of arachidonic acid in rats receiving pyridoxine. However, Witten and Holman (63) submitted evidence to show that rats fed on a fat-deficient, pyridoxine-deficient diet developed acrodynia which can be relieved best by pyridoxine plus linoleate, suggesting that pyridoxine-deficient animals may suffer a secondary deficiency of arachidonic acid.

Preliminary observations in our laboratory indicate that the relationship between pyridoxine and essential fatty acid metabolism may be confined to the role of pyridoxal phosphate in the elongation of fatty acids. When soluble extracts of the mitochondrial system were dialyzed and treated with charcoal, a partial requirement for pyridoxal phosphate was noted (Table 3). The effect of pyridoxal phosphate can be demonstrated not only in soluble extracts but also in particles derived from mitochondria. A two- to threefold increase in the amount of C^{14}-acetyl CoA incorporated into the longer chain fatty acids was observed when the pyridoxal phosphate concentration in the reaction mixture was about 10^{-3} M. Pyridoxamine phosphate can substitute for pyridoxal phosphate but with less efficiency, whereas pyridoxine or pyridoxal hydrochloride are ineffective (Table 3). The response to added pyridoxal phosphate could be demonstrated in some, but not in all, preparations.

The precise role of pyridoxal phosphate in the proposed sequential reactions of fatty acid elongation (Scheme 2) cannot be defined at present. The most likely possibility appears to be in the condensation step of acetyl CoA with the fatty acyl CoA acceptor (65), thus avoiding the requirement of thiolase for this condensation reaction. Thiolase has two drawbacks: the first is the extremely low equilibrium constant of the condensation reaction (1.6×10^{-5} M) at pH 7.0 (25), and the second is the greater tendency of thiolase to catalyze the condensation of two acetyl CoA molecules to form acetoacetyl CoA, rather than the condensation of acetyl CoA with a higher acyl CoA

(33, 60). The involvement of pyridoxal phosphate in this reaction may overcome these drawbacks as follows: first, by the introduction of specificity to the reaction favoring the condensation of acetyl CoA with an intermediate-chain acyl CoA; and second, by the formation of a complex pyridoxal or pyridoxamine with acetyl CoA resulting in the formation of a Schiff base (Fig. 4) similar to the general pyridoxal coenzyme models proposed by Snell and his colleagues[2] (66). This may activate the methyl group of acetyl CoA by imposing an electronegative charge, thus favoring condensation with the relatively positively-charged carbonyl group of acyl CoA resulting in the elongation of the carbon chain as shown in Scheme 3:

SCHEME 3. A hypothetical mechanism of action of pyridoxal phosphate in fatty acid elongation. *R* represents the rest of the pyridoxamine phosphate molecule.



Thus the role of pyridoxal phosphate in this reaction may be as an additional means for the specific activation of the methyl group of acetyl CoA, other than the formation of malonyl CoA. This also would result in changing the equilibrium of the reaction in favor of carbon-carbon chain formation rather than cleavage.

Another alternative for the action of pyridoxal phosphate may be in the reduction of the α,β-unsaturated fatty acyl CoA derivatives by TPNH. This possibility appears very unlikely at present and must await further experimentation.

THE NON-MITOCHONDRIAL SYSTEM FOR FATTY ACID SYNTHESIS

A decade ago Gurin and his co-workers (14, 15, 16) reported the synthesis of long-chain fatty acids from acetate, first in homogenates and later in particle-free extracts prepared from pigeon liver. They found that a water extract of mitochondria was required in addition to the particle-free supernatant fluid, and, after treatment of such an extract with charcoal, could demonstrate a requirement for ATP, DPN^+, and CoA. The addition of citrate to this system stimulated the process, as did Mg^{++}; acetyl CoA, however, was less

[2] For comprehensive reviews on this subject, see Snell (66), Westheimer (103), and Braunstein (104).

TABLE 2. FATTY ACID SYNTHESIS BY SOLUBLE MITOCHONDRIAL EXTRACTS

	Acetyl CoA Incorporated into Fatty Acids
	mμmoles
Complete system*	5.5
No ATP	0.1
No TPNH	2.0
No DPNH	1.8
No TPNH; no DPNH	0.0
Complete system + HCO_3^-	5.0

* The complete system contained 58.6 mμmoles 1-C^{14}-acetyl CoA (152,000 cpm), 2 μmoles ATP, 0.125 μmole DPNH, 0.125 μmole TPNH, 30 μmoles phosphate buffer (pH 6.5), and 1.0 mg of soluble extract (prepared by extracting the acetone powder of beef liver mitochondria with 10 volumes of 0.1 M phosphate buffer, pH 7.0, for 1 hour at 0°). Four μmoles $KHCO_3$ was added where indicated. The reaction mixture was incubated under N_2 at 38° for 1 hour.

efficient as a precursor of fatty acid synthesis than acetate. This system incorporated labeled acetate predominantly into fatty acids rather than into glycerides.

The pigeon liver system of Gurin and his collaborators was the basis for our studies on the mechanism of fatty acid synthesis (1, 2, 3, 5, 6, 7, 9, 10). This system can be prepared from chicken liver (67), rat liver, and rat kidney, as well as pigeon liver. When any of these tissues were extracted in a Potter-Elvehjem homogenizer with 0.1 M phosphate, 0.25 M sucrose, or 0.88 M sucrose and the extract fractionated into mitochondria, microsomes, and soluble fractions, the soluble fraction invariably contained all the enzymes required for the conversion of acetate or acetyl CoA to fatty acids (Table 4). The 100,000 × *g* supernatant

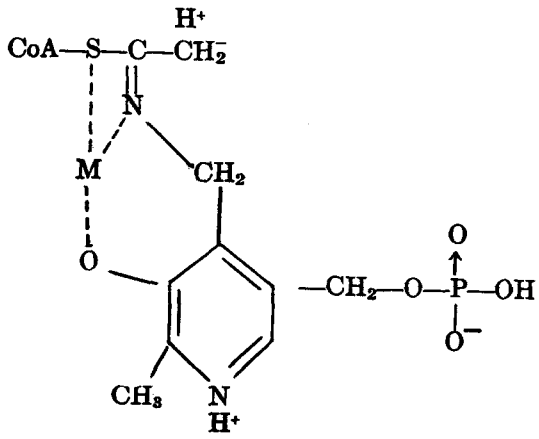


FIG. 4. A proposed model system for acetyl CoA, pyridoxamine phosphate, and metal (M).

TABLE 3. FACTORS AFFECTING FATTY ACID SYNTHESIS BY SOLUBLE MITOCHONDRIAL EXTRACTS

Exp.	Additions	Acetyl CoA Incorporated into Fatty Acids
		mμmoles
I	None	0.00
	ATP	0.29
	ATP and octanoic acid	3.00
	ATP and pyridoxal phosphate	0.79
	ATP and octanoic acid and pyridoxal phosphate	4.50
	ATP and octanoic acid and pyridoxamine phosphate	4.50
II	None	0.00
	ATP	0.20
	ATP and octanoic acid	1.88
	ATP and octanoic acid and pyridoxal phosphate	3.54
	ATP and octanoic acid and pyridoxamine phosphate	3.16
III	ATP	0.2
	ATP and pyridoxal phosphate	0.6
	ATP and boiled enzyme extract	1.3
	ATP and boiled enzyme extract and pyridoxal phosphate	2.61
	ATP and boiled enzyme extract and pyridoxamine phosphate	2.28
	ATP and boiled enzyme extract and pyridoxine HCl	1.5
	ATP and boiled enzyme extract and pyridoxamine HCl	1.88
IV	None	.08
	Palmityl CoA	1.30
	Palmityl CoA plus pyridoxal phosphate	2.00
V	Palmityl CoA plus ATP	1.0
	Palmityl CoA plus ATP and pyridoxal phosphate	1.6

The reaction mixture contained 30 μmoles of phosphate buffer (pH 6.5), 58 mμmoles of 1-C^{14}-acetyl CoA (152,000 cpm), 0.125 μmole DPNH, 0.125 μmole TPNH, and water to a final volume of 0.5 ml. Where indicated, the following were added: 2 μmoles ATP, 25 mμmoles octanoic acid, 50 mμmoles pyridoxal phosphate, 150 mμmoles pyridoxamine phosphate, 150 mμmoles pyridoxine HCl, 150 mμmoles pyridoxamine HCl, 10 mμmoles palmityl CoA, and 0.05 ml of boiled enzyme extract (prepared by boiling the soluble extract of mitochondria [cf. Table 2] for 5 minutes at 100°).

Five different dialyzed enzyme preparations were added as follows: In experiment I, 0.13 mg of charcoal-treated soluble extracts of beef liver mitochondria; in experiment II, 0.2 mg of charcoal-treated soluble extracts of beef liver mitochondria; in experiment III, 0.62 mg of soluble extract of beef liver mitochondria; in experiment IV, 0.55 mg of soluble extract after sonic oscillation of rat liver mitochondria, and in experiment V, 1.5 mg of mitochondria after sonic oscillation.

All tubes were incubated under N_2 gas for 1 hour at 38°.

fraction shows higher activity per mg of protein than the whole homogenate minus cell debris. This is probably because of the removal of the inactive particles (mitochondria and microsomes) from the mixture. The results in Table 4 also show that addition of mitochondria or microsomes decreased the activity of the supernatant fluid. Similar results were obtained when the tissues were homogenized in hypertonic sucrose solution (0.88 M), which minimizes the destruction of

the mitochondria during the homogenization. Furthermore, the type of fitting (loose or tight) of the homogenizer appeared to have very little effect on the extraction of the fatty acid synthesizing system from these tissues.

From these experiments it was concluded that the enzymes of fatty acid synthesis are localized in the soluble cytoplasmic portion of the pigeon and rat liver cells. A similar distribution of the fatty acid synthesizing system was found by Brady *et al.* in pigeon liver (68), by Langdon (17) in rat liver, and by Popják and Tietz (69, 70) in lactating mammary gland. Therefore, we would like to designate this pathway as the "non-mitochondrial system" in order to differentiate it from the mitochondrial system" discussed previously. Klein (71) studied the fatty acid synthesizing system from yeast (*S. cervisiae*) and reported that the conversion of acetate to long-chain fatty acids required both the soluble fraction plus what he called "small particle fraction" sedimented between 25,000 and 60,000 $\times$ *g*, free from the cytochromes and cytochrome oxidase). This also supports our conclusion that the fatty acid synthesizing system is a non-mitochondrial system in origin.

The fatty acid synthesizing system from pigeon liver, rat liver, or rat kidney can be separated from the 100,000 $\times$ *g* supernatant fluid by centrifugation of this extract at 140,000 $\times$ *g* for 2 to 4 hours (7). The resulting supernatant fluid was inactive while the red-colored pellets contained all the enzymes required for the conversion of acetate to fatty acids (cf. Table 4). The structural relationship of the various enzymes of the fatty acid synthesizing sequence in these pellets are not as yet understood and must await further experimentation.

Purification of the Non-mitochondrial Enzyme System. The soluble extracts of pigeon liver were fractionated with ammonium sulfate into two fractions by Wakil *et al.* (1). The first fraction (designated as R_1) is precipitated between 0% and 25% saturation of ammonium sulfate, and the second fraction (designated as R_2) is precipitated between 25% to 40% saturation. Both of these fractions are required for the conversion of acetyl CoA to fatty acids. Earlier work (2, 7) indicated a requirement for a third fraction (R_4), precipitating between 50% to 65% saturation of ammonium sulfate. This extra fraction was shown to contain acetate thiokinase and was no longer necessary when acetyl CoA was used as the starting substrate instead of acetate.

The two fractions R_1 and R_2 were further purified by adsorption on calcium phosphate gel and subsequent elution with phosphate buffer (1, 7) followed by chromatography on a cellulose column (9), according to the general procedure of Sober and Peterson (72). The final purified fractions obtained are referred to as R_{1gc} (derived from R_1 fraction) and R_{2gc} (derived from R_2). The over-all purification of the fatty acid synthesizing system amounts to more than 1,000-fold based on the original 100,000 $\times$ *g* supernatant extract. This is a minimum value, since it is difficult to give an exact value in a multi-enzyme system of this nature.

Fractions R_{1gc} and R_{2gc} are free of some of the various enzymes of the β-oxidation cycle: fatty acid activating enzyme (73), acetic thiokinase (74, 75), butyryl dehydrogenase (76), palmityl dehydrogenase (77, 78), the electron-transferring factor (79), and thiolase (80-82). They do contain, however, traces of enoyl hydrase (83, 84) and β-hydroxyacyl dehydrogenase (80, 85). The TPNH α,β-unsaturated acyl CoA reducing enzyme (17), which is a key enzyme in the synthesis of fatty acids by the reversal of the β-oxidation sequence (35), is absent from R_{1gc} and R_{2gc}. These findings, in addition to the studies on cellular distribu-

TABLE 4. INCORPORATION OF LABELED ACETATE INTO FATTY ACID BY PIGEON LIVER FRACTIONS

Fraction Tested*	C^{14}-acetate Incorporated/hour/mg of Protein
	mμmoles
Mitochondria	2.0
Microsomes	0.8
Clear supernatant	18.0
Mitochondria + microsomes	1.2
Mitochondria + clear supernatant	5.0
Microsomes + clear supernatant	8.0
Pellets	42.0

* The various fractions were prepared by homogenizing pigeon liver in 0.25 M sucrose with the Potter-Elvehjem homogenizer. The whole homogenate was centrifuged at 600 $\times$ *g* to remove the nuclei and the cell debris. The mitochondria and the microsomes were isolated by the usual centrifugal fractionation at 9000 $\times$ *g* and 100,000 $\times$ *g*, respectively. The resulting clear supernatant solution was used as indicated above. The pellets were obtained by further centrifuging the clear supernatant solution for 4 hours at 100,000 $\times$ *g*. Each reaction mixture contained 5.0 μmoles of acetate-1-C^{14} (300,000 cpm total activity), 50 μmoles of potassium phosphate buffer (pH 6.5), 4 μmoles of ATP, 0.3 μmole of Mn^{++}, 0.1 μmole of TPN^+, 8 μmoles of isocitrate, 0.04 μmole of CoA, 6.0 μmoles of cysteine, and 10 μmoles of $KHCO_3$. Final volume was 0.5 ml, and the reaction was started by the addition of 2.5 mg of protein of each fraction where indicated. The reaction mixture was incubated at 38° for 2 hours.

tion, further differentiate the non-mitochondrial system from the mitochondrial system.

Products of the Reaction. Considerable experimental evidence has been obtained which supports the concept that fatty acids are synthesized by successive head-to-tail condensations of two carbon units (47, 86). Much of this information has been derived from earlier studies with whole animals or tissue slices (47, 48, 49, 86). Brady and Gurin (14) separated the long-chain fatty acids ($>C_{10}$) that were synthesized by soluble preparations of pigeon liver, and on decarboxylation of these fatty acids (synthesized from acetate-1-C^{14}), the radioactivity in the carboxyl carbon atom (cpm per C) of the fatty acid was found to be only slightly above the specific activity of the fatty acid (cpm per C), which is to be expected from successive condensations of acetate units. Popják and Tietz (69, 70) separated and identified the products of fatty acid synthesis in mammary gland homogenates, and these were shown to be predominately long-chain fatty acids (C_{10} to C_{18}). Similar results were reported by Klein (71) in the fatty acid synthesizing system from yeast, and by Stumpf and Barber (4) utilizing extracts of avocado fruits.

The products arising from the conversion of acetate to fatty acids by the relatively crude avian liver fractions (R_1 and R_2) have been separated and identified by a variety of techniques (38, 39). The major components were palmitic acid (80%) with myristic and lauric acids constituting the remainder; short-chain acids (C_4 and C_{10}) did not accumulate under the reaction conditions employed (3).

Palmitic acid synthesized from acetate-1-C^{14} was isolated by dilution with unlabeled palmitic acid and recrystallized from various solvents to a constant specific activity. The resulting acid was decarboxylated by the Schmidt reaction as described by Phares (40), and the liberated CO_2 was found to contain twice the average specific radioactivity of the other carbon atoms of the palmitic acid, indicating a *de novo* synthesis of palmitic acid from acetate.

Components and Properties of the Non-mitochondrial System. As mentioned above, Gurin and his coworkers (14, 15, 16) showed that the synthesis of long-chain fatty acids from acetate by the soluble extract from pigeon liver was markedly stimulated by Mg^{++} and citrate. When this extract was treated with charcoal, the synthesis was stimulated by the addition of CoA, ATP, and DPN^+ in the presence of Mg^{++} and citrate. However, after dialysis of the extract, the activity declined almost completely and could not be restored by the addition of the above cofactors. These observations were confirmed by the investigators at the Enzyme Institute (1, 2, 3), where earlier studies had demonstrated the following cofactors to be required for the conversion of acetate to fatty acids by the reconstituted crude system: ATP, CoA, GSH, DPN^+, glucose-1-phosphate, isocitrate, TPN^+, lipoic acid, Mg^{++}, and Mn^{++}. Some of these factors dropped out as purification of the enzyme system proceeded, whereas several new requirements emerged (5, 6, 7).

The purified system of Wakil and Gibson (R_{1gc} and R_{2gc}) required ATP, Mn^{++}, HCO_3^-, and TPNH (Table 5) for optical conversion of acetyl CoA to long-chain fatty acids. Very little or no synthesis took place in the absence of any one of these factors. The additional components required in the crude system, such as glucose-1-phosphate and isocitrate, may have provided a source for both CO_2 and reduced pyridine nucleotides. Furthermore, isocitrate may play a role similar to other di- and tricarboxylic acids such as malonate, α-ketoglutarate, succinate, fumarate, citrate, etc. (1, 2, 69, 70), which stimulate the synthetic process without being incorporated partly or wholly into the products (7). The exact mechanism of this effect is not yet clear. One possible interpretation for the stimulatory action of these polycarboxylic acids may be the protection of the malonyl CoA (itself a dicarboxylic acid derivative of CoA) against enzymatic destruction, e.g., deacylation to free acid and CoA, or decarboxylation to acetyl CoA, etc.

Earlier assays of the synthesis of fatty acids from acetate were based exclusively on the use of radioisotopes (1, 2, 3). However, in the purified enzyme sys-

TABLE 5. COMPONENTS OF THE FATTY ACID SYNTHESIZING SYSTEM OF PIGEON LIVER

	Acetyl CoA Incorporated into Fatty Acid
	μmoles
Complete system*	0.394
No ATP	0.000
No TPNH	0.000
No Mn^{++}	0.030
No HCO_3^-	0.010
No R_{1g} (or R_{2g})	0.000

* Complete system: 50 μmoles histidine buffer (pH 6.5); 2 μmoles ATP; 0.6 μmole $MnCl_2$; 0.6 μmole acetyl CoA; 0.8 μmole TPNH; 8 μmoles $KHCO_3$; 1 mg of R_{1g}; and 0.8 mg R_{2g}. Time: 30 minutes at 38°. Final volume, 1 ml.

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tem, an equally sensitive and far more convenient assay method was developed, based upon the spectrophotometrically measured rate of oxidation of TPNH by the fatty acid synthesizing system (87). Figure 5

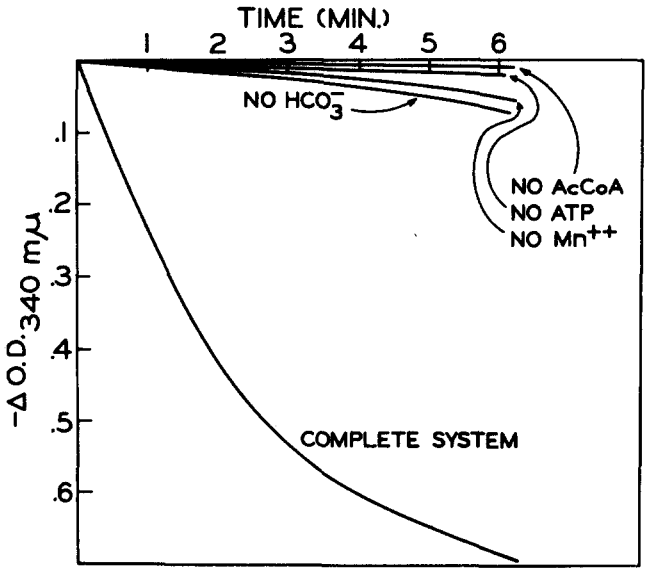

FIG. 5. Oxidation of TPNH. Each cuvette contained the following reagents (except when omitted as indicated): 25μmoles of potassium phosphate buffer (pH 6.5), 1.0 μmole of ATP, 0.3 μmole of $MnCl_2$, 4.0 μmoles of $KHCO_3$, 0.05 μmole of 1-C^{14}-acetyl CoA, and 0.08 μmole of TPNH in a final volume of 0.50 ml. The reaction was started by the addition of 0.7 mg of R_{2gc}. The temperature was maintained at 38°. At the end of 5 minutes 0.032 μmole of acetyl CoA was incorporated into fatty acids.

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shows that the rate of oxidation of TPNH is strictly dependent upon the presence of all the components of the complete system. This rate parallels the rate of incorporation of C^{14}-1-acetyl CoA into the long-chain fatty acids as determined by the isolation of the fatty acids from the reaction mixture (1).

DPNH can substitute for TPNH in fatty acid synthesis, but the rate of oxidation of DPNH is about one-third the rate of oxidation of TPNH (87). This cannot be caused by the presence of DPNH-TPN^+ transhydrogenase (88), since we were unable to demonstrate transhydrogenase activity in the two enzyme preparations.

The lack of specificity for pyridine nucleotides distinguishes the non-mitochondrial system from the mitochondrial system (19), in which both TPNH and DPNH are required for the synthesis of fatty acids from acetyl CoA and fatty acyl CoA.

Acetyl CoA Carboxylase. The requirement of bicarbonate for fatty acid synthesis could be displayed by highly purified enzyme preparations obtained by Gibson *et al.* (5, 7). They demonstrated that this requirement was absolute for all stages of purification and that it applied to the synthesis of fatty acids not only in the avian liver preparation but also in crude preparations from rat liver and rat kidney. Figure 6

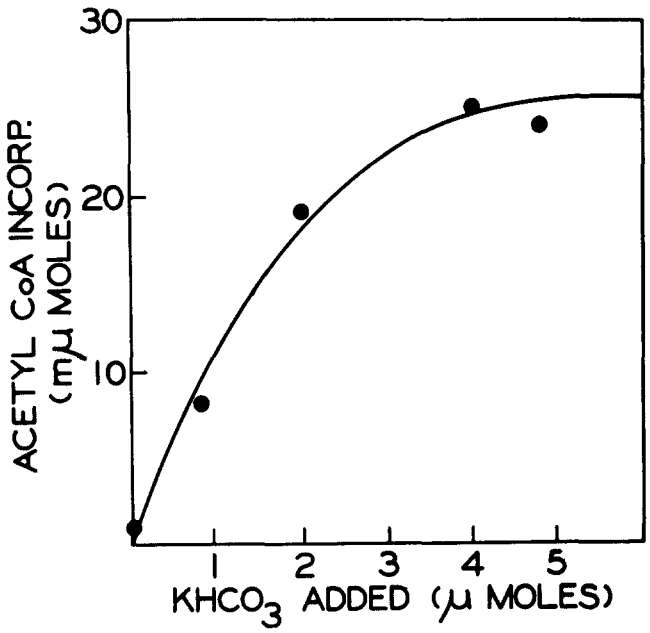

FIG. 6. Each experimental tube contained in a final volume of 0.5 ml the following reagents: 50 μmoles of potassium phosphate buffer (pH 6.5), 1.0 μmole of ATP, 0.3 μmole of $MnCl_2$, 0.08 μmole of TPNH, and 50 μmoles of acetyl-1-C^{14} CoA. The reaction was started by addition of 0.7 mg R_{1g} and 0.4 mg R_{2g}. All samples were incubated for 5 minutes at 38°. At a bicarbonate concentration of 8×10^{-3} M, the rate of acetyl CoA incorporation is equivalent to 0.3 μmole/mg enzyme/hour.

shows the effect of increasing concentration of bicarbonate on the synthesis of fatty acids in the purified system.

$HC^{14}O_3^-$ did not incorporate into the fatty acids during active synthesis from unlabeled acetyl CoA, as shown in Table 6. This is not surprising, since it had reason a "catalytic" role for HCO_3^- had been proposed (5, 7).

A similar requirement for bicarbonate was reported by Klein (71) in studies on the synthesis of fatty acids by particulate preparations from yeast cells, as well as by Stumpf and co-workers (8) in their studies on fatty acid synthesis in extracts from avocado fruit. This common requirement for fatty acid synthesis establishes the universality of the fatty acid synthesizing system in living cells.

Recently Wakil (9) was able to isolate the first intermediate in the synthesis of long-chain fatty acids from acetyl CoA and thus split the reaction sequence into two parts. When acetyl CoA was incubated with R_{1gc} in the presence of HCO_3^-, Mn^{++}, and ATP, an intermediate was isolated from the reaction mixture which could be converted to palmitate in the presence of R_{2gc} and TPNH. This intermediate incorporated both C^{14}-acetyl CoA and $HC^{14}O_3^-$ in a ratio of approximately 1:1. After hydrolysis with alkali, the saponifiable fraction was extracted with diethyl ether and was shown to contain all of the radioactivity. This compound was identified as malonate (9) by its R_f in two different chromatographic systems, melting point, and melting point of the *p*-nitrobenzyl ester. Formica and Brady (89) were also able to demonstrate the formation of malonyl CoA by the carboxylation of acetyl CoA by crude extracts of pigeon liver and pig heart.

The formation of the malonyl derivative by the purified enzyme preparation R_{1gc} is dependent on the presence of Mn^{++}, ATP, and HCO_3^- (Table 7). Thus it appears that ATP activates the HCO_3^- (or any of its equilibrium species, CO_2 and H_2CO_3), which is then condensed with the methyl group of acetyl CoA to form malonyl CoA. For this reason the name "acetyl CoA carboxylase" (referred to previously as R_{1gc}) has been suggested for this enzyme.

The product of the carboxylation reaction has been identified as malonyl CoA by paper chromatography in two different systems (isobutyric acid-ammonia and ethanol-sodium acetate). Its behavior was identical to that of chemically prepared malonyl CoA. This evidence led us to conclude that the first step in fatty acid synthesis is the formation of malonyl CoA from bicarbonate and acetyl CoA.

The reaction was absolutely dependent upon ATP for the activation of CO_2 prior to its condensation with acetyl CoA, with a simultaneous phosphorolysis of ATP into ADP and inorganic phosphate. The stoichiometry of the reaction showed that for each mole of malonyl CoA formed there was 1 mole of ATP utilized and 1 mole each of ADP and inorganic phosphate formed as shown in the following equation:

TABLE 6. FAILURE OF $H^{14}CO_3^-$ TO BECOME INCORPORATED INTO THE SYNTHESIZED FATTY ACIDS

Exp.	Complete System Plus*	TPNH (ΔA† 340 mμ)	Acetyl CoA Incorp. into F.A. (cpm)	$HC^{14}O_3^-$ Incorp. into F.A. (cpm)
I	Acetyl-1-C^{14} CoA and unlabeled HCO_3^-	0.460	8430	
	Unlabeled acetyl CoA and $HC^{14}O_3^-$ (6×10^5 cpm)	0.420		50
II	Acetyl-1-C^{14} CoA and unlabeled HCO_3^-	0.375	6800	
	Unlabeled acetyl CoA and $HC^{14}O_3^-$ (2×10^6 cpm)	0.370		0.00

* Each cuvette contained the following components of the complete system: 25 μmoles of potassium phosphate pH 6.5; 1 μmole ATP; 0.3 μmole of $McCl_2$; and 0.05 μmole of TPNH. As indicated, the following reagents were also added: 50 mμmoles of acetyl-1-C^{14} CoA (37,000 cpm) or 45 mμmoles of unlabeled acetyl CoA; 4 μmoles of either unlabeled HCO_3^- or $HC^{14}O_3^-$. Total volume was 0.5 ml. The reaction was started by the addition of 0.6 mg of R_{1g} and 0.5 mg R_{2g}.

† ΔA represents the change in absorbancy at 340 mμ, which was followed for 5 minutes at 30° in the Beckman DUR, and the amount of fatty acid synthesized was determined as usual at the end of this time.

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Our earlier reports on the stoichiometry of the overall conversion of acetyl CoA to palmitate employing relatively cruder fractions of R_{1g} and R_{2g} indicated that 2 moles of ATP were utilized for every mole of acetyl CoA converted to fatty acids (87). When the highly purified fraction R_{1gc} was used in the malonyl CoA formation, only 1 mole of ATP was consumed for every mole of acetyl CoA carboxylated to malonyl CoA. Brady (11) has recently reported on the possible formation of higher fatty acids (as identified by the R_f of their hydroxamic acids) from malonyl CoA by either crude R_1 or R_2 fractions. Furthermore, Lynen and his colleagues were able to confirm these observations, using purified enzyme preparations from yeast cells (12, 90).

$$CO_2 + CH_3\overset{\overset{O}{\|}}{C}SCoA + ATP \xrightarrow[(\text{bound-biotin}) + Mn^{++}]{\text{acetyl CoA carboxylase}} CH_2\begin{matrix}COOH\\COSCoA\end{matrix} + ADP + P_i$$

TABLE 7. REQUIREMENTS OF ACETYL CoA CARBOXYLASE REACTION

	Malonyl CoA Formed
	mμmoles
Complete system*	12.0
No AcCoA	0.0
No ATP	0.0
No HCO_3^-	0.1
No Mn^{++}	0.1
No enzyme	0.0

* The complete system contained 20 mμmoles acetyl CoA, 1.0 mμmole ATP, 0.3 mμmole $MnCl_2$, 4 mμmoles $KHCO_3$, 30 mμmoles phosphate buffer (pH 6.5), and 0.100 mg of acetyl CoA carboxylase in a total volume of 0.4 ml. The reaction mixture was incubated for 10 minutes at 38° and the reaction was stopped by heat denaturation of the enzyme. Malonyl CoA was assayed by its oxidation of TPNH in the presence of the R_{2gc} enzyme fraction.

With the aid of malonyl CoA, Ganguly (91) was able to study the distribution of the non-mitochondrial system in various tissues. The results (Table 8) show that there is a wide distribution of this system in animal tissue, suggesting that this system is the main pathway for synthesis of fatty acids.

TABLE 8. DISTRIBUTION OF THE NON-MITOCHONDRIAL SYSTEM IN CRUDE EXTRACTS OF VARIOUS TISSUES

Tissue (Crude Extract)	Malonyl CoA Converted to Palmitate per mg of Protein per 10 Minutes
	mμmoles
Beef liver	0.15
Beef brain	1.8
Beef pancreas	0.3
Beef lung	0.23
Beef kidney	0.06
Beef small intestine	0.02
Beef mammary gland	5.3
Beef adipose tissue	2.3
Beef suprarenal fat	7.0
Beef aorta	0
Chicken liver	14.5
Chicken ovary	0.04
Chicken oviduct	0
Pigeon liver	28.9

Each assay tube contained 20 μmoles of potassium phosphate buffer (pH 6.5), 50 mμmoles of TPNH, 6 mμmoles (12,000 cpm total activity) of labeled malonyl CoA, limiting amount of the crude extract of the tissues indicated, and water to 0.40 ml. The The reaction mixture was incubated at 38° for 10 minutes.

The Role of Biotin in Fatty Acid Synthesis (Biotin in Acetyl CoA Carboxylase). Available information about the role of biotin in metabolic reactions points to a close relationship between this vitamin and the metabolism of carbon dioxide in the carboxylation-decarboxylation type reactions (for a comprehensive review see György (92)). A relationship has also been reported between biotin and fatty acid metabolism. Unsaturated fatty acids such as oleic, linoleic, etc., can promote growth of many microorganisms in the absence of biotin (93). Saturated fatty acids, although inactive alone, have a sparing effect on the unsaturated fatty acids. Until recently (6, 94) this relationship between biotin and fatty acid metabolism was very obscure and could not be related, on the surface at least, to the more generalized effect of biotin on the metabolism of carbon dioxide. The discovery by Gibson *et al.* (5, 7) that bicarbonate is an absolute requirement for fatty acid synthesis by a purified enzyme system suggested a possible role for biotin. When such enzyme fractions were assayed for biotin content by the procedure of Wright and Skeggs (95, 96), there was a significant concentration of this vitamin in one of the fractions, namely, R_{1gc} (acetyl CoA carboxylase) (6, 7, 94). Biotin concentrated with the active protein of this fraction all along the steps of purification (Fig. 7), and the ratio of enzymatic activity to biotin content remained essentially the same throughout the purification. The final concentration

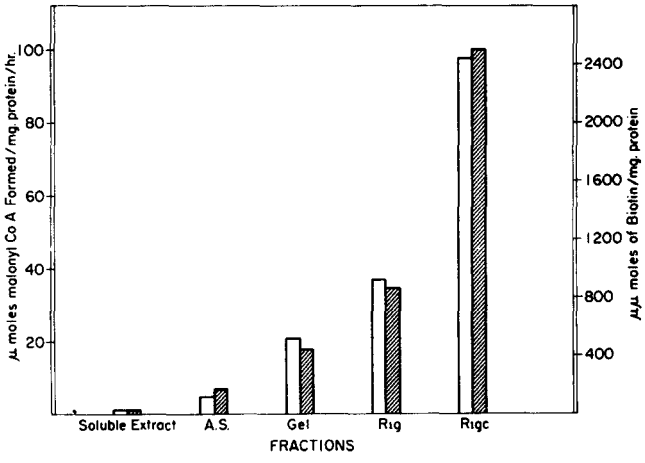

FIG. 7. The ratio of biotin content to specific activity of acetyl CoA carboxylase during the various steps of purification. The specific enzyme activity (μmoles acetyl CoA converted to malonyl CoA per mg protein per hour) of acetyl CoA carboxylase (left column) is compared with the protein-bound biotin concentration (right column).

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of biotin in the most purified preparation (after purification by ion exchange column) amounts to about 2 or 3 moles of biotin per 10^6 of protein or 1 mole of biotin per 300,000 to 500,000 of protein. This is the highest concentration of protein-bound biotin reported so far.

Biotin is tightly bound to the protetin and can be released only by tryptic digestion or acid hydrolysis (94). The product of tryptic digestion is not free biotin but a conjugated derivative of biotin, possibly biocytin (94), as shown by microbiologic assay (95) and paper chromatography (96). Free biotin is released by the acid hydrolysis of the protein fractions.

Wakil *et al.* (6, 94) have presented evidence to show that the protein-bound biotin does indeed participate in the over-all synthesis of long-chain fatty acids from acetyl CoA. They have shown that the conversion of acetyl CoA to palmitate is inhibited by avidin (92), an egg-white protein which specifically binds biotin (Fig. 8). This inhibition can be relieved by the addition of free *d*-biotin as shown in Figure 8. The effect of avidin can also be demonstrated spectrophotometrically as shown in Figure 9.

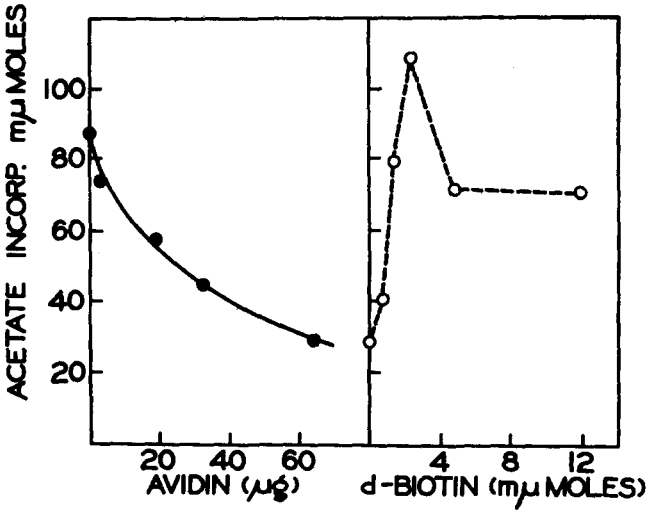

FIG. 8. Reversal of the avidin inhibition of fatty acid synthesis by D-biotin. In the left curve, increasing amounts of purified avidin are added to a series of identical incubation mixtures. The maximum inhibitory effect in this sequence was obtained with 64 μg avidin. The right curve demonstrates that by adding increasing quantities of free *d*-biotin to a second series of incubation mixtures, each containing 64 μg avidin, the inhibitory effect of avidin on synthesis is progressively eliminated. Each experimental system contained in a total volume of 0.5 ml the following reagents: 40 μmoles of phosphate buffer (pH 6.5); 0.3 μmole of $MnCl_2$; 6.0 μmoles of cysteine; 2.7 μmoles of ATP; 0.38 μmole of TPN; 6.0 μmoles of isocitrate; 0.04 μmole of CoASH; 1.32 μmoles of 1-C^{14}-acetate; 10 μmoles of $KHCO_3$; 0.3 mg acetic thiokinase (contains isocitric dehydrogenase); 0.9 mg R_{2g}. The reactions were initiated by the addition of 0.24 mg R_{1g}. Incubations were carried out at 38° for 30 minutes in an atmosphere of nitrogen.

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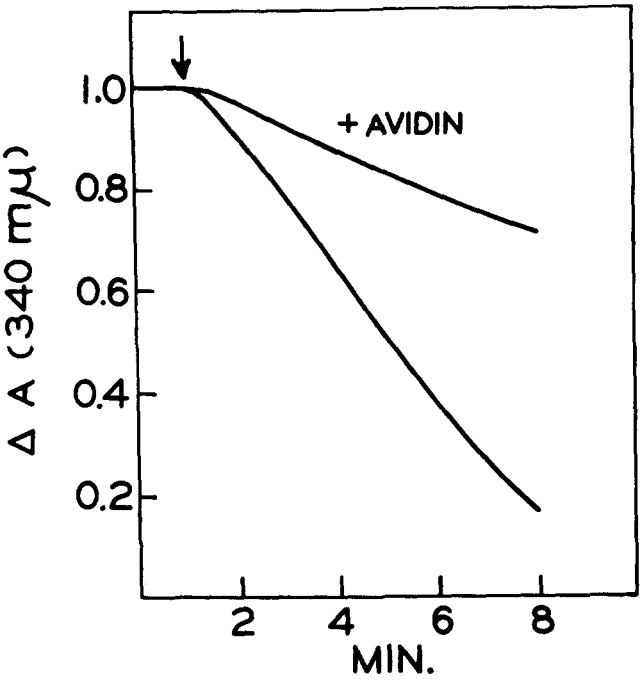

FIG. 9. Avidin inhibition of the oxidation of TPNH associated with fatty acid synthesis. Purified avidin (46 μg) was added to one of two identical systems each of which contained in a total volume of 0.50 ml the following reagents: 50 μmoles potassium phosphate buffer (pH 6.5); 0.3 μmole $MnCl_2$; 1.0 μmole ATP; 0.21 μmole acetyl CoA; 5.0 μmoles $KHCO_3$; 0.17 μmole TPNH; and 1.5 mg R_{2g}. The reaction was initiated in both systems by the addition of 0.26 mg R_{1g} (indicated by arrow). The optical density at 340 mμ (1.0 cm cell) was followed continuously in the Beckman DUR spectrophotometer (at 38°).

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Biotin-deficient animals (rats and chicks) were prepared[3] and their livers were used to prepare the fatty acid synthesizing system. The results show that the levels of acetyl CoA carboxylase in the livers of these animals is lower than the amount of carboxylase in the normal animals, but it is by no means absent. On purification of the enzyme from deficient animals, the activity of the enzyme increases, as does the concentration of biotin. At the highest purity level the biotin content of such enzyme preparations appears to be comparable to the preparations from normal animals. This observation indicates that biotin deficiency, however severe it may be, results in the decrease of the

[3] S. J. Wakil, D. M. Gibson, and A. E. Harper. Unpublished observations.

total level of the acetyl CoA carboxylase, but it does not completely eliminate this vital enzyme from the liver.

Lynen and his collaborators (12, 97) have recently isolated another biotin-containing enzyme which carboxylates β,β-dimethyl acrylyl CoA to form β-methyl glutaryl CoA. They presented evidence to show that biotin is an integral part of this enzyme, and that CO_2-biotin-enzyme is an intermediate in this reaction. The proposed scheme is as follows:

$$CO_2 + ATP + \text{biotin-enzyme} \longrightarrow CO_2 \sim \text{biotin-enzyme} + P_i + ADP$$

$$CO_2 \sim \text{biotin-enzyme} + \beta,\beta\text{-dimethyl acrylyl CoA} \longrightarrow \beta\text{-methylglutaryl CoA} + \text{biotin-enzyme}$$

A similar mechanism may be operative in the carboxylation of acetyl CoA to form malonyl CoA.

Conversion of Malonyl CoA to Palmityl CoA. Wakil and Ganguly (10) have shown that the second enzyme fraction (R_{2gc}) is able to convert malonyl CoA to palmityl CoA in the presence of acetyl CoA and TPNH. This conversion could be followed either spectrophotometrically by measuring the oxidation of TPNH, or isotopically by measuring the incorporation of C^{14}-labeled malonyl CoA into palmitate. The requirement for acetyl CoA in the conversion of malonyl CoA to palmitate is absolute, as shown in Figure 10.

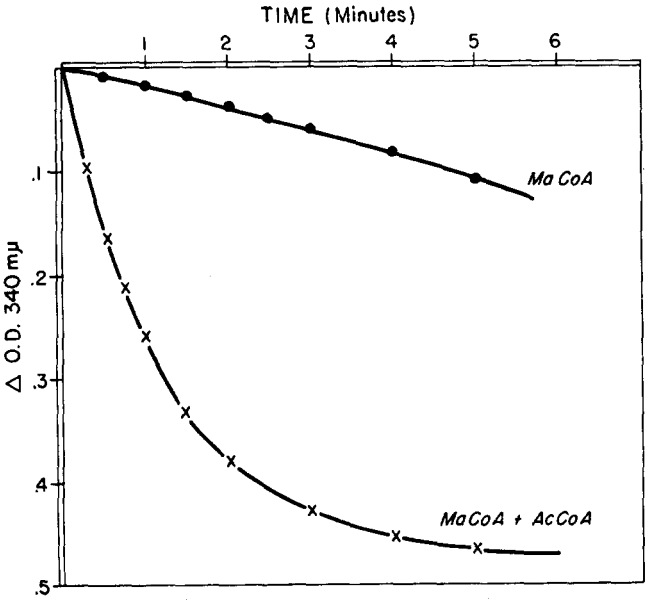


FIG. 10. The requirement for acetyl CoA in the oxidation of TPNH by malonyl CoA. Each cuvette contained 30 μmoles of phosphate buffer (pH 6.5), 0.015 μmole acetyl CoA where indicated, 0.016 μmole malonyl CoA, .05 μmole TPNH and water to a final volume of 0.4 ml. The reaction started with the addition of 0.2 mg of R_{2gc} fraction.

Furthermore, a significant amount of C^{14}-acetyl CoA was incorporated into palmitate when unlabeled malonyl CoA was used, as shown in Table 9.

The tendency of the reaction to proceed toward palmitate synthesis after incubation of malonyl CoA with enzyme in absence of acetyl CoA (Fig. 10) may result from the presence of a contaminating enzyme in the R_{2gc} preparation which decarboxylates malonyl CoA to acetyl CoA plus CO_2. Indeed, such an enzyme could be demonstrated in the cruder fractions of R_{2gc} by the formation of citrate from malonyl CoA in the presence of oxalo-acetate and Ochoa's condensing enzyme (98).

The data in Table 9 also show the stoichiometric relationship (based on 1 mole of palmityl CoA formed) between acetyl CoA, malonyl CoA, and TPNH in the synthetic process. The results show that for each mole of palmityl CoA synthesized, 1 mole of

TABLE 9. STOICHIOMETRY OF THE PALMITYL CoA FORMATION FROM MALONYL CoA PLUS ACETYL CoA

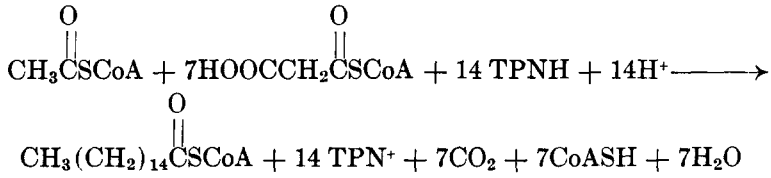
Acetyl CoA	Malonyl CoA	TPNH	Palmitate	CO_2	CoASH
mμmoles	*mμmoles*	*mμmoles*	*mμmoles*	*mμmoles*	*mμmoles*
	−8.2	−16.7	+1.1		+7.9
	−6.0	−12.3		+5.8	
−2.2		−25.8	+1.9		

Three separate experiments were used for the determination of the stoichiometry of the reaction. In the first experiment the reaction mixture contained 8.5 mμmoles C^{14}-malonyl CoA ($HOOCCH_2C^{14}OCoA$, 20,000 cpm), 50 mμmoles TPNH, 13 mμmoles acetyl CoA, 30 μmoles phosphate buffer (pH 6.5), 0.120 mg of R_{2gc}, and water to a final volume of 0.4 ml. The oxidation of TPNH was followed spectrophotometrically (cf. Fig. 10). At the end of the reaction (5 minutes, at 38°), aliquots were withdrawn for (a) CoASH analysis by the procedure of Wakil and Hübscher (105), (b) palmitate analysis by C^{14}-incorporation, and (c) malonyl CoA as determined by the amount of C^{14} left in the reaction mixture after extraction of C^{14}-palmitate.

In the second experiment the reaction mixture contained exactly the same reagents as in the first except that 6.0 mμmoles carboxyl labeled malonyl CoA ($HOOC^{14}CH_2COCoA$, 13,000 cpm) were used. The reaction was followed by TPNH oxidation. The $C^{14}O_2$ was trapped in NaOH and the radioactivity was determined as $BaC^{14}O_3$. Palmitate did not incorporate any C^{14}.

In the third experiment the reaction mixture was the same as in the first except that 13 mμmoles 1-C^{14}-acetyl CoA (32,000 cpm) and 30 mμmoles of unlabeled malonyl CoA were used. The remaining acetyl CoA was determined by both radioactivity and by the citrate condensing enzyme.

acetyl CoA, 7 moles of malonyl CoA, and 14 moles of TPNH are consumed and 7 moles each of CO_2, CoA, and water were formed. The over-all reaction can be presented as follows:



$$CH_3\overset{O}{\overset{\|}{C}}SCoA + 7HOOCCH_2\overset{O}{\overset{\|}{C}}SCoA + 14\,TPNH + 14H^+ \longrightarrow$$

$$CH_3(CH_2)_{14}\overset{O}{\overset{\|}{C}}SCoA + 14\,TPN^+ + 7CO_2 + 7CoASH + 7H_2O$$

In short, one "C_2-unit" of palmitate is derived from acetyl CoA, and the remaining 14 carbon atoms are derived from malonyl CoA.

Acetyl CoA contributes carbons 15 and 16 of palmitate as shown by degradation studies of the carboxyl group of palmitic acid derived from 1-C^{14}-acetyl CoA and 1-C^{14}-malonyl CoA, respectively. The carboxyl group of palmitic acid derived from 1-C^{14}-acetyl CoA had no radioactivity, while the carboxyl group of palmitic acid synthesized from malonyl CoA ($HOOCCH_2C^{14}OSCoA$) had the same specific activity as that of the labeled carbonyl group of malonyl CoA.

Acetaldehyde does not substitute for acetyl CoA under these conditions (10) nor does it dilute the amount of C^{14}-acetyl CoA incorporated into palmitate. These results are contrary to Brady's proposed mechanism for fatty acid synthesis (11), which implicated acetaldehyde as an intermediate.

Propionyl CoA can substitute for acetyl CoA, but other acyl CoA's, such as hexanoyl or octanoyl CoA, which do not incorporate into palmitate, cannot. 1-C^{14}-Butyryl CoA is incorporated into palmitate to an extremely low extent, as shown in Figure 11. This is contrary to our earlier reports, based on experiments utilizing cruder enzyme preparations, that butyryl CoA or octanoyl CoA (10 can be substituted for acetyl CoA. The pathway of incorporation of butyryl CoA or octanoyl CoA into palmitate in these experiments is not yet clear; nevertheless, with highly purified subfractions of R_2 only acetyl CoA or propionyl CoA can be incorporated into long-chain fatty acids, as shown in Figure 11.

When the rate of palmitate formation, as measured by the rate of oxidation of TPNH, was plotted against the concentration of acetyl CoA or propionyl CoA, the usual hyperbolic plot was obtained (Fig. 12). The same data could be plotted by the method of Lineweaver and Burk (Fig. 13), yielding two straight lines which intercept the abscissa at a single point. From such plots the Michaelis-Menten constants for acetyl CoA, propionyl CoA, and butyryl CoA were determined and found to be 2.3×10^{-6} M, 5.7×10^{-5} M, and 3.9×10^{-4} M, respectively. These values indicate that the affinity of the enzyme for propionyl CoA is about one twenty-fifth that of acetyl CoA, whereas with butyryl CoA the value is one one-hundred-and-seventieth that of acetyl CoA (Fig. 12).

When propionyl CoA was used instead of acetyl CoA, the product of the synthesis was an odd-chain fatty acid with 17 carbon atoms, indicated by the slightly lower R_f of this acid as compared to palmitate. This observation, therefore, may explain the occurrence of odd-chain fatty acids in animal tissues. This appears to be dependent on the availability of propionyl CoA to the cell rather than on the presence of another enzyme system for the synthesis of odd-chain fatty acids.

Incorporation of Tritiated Acetyl and Malonyl CoA into Palmitate.[4] In 1937 Sonderhoff and Thomas (42) demonstrated for the first time that deuterium-labeled acetic acid (CD_3COOH) could be incorporated into long-chain fatty acids by yeast cells. These results were confirmed by Barker *et al.* in 1945, and were extended by Rittenberg and Bloch to include experiments on whole animals (rats and mice) in which they showed that deuterium-labeled acetate incorporated in cholesterol as well as fatty acids. We recently studied the incorporation of tritium-labeled acetyl CoA ($CT_3COSCoA$) and tritium-labeled malonyl CoA ($HOOCCT_2COSCoA$) into palmitate by the purified enzyme preparation (R_{2gc}).[5] We hoped that such studies might help us formulate and understand the mechanism of fatty acid synthesis.

Tritium-labeled acetyl CoA was prepared enzymatically from tritiated acetate (CT_3COOH) and the acetic thiokinase system (74, 75). The tritiated acetyl CoA was then used to prepare the tritium-labeled malonyl CoA ($HOOCCT_2COSCoA$) employing the acetyl CoA carboxylase system. When tritium-labeled acetyl CoA ($CT_3COSCoA$) was added to the reaction

[4] This section dealing with tritiated substrates was received for publication on November 2, 1960.

[5] S. J. Wakil and R. Bressler, Unpublished results.

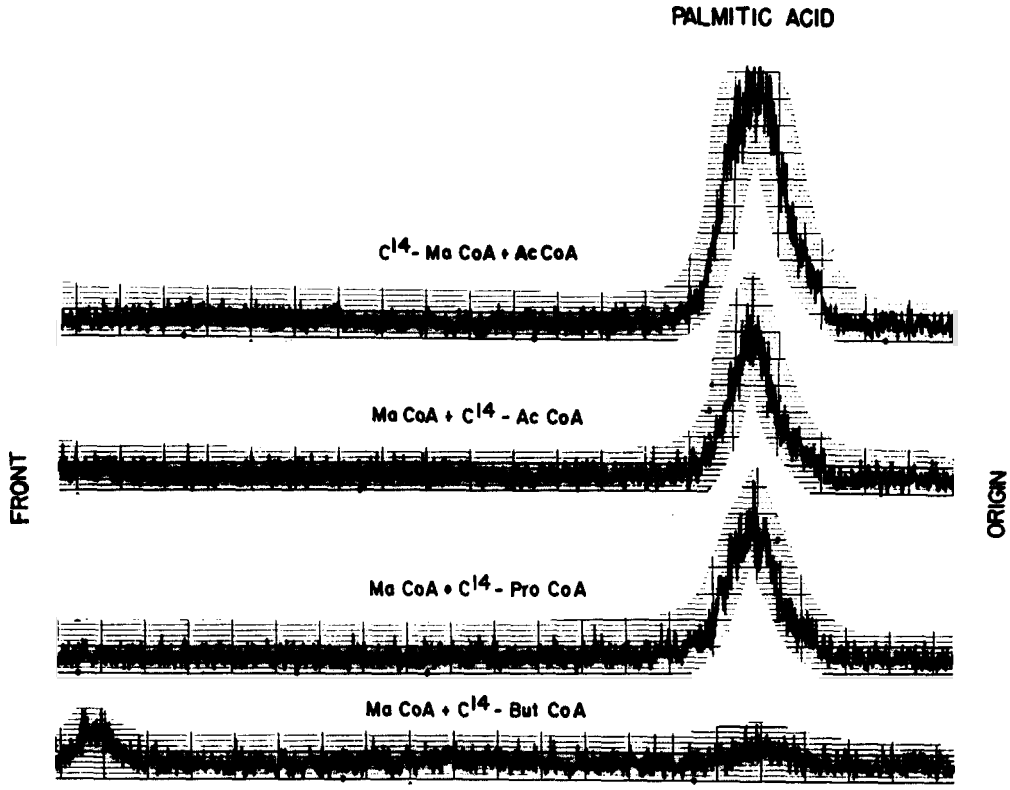


FIG. 11. Scanning of the paper chromatograms of the fatty acids synthesized from various labeled substrates. Four separate tubes contained 30 μmoles phosphate buffer (pH 6.5) and .05 μmole TPNH in a final volume of 0.4 ml. The following were also added: To the first tube, 0.01 μmole C^{14}-malonyl CoA ($HOOCCH_2C^{14}OSCoA$, 18,000 cpm) and 0.01 μmole acetyl CoA; to the second tube, 0.01 μmole of malonyl CoA and 0.013 μmole 1-C^{14}-acetyl CoA (32,000 cpm); to the third tube, 0.01 μmole malonyl CoA and 0.065 μmole 1-C^{14}-propionyl CoA (135,000 cpm); to the fourth tube, 0.01 μmole malonyl CoA and 0.08 μmole 1-C^{14}-butyryl CoA (170,000 cpm). The reaction was started by the addition of 0.180 mg of protein (R_{2gc}) and incubated at 38° for 5 minutes. The fatty acids were extracted in pentane after hydrolysis of the acyl CoA derivatives (1) and were chromatographed in the reverse phase system of Kaufmann and Nitsch (39). "Palmitic acid" is the only acid formed. The extra peak in the experiment of C^{14}-butyryl CoA is due to C^{14}-butyric acid.

mixture containing nonlabeled malonyl CoA, TPNH, and the R_{2gc} enzyme, tritium-labeled fatty acids were isolated. The incorporation of tritium from acetyl CoA was absolutely dependent upon the presence of malonyl CoA. The amount of tritium incorporated into the palmitate corresponded to about 1 mole of acetyl CoA incorporated into 1 mole of palmitate (as measured by TPNH oxidation), or 3 atoms of tritium were incorporated per molecule of palmitate. This is in complete agreement with the formulation given above for the synthesis of palmityl CoA from acetyl CoA and malonyl CoA. It would also indicate that the methyl group (CT_3) of acetyl CoA is incorporated into palmitate as a unit (presumably as carbon 16 of palmitate) with no loss of tritium atoms during this transformation.

When tritium-labeled malonyl CoA ($HOOCCT_2$-COSCoA) was added to nonlabeled acetyl CoA, TPNH, and the R_{2gc} enzyme, tritium-labeled palmitic acid was also isolated. The amount of tritium labeling in palmitic acid in three such experiments amounted to about 5 to 6.8 microatoms of tritium per μmole of palmitic acid synthesized. Since our stoichiometric formulation for palmitic acid synthesis indicated the consumption of 7 moles of malonyl CoA for each mole of palmityl CoA formed, then it is reasonable to conclude that there was approximately 1 atom of tritium incorporated into palmitate per "C_2-unit" derived from malonyl CoA. In other words, of two possible tritium atoms that might incorporate into palmitate from $HOOCCT_2COSCoA$, only one tritium atom was found in palmitate, while the other was presumably lost to the medium in the form of water (THO) in the course of the reaction.

These results did not lend support to our earlier suggestion that dicarboxylic acyl CoA's may be involved as intermediates in the conversion of acetyl CoA and malonyl CoA to palmitate. If the latter were the case, then we could not expect any incorporation of tritium atoms from malonyl CoA into palmitate. Therefore a modification of the scheme of Wakil and Ganguly must be made in order to take these observations into account. Such a modification must assume that the release of CO_2 from malonyl CoA takes place prior to the reduction of the carboxyl group, elimination of H_2O, and the reaction of the carbon-carbon double bond.

A likely possibility is that the condensation and decarboxylation of malonyl CoA may take place simultaneously. It is also conceivable that a negatively charged hypothetical intermediate such as this ($^-CT_2COSCoA$) may be formed on the enzyme surface as a result of the interaction of the enzyme with malonyl CoA. Such intermediate is then coupled with the partially positive charged carbonyl group of acetyl CoA ($CH_3-\overset{O^-}{\overset{|}{C^+}}-SCoA$) to form the keto derivative according to the following formulation:

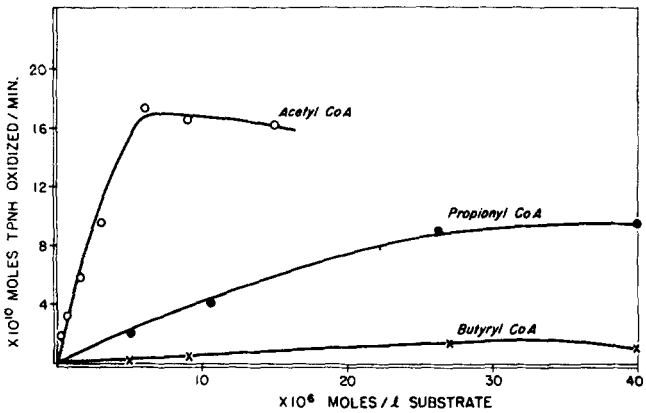
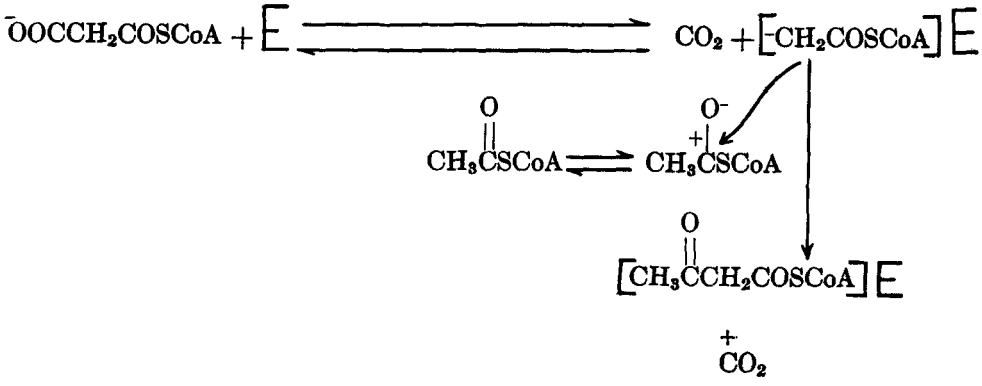


FIG. 12. The relationships between the rate of fatty acid synthesis (as measured by the rate of TPNH oxidation) and acetyl CoA, propionyl CoA, or butyryl CoA concentrations. Each reaction mixture contained 30 μmoles of phosphate buffer (pH 6.5), 0.016 μmole malonyl CoA, .05 μmole TPNH, the indicated amounts of acetyl CoA, propionyl CoA or butyryl CoA, and water to a final volume of 0.4 ml. The reactions were started by the addition of 0.1 mg of R_{2gc}. Temperature was 38°. The rate of the reaction was linear for the first 3 minutes.

Other possibilities cannot be ruled out at the present. Some of these may be the formation of polyketo acyl CoA (12) or an acyl derivative of a coenzyme other than CoA.



Very little is known about the nature of the β-keto derivatives. It probably is not acetoacetyl CoA, since the latter compound does not oxidize TPNH in the presence of the enzyme R_{2gc}; neither does it incorporate into palmitate. Lynen (90) has suggested that it is an acyl derivative of the enzyme

$$(CH_3-\overset{O}{\overset{\|}{C}}-CH_2-\overset{O}{\overset{\|}{C}}-SE)$$

which can be formed as a result of the condensation of acetyl CoA and malonyl-S-enzyme, but it is not formed by the interaction of acetoacetyl CoA and the enzyme.

The loss, as water, of one of the tritium atoms of the α-carbon of malonyl CoA during the synthesis of palmitic acid is of extreme interest and represents the most direct evidence so far for the dehydration of the intermediate(s) during the sequential transformation of malonyl CoA to fatty acids.

The elimination of water would undoubtedly yield an α,β-unsaturated acyl compound which is then reduced by TPNH to form the saturated derivative. It is too early as yet to say whether the reduction of (>C=C<) by TPNH takes place by direct transfer

of electrons from the pyridinenucleotide or through the intermediate oxido-reduction of another coenzyme (such as flavins in flavoproteins). It is interesting to note in this regard that all the known oxido-reduction reactions of DPN^+ and TPN^+ involve substrates with substituted carbons (e.g, >CHOH, $>CHNH_2$, etc.) and none that are >CH—CH<. On the other hand, there are numerous examples where flavoproteins are involved in the oxido-reduction of the carbon-carbon bond.

The Possible Mechanisms of Fatty Acids Synthesis. In a preliminary communication (10) we proposed a scheme for palmityl CoA synthesis from acetyl CoA and malonyl CoA which at the time appeared to explain our observations. This hypothesis proposed the formation of a C_5 intermediate (possibly acetomalonyl CoA) as the compound formed from the condensation of acetyl CoA and malonyl CoA. This C_5 compound was then thought to be successively reduced, dehydrated, reduced again, and decarboxylated to yield butyryl CoA. Thus in five successive steps, butyryl CoA was thought to be formed from acetyl CoA plus malonyl CoA. The butyryl CoA could condense with malonyl CoA to yield a C_7 dicarboxylic acyl CoA which would undergo the same successive transformations to yield a C_6 CoA, and the process would be repeated until finally palmityl CoA was formed. This hypothesis was based primarily on the ability of butyryl CoA and octanoyl CoA to be incorporated into palmitate (experiments performed with relatively crude enzyme preparations) and the inability of acetoacetyl CoA, β-hydroxyacyl CoA, and crotonyl CoA to oxidize TPNH and be incorporated into palmitate.

Lynen (90) subsequently offered another proposal, embodying the formation of tightly bound acyl-S-enzyme as an intermediate in order to explain the aforementioned observations. Lynen's scheme assumed that malonyl-S-enzyme is first formed from malonyl CoA and enzyme-SH, which is then condensed with an acyl CoA (acetyl CoA, butyryl CoA, etc.) and simultaneously decarboxylated to form a β-ketoacyl-S-enzyme. The latter derivative would be reduced, dehydrated, and reduced again to form the saturated acyl-S-enzyme which would then undergo an ester interchange reaction with CoASH to yield acyl CoA and free HS-enzyme. This scheme assumed the presence of only one enzyme responsible for the entire sequence, and required that the saturated acyl CoA's (C_4CoA, C_6CoA, etc.) were intermediates in the synthesis of palmityl CoA.

Our recent experiments on the mechanism of fatty acid synthesis employing highly purified enzyme preparations from pigeon liver appear to be somewhat different from those with crude enzyme preparations. The new observations can be summarized as follows: (*a*) Only acetyl CoA and propionyl CoA can be incorporated into long-chain fatty acids in the presence of malonyl CoA, TPNH, and enzyme. (*b*) One mole of acetyl CoA or propionyl CoA is incorporated into 1 mole of the fatty acid and constitutes the last 2 or 3 carbons, respectively, of the synthesized C_{16}- or C_{17}-fatty acid. (*c*) Butyryl CoA substitutes for acetyl CoA only to a very small extent (Figs. 11, 12), whereas hexanoyl CoA, octanoyl CoA, etc., do not incorporate at all into "palmitate." (*d*) With highly purified enzyme there is only one fatty acid synthesized from acetyl CoA plus malonyl CoA or propionyl CoA plus malonyl CoA, namely, C_{16}- or C_{17}-acids, respectively (Fig. 11). (*e*) Experiments (including trapping and dilution techniques) designed to isolate short-chain acyl CoA's (butyryl CoA, hexanoyl CoA, etc.) from the reaction mixture were not successful. (*f*) We have been unable to isolate the first condensation product (C_5 CoA) or any other dicarboxylic acyl CoA. Steberl *et al.* (99) reported the isolation of a "C_5" and "C_7" dicarboxylic

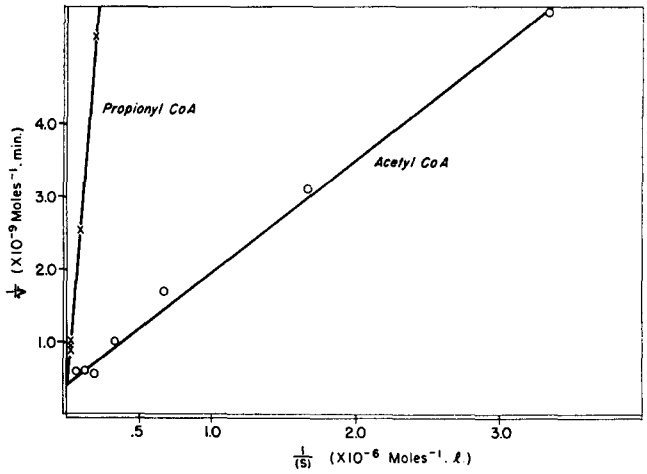


FIG. 13. Lineweaver and Burk plot of the data in Fig 12. for acetyl CoA and propionyl CoA.

acyl CoA from the condensation of acetyl CoA and butyryl CoA with malonyl CoA, respectively. However, the exact structures of these intermediates and their role in fatty acid synthesis must await additional information. (*g*) Substituted acyl CoA's (acetoacetyl CoA, β-hydroxybutyryl CoA, crotonyl CoA) do not oxidize TPNH in the presence of purified enzyme. They do not incorporate into the fatty acid nor do they dilute the amount of C^{14}-malonyl CoA converted to palmitate. (*h*) For each mole of palmityl CoA synthesized, 1 mole of acetyl CoA, 7 moles of malonyl CoA,

and 14 moles of TPNH were consumed and 7 moles of CO_2, 7 moles of CoASH, 14 moles of TPN, and 7 moles of water were formed. (*i*) Three tritium atoms from tritium-labeled acetyl CoA ($CT_3COSCoA$) were incorporated into 1 molecule of palmitate, indicating that the α-carbon of acetyl CoA constituted carbon 16 of palmitate. (*j*) One tritium atom of the tritium-labeled malonyl CoA $HOOCCT_2COSCoA$ was incorporated into palmitate per one "C_2-unit" derived from malonyl CoA, while the other tritium atom was presumably lost as water during the reactions.

These observations do not lend complete support to either Wakil and Ganguly's hypothesis based on a reaction sequence similar to β-oxidation and involving dicarboxylic acyl CoA derivatives, nor Lynen's acyl-S-enzyme hypothesis, which requires a multicatalytic enzyme which is without precedent in such varied and cyclic reactions.

were no loss of tritium from the methyl group of the polyketo acid.

Mammary Gland System. Popják and Tietz (69, 70, 100) have studied fatty acid synthesis in homogenates and in soluble preparations from the mammary gland of lactating rats. The soluble supernatant fraction shows greater activity in synthesizing fatty acids from acetate than does the full homogenate. Neither the mitochondrial nor the microsomal fractions were required for fatty acid synthesis. ATP was absolutely required for synthesis, and when the enzyme preparation was treated with Dowex-1 resin, a requirement for CoA and DPN was noted. Oxalacetate, α-ketoglutarate, and succinate markedly stimulated the synthesis. Malonate stimulated the synthesis of fatty acids markedly, and in combination with β-ketoglutarate it gave as much as a thirtyfold increase in the amount of C^{14}-acetate incorporated into the fatty acids. The

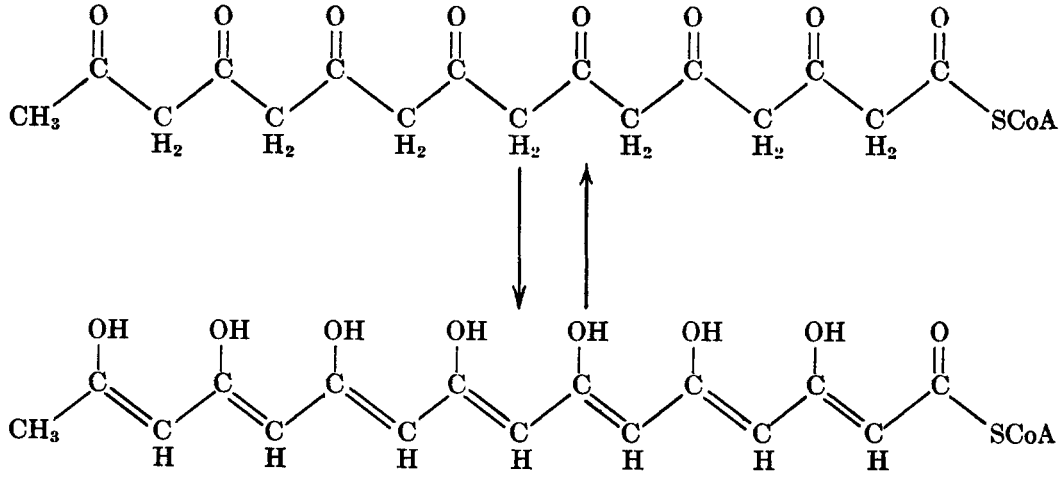


FIG. 14. Poly-keto palmityl CoA and its enol-form as one of the suggested intermediates in synthesis of palmityl CoA.

Further information is necessary in order to substantiate this mechanism. One of the attractive hypotheses is that proposed earlier by Lynen and later dropped in favor of the above. This involves the formation of a polyketo polymer of C_2-units (polyketo palmityl CoA; Fig. 14), which is then reduced completely to palmityl CoA. Such a compound might be formed by the condensation of 1 mole of acetyl CoA and 7 moles of malonyl CoA with concomitant formation of 7 moles of CO_2 and CoASH. The polyketo palmityl CoA formed could be reduced by TPNH to palmityl CoA. Although it is very difficult to conceive of the complete enzymic reduction of the polyketo acid occurring in a single step, it is conceivable that such a reduction of the undoubtedly predominant enol form may take place with the simultaneous removal of water, provided that during the tautomeric shifts there

mammary gland extract synthesizes both short (C_4 to C_8) and long-chain fatty acids (C_{12} to C_{18}), while the whole homogenate preparation synthesizes predominantly long-chain fatty acids (1).

Hele *et al.* (101) prepared a fatty acid synthesizing system from the mammary glands of lactating rabbits which had essentially the same characteristics as previous systems from rat and sheep. On fractionation with ammonium sulfate (35% to 65%) an enzyme preparation was obtained which synthesized short-chain fatty acids from acetate in the presence of ATP, CoA, Mg^{++}, cysteine, and DPNH. They were unable to demonstrate any requirement for TPN^+ or for TPNH. Furthermore, the authors (101, 102) found that predominantly short-chain fatty acids were synthesized by the ammonium sulfate fractions of the mammary gland. According to the data of Hele (102),

there was much less octanoate and hexanoate synthesized than β-hydroxy octanoate. In other words, the amounts of saturated fatty acids formed, except for butyrate, were extremely small compared to the β-hydroxy and unsaturated acids. It is of great interest to note that in crude homogenates Popják and Tietz's description (69, 70, 100) of the fatty acid synthesizing system of mammary gland is remarkably similar to that of the pigeon, rat and chicken liver system described earlier. It may be possible that the mammary gland has two soluble fatty acid synthesizing systems, one for the synthesis of the short-chain acids (the system described by Hele and Popják (101)) and the other, the non-mitochondrial system similar to that described by Wakil *et al.* (1).

Further information is needed in order to ascertain this point.

SUMMARY AND CONCLUSION

Evidence has been presented to show that there are two distinct systems for the synthesis of fatty acids.

1. The mitochondrial system, which may involve some enzymes of the β-oxidation system (enoyl hydrase, β-hydroxyacyl dehydrogenase) working in reverse plus the TPNH-α, β-unsaturated fatty acyl CoA reductase and perhaps a new condensing enzyme. Both TPNH and DPNH are required for the synthesis. Essentially this system is for the elongation of the existing fatty acids by the addition of two-carbon units at a time. It is possible that this system may be responsible for the formation of stearate from palmitate, arachidonate from linoleate, etc.

2. The non-mitochondrial system, which is located in the cytoplasm of the cell and catalyzes the conversion of acetyl CoA to palmitate in the presence of ATP, Mn^{++}, HCO_3^-, and TPNH (DPNH may be substituted for TPNH with a slower rate). Acetyl CoA is condensed with HCO_3^- to form malonyl CoA in a reaction catalyzed by acetyl CoA carboxylase (a biotin-containing enzyme) in the presence of ATP and Mn^{++}. The biotin is bound to the protein, and evidence has been presented to show that it does participate in the formation of malonyl CoA. Malonyl CoA condenses with acetyl CoA or propionyl CoA and yields, on reduction by TPNH, saturated fatty acids, CO_2, and CoA.

This system appears to be the main pathway for fatty acid synthesis and is widely distributed in living organisms. So far this system has been isolated from pigeon liver, chicken liver, rat liver, rat kidney, yeast cells, and avocado fruits.

The enzymatic system for the synthesis of short-chain fatty acids in mammary gland may be different from the above two systems, but final judgment has to await more information.

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